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Iron regulated surface determinants of *Staphylococcus aureus*
and *S. lugdunensis* –their roles in pathogen-host interactions

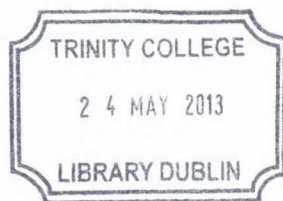
A thesis submitted for degree of Doctor in Philosophy

by

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February 2013



Thesis 9831

Declaration

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Summary

Staphylococcus aureus is a human pathogen that causes invasive infections. The ability to internalize into and persist within host cells is thought to contribute to pathogenesis. The study described here has identified a novel role for the well-characterized haemoglobin receptor of *S. aureus*, the iron regulated surface determinant B (IsdB). IsdB_{SA} was shown to promote bacterial adherence to human embryonic kidney cells (293T), human cervical cancer cells (HeLa) and platelets. IsdB_{SA}-mediated adherence is independent of its ability to bind haemoglobin and can be inhibited by the integrin blocking RGD peptide. IsdB_{SA} was shown to bind the active form of the platelet integrin $\alpha_{IIb}\beta_3$, both on platelets and when the integrin was expressed by CHO-K1 cells. IsdB_{SA}-promoted adhesion to human cells correlated with increased levels of bacterial invasion. This was demonstrated with bacteria lacking fibronectin binding proteins (FnBPs), which are known to promote invasion in the presence of fibronectin. However, IsdB_{SA} also contributed to invasion by cells expressing FnBPs in the presence of fibronectin. These results suggest that IsdB_{SA} is able to interact with a family of integrins that can bind ligands *via* the RGD recognition motif. IsdB_{SA}-dependent binding to integrins may provide a back up mechanism to promote *S. aureus* interactions with human cells.

Staphylococcus lugdunensis is the only species of coagulase negative staphylococcus that carries a locus encoding an Isd system. In *S. aureus* the Isd proteins capture haem from haemoglobin, transfer it across the cell wall to a membrane-bound transporter which delivers it into the cytoplasm where haem oxygenases release iron. This study demonstrated that, like *S. aureus*, the Isd proteins of *S. lugdunensis* were expressed under iron-restricted conditions and were down-regulated upon an increase in the concentration of iron in the environment. It is shown that *S. lugdunensis* IsdB and IsdC proteins performed the same functions as those of *S. aureus*. IsdB_{SL} is the only haemoglobin receptor encoded by the *S. lugdunensis* *isd* locus. It specifically bound human haemoglobin with a K_D of 23 nM and transferred haem to IsdC_{SL}. IsdB_{SL} expression promoted bacterial growth in an iron-restricted medium supplemented with human haemoglobin but not mouse haemoglobin. This correlated with weak binding of recombinant IsdB_{SL} to mouse haemoglobin *in vitro*, as measured by surface plasmon resonance. Unlike IsdB_{SL} and IsdC_{SL}, the *S. lugdunensis* IsdJ and IsdK proteins were not sorted to the cell wall.

However IsdJ became anchored to the cell wall and was surface-exposed when expressed in *S. aureus* and *Lactococcus lactis*. This suggests that *S. lugdunensis* sortase, which is responsible for anchoring proteins to bacterial cell wall peptidoglycan, may differ in its ability to recognize the sorting sequence at the proteins' C-termini. IsdJ and IsdK were found in the culture supernatant suggesting they could also acquire haem from the external milieu.

The IsdA protein of *S. aureus* protects bacteria from bactericidal lipids in human skin sebum due to the hydrophilic C-terminal domain. This domain is similar to the C-terminus of IsdJ. The ability of IsdJ to provide resistance to a skin fatty acid (linoleic acid) was studied. When expressed by *S. aureus* or *L. lactis* IsdJ protected bacteria as efficiently as IsdA, but was less effective when expressed by *S. lugdunensis* possibly due to its cellular localization.

IsdJ, like its *S. aureus* homolog – IsdA was also shown to bind to fibrinogen, and proteins from the cornified envelope of human skin, including loricrin and cytokeratin 10. However, expression of IsdJ or IsdA was not sufficient to promote bacterial adhesion to these ligands. IsdB_{SL}, unlike its orthologue in *S. aureus*, neither promoted platelet adhesion or aggregation nor adhered and invaded human cells.

Publications

Miajlovic H, Zapotoczna M, Geoghegan JA, Kerrigan SW, Speziale P, Foster TJ (2010) Direct interaction of iron-regulated surface determinant IsdB of *Staphylococcus aureus* with the GPIIb/IIIa receptor on platelets. *Microbiology* 156 (3): 920-928.

Zapotoczna M, Heilbronner S, Speziale P, Foster TJ (2012) Iron regulated surface determinant (Isd) proteins of *Staphylococcus lugdunensis*. *J Bacteriol* 194 (23): 6453-67.

Zapotoczna M, Jevnikar Z, Kos J, Foster TJ (2012) Iron regulated surface determinant B (IsdB) promotes *Staphylococcus aureus* adherence to and internalization by human non-phagocytic human cells. *Cell Microbiol* doi: 10.1111/cmi.12097

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Key to abbreviations

Single / triple amino acid code

A / Ala	Alanine
C / Cys	Cysteine
D / Asp	Aspartic acid
E / Glu	Glutamic acid
F / Phe	Phenylalanine
G / Gly	Glycine
H / His	Histidine
I / Iso	Isoleucine
K / Lys	Lysine
L / Leu	Leucine
M / Met	Methionine
N / Asp	Asparagine
P / Pro	Proline
Q / Glt	Glutamine
R / Arg	Arginine
S / Ser	Serine
T / Thr	Threonine
V / Val	Valine
W / Trp	Tryptophan
Y / Tyr	Tyrosine

Nucleotides

A	Adenine
T	Thymine
C	Cytosine
G	Guanine

Antibiotics

Amp	Ampicillin
Chm	Chloramphenicol
Erm	Erythromycin
aTc	anhydrotetracycline

Key to abbreviations

aa	amino acid
bp	base pair(s)
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
dNTP	deoxy nucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
Fg	fibrinogen
Fn	fibronectin
Vn	vitronectin
Hb	haemoglobin
MetHb	methaemoglobin
Ig	immunoglobulin
kb	kilobase pair
kDa	kilodalton
min	minute(s)
h	hour(s)
nt	nucleotides
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRP	platelet rich plasma
PPP	platelet poor plasma
r.p.m.	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	- II - polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl)aminomethane
v/v	volume per volume
w/v	weight per volume
wt	wild-type

Chapter 1
Introduction

1.1 Biology of the staphylococci.

1.1.1 Classification and identification.

Bacteria of the genus *Staphylococcus* are non-motile, spherical, gram-positive organisms that are ca. 1 μm in diameter and characteristically form grape-like irregular clusters (Ludwig *et al.*, 1985; Stackebrandt, 1988; Stackebrandt *et al.*, 1988). The name *Staphylococcus* proposed by Alexander Ogston originated from the Greek *staphyle* for 'bunch of grapes' and *kokkos* for 'berry'. Formal description of the genus was provided by Rosenbach, who also divided it into two species, *Staphylococcus aureus* and *Staphylococcus albus*. The genus *Staphylococcus* now comprises at least 45 species and 21 subspecies (Drancourt *et al.*, 2002; Hauschild *et al.*, 2010; Novakova *et al.*, 2010; Supre *et al.*, 2010).

Taxonomic studies using molecular typing methods placed the staphylococci in the *Bacillus-Lactobacillus-Streptococcus* cluster of the *Micrococcaceae* (Drancourt *et al.*, 2002; Ludwig *et al.*, 1985; Stackebrandt *et al.*, 1988). Staphylococci are most closely related to *Enterococcus*, *Bacillus*, or *Listeria* and are facultative anaerobes producing catalase. Other characteristics of the genus include the low G + C content (30 – 39 %) of their genomic DNA. They are extremely halotolerant as they grow at up to 3.5 M NaCl, and are resistant to dessication.

The production of coagulase is used to divide the staphylococci into two major groups, coagulase-positive staphylococci and coagulase-negative staphylococci (CoNS). Coagulase is a secreted zymogen that binds to and activates prothrombin, thereby converting fibrinogen to fibrin and promoting clotting of plasma. The coagulase-positive staphylococci have always been regarded as more virulent (Fahlberg *et al.*, 1960; Marston *et al.*, 1960).

The coagulase positive *Staphylococcus aureus* (from the Latin *aurum* for 'golden') was named after its characteristic golden pigment. Among other phenotypic features allowing separation of *S. aureus* from other species within the genus are production of thermostable DNase and the ability to ferment mannitol.

Staphylococcus lugdunensis is a CoNS, which was first distinguished from other organisms through DNA relatedness studies based on 11 clinical strains by Freney *et al.* in 1988 (Freney *et al.*, 1988). The new species was named after Lyon, the French city where the organism was first isolated (from the Latin *Lugdunum* for Lyon). *S. lugdunensis* can be differentiated from other CoNS by its ability to produce ornithine decarboxylase and pyrrolidonyl arylamidase (Tan *et al.*, 2009). Standard identification

procedures lead to misidentification of this organism as *S. aureus*, as its colony morphology resembles that of *S. aureus* and it expresses a clumping factor that yields a positive result in latex agglutination tests (Lambe *et al.*, 1990; Pereira *et al.*, 2011).

1.1.2 Colonisation and disease.

1.1.2.1 *Staphylococcus aureus*.

S. aureus permanently colonizes approximately 20% of the population whilst the remaining can be colonised transiently (van Belkum *et al.*, 2009). The primary ecological habitat of *S. aureus* in humans is the moist squamous epithelium of the anterior nares, which is composed of layers of cells that are differentiating as they progress from lower layers to the exposed surface of the *stratum corneum*. This involves changes in shape, loss of the nucleus and keratinisation. *S. aureus* adheres strongly to the flat, mature squames found in the superficial layer (Peacock *et al.*, 2001).

Bacterial factors that promote adhesion to squames are the surface proteins clumping factor B (ClfB), iron-regulated surface determinant A (IsdA), serine-aspartate repeat proteins (SdrC and SdrD) and the *S. aureus* surface protein G (SasG) (Corrigan *et al.*, 2009b), but only the first two interactions have been studied in any detail.

The ClfB protein mediates adhesion to squames by binding to cytokeratin 10 and loricrin, proteins that are present in desquamated epithelial cells (Mulcahy *et al.*, 2012; O'Brien *et al.*, 2002b; Walsh *et al.*, 2004). Cytokeratins belong to the family of intermediate filaments which provide mechanical strength to vertebrate epithelial cells whereas loricrin is an insoluble glycine and serine - rich transglutaminase substrate protein that is cross-linked in the cornified envelope of squames. Active immunization with ClfB and application of a monoclonal antibody directed against ClfB reduced *S. aureus* nasal colonization in a murine model (Schaffer *et al.*, 2006). Also *S. aureus* deficient in *clfB* was cleared faster from the human nares than the wild-type strain (Wertheim *et al.*, 2008). The crucial role of loricrin as the substrate for ClfB-promoted colonization was revealed by demonstrating reduced colonization of loricrin-deficient mice by the wild-type but not a *clfB* mutant (Mulcahy *et al.*, 2012).

IsdA is best known for its ability to bind haem but it has also been shown to promote bacterial adhesion to squames (Clarke *et al.*, 2009; Corrigan *et al.*, 2009b). Expression of IsdA is up-regulated under the iron-restrictive conditions of the nasal mucosa allowing it to associate with proteins that occur in the outer envelope of

cornified epithelial cells, most probably *via* loricrin, cytokeratin 10 or involucrin (Clarke *et al.*, 2009). Vaccination of cotton rats with IsdA reduced nasal colonisation (Clarke *et al.*, 2006).

Another type of *S. aureus* cell wall component implicated in colonization is the wall teichoic acid (WTA). The WTA-defective mutant exhibited decreased adherence to nasal cells and colonisation of the nares of cotton rats (Weidenmaier *et al.*, 2004). The WTA biosynthesis genes *tagO* and *takK* were up-regulated during first four days of colonisation of the nares of cotton rats which may increase the effect of bacteria settling. At later stages of colonization the expression profile switched to production of the adhesive proteins ClfB and IsdA (Burian *et al.*, 2010).

In addition, recent evidence suggests that polymicrobial interactions play a role in *S. aureus* nasal colonization. Secretion of serine protease Esp by *S. epidermidis* correlated with the absence of *S. aureus* in nasal cavities (Iwase *et al.*, 2010). Purified Esp was also shown to inhibit biofilm formation by *S. aureus* and destroy pre-existing biofilms (Iwase *et al.*, 2010).

Healthy individuals have a small but finite chance of contracting an invasive *S. aureus* infection. The risk is increased among carriers both in the hospital and the community (von Eiff *et al.*, 2001a; Wertheim *et al.*, 2005; Wertheim *et al.*, 2004). Colonisation of the nares depends on the ability of *S. aureus* to adhere to desquamated epithelial cells and to overcome the host innate and adaptive immune responses, which are controlled by nasal-associated lymphoid tissue (NALT). Host factors associated with immune responses are believed to play a role in determining carriage status. For instance, increased rates of persistence in carriage were found in individuals with a polymorphism in the interleukin-4 (IL-4) gene promoter which is associated with lower IL-4 serum concentrations and reduced mucin (Emonts *et al.*, 2008). Nasal carriage is also influenced by glucocorticoid receptor gene polymorphisms. Glucocorticoid receptors are involved in immune modulation. For example, individuals with haplotype 5, which is associated with immune suppression, have an 80% increased *S. aureus* carriage rate, whereas individuals with haplotype 3 were characterized by enhanced immunity and 68% lower persistence in carriage rates (van den Akker *et al.*, 2006).

Different carriage rates have been also observed among different ethnic groups and families (Noble, 1974; Noble *et al.*, 1967). Moreover, human nasal secretions show variability in supporting growth or having antimicrobial activity against *S. aureus*. It was suggested that haemoglobin present in nasal secretions promotes *S. aureus*

colonization by inhibiting expression of the Agr effector molecule, RNAIII (Pynnonen *et al.*, 2011). Taken together these studies suggest that nasal carriage is a multifactorial process that is influenced by host determinants, bacterial products, and polymicrobial interactions.

Infections are initiated when the mucosal or skin barriers are breached allowing staphylococci access to underlying tissues or the bloodstream. Whether an infection is contained or spreads depends on the complex interplay between *S. aureus* virulence determinants and host defence mechanisms. The most common types of *S. aureus* infection are superficial skin lesions such as boils, impetigo and abscesses. If the organism gains access to the bloodstream (bacteraemia), it can infect internal tissues such as bone (osteomyelitis), joints (septic arthritis), lungs (pneumonia), and heart valves (endocarditis) (Lowy, 1998). Many instances of nosocomial *S. aureus* bacteraemia are attributable to an endogenous source (von Eiff *et al.*, 2001a), although it has been noted that bacteraemia-related death was significantly higher in infected non-carriers compared to infected carriers, suggesting immunological adaptation to the harboured strain of *S. aureus* (Wertheim *et al.*, 2004).

1.1.2.2 *Staphylococcus lugdunensis*.

S. lugdunensis is an integral part of normal skin flora. It colonizes several distinct niches in the human body, primarily in the lower abdomen and the extremities (Bieber *et al.*, 2010) and, with lower frequency, the anterior nares or nasal cavities (Rasmussen *et al.*, 2000). *S. lugdunensis* is unique among CoNS in having an elevated degree of virulence. It can cause severe infections such as urinary tract infections, peritonitis, osteomyelitis, skin and soft tissue infections, and native or prosthetic valve endocarditis (Frank *et al.*, 2008). These infections occur in patients with underlying diseases, especially those who are immunosuppressed (Kourbeti *et al.*, 2007; Murdoch *et al.*, 1996; Patel *et al.*, 2000; Pirila *et al.*, 2006) but have also been described in otherwise healthy people (Bellamy *et al.*, 2002; Farrag *et al.*, 2001; Sotutu *et al.*, 2002). It is known for causing aggressive native valve infective endocarditis with a high mortality rate (Anguera *et al.*, 2005).

1.1.3 Cell wall components.

1.1.3.1 Peptidoglycan.

The main component of cell wall is peptidoglycan. It is synthesized in the bacterial cytoplasm from the modified amino sugars N-acetylmuramic acid (MurNAc) and D- or L-amino acids (L-Ala-D-iGlu-m-Dpm-D-Ala-D-Ala, abbreviated AGDA₂) forming Park's nucleotide (UDP—MurNAc-AGDA₂) (Fig. 1.1) (Park, 1952; Park *et al.*, 1949). Park's nucleotide is linked to the lipid carrier undecaprenol pyrophosphate generating lipid I [C₅₅-PP-MurNAc-AGDA₂], which is then modified with N-acetylglucosamine and pentaglycine cross bridge formation (UDP-GlcNAc) at the L-Lys with tRNA^{Gly} producing lipid II [C₅₅-PP-MurNAc(AGDA₂)-GlcNAc] (Fig. 1.1) (Higashi *et al.*, 1967). Lipid II is polymerized by translocases and transpeptidases (penicillin binding proteins) to generate peptidoglycan strands [MurNAc(AGDA₂)-GlcNAc-MurNAc(AGDA₂)-GlcNAc] that are crosslinked with other strands (Fig. 1.1 and 1.2) (Schneewind *et al.*, 2012; Strominger, 1968).

The peptidoglycan layer in gram-positive bacteria is much thicker than that of gram-negatives. In some cases it can be 50-100 nm in diameter (Giesbrecht *et al.*, 1998). This rigid structure protects gram-positive bacteria from osmotic lysis and serves as a barrier against hydrolases or membrane damaging compounds (Shockman *et al.*, 1983). Peptidoglycan also provides scaffolding for bacterial surface components such as capsular polysaccharides, wall teichoic acids and sortase-anchored proteins which additionally protect bacteria and aid in their interaction with the environment, most notably the tissue and immune cells of an infected host. The cell wall anchored proteins of *S. aureus* can be released by treatment with the endopeptidase lysostaphin, the muramidase mutanolysin or amidases which cleave at specific sites in the peptidoglycan structure (Fig. 1.2) (Schleifer *et al.*, 1972).

1.1.3.2 Teichoic acids.

The teichoic acids (TAs) are either connected to peptidoglycan (wall teichoic acids, WTA) or to the cytoplasmic membrane (lipoteichoic acid, LTA). WTA is covalently linked to the N-acetylmuramic acid (MurNAc) *via* a disaccharide composed of N-acetylglucosamine (GlcNAc)-1-P and N-acetylmannosamine (ManNAc) which is followed by two units of glycerol phosphate (Gro-P) (Fig. 1.3). In most *S. aureus* strains the actual WTA polymer is composed of units of ribitol phosphate (Fig. 1.3). LTA polymers of *S. aureus* are built of glycerol phosphate units substituted with D-

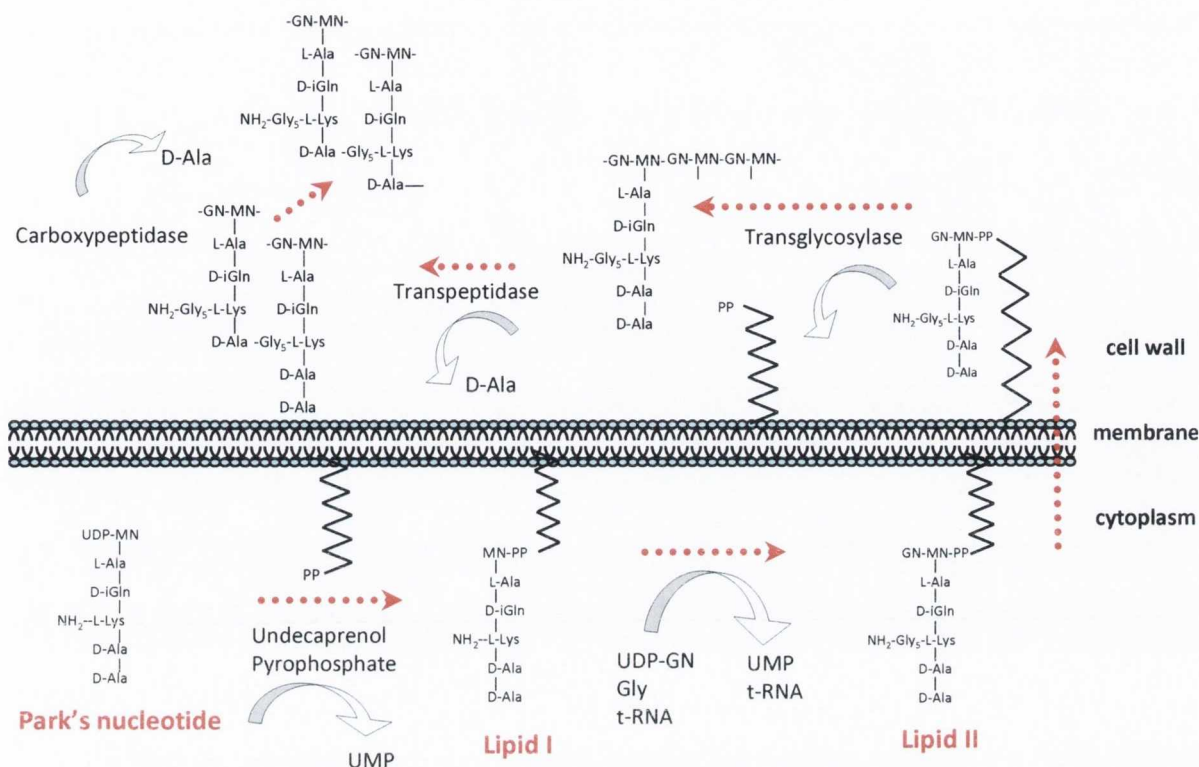


Figure 1.1 Peptidoglycan synthesis in *S. aureus*.

Park's nucleotide is a soluble nucleotide precursor synthesized in the cytoplasm by sequential addition of amino acids (L-Ala and L-Lys) as well as (D-isoglutamine [D-iGln] and D-Ala) to UDP-N-acetylmuramic acid (UDP-NM). Precursor transfer to undecaprenol pyrophosphate, a bacterial membrane carrier, generates lipid I and removes the UMP nucleotide. Lipid I is modified with N-acetylglucosamine (GN) and by pentaglycine cross bridge formation at the L-Lys with tRNA^{Gly} forming lipid II. Following translocation across the cytoplasmic membrane, lipid II serves as substrate for transglycosylation, transpeptidation, and carboxypeptidation. Transglycosylases polymerize MN-GN subunits into repeating glycan strands. Transpeptidases cleave the amide bond of the terminal D-Ala in pentapeptide precursors generating an amide bond between the carboxyl group of D-Ala at position four and the amino group of pentaglycine cross bridges in wall peptides. Carboxypeptidases hydrolyze the C-terminal D-Ala of most non-cross-linked pentapeptides to yield mature peptidoglycan. Figure adapted from Marraffini *et al.*, 2006.

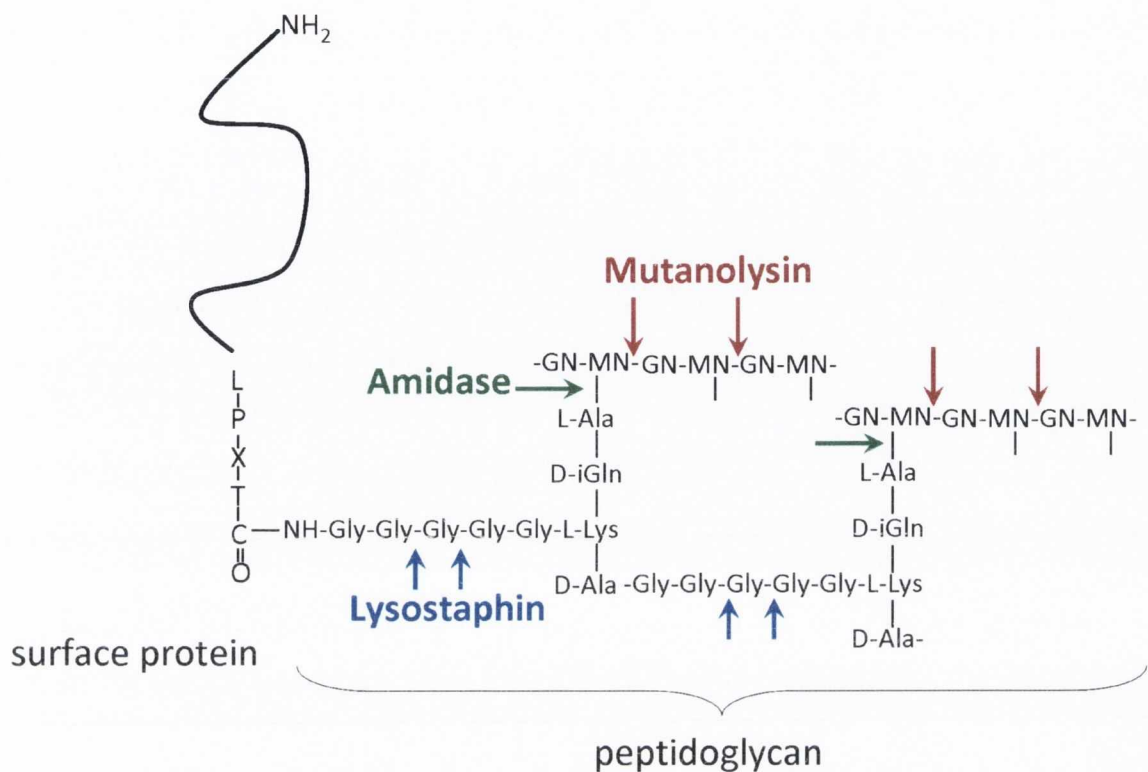


Figure 1.2 Schematic representation of sites for digestion of staphylococcal peptidoglycan.

Cleavage sites are indicated for lysostaphin (glycine-glycine endopeptidase), mutanolysin [N-acetylmuramidase that cleaves the (1-4) O-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine (GN)] and amidase (N-acetylmuramoyl-L-Ala amidase). The C-terminal threonine of surface proteins, generated by sortase A-mediated cleavage between the threonine and the glycine of the LPXTG motif, is amide linked to the pentaglycine cross bridge of *S. aureus* cell wall peptidoglycan. Treatment of the staphylococcal peptidoglycan with those enzymes releases surface protein(s) with the characteristic C-terminal cell wall peptidoglycan fragment. Figure adapted from Marraffini *et al.*, 2006.

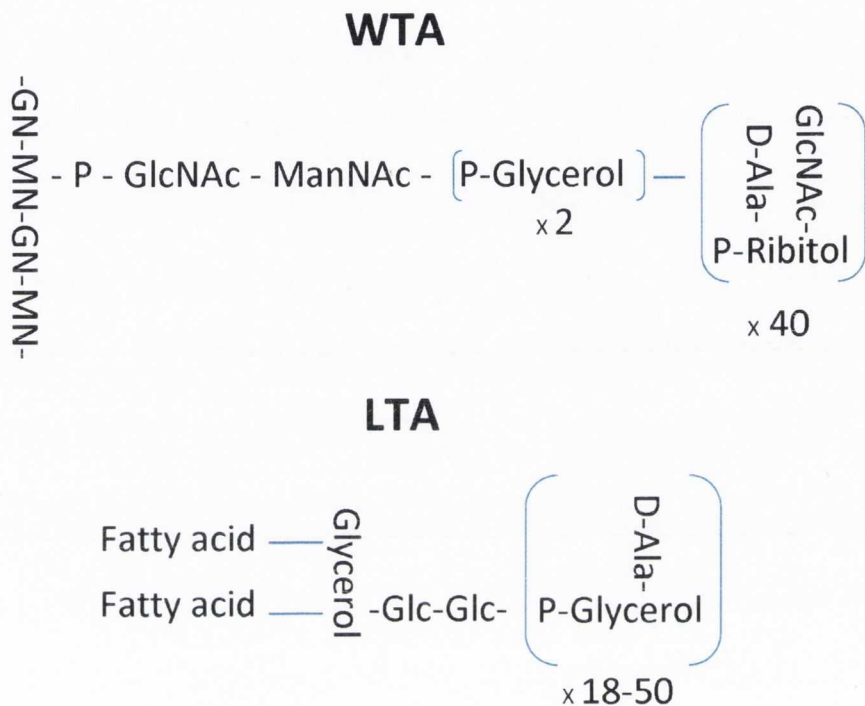


Figure 1.3 Structures of *S. aureus* wall teichoic acid and lipoteichoic acid.
 Components of the wall teichoic acid (WTA) and the lipoteichoic acid (LTA) are presented schematically. Structures components, such as (P) phosphate, (D-Ala) D-alanine; (GlcNAc) N-acetylglucosamine; (ManNAc) N-acetylmannosamine; (MN) N-acetylmuramic acid and (Glc) glucose are indicated. Adapted from Xia *et al.*, 2010.

alanine and attached to the cytoplasmic membrane *via* a glycolipid anchor which is a digalactosyldiacylglycerol in *S. aureus* and most other staphylococcal species (Fig. 1.3) (Xia *et al.*, 2010).

Higher levels of D-alanine substitution of WTA or L-lysine of LTA were shown to promote resistance to antimicrobial peptides (CAMPs), including defensins, kinocidins and cationic antibiotics like vancomycin (Collins *et al.*, 2002; Peschel *et al.*, 2001; Peschel *et al.*, 1999), by neutralizing negatively charged bacterial envelope. The same mechanism may occur in *S. lugdunensis*, as genes encoding the apparatus for D-alanine substitution to WTA (*dlt* genes) and L-lysine addition to phosphatidyl glycerol (*mrpG* gene) were annotated in the genomes of *S. lugdunensis* N920143 and HKU09-01 (Heilbronner *et al.*, 2011). The role of WTA in colonisation was discussed in section 1.1.2.1. Other functions of WTA include protection against antimicrobial molecules like lysozyme and human skin fatty acids (Collins *et al.*, 2002; Peschel *et al.*, 2001; Weidenmaier *et al.*, 2005a; Weidenmaier *et al.*, 2005b). Resistance to heat stress and low osmolarity was also shown to be enhanced by both types of TA (Oku *et al.*, 2009; Vergara-Irigaray *et al.*, 2008). WTA promotes adherence to epithelial and endothelial cells (Weidenmaier *et al.*, 2005a; Weidenmaier *et al.*, 2005b). LTA interacts with ectodomains of CD36 and CD14 host membrane glycoproteins that trigger the processes of inflammation (Draing *et al.*, 2008; Jimenez-Dalmaroni *et al.*, 2009; Morath *et al.*, 2001; Xia *et al.*, 2010). Both TAs can promote attachment to biomaterials and formation of biofilm (Fedtke *et al.*, 2007; Gross *et al.*, 2001; Vergara-Irigaray *et al.*, 2008).

1.1.3.3 Capsular polysaccharide.

Capsular polysaccharide (CP) or capsule is produced by a majority of *S. aureus* clinical isolates (O'Riordan *et al.*, 2004; Roghmann *et al.*, 2005). Although 11 *S. aureus* CP types were described, only type 5 and 8 are clinically relevant in that they are predominant among clinical isolates and are associated with increased virulence in animal infection models (Luong *et al.*, 2002; Nilsson *et al.*, 1997; Roghmann *et al.*, 2005; Thakker *et al.*, 1998). *In vitro*, CP is expressed in the stationary phase of growth and has been detected *in vivo* in endocardial vegetations (Lee *et al.*, 1993). Expression of capsule inhibits *S. aureus* clumping factor A-mediated binding to fibrinogen and platelets (Risley *et al.*, 2007). Capsule also reduced uptake of bacterial cells by neutrophils by masking opsonins in animal serum (O'Riordan *et al.*, 2004).

Orthologue of the *cap* genes were identified in the genomes of *S. lugdunensis* N920143 and HKU09-01, indicating production of capsule by members of this species (Heilbronner *et al.*, 2011).

1.1.3.4 Anchoring of surface proteins of *S. aureus*.

The surface proteins of *S. aureus* play key roles in mediating interactions with the host, including adhesion to tissues, uptake into host cells, evasion and suppression of the immune responses and acquisition of nutrients (Speziale *et al.*, 2009). Some of these functions are discussed in subsequent chapters. This section reviews the process of covalent anchorage of surface proteins to *S. aureus* cell wall peptidoglycan.

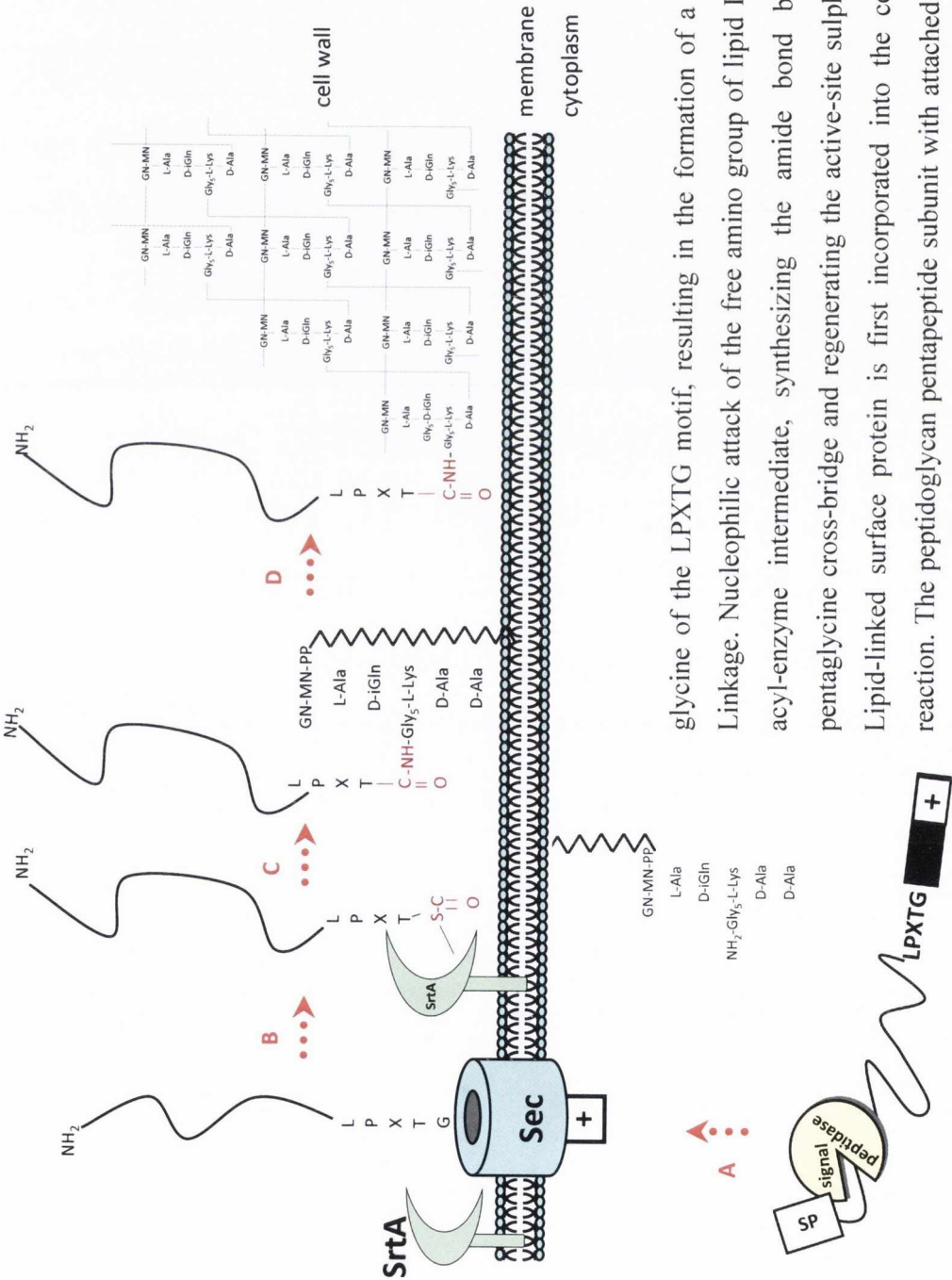
Gram-positive bacteria use sortases to anchor surface proteins to the cell wall. Sortases are cysteine transpeptidases which link a specific sorting signal at a protein C-terminus with cell wall peptidoglycan (Schneewind *et al.*, 2012; Spirig *et al.*, 2011). The mechanism of sortase A (SrtA) protein anchoring to *S. aureus* peptidoglycan was the first to be described over a decade ago and is the best understood to date (Mazmanian *et al.*, 1999). During polypeptide secretion *via* a Sec-dependent pathway, SrtA scans the amino acid chain for a recognition sequence comprising Leu–Pro–X–Thr–Gly (LPXTG) (Fig. 1.4) (Ilangoan *et al.*, 2001). The LPXTG-motif is the dominant sorting motif of *S. aureus* cell wall proteins, including the virulence factors discussed in Chapter 1.2 (Fig. 1.5). The LPXTG motif is followed by a segment of hydrophobic amino acids and a tail of primarily positively charged residues which presumably retards the export and positions the protein for cleavage by the membrane-associated SrtA (Fig. 1.4A). The active site of SrtA nucleophilically attacks the backbone carbonyl carbon of the threonine residue in the LPXTG motif, breaking T-G peptide bond and forming a thioester-linked intermediate with the carboxyl group at the C-terminal end of the protein (Fig. 1.4B). A free amino group within lipid II nucleophilically attacks the thioacyl linkage to create a protein-lipid II product and restores the enzyme's active site (Fig. 1.4C). The surface protein linked to lipid II is then incorporated into the cell wall by transglycosylation and transpeptidation reactions of cell wall peptidoglycan biosynthesis (Fig. 1.1 and 1.4D).

Sortases are classified into six groups (A to F) (Spirig *et al.*, 2011). Each recognizes a distinct sorting signal. SrtA performs a housekeeping role by anchoring a large number of functionally distinct proteins. SrtB recognizes the NP(Q/K)(T/S)(N/G/S)(D/A) sorting motif and anchors the iron-regulated surface

Figure 1.4 Surface protein anchoring in *S. aureus*.

(A) Export and retention. Precursor proteins with an N-terminal signal peptide (SP) are initiated into the secretory (Sec) pathway and the signal peptide is removed by signal peptidase. The C-terminal sorting signal retains polypeptides within the secretory pathway. **(B)** Cleavage. Sortase cleaves between the threonine and the

glycine of the LPXTG motif, resulting in the formation of a thioester enzyme intermediate. **(C)** Linkage. Nucleophilic attack of the free amino group of lipid II by the thioester bond resolves the acyl-enzyme intermediate, synthesizing the amide bond between surface proteins and the pentaglycine cross-bridge and regenerating the active-site sulphhydryl. **(D)** Cell wall incorporation. Lipid-linked surface protein is first incorporated into the cell wall via the transglycosylation reaction. The peptidoglycan subunit with attached surface protein is then cross-linked to other cell wall peptides, generating a mature peptidoglycan tetrapeptide.



protein C (IsdC). In contrast to SrtA, SrtB is expressed only under iron restricted conditions (Mazmanian *et al.*, 2002). SrtA and SrtB attach their substrates to different sites within the cell wall. SrtA attaches its substrates to surface-exposed sites that are heavily cross-linked while SrtB attaches IsdC to a buried site within the peptidoglycan that is not heavily cross-linked (Marraffini *et al.*, 2005; Mazmanian *et al.*, 2003). *isdC* and *srtB* are encoded within the *isd* locus in *S. aureus* and orthologs were annotated in *S. lugdunensis* N920143 and HKU09-01 genomes (Heilbronner *et al.*, 2011).

The distribution of surface proteins in the cell wall is determined by the YSIRK/GS motif in the N-terminal signal sequence. The motif directs proteins to be secreted to the cross wall formed to separate daughter cells upon cell division. This results in a ring-like distribution of the protein on the bacterial surface and is characteristic of clumping factor A, protein A and fibronectin-binding proteins. Signal peptides without YSIRK/GS, like IsdA direct proteins to the cell pole and discrete assembly sites on the cell surface (DeDent *et al.*, 2008).

1.2 Surface proteins-mechanisms of ligand binding.

As a successful and diverse pathogen, *Staphylococcus aureus* has been widely studied over the years leading to identification of an arsenal of surface proteins which mediate interactions with the host. The Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) bind multiple host proteins such as fibrinogen, fibronectin and collagen, enabling colonization of host tissues. Other surface proteins bind immunoglobulins and complement molecules evading host immune responses. Another group of cell wall proteins provides *S. aureus* with iron during infection from host haemoproteins and haem.

1.2.1 IgG-binding protein A.

Surface protein A (Spa) was the first cell wall protein of *S. aureus* to be characterized (Oeding *et al.*, 1964). It is expressed by at least 95% of *S. aureus* strains (Forsgren *et al.*, 1974). The N-terminal region of Spa contains five homologous subdomains labelled E, D, A, B and C (Fig. 1.5). Each subdomain consists of three anti-parallel α -helices that form three helix bundles capable of high affinity binding to multiple host ligands. These ligands include 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

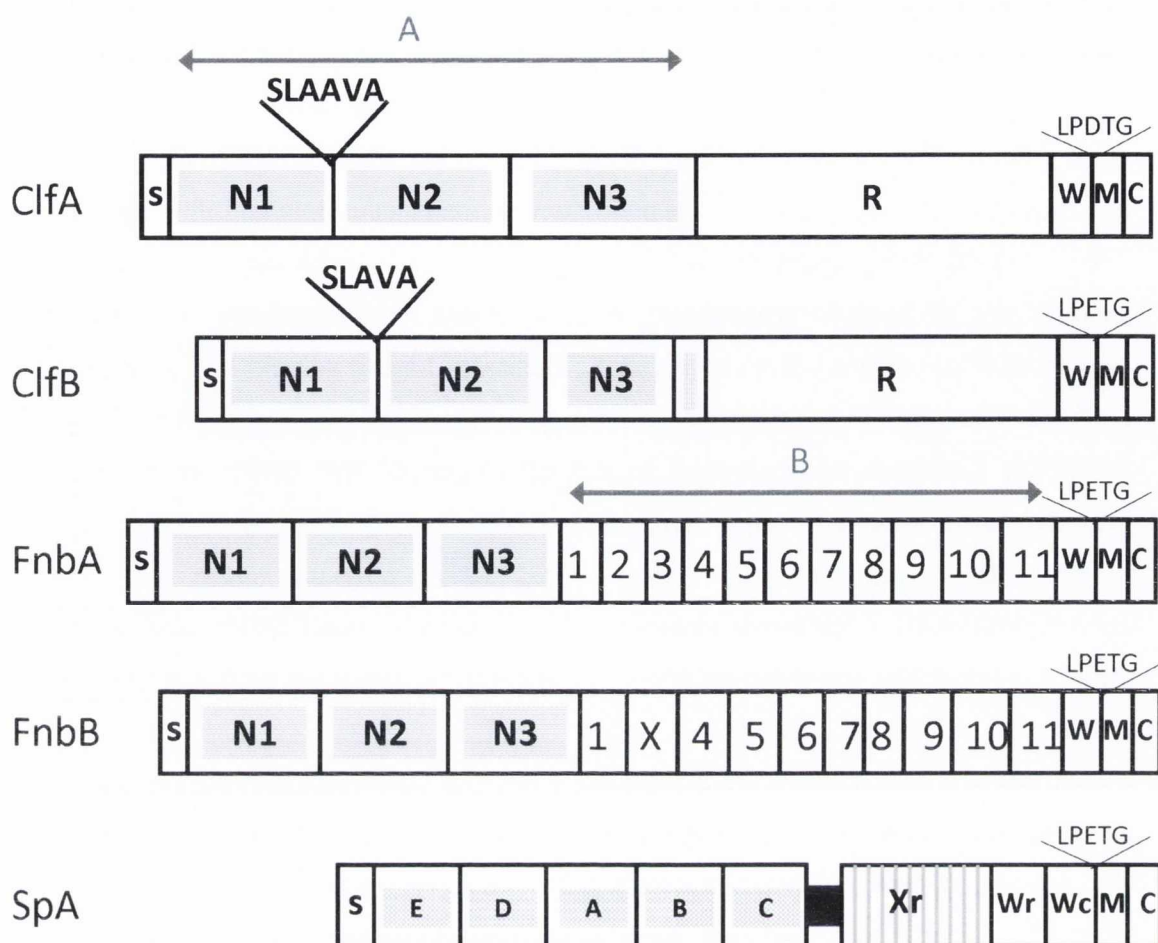


Figure 1.5 Staphylococcal surface proteins. Structural organisation of surface proteins from *S. aureus*. The signal sequence (S), A domain (A), SD-repeat region (R), proline-rich octapeptide repeats (Xr domain) and cell wall/membrane/cytoplasm-spanning regions (WMC) are shown. LPXTG motifs for recognition by sortase A are indicated. The structural organization of ClfA is similar to that of ClfB, but the proteins are only 27% identical. ClfA and ClfB contain metalloprotease recognition SLA(A)VA motifs at the end of N1 subdomains. The only characterized surface protein of *S. lugdunensis* – Fbl is an ortholog of ClfA and shares its structural organisation.

von Willebrand factor (vWF) (Hartleib *et al.*, 2000; O'Seaghdha *et al.*, 2006). The N-terminal domain is followed by a region that contains a variable number of proline-rich octapeptide repeats (Xr domain) and a C-terminal region which is conserved across the cell wall proteins (Fig. 1.5) (Moks *et al.*, 1986).

Spa binding to the immunoglobulin Fc region results in bacterial cells being coated with IgG molecules in an incorrect orientation for recognition by the neutrophil Fc receptors providing a mechanism for avoidance of phagocytosis (Foster, 2009). Moreover, Spa-bound IgG cannot stimulate complement fixation by the classical pathway. Spa binding to IgM bearing V_H3 displayed on B lymphocytes induces their proliferation resulting in immunosuppressive depletion of the B cell repertoire (Silverman, 1992). Spa binding to the tumour necrosis factor receptor-1 (TNFR-1), which is widely distributed in the airway epithelium, is important in the pathogenesis of *S. aureus* pneumonia (Gomez *et al.*, 2004). Protein A acts as a ligand mimicking TNF- α to activate TNFR-1 and subsequent proinflammatory signalling. It stimulates cleavage of TNFR-1 from the surface of epithelial cells and macrophages, which serves to limit TNF- α signalling (Gomez *et al.*, 2006; Gomez *et al.*, 2007). The TNFR-1 receptor is expressed on pre-osteoblastic cells. The Spa-mediated interaction with TNFR-1 results in generation of a signal leading to osteoblast apoptosis and bone loss. It also prevents expression of key compounds of osteoblast growth and division, such as collagen type I, osteocalcin and osteopontin inhibiting *de novo* bone formation (Claro *et al.*, 2011; Widaa *et al.*, 2012). Spa also recognizes A1 and D'-D3 domains of von Willebrand factor (vWF) (O'Seaghdha *et al.*, 2006). Binding of protein A to vWF contributes to bacterial adhesion to platelets under shear conditions which may contribute to bloodstream infection (Hartleib *et al.*, 2000). This is described in more detail in section 1.5.2. *S. lugdunensis* binding to Fc region of IgG has not been identified (Paulsson *et al.*, 1993). The bacterium does not encode Spa or the non-covalently associated IgG-binding Sbi protein (Heilbronner *et al.*, 2011).

1.2.2 Fibrinogen-binding proteins.

Fibrinogen (Fg) is a 340 kDa glycoprotein synthesized by hepatocytes and found in plasma. It is made up of two subunits that each consist of three polypeptide chains (A α , B β and γ) which are linked by 29 disulphide bonds forming a trimodular rod (Fig. 1.6) (Procyk *et al.*, 1990). The N-termini of the A α -, B β - and γ -chains form a cysteine-rich knot which makes up the central E domain (Fig. 1.6) (Herrick *et al.*,

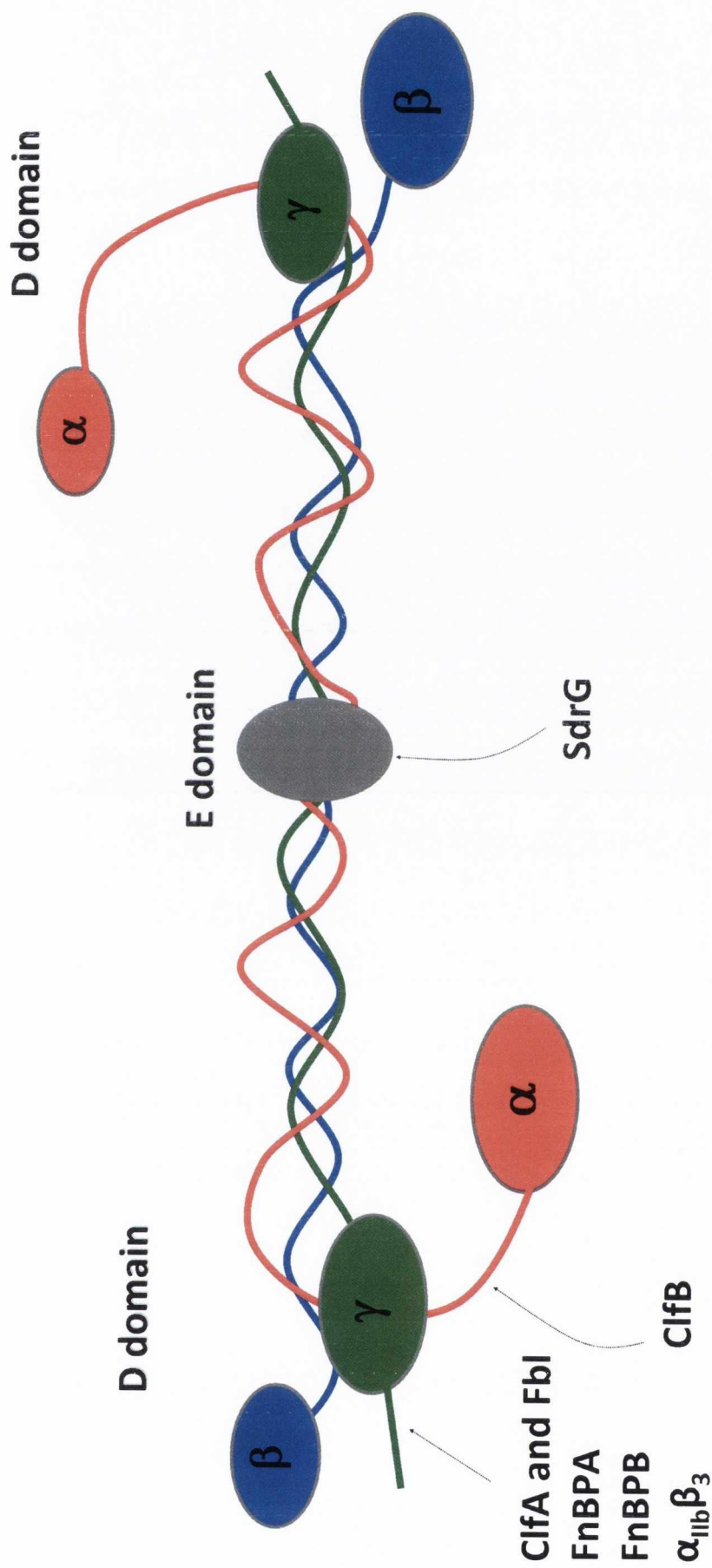


Figure 1.6 Structure of human fibrinogen.

Binding sites for *S. aureus* surface proteins, *S. lugdunensis* Fbl and the *S. epidermidis* SdrG proteins are indicated. ClfA, FnBPA, FnBPB, Fbl as well as the platelet integrin $\alpha_{11b}\beta_3$ recognize the same unfolded peptide at the C-terminus of the Fg γ -chain. SdrG binds to an N-terminus site in the β -chain, blocking the cleavage site of thrombin. ClfB binds to unfolded C-terminal fragment of the α -chain.

1999). The two distal D domains consist of the globular C-termini of the B β - and the γ -chains and the middle section of the A α -chain (Fig. 1.6). Thrombin cleaves fibrinopeptides A and B from the N-termini of the A α and B β chains inducing the assembly of the long, insoluble homopolymeric fibrin. Fibrinogen is a ligand for the platelet integrin, $\alpha_{IIb}\beta_3$. The interaction of Fg with high-affinity form of the integrin results in formation of platelet-fibrin thrombi. Staphylococcal surface proteins can bind to several different regions of Fg (summarized in Fig. 1.6).

1.2.2.1 Clumping factor A.

Clumping factor A (ClfA) is the major fibrinogen binding protein of *S. aureus*. It mediates bacterial adherence to immobilized Fg and clumping of blood plasma fibrinogen (Geoghegan *et al.*, 2010; McDevitt *et al.*, 1997). Expression of ClfA is induced predominantly in the stationary growth phase from the alternative, SigB-dependent promoter (Nicholas *et al.*, 1999). Lower levels of expression can be detected in the exponential growth phase from a housekeeping, SigA-dependent promoter (Higgins, 2005).

ClfA is the archetype of a family of surface-associated proteins with similar structural organization. The primary translation product comprises a secretory signal sequence followed by a 520 residue ligand-binding A domain, then repeats (R) of the serine-aspartate (SD) dipeptide, a short cell-wall-spanning region and a C-terminal domain that comprises a sortase signal LPXTG, a hydrophobic membrane-spanning domain and a series of positive-charged residues (Fig. 1.5) (McDevitt *et al.*, 1994). The SD repeats act as a flexible stalk to extend the ligand-binding A domain from the cell surface (Hartford *et al.*, 1997). The A domain of ClfA is composed of three subdomains (N1, N2 and N3). The N2 and N3 subdomains comprise nine β -strands that form an IgG-fold (Deivanayagam *et al.*, 2002). Fibrinogen binding to ClfA occurs between the N2 and N3 domains by the “dock, lock and latch” mechanism, which is described in section 1.2.2.4 (Fig. 1.7). The protein binds to a flexible unfolded peptide at the C-terminus of the γ -chain of fibrinogen (Fig. 1.6) (McDevitt *et al.*, 1997). This interaction can be inhibited by Ca^{2+} (O'Connell *et al.*, 1998). ClfA is a major mediator of *S. aureus* interaction with platelets which is discussed in section 1.5.2.

1.2.2.2 Clumping factor B.

Clumping factor (ClfB) was the second fibrinogen-binding cell wall protein of *S. aureus* to be identified (Ni Eidhin *et al.*, 1998). Like ClfA, it mediates clumping in a Fg solution and promotes adherence of cells to immobilized Fg. However, unlike ClfA, which is maximally expressed in the stationary growth phase, ClfB is mainly expressed in the exponential phase of growth (McAleese *et al.*, 2001; Ni Eidhin *et al.*, 1998). ClfB on stationary phase cells is degraded by the metalloprotease aureolysin, which recognizes the SLAVA motif (Fig. 1.5). Degradation by aureolysin causes loss of ClfB ligand binding ability (McAleese *et al.*, 2001; Perkins *et al.*, 2001).

ClfB binds to the A α -chain of fibrinogen (Fig. 1.5 and 1.6) and seemingly disparate ligands loricrin and cytokeratin 10 (Clarke *et al.*, 2009) (Chapter 1.1.2.1). Recently it has been revealed that the protein recognizes a Gly-Ser rich sequence (GSSGXGXXG) in Ω loops in the C-terminal region of the cytokeratin 10 (Ganesh *et al.*, 2011; Walsh *et al.*, 2004) which is similar to repeat 5 of the flexible region of Fg α -chain (Ganesh *et al.*, 2011; Xiang *et al.*, 2012).

1.2.2.3 A fibrinogen-binding protein from *S. lugdunensis*.

The fibrinogen binding protein from *S. lugdunensis* (Fbl) is organizationally similar to that described above ClfA and ClfB of *S. aureus* (Fig. 1.5). Its N2N3 subdomains share 60% identity with the ligand-binding N2N3 domains of ClfA. Residues that are known to be required for binding to fibrinogen by ClfA are conserved in Fbl (Deivanayagam *et al.*, 2002; Hartford *et al.*, 1997; Hartford *et al.*, 2001). Differences between ClfA and Fbl include the metalloprotease cleavage motif SLAAVA which is not present in Fbl and repeated region which in Fbl comprises a SDSDSA hexapeptide motif rather than the SD repeats in ClfA (Mitchell *et al.*, 2004).

1.2.2.4 “Dock, lock and latch”.

The crystal structure of ClfA revealed that the N2N3 domains are organised into a novel immunoglobulin-type fold which was named the D-variant or DEv-type IgG fold (Deivanayagam *et al.*, 2002). This structural organization is also valid for N2N3 domains of ClfB, FnBPA and *S. epidermidis* fibrinogen-binding protein SdrG.

SdrG binds to the N-terminus of the fibrinogen β -chain (Fig. 1.6) (Davis *et al.*, 2001). A dynamic mechanism of Fg binding termed “dock, lock and latch” (DLL) was

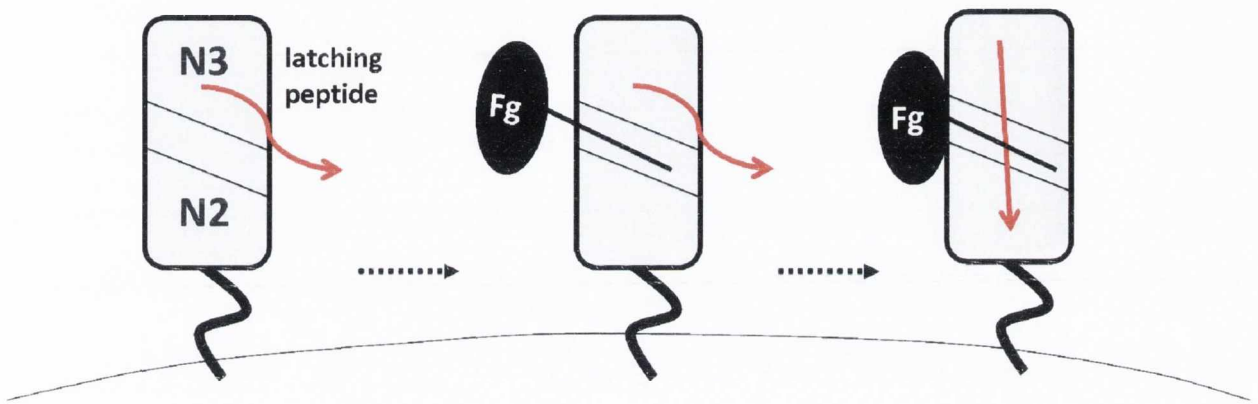


Figure 1.7 Schematic representation of the “dock, lock and latch” mechanism of fibrinogen binding.

The N2 and N3 subdomains of a fibrinogen-binding protein are indicated. The flexible N3 latching peptide is represented by a red arrow. Fibrinogen (Fg) docks in the trench located between N2 and N3, peptide docking redirects the N3 latching peptide which crosses over the binding trench and inserts into the N2 subdomain complementing a β -sheet.

proposed based on a comparison of the crystal structures of SdrG N2N3 as an apo-protein and in complex with a synthetic peptide mimicking the target site in Fg (Ponnuraj *et al.*, 2003). The apo-form of the protein adopts an open conformation that allows the Fg ligand to access the binding trench between the N2 and N3 domains (Fig. 1.7). As the ligand peptide docks into the trench, a flexible C-terminal extension of the N3 domain is redirected to cover the ligand peptide and “lock” it in place. The C-terminal part of this extension interacts with the N2 domain by β -strand complementation with a β -sheet in the N2 domain forming a stable complex (Fig. 1.7) (Ganesh *et al.*, 2008; Ponnuraj *et al.*, 2003).

The “dock, lock and latch” model has been proposed to be a common mechanism for ClfA, ClfB, FnBPA and FnBPB (Deivanayagam *et al.*, 2002; Hartford *et al.*, 2001). The mechanism of Fg “docking” into SdrG requires an open conformation that allows the Fg ligand to access the binding trench. In contrast, in ClfA the γ chain of fibrinogen can thread into a stabilized closed configuration of the N2N3 subdomains therefore is referred to as “latch and dock” model (Ganesh *et al.*, 2008). ClfA and Fbl are highly conserved and have very similar affinities for fibrinogen suggesting they use the same binding mechanism to bind to Fg (Geoghegan *et al.*, 2010).

1.2.3 Fibronectin-binding proteins.

Fibronectin (Fn) is a glycoprotein found at high concentrations in plasma and extracellular fluids. It is secreted primarily by hepatocytes and fibroblasts as a disulfide-bonded dimer consisting of subunits of 250 kDa containing three types of repeating modules: type I, type II, and type III (Fig. 1.8). All three types of modules are composed of two anti-parallel β -sheets. The type I and type II modules are stabilized by intra-chain disulfide bonds, while type III modules do not contain any disulfide bonds, which allows them to partially unfold under applied force. At least 20 different human Fn chains have been identified, all of which arise from alternative splicing of the RNA transcript of single gene (Pankov *et al.*, 2002).

Fn promotes cell adhesion and spreading, regulates cell shape and assembly of cytoskeleton and is essential for migration and differentiation of many types of cells during embryogenesis. The interaction of $\alpha_5\beta_1$ or α_v integrins with the RGD motif of type III repeat 10 is an essential step in the assembly of Fn fibrils (Fig. 1.8).

The majority of *S. aureus* strains express two fibronectin-binding proteins A and B (FnBPA and FnBPB) (Peacock *et al.*, 2000). FnBPA and FnBPB are 68% identical

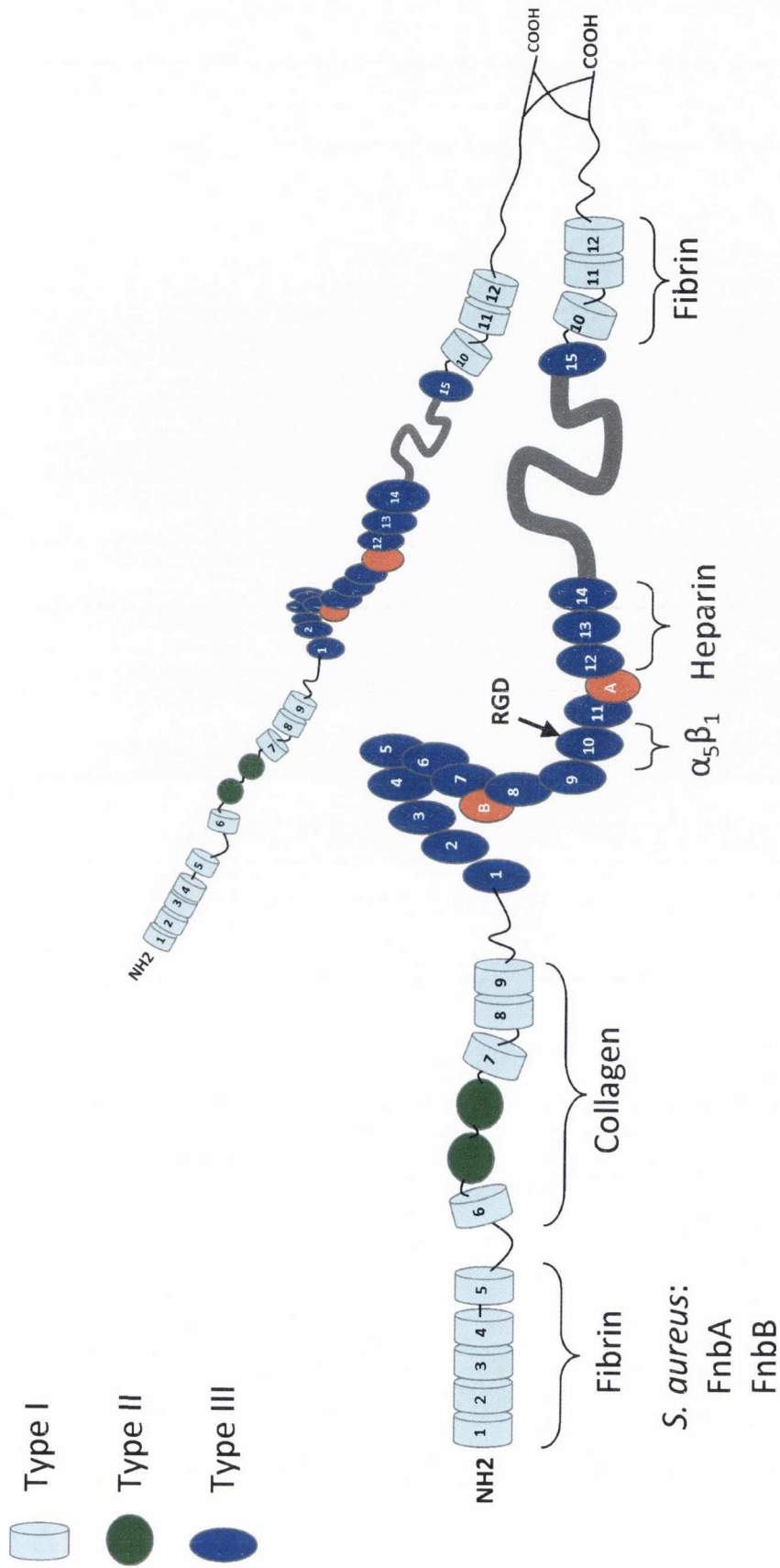


Figure 1.8 Structure of human fibronectin (Fn).

Schematic representation of dimeric fibronectin consisting on three types of structural modules (type I, type II and type III). Binding sites for *S. aureus* FnbA and FnbB, the integrin α₅β₁ and various ligands (fibrin, heparin and collagen) are indicated.

and are encoded by two conserved genes the *fnbA* and the *fnbB* (Jonsson *et al.*, 1991; Signas *et al.*, 1989). Seven different allelic variants of each FnBP have been identified (isotypes I-VII) based on sequence divergence in their N2N3 subdomains (Burke *et al.*, 2010; Loughman *et al.*, 2008). Both FnBPs are expressed in the exponential phase of growth.

FnBPA and FnBPB have structurally similar N-terminal A domains which, like ClfA, mediate binding to α -chain of fibrinogen by “dock, lock and latch” (see section 1.2.2.4) (Fig. 1.5) (Burke *et al.*, 2011; Ganesh *et al.*, 2008; Roche *et al.*, 2004). Unlike ClfA, the A domain of FnBPA and FnBPB bind elastin (Keane *et al.*, 2007). Both FnBPs contain tandemly arrayed fibronectin binding repeats (FnBRs) in the C-terminus. FnBA contains 11 whereas FnBB has 10 repeats (Fig. 1.5) (Penkett *et al.*, 2000). The repeats do not have an ordered structure until ligand binding takes place (House-Pompeo *et al.*, 1996; Schwarz-Linek *et al.*, 2003) at the N-terminal type I modules of Fn (Fig. 1.8) (Sottile *et al.*, 1991). An additional binding site in Fn occurs in the heparin-binding type III module 14 (Bozzini *et al.*, 1992). Six out of the 10 or 11 repeats bind Fn with high affinity whereas remaining ones bind weakly (Meenan *et al.*, 2007; Speziale *et al.*, 2009). The A-domain of FnBPB but not that of FnBPA was also shown to bind to Fn with reduced affinity compared to the main mechanism (Burke *et al.*, 2010).

A tandem β -zipper mechanism of binding to Fn has been proposed for FnBPs. The FnBRs form anti-parallel strands along sequential type I modules of Fn. A new β -strand is added to a small β -sheet in each type I module. The disordered nature of the repeats is believed to facilitate the formation of large intermolecular interfaces allowing one FnBP to bind up to 9 monomers of Fn (Bingham *et al.*, 2008; Matsuka *et al.*, 2003).

The ability of *S. aureus* to adhere to and to invade endothelial and epithelial cells is mediated by FnBPs (Dziewanowska *et al.*, 1999; Edwards *et al.*, 2010). The FnBPs form a fibronectin - bridge between *S. aureus* and the $\alpha_5\beta_1$ integrin on host cells (Fowler *et al.*, 2000; Sinha *et al.*, 1999). Invasion into the cells may provide a way of evading the host immune response and persistence in the host. This is further discussed in Chapter 1.5.

FnBPs can promote activation and aggregation of platelets which is an important factor in the pathogenesis of infective endocarditis (see section 1.4.2). Bacterial cells expressing FnBPA colonized sterile thrombi on heart valves and invaded

the surrounding endothelium thus contributing to pathogenesis in an experimental endocarditis infection model (Que *et al.*, 2001; Que *et al.*, 2005). The FnBPs are believed to be important in establishing infections by promoting adhesion of *S. aureus* cells to implanted devices coated in fibronectin (Arrecubieta *et al.*, 2006; Vaudaux *et al.*, 1993).

FnBPs are also involved in the intracellular accumulation phase of biofilm formation by some MRSA strains. Methicillin-sensitive *S. aureus* strains mediate biofilm formation by a different mechanism (O'Neill *et al.*, 2008).

1.2.4 Iron regulated surface determinants.

Iron regulated surface determinant (Isd) proteins are primarily involved in binding haemoproteins and haem and the following transfer of haem across the cell envelope to provide a source of iron. Isd expression, localization and the haem-iron uptake pathway are discussed in Chapter 1.4. The IsdA, IsdB and IsdH are up-regulated under iron-deprived conditions and abundantly displayed on the surface of iron-starved *S. aureus*. All three proteins contain an LPXTG sequence and are anchored to the peptidoglycan by sortase A. Also all the proteins are multifunctional promoting bacterial adhesion and resistance to host defences which is discussed in this section.

S. lugdunensis, like *S. aureus* encodes a conserved *isd* locus (Heilbronner *et al.*, 2011). This locus is duplicated in the genome of strain HKU09-01. *S. lugdunensis* is the only coagulase negative *Staphylococcus* known to encode an Isd system.

1.2.4.1 NEAr Transporter domains.

The cell wall-anchored IsdA, IsdB, IsdC and IsdH proteins contain one to three copies of NEAr Transporter (NEAT) domains. NEAT domains are responsible for binding to haemoproteins and haem. They are also multifunctional and recognize other host ligands. NEAT domains consist of ~ 125 amino acids and even though their primary sequences are often unrelated their structures are conserved, forming superimposed IgG-like beta sandwich folds of 7 or more β -strands in 2 β -sheets (Andrade *et al.*, 2002; Grigg *et al.*, 2011; Grigg *et al.*, 2010b). Based on amino acid sequences, NEAT domains can be divided into either haemoprotein or haem binding receptors. The haem binding domains of IsdA, IsdC, IsdB NEAT2 and IsdH NEAT2 use a conserved mechanism to bind the porphyrin ring of haem. They coordinate it in a hydrophobic pocket *via* a conserved Tyr (Grigg *et al.*, 2011). Haemoglobin binding by

IsdB and IsdH requires aromatic residues in the loop (β 1- β 2) for recognition of the α -chain of Hb (Krishna Kumar *et al.*, 2011; Pilpa *et al.*, 2006).

1.2.4.2 Iron regulated surface determinant A.

Iron regulated surface determinant A (IsdA) is a multifunctional protein which can bind to a diverse array of host ligands promoting *S. aureus* colonisation and invasion of the host. It mediates *S. aureus* adhesion to desquamated epithelial cells of human nares (Corrigan *et al.*, 2009), possibly by binding to loricrin, cytokeratin 10 and involucrin (see Chapter 1.1.2.1) (Clarke *et al.*, 2009; Corrigan *et al.*, 2009b). Expression of IsdA confers resistance to killing by lactoferrin which is an abundant peptide in human nasal secretions with an anti-bacterial activity (Clarke *et al.*, 2008). Additionally, the C-terminal hydrophilic domain of IsdA is responsible for decreased cellular hydrophobicity rendering *S. aureus* resistant to bactericidal human skin fatty acids and peptides and thus promotes bacterial survival on human skin (Clarke *et al.*, 2007).

IsdA binds haem transfer and therefore can promote *S. aureus* survival in the bloodstream. It contains a single NEAT motif which mediates haem binding *via* a hydrophobic pocket with propionate groups at the molecular surface. The conserved Tyr166 (in β 6) coordinates haem-iron and forms a H-bond with Tyr170 which stabilizes the Tyr166-haem-iron interaction (Fig. 1.9). Residues located in adjacent loops, including Lys75, Ser82 and His83, additionally coordinate haem by forming H-bonds with haem propionates (Fig. 1.9) (Grigg *et al.*, 2011; Grigg *et al.*, 2007; Pilpa *et al.*, 2006). The NEAT domain of IsdA also binds to several human plasma glycoproteins including fetuin, fibronectin and the B β - and γ -chains of fibrinogen which may further support *S. aureus* invasion of the host (Clarke *et al.*, 2004). IsdA was also shown to act in concert with IsdB in promoting resistance to neutrophil killing. Microbicides of human neutrophils, like hydrogen peroxide (H₂O₂) triggered up-regulation of the *isdA* and *isdB* in *S. aureus* MW2. Mutation of *isdA* and *isdB* resulted in increased susceptibility to H₂O₂ and human neutrophils compared with the wild-type parental strain (Palazzolo-Ballance *et al.*, 2008).

Inactivation of *isdA* lead to decrease in bacterial load in murine kidneys five days post infection in the murine model of abscess formation implying an important role of IsdA in *S. aureus* pathogenesis (Cheng *et al.*, 2009; Clarke *et al.*, 2007).

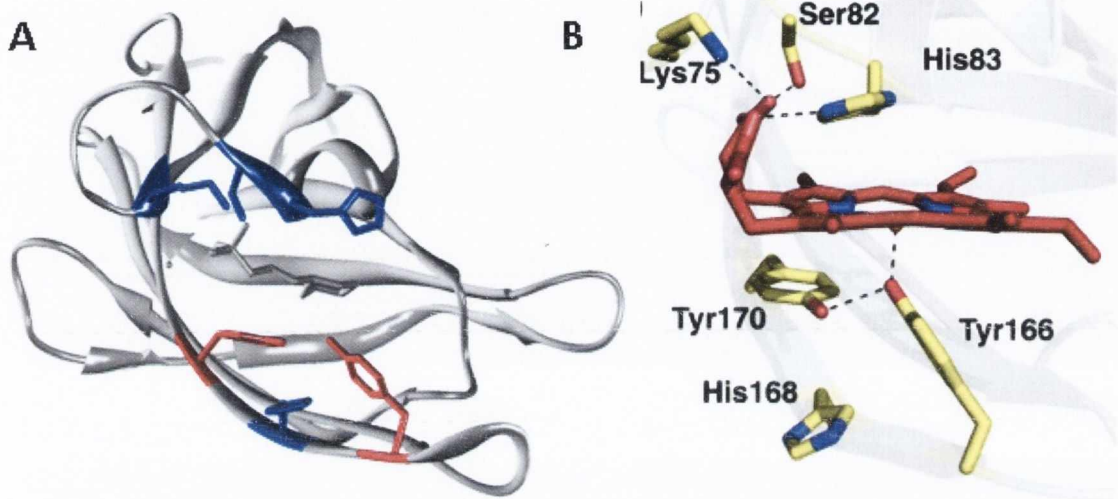


Figure 1.9 Structure of IsdA NEAT domain and haem-coordinating pocket.

(A) Overall structure of the IsdA NEAT domain with highlighted residues involved in haem coordination (red) Tyr166, Tyr170 and (blue) Lys 75, Ser82, His83, His168. Structure was modeled using chimera software (PDB:2ITF) (Grigg *et al.*, 2007)

(B) Close-up wild-type binding pocket. IsdA residues involved in haem coordination (yellow) are indicated. The porphyrin ring of haem is indicated in red. Taken from Grigg *et al.*, 2011.

1.2.4.3 Iron regulated surface determinant B.

Iron regulated surface determinant B (IsdB) contains two NEAT domains which bind to haemoglobin (NEAT1) and haem (NEAT2). The *isdB* mutant is impaired in use of haemoglobin as an iron source. This is not the case with an *isdH* mutant indicating that *in vivo* IsdB is the primary haemoglobin receptor of the Isd system (Torres *et al.*, 2006). IsdB binds human haemoglobin (hHb) with a ca. 20-fold higher affinity than the mouse haemoglobin (mHb). This is consistent with the preference of *S. aureus* for hHb over mHb as an iron source a characteristic of bacteria that are associated with humans, like *S. aureus*, *S. simulans* and *Corynebacterium diphtheriae*. In contrast, environmental bacteria that infect numerous hosts like *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Bacillus anthracis* and *B. cereus* use hHb and mHb with equal efficiencies (Pishchany *et al.*, 2010; Pishchany *et al.*, 2012; Torres *et al.*, 2006).

IsdB coordinates haem by Tyr440 which forms a hydrogen bond with the phenolate of Tyr444, an interaction that is conserved among haem binding NEAT domains (Grigg *et al.*, 2011). Haem that is coordinated by IsdB exists as a mixture of penta- and hexa-coordinated iron, with the sulphur from Met362 occupying the sixth-coordinate position. This is different to haem binding by IsdA. Additionally, in IsdB the haem propionate groups form hydrogen bonds with the hydroxyl group of Ser361 whereas Val431, Val433, Val 435, Val446, Tyr391 and Trp392 contribute to hydrophobic contacts (Gaudin *et al.*, 2011).

Torres *et al.* used a mouse model of abscess formation to evaluate the contribution of IsdB - mediated haemoglobin binding to pathogenesis of *S. aureus* infections. They observed a great reduction in the load of *isdB* mutant in both the spleen and kidneys of infected animals compared to that in the wild-type Newman strain, indicating an importance of IsdB in *S. aureus* virulence. Mutation of *isdH* did not affect the ability of the bacterium to colonize these organs suggesting that IsdH, unlike IsdB, is not required for abscess formation (Torres *et al.*, 2006).

IsdB and IsdA are differentially expressed within organs of mice infected with *S. aureus*. IsdB is expressed at low levels in livers and is strongly up-regulated in hearts of infected animals (Pishchany *et al.*, 2009). Mutation of *isdB* resulted in strongly reduced ability to colonize murine hearts. Consistent with the low IsdB expression in murine livers, strains lacking IsdB colonized this organ as efficiently as the wild-type (Pishchany *et al.*, 2009).

This is also consistent with studies of IsdB- and IsdA-mediated contribution to sepsis and abscesses formation in murine models of *S. aureus* infection (Cheng *et al.*, 2009; Kim *et al.*, 2010; Torres *et al.*, 2006) but was contradicted by Hurd *et al.* who observed redundancy of the IsdA, IsdB and IsdH proteins in *S. aureus* pathogenesis (Hurd *et al.*, 2012).

Due to the high affinity of IsdB to human Hb the susceptibility of transgenic $\alpha^H\beta^A$ mice that express normal adult human haemoglobin to systemic staphylococcal infection was examined. The $\alpha^H\beta^A$ mice were more efficiently colonized as compared to wild-type animals suggesting that murine models of infection which were used previously could have underestimated the potential of contribution of the IsdB to *S. aureus* virulence (Pishchany *et al.*, 2010).

1.2.4.4 Iron regulated surface determinant H.

Iron regulated surface determinant H (IsdH) consists of three NEAT domains. The N-terminal NEAT1 and NEAT2 are 53% identical and recognize haemoglobin (Hb), haptoglobin and haemoglobin-haptoglobin complexes. The C-terminal NEAT3 domain is only 16% identical to NEAT1 and NEAT2 and is responsible for capturing haem from Hb (Pilpa *et al.*, 2006; Pilpa *et al.*, 2009).

The first IsdH NEAT1 domain structure revealed an IgG fold adopting a β -sandwich fold consisting of two five-stranded anti-parallel β -sheets (Pilpa *et al.*, 2006). A co-crystal structure of IsdH NEAT1 and IsdH NEAT2 with human Hb demonstrated that the domains interact with α -chains (α Hb) (Fig. 1.10) (Krishna Kumar *et al.*, 2011). Hb is coordinated by an N-terminally located loop 2 which contains a string of aromatic residues unique in the Hb-binding type NEAT domains. The loop 2 of IsdH NEAT1 contains Tyr125, Tyr126, His127, Phe128 and Phe129 which are crucial for the Hb binding (Fig. 1.10). Their aromatic side chains make van der Waals contacts with non-polar surfaces on α Hb and are buried from the solvent. Additionally, loops 4 and 6 account for the majority of electrostatic interactions with α Hb. The Hb interface includes the α -chain residues of Lys7, Lys 11, and Gly18 (Fig. 1.10). Loops 2 and 4 account for the majority of IsdH NEAT1 interactions with Hb and are largely conserved in IsdH NEAT2 and IsdB NEAT1, suggesting that like IsdH NEAT1 they bind α Hb (Fig. 1.10).

IsdH was shown to induce accelerated degradation of serum opsonin C3b which led to a reduction in *S. aureus* uptake by human neutrophils. IsdH expression by *S.*

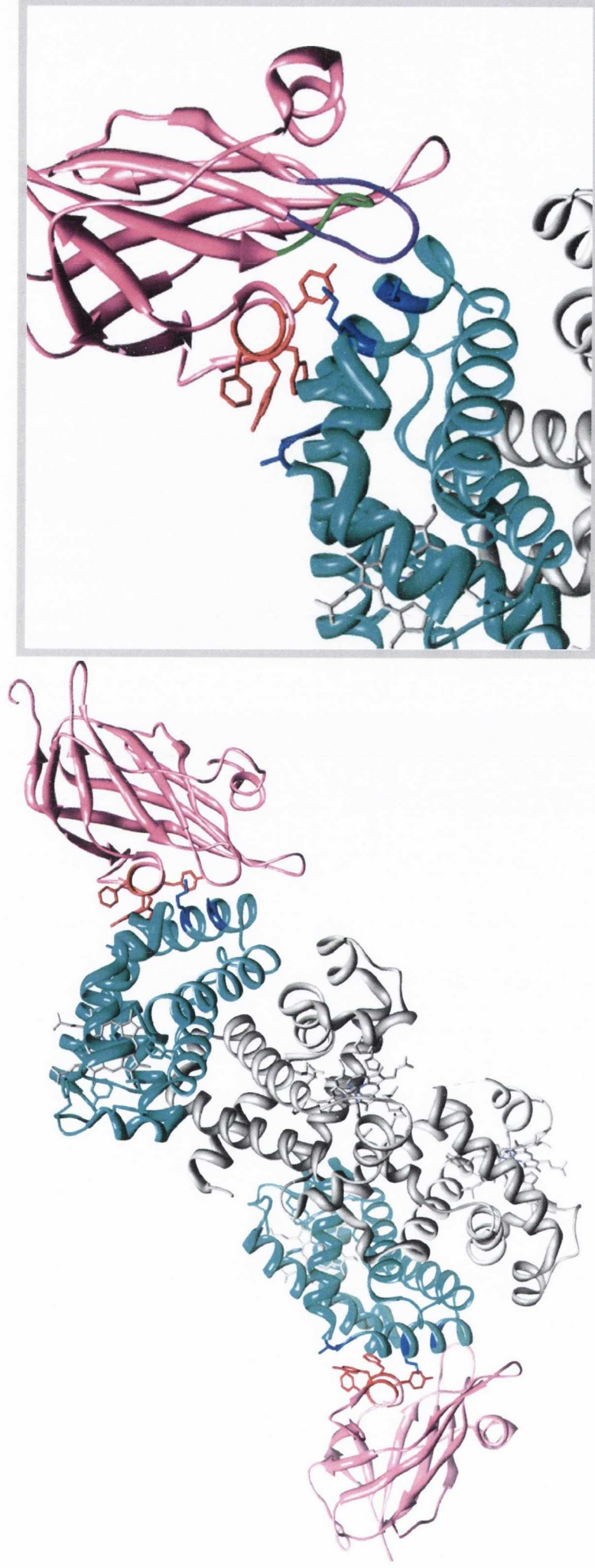


Figure 1. 10 The crystal structure of IsdH NEAT1 $\times$ haemoglobin complex.

Visualisation of the co-structure of IsdH NEAT1 domain binding to haemoglobin (Hb) (PDB ID 3S48) (Krishna Kumar *et al.*, 2011) was generated using Chimera software. Tetrameric Hb consist of 2 α -chains (light blue) and 2 β -chains (grey). NEAT1 domains (pink) interact with α -chains of Hb. Interaction interface is indicated, including the aromatic side chains of Tyr125, Tyr126, His127, Phe128 and Phe129 (red) in loop 2 of IsdH NEAT1 and Lys7, Lys11 and Gly18 (blue) on α -chains of Hb. Right: NEAT1 loops 4 and 6 are indicated in green and purple, respectively.

aureus promoted the survival of bacteria in human blood (Smith *et al.*, 2011; Visai *et al.*, 2009) and virulence in a mouse model of sepsis (Visai *et al.*, 2009).

1.2.5 *S. aureus* vaccine candidates.

The emergence of multidrug resistance among *S. aureus* strains, including resistance to vancomycin, the antibiotic of last resort, has been encouraging development of active and passive immunization. Vaccination with surface components or toxins of *S. aureus* has shown protective results in animal models, especially murine models of infection, which so far have not translated into increased protection in clinical trials in humans (Proctor, 2012; Schaffer *et al.*, 2008).

The first anti-staphylococcal vaccine tested in humans (StaphVax® by Nabi Biopharmaceuticals) contained capsule (CP) types 5 and 8 conjugated with recombinant *Pseudomonas aeruginosa* exotoxin A. Although active and passive immunization with CP5 were shown to be protective in animal models of staphylococcal infection (Fattom *et al.*, 1996; Lee *et al.*, 1997), there was no consistent protection associated with CP8 immunization (Cook *et al.*, 2009; Tuchscherer *et al.*, 2008). The vaccine passed an initial phase III trial in haemodialysis patients, but was found to be ineffective in a confirmatory trial (Fattom *et al.*, 2004; Shinefield *et al.*, 2005).

As a virulence factor conserved and expressed by different *S. aureus* strains IsdB shows favourable characteristics as a vaccine candidate. Mice and *Rhesus macaques* actively immunized with purified IsdB generated high titers of IgG. Vaccination with only IsdB or the protein in combination with IsdA, SdrE and SdrD was shown protective in mice (Kuklin *et al.*, 2006; Stranger-Jones *et al.*, 2006) inducing both humoral and cellular mediated responses (Stranger-Jones *et al.*, 2006). Recent studies demonstrated that protection mediated by the IsdB vaccine in a disseminated lethal challenge model is mediated by T helper 17 cells (Th17) response where interleukin 17A (IL-17A) plays an important role (Joshi *et al.*, 2012). IL-17, produced by Th17, is a cytokine that acts as a potent mediator in delayed-type reactions by increasing chemokine production in various tissues and recruiting monocytes and neutrophils to the site of inflammation. Antibodies induced by the IsdB-based vaccine (V710 by Merck, Intercell) had opsonic activity. However, the phase III trial was terminated due to lack of efficacy (Daum *et al.*, 2012).

Inhibitex has developed an antibody based immunotherapy approach with Veronate®, a hyper-immunoglobulin against ClfA and *S. epidermidis* SdrG (Vernachio

et al., 2003). It aimed the potential prevention of *S. epidermidis*, *S. aureus* and *Candida* infections in very low-birth-weight infants (Kaufman, 2006). Immunotherapy with Veronate® resulted in elevated levels of antibodies to the staphylococcal surface adhesins ClfA and SdrG and made it into phase III testing, where it failed to show a clinical benefit in neonates (Kaufman, 2006).

Aurexis®, known also as tefibazumab (Inhibitex), is a humanised murine antibody against the A domain of ClfA, which protected mice in a lethal sepsis model (Hall *et al.*, 2003). Immunotherapy with Aurexis® in combination with vancomycin resulted in reduced numbers of bacteraemia in rabbit endocarditis model (Patti, 2004; Proctor, 2012). A fewer bacteria were found in the spleens and kidneys of immunized animal than in animals given vancomycin alone (Patti, 2004; Proctor, 2012). The vaccine failed phase II trial on hospitalised patients with documented *S. aureus* bacteraemia (Weems *et al.*, 2006).

1.3 Iron uptake in staphylococci.

1.3.1 Iron sequestration in vertebrates.

Iron is one of the most abundant metals in nature. It rarely occurs free within living organisms. This is due to the insolubility of the ferric form (Fe^{3+}) at neutral pH as well as risks of oxidative damage caused by the ferrous iron (Fe^{2+}) forming reactive oxygen and hydroxyl radicals *via* the Fenton reaction. On the other hand, due to its unique redox potential iron is used as a cofactor in diverse biochemical reactions and therefore is critical in many biological processes vital for mammals and bacteria (Gutteridge, 1994; Tuomainen *et al.*, 2007; Wilks *et al.*, 2007).

In humans and other higher vertebrates iron is assimilated from dietary components *via* duodenal enterocytes by the iron membrane transporter ferroportin. In the bloodstream iron is bound by ubiquitous transferrins which transport and donate iron to various types of cells for subsequent assimilation into proteins such as the storage protein ferritin (Wang *et al.*, 2011). Iron levels in lymph and mucosal secretions are limited through binding to lactoferrins. Additionally, lactoferrin occurs within phagocytes, ensuring that engulfed pathogens have limited access to intracellular iron. In healthy individuals both lactoferrin and transferrin are 40% saturated and ready to bind any free iron (Bullen *et al.*, 2005; Haley *et al.*, 2012).

Over 80% of mammalian iron is incorporated into a porphyrin ring as ferriprotoporphyrin IX (or haem) in a tightly regulated biosynthetic process (Ajioka *et*

al., 2006). The intracellular haem concentration within vertebrates is controlled by balancing the rates of haem biosynthesis and catabolism (Ponka, 1999). The majority of haem is bound by haemoglobin, a complex oxygen transport protein found in erythrocytes. The less abundant haemoprotein myoglobin is responsible for oxygen delivery in myocytes that are abundant in cardiac muscle. Therefore more than 90% of iron is sequestered intracellularly depriving infecting pathogens unless it can be liberated from the host cells. The serum concentration of iron is extremely low (ca. 10^{-24} M) (Raymond *et al.*, 2003). Upon erythrocyte haemolysis an acute phase glycoprotein haptoglobin binds released haemoglobin forming haemoglobin-haptoglobin complexes which are then removed by the reticuloendothelial system. This prevents iron loss through urinary excretion and subsequent kidney damage. Haptoglobin is plentiful in serum and becomes more abundant during infection. Furthermore vertebrates rapidly remove free haem from plasma *via* haemopexin, a glycoprotein produced in hepatocytes. Haemopexin-bound haem is delivered to the liver and taken up by receptor mediated endocytosis (Haley *et al.*, 2012).

This limited availability of free iron and haem creates a defence mechanism called nutritional immunity which protects the host against infecting pathogens. In support of this idea the virulence of many pathogens has been shown to be considerably enhanced in experimental infections by injecting iron compounds into the animal host (Cassat *et al.*, 2012; Payne, 1993; Weinberg, 1993). Moreover, inflammation upon bacterial entry further decreases free iron by reducing intestinal iron absorption and sequestering iron in macrophages. The master regulator of these processes is hepcidin, the iron-regulatory hormone secreted by hepatocytes. Inflammation is one of stimulatory signals that affect hepcidin concentrations in the serum. Specifically, induction of Il-6 production and LPS-mediated TLR4 signaling both cause increases in circulating hepcidin. Hepcidin causes internalization and degradation of ferroportin, thereby decreasing iron transfer into blood plasma from the duodenum, from macrophages involved in recycling senescent erythrocytes and from iron-storing hepatocytes (Ganz *et al.*, 2011). Furthermore, during the acute phase responses to bacterial infection the host Toll-like receptors stimulate lipocalin-2 expression in neutrophils. This factor is also referred to as siderocalin or neutrophil gelatinase-associated lipocalin (NGAL). Lipocalin-2 binds enterobactin, the primary siderophore of many enteric bacteria, and sequesters the siderophore - iron complex, preventing bacterial uptake (Flo *et al.*, 2004; Skaar, 2010).

Disruption of the host's nutritional immunity affects the outcome of bacterial infections. Genetic disorders caused by mutations in host genes encoding factors involved in maintaining iron haemostasis like Hereditary Haemochromatosis and β Thalassaemia are associated with increased risks of bacterial infections (Gardenghi *et al.*, 2010; Khan *et al.*, 2007).

1.3.2 The response of *S. aureus* to iron starvation.

Iron limitation is one of environmental signals sensed by bacteria during infection. Both gram-positive and gram-negative bacteria are known to sense iron-limited environments *via* the conserved ferric uptake regulator (Fur). Under iron-rich conditions Fur binds a consensus sequence in so-called Fur boxes located upstream of the Fur regulated genes. During iron starvation Fur repression is alleviated and transcription of a number of genes like those involved in iron acquisition, glycolysis and virulence are up-regulated (Friedman *et al.*, 2006). Fur-dependent expression embraces the global regulators of *S. aureus*, including Sae, Agr or Rot, implying a wide scope of adjustments in gene expression due to iron restriction (Johnson *et al.*, 2011).

In vivo imaging of *S. aureus*-infected mice has demonstrated that Fur-regulated genes are expressed in heart and kidney abscesses, suggesting that staphylococci are starved for iron in these organs (Pishchany *et al.*, 2009; Reniere *et al.*, 2008). Fur-mediated changes in gene expression were shown to be necessary for protection against killing by neutrophils, and for full virulence in a murine model of infection. The observed effect was explained by decreased expression of immunomodulatory proteins and increased expression of cytolytins (Hammer *et al.*, 2011; Nizet, 2007; Pishchany *et al.*, 2009). In order to adapt to iron-depleted environments *S. aureus* modulates its metabolism by reducing expression of TCA enzymes and simultaneously elevating expression of glycolytic and fermentative enzymes. These participate in the production of lactate which lowers the pH of the microenvironment resulting in weaker affinity of host transferrins for iron and higher iron availability to the infecting bacterium (Friedman *et al.*, 2006; Haley *et al.*, 2012). Moreover, several TCA enzymes contain iron sulphur clusters and down-regulating the TCA cycle may decrease the dependence of *S. aureus* on enzymes that utilize iron (Friedman *et al.*, 2006).

1.3.3 Haem acquisition by *S. aureus*.

To overcome the limited bio-availability of iron and haem within the host, pathogens have developed sophisticated mechanisms to access and utilize haem as their preferred source of iron (Skaar *et al.*, 2004). Bacterial pathogens can circumvent nearly every vertebrate form of haem sequestration including haemopexin, haem-albumin, haemoglobin, and haemoglobin-haptoglobin complexes (Anzaldi *et al.*, 2010). Gram-positive bacteria utilize haem uptake systems consisting of surface-exposed receptors for haemoproteins and haem that scavenge and then shuttle haem through the cell wall to an ABC transporter for delivery into the cytoplasm.

The paradigm for Gram-positive bacterial haem uptake is the *S. aureus* iron-regulated surface determinant (Isd) system. This includes cell wall anchored haem binding proteins (IsdA and IsdC), a haemoglobin receptor (IsdB), a haemoglobin and haptoglobin receptor (IsdH), a membrane transporter (IsdEF), and two cytoplasmic heme oxygenases (IsdG and IsdI) (Fig.1.11) (Grigg *et al.*, 2010b; Hammer *et al.*, 2011).

The membrane transporter, composed of the substrate binding proteins (IsdE) and permease (IsdF) have been predicted to act in concert with IsdD. However the function of IsdD has yet to be elucidated. The Isd system also includes sortase B, an enzyme dedicated to anchoring IsdC to the cell wall (Mazmanian *et al.*, 2002). Conversely, IsdA, IsdB and IsdH are anchored to the cell wall by sortase A. These Isd proteins cooperate to enable *S. aureus* to utilize vertebrate haemoglobin as source of nutrient iron. The ligand specificities and roles in bacterial virulence of IsdA, IsdB and IsdH were discussed in section 1.2.4.

According to the current *S. aureus* haem acquisition model the bacterium secretes cytolytic toxins (eg. α -, β -, γ - and δ -haemolysins), which lyse host erythrocytes releasing haemoglobin. Free haemoglobin is toxic and is rapidly cleared from plasma by the haemoglobin carrier molecule, haptoglobin. Free haem is highly reactive and is promptly cleared from the circulation by haemopexin. The *S. aureus* IsdH receptor, which binds haemoglobin and haemoglobin-haptoglobin complexes strips haem from the haemoproteins and passes it bidirectionally to the IsdB NEAT2 domain and/or unidirectionally to the NEAT domain of IsdA. The haem is then transferred to IsdC followed by IsdE. Similarly, the second haemoglobin receptor of the Isd system - IsdB scavenges haem from the haemoprotein and passes it unidirectionally to IsdA or directly to IsdC. The NEAT domain of IsdC can transfer haem to IsdE. Haem is not transferred between IsdA and IsdE. Transfer of haem through Isd proteins was

confirmed *in vitro* using magnetic circular dichroism and electrospray ionization mass spectrometry. The ABC transport system of IsdE, IsdF and possibly FhuC imports haem through the membrane into the cytoplasm (Fig. 1.11) (Grigg *et al.*, 2010b; Haley *et al.*, 2012; Liu *et al.*, 2008; Muryoi *et al.*, 2008; Zhu *et al.*, 2008).

Haem degradation catalyzed in the cytoplasm by the IsdG and IsdI haem monooxygenases gives a mixture of β - and σ -isomers of oxo-bilirubin, molecules collectively referred to as staphylobilins (Fig. 1.11). The electron donor required to catalyze IsdG-mediated haem degradation *in vivo* is unknown. Mutants of *isdG* and *isdI* are attenuated in a murine model of systemic infection, implying that haem degradation and staphylobilin formation are important for pathogenesis (Reniere *et al.*, 2008).

1.3.4 *S. aureus* siderophores.

Siderophores are small molecules with a high-affinity for iron. Their expression is Fur-regulated and is consequently elevated during infection to target transferrins and lactoferrins (Friedman *et al.*, 2006; Lindsay *et al.*, 1994). Bacterial siderophores are biosynthesised by one of two pathways, the nonribosomal peptide synthetase pathway or the nonribosomal peptide synthetase independent pathway (NIS). Staphyloferrins A and B are synthesized *via* the NIS pathway and belong to the carboxylate family of siderophores (Fig. 1.11) (Haley *et al.*, 2012). A third *S. aureus* siderophore, aureochelin, has been suggested but has not been characterized (Courcol *et al.*, 1997).

Staphyloferrin A (479 Da) is encoded by the *sfaABC* locus (Fig. 1.11C). It is composed of a molecule of D-ornithine that links together two molecules of citrate *via* amide bonds (Cotton *et al.*, 2009; Konetschny-Rapp *et al.*, 1990). Staphyloferrin B (448 Da) is an α -hydroxycarboxylate siderophore composed of L-2,3-diaminopropionic acid, 1,2-diaminoethane, and α -ketoglutaric acid. The synthesis of staphyloferrin B is encoded by the *sbnABCDEFGHI* operon (Fig. 1.11) (Cheung *et al.*, 2009). After binding to extracellular iron, siderophore-iron complexes are transported back into the cytoplasm through dedicated ABC transporters Hts for (for staphyloferrin A) and Sir for (staphyloferrin B) (Grigg *et al.*, 2010a; Haley *et al.*, 2012; Hammer *et al.*, 2011).

Additionally, the FhuBCDG iron transporter allows *S. aureus* to utilize hydroxamate type siderophores (or xenosiderophores) as a source of iron. The bacterium does not produce this type of siderophore but utilizes the Fhu system to acquire xenosiderophores produced by other bacterial species including *Pseudomonas spp.*, *Neisseria spp.* and *Serratia spp.* Moreover, this may have clinical consequences

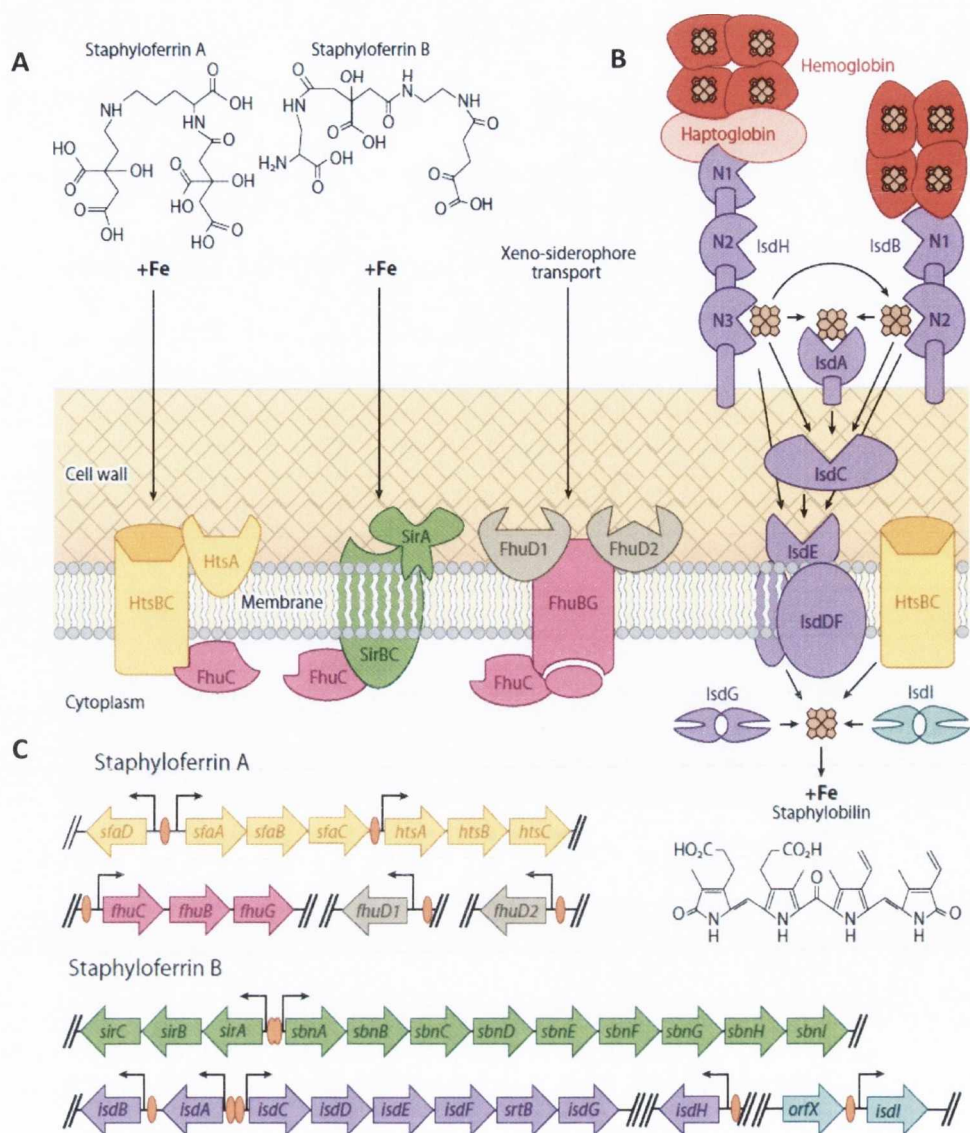


Figure 1.11 *Staphylococcus aureus* iron acquisition pathways.

(A) *S. aureus* produces two siderophores, staphyloferrin A and staphyloferrin B. Staphyloferrin A import is mediated by the HtsA lipoprotein and HtsBC permease. The SirA lipoprotein is the receptor for staphyloferrin B, and the SirBC permease mediates the translocation of staphyloferrin B across the membrane. *S. aureus* imports xenosiderophores produced by other bacteria through the binding activity of FhuD1 and FhuD2 receptor lipoproteins and the FhuBG permease. The energy needed for siderophore uptake is provided by the FhuC ATPase. (B) Isd system for haem acquisition. IsdH binds the haemoglobin-haptoglobin complex and IsdB binds haemoglobin. Haem is passed through the NEAT domains of IsdH (N3), IsdB (N2), IsdA, and IsdC. Across the membrane haem is transported by either the IsdDF or the HtsBC permeases. Once in the cytoplasm haem is a substrate for the haem monooxygenases IsdG and IsdI leading to release of iron and the production of staphylobilin. (C) Genetic loci involved in *S. aureus* iron acquisition pathways. The *sfa* operon encodes the genes required for staphyloferrin A biosynthesis, and the *sbn* operon encodes the genes required for staphyloferrin B synthesis. Promoter regions containing a consensus *fur* box are indicated by an orange oval. Taken from Hammer *et al.*, 2011.

for patients who have been administered siderophore-based therapy to treat iron-overload diseases, like Thalassemia (Brock *et al.*, 1983; Hammer *et al.*, 2011; Rahav *et al.*, 2006).

In vitro studies have demonstrated that siderophore-mediated iron acquisition is the dominant mechanism by which *S. aureus* acquires iron from transferrin. The IsdA protein which can bind transferrin was shown not to be involved in iron scavenging from transferrin (Park *et al.*, 2005).

1.3.5 Haem synthesis and toxicity in *S. aureus*.

Staphylococcus belongs to a group of bacteria which both import haem from the extracellular environment and synthesize haem *de novo*. The haem precursor δ -aminolevulinic acid is synthesized from glutamyl-tRNA^{Glu} and is then converted into protohaem by enzymes encoded by the *hemBCDEFGHNY* locus. Protohaem can be used directly or modified further before being employed as a prosthetic group in haemoproteins (Ajioka *et al.*, 2006).

Disruption of the haem synthesis pathway in *S. aureus* by inactivation of *hemB* results in an aberrant growth phenotype called the small colony variant (SCV). SCVs have a slow growth rate, lack pigmentation, their range of carbohydrate usage is reduced and they lack expression of virulence factors (Haley *et al.*, 2012; McNamara *et al.*, 2000; von Eiff, 2008). SCVs are clinically relevant as they are associated with an increase in persistence and have reduced susceptibility to antibiotics (Begic *et al.*, 2009). The SCV phenotype of a *hemB* mutant is due to the uncoupling of electron transport and a resulting decrease in ATP production caused by a defect in cytochrome maturation. This SCV phenotype can be reversed upon acquisition of exogenous haem (Haley *et al.*, 2012; McNamara *et al.*, 2000; von Eiff *et al.*, 2006).

Bacteria control the level of intracellular haem by regulating its biosynthesis and uptake. When these mechanisms fail other mechanisms including export, sequestration and/or degradation are utilized. When *S. aureus* is exposed to sublethal concentrations of haem, it upregulates an ABC transport system of the haem-regulated transporter (HrtAB). HrtAB which is an efflux pump that expels haem or a toxic metabolite that accumulates as a result of the presence of excess of haem. The up-regulation of HrtA in response to haem is dependent on a two-component signal transduction system known as the haem sensor system (HssRS). Haem exposure activates the histidine kinase HssS, resulting in autophosphorylation. HssS then transphosphorylates HssR, the response

regulator component of the system. Phosphorylated HssR binds to a direct repeat sequence within the *hrtAB* promoter in order to induce transcription of *hrtAB* (Haley *et al.*, 2012; Hammer *et al.*, 2011; Stauff *et al.*, 2007).

1.4 Bacteria, platelets and cardiovascular disease.

Vascular damage allows opportunistic bacterial pathogens entry into the circulatory system causing bacteraemia. This can lead to development of serious cardiovascular complications such as infective endocarditis (IE), disseminated intravascular coagulation (DIC), thrombocytopenia, atherosclerosis and myocardial infarction. The interactions of bacterial pathogens and platelets circulating along the vessel wall are postulated to play an important role in the development of IE, which is characterized by the development of a large septic thrombus on cardiac valves (Beynon *et al.*, 2006; Moreillon *et al.*, 2004). Many pathogens have been shown to cause IE (Baddour *et al.*, 2005; Kerrigan *et al.*, 2010) but the vast majority of cases with an identified pathogen were due to staphylococci (primarily *S. aureus*) or streptococci (primarily *S. sanguinis* and *S. oralis*). Increased use of intravascular implants and invasive procedures in hospital settings has led to growing numbers of bacteraemic infections caused mainly by staphylococci, forming a new population at risk of IE (Rasmussen *et al.*, 2011).

There are three types of interaction of bacteria with platelets: (I) the direct binding of bacterial surface components to platelet receptor(s), (II) indirect binding of those components *via* a bridging molecule (like the plasma fibrinogen) and (III) interactions of molecules secreted by bacteria with platelets. These interactions can mediate bacterial adherence to platelets and/or induce activation of platelets. Thus, bacteria can promote platelet adhesion and/or trigger platelet activation. Platelet activation and aggregation are necessary steps in thrombus formation. Therefore bacteria-platelet interactions causing platelet activation have a primary role in development of disease during sepsis (Fitzgerald *et al.*, 2006a; Kerrigan *et al.*, 2010).

A role for platelets in the progression of IE was implied by studies using the rabbit model of *S. aureus* endocarditis. Animals treated with aspirin, a cyclo-oxygenase inhibitor that prevents platelet aggregation, had significantly lower bacterial titres and smaller vegetations than untreated controls (Kupferwasser *et al.*, 1999; Nicolau *et al.*, 1993). Strains of *S. aureus* that were defective in platelet binding and activation were less virulent in animal models of IE. *S. aureus* is thought to have a high affinity for pre-

existing lesions on heart valve surfaces (Niemann *et al.*, 2004). Bacteria can colonize sterile developing thrombi on damaged heart valves and subsequently enhance thrombi by capturing and activating circulating platelets. In patients with no prior heart damage IE is believed to develop when small circulating thrombi consisting of bacteria and platelets are deposited on the heart valve surface.

Light aggregometry is one of most popular methods to study platelet activation *in vitro*. In this method bacteria (aggregating agent) are added to platelet rich plasma and changes in the optical densities (OD) are measured overtime. A decreasing OD corresponds to increasing levels of aggregation. Bacteria-mediated platelet aggregation is preceded by a lag phase which reflects the time taken for aggregation to occur after bacteria and platelets come into contact. Lag times depend on the bacterial species, the cell density and the levels of expression of bacterial components which stimulate platelets.

1.4.1 Biology and function of platelets

Platelets are the smallest and the most abundant cells in blood at 1.5×10^8 to 4.5×10^8 per ml. They are produced as fragments of megakaryocytes (Clemetson, 2012). Their primary role is to promote haemostasis. As a result of damage to the vascular endothelium, platelets bind to exposed collagens and von Willebrand factor (vWf). They are irreversibly transformed from a resting discoid shape to an irregular shape with numerous pseudopodia. The resulting activated platelets are highly adhesive and they rapidly aggregate. The $\alpha_{IIb}\beta_3$ integrin is up-regulated and mediates platelet aggregation by binding plasma fibrinogen, a divalent molecule that can bind integrins on different platelets (discussed in chapter 1.4.1.1). The aggregates form a haemostatic plug that seals the damaged vessel and arrests bleeding. Deficiencies in either the platelet GPIb/V/IX complex (Bernard-Soulier Syndrome) or the platelet integrin $\alpha_{IIb}\beta_3$ (known also as GPIIb/IIIa) (Glanzmann thrombasthenia) lead to bleeding disorders as a result of impaired platelet function (Clemetson, 2002).

Platelets are highly responsive and can be activated by many different agonists. They have two different collagen receptors ($\alpha_2\beta_1$ and GPVI), two ADP receptors (P2X1 and P2Y12), a serotonin receptor, an adrenergic receptor and a thromboxane A2 receptor. There are also three thrombin receptors protease-activated receptor (PAR) -1, PAR-4 and GPIb α . Platelet activation is dependent on the sum of the generated signals from all of these receptors and not on any specific receptor. Signalling is either

mediated by phospholipase A2/cyclooxygenase (COX), resulting in the generation of thromboxane A2 or by protein kinase C (Clemetson, 2002).

Upon becoming activated platelets release α -granules, dense granules and lysosomes. Dense granules are rich in small molecules especially ATP/ADP and serotonin whereas α -granules contain numerous plasma proteins such as vWf, platelet-derived growth factor, vascular endothelial growth factor, endostatin, thrombospondin, and basic fibroblast growth factor, which are typically associated with haemostasis. Lysosomes contain acid hydrolases. These secreted products act to enhance haemostasis as ADP activates other platelets, serotonin causes vasoconstriction and the secreted proteins trigger platelet aggregation (Kerrigan *et al.*, 2010).

Platelets play a key role in innate immunity by directly acting to kill bacteria as well as coordinating the response to pathogens. Activated platelets secrete anti-microbial peptides (platelet microbicidal proteins, PMPs) including the chemokine platelet factor 4 (PF-4), platelet basic peptide and its derivatives, as well as the chemokine RANTES. These molecules have been termed kinocidins, reflecting their complementary roles as microbicides and chemokines. They coordinate molecular and cellular antimicrobial host defenses (Antczak *et al.*, 2010; Fitzgerald *et al.*, 2006a; Mercier *et al.*, 2004; Yeaman, 1997). *S. aureus* endocarditis isolates are more resistant to PMPs than isolates from soft-tissue infections (Bayer *et al.*, 1998; Dhawan *et al.*, 1998; Yeaman *et al.*, 1998). Platelets are also capable of inducing proinflammatory response against pathogen as they contain pattern recognition receptors, like the Toll-like receptors (TLRs) (Cox *et al.*, 2011; Semple *et al.*, 2011).

1.4.1.1 Platelet integrin $\alpha_{IIb}\beta_3$.

Upon platelet activation the resting form of integrin $\alpha_{IIb}\beta_3$ undergoes conformational changes allowing it to bind to fibrinogen. The conformationally active integrin binds specifically to the distal ends of the dimeric fibrinogen molecule in an unstructured region of the γ subunit (Fig. 1.6) (Podolnikova *et al.*, 2003). The large separation between the two $\alpha_{IIb}\beta_3$ - binding sites on fibrinogen (~ 440 Å) is well suited for cross-linking of platelets. This results in platelet aggregation and formation of platelet plugs to control bleeding. Later the N-terminal peptides of the Fg α and β subunits are cleaved which stimulates the assembly of fibrinogen into fibrin. Adjacent fibrinogen molecules within fibrin are then cross-linked through their γ C peptides by the factor XIIIa transglutaminase (Bennett, 2001; Hawiger, 1995; Springer *et al.*, 2008).

$\alpha_{IIb}\beta_3$ is formed from an α and a β subunit, each of which has a large extracellular domain and a smaller C-terminal transmembrane domain (Fig. 1.12). The extracellular part of the α -subunit is composed of an N-terminal β propeller, the thigh domain and two calf domains (Fig. 1.12). The extracellular portion of β_3 subunit is formed from an A domain, a PSI (plexin/semaphoring/integrin) domain, a hybrid domain, four integrin epidermal growth factor (IEGF) domains and a membrane proximal β -TD domain (Arnaout *et al.*, 2005; Zhu *et al.*, 2010). The subunits come together in a large interface between the α subunit's β propeller domain and the β subunit's A domain to form the ligand - binding head (Fig. 1.12). Other domains form upper and lower legs in each subunit to connect the heads to the membrane. Integrins have a bent conformation in the low affinity state with the bend occurring between the upper and lower legs. Upon activation, integrins extend, and a major reorientation at the interface between the β A and hybrid domains occurs that is linked to remodelling of the ligand-binding site in the β A domain (Fig. 1.12) (Luo *et al.*, 2007). The β A domain has three metal ion – binding sites, with a Mg^{2+} ion in the central metal ion – dependent adhesion site (MIDAS) flanked by two Ca^{2+} ions, one of which is in a site termed adjacent to MIDAS (ADMIDAS) (Harburger *et al.*, 2009; Springer *et al.*, 2008).

$\alpha_{IIb}\beta_3$ is the most abundant integrin on platelets (approximately 80,000 per cell) with an additional pool that can be recruited from internal membranes upon thrombin-induced platelet activation (Kasirer-Friede *et al.*, 2007). Conformationally active $\alpha_{IIb}\beta_3$ can bind several ligands containing an RGD (Arg-Gly-Asp) motif, including von Willebrand factor, fibronectin, vitronectin and thrombospondin. Although fibrinogen contains 2 pairs of RGD sequences, the primary binding sites for $\alpha_{IIb}\beta_3$ necessary for platelet aggregation are at the C-termini of the 2 fibrinogen γ -chains. Less abundant and less physiologically relevant platelet integrins include $\alpha_V\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ (Kasirer-Friede *et al.*, 2007).

$\alpha_{IIb}\beta_3$ is in a low-affinity state on unstimulated platelets such as those in normal circulation and is unable to bind soluble ligands. Platelet activation leading to conversion of the integrin from the resting to the conformationally active state is induced by multiple intracellular signalling pathways. The origin of the signal determines the direction of the pathway as either inside-out or outside-in. Inside-out signalling depends on either soluble agonists such as adenosine diphosphate (ADP), thrombin and thromboxane A2 (TxA2) signalling through G-protein coupled receptors or immobilized agonists such as von Willebrand factor or collagen that initiate the

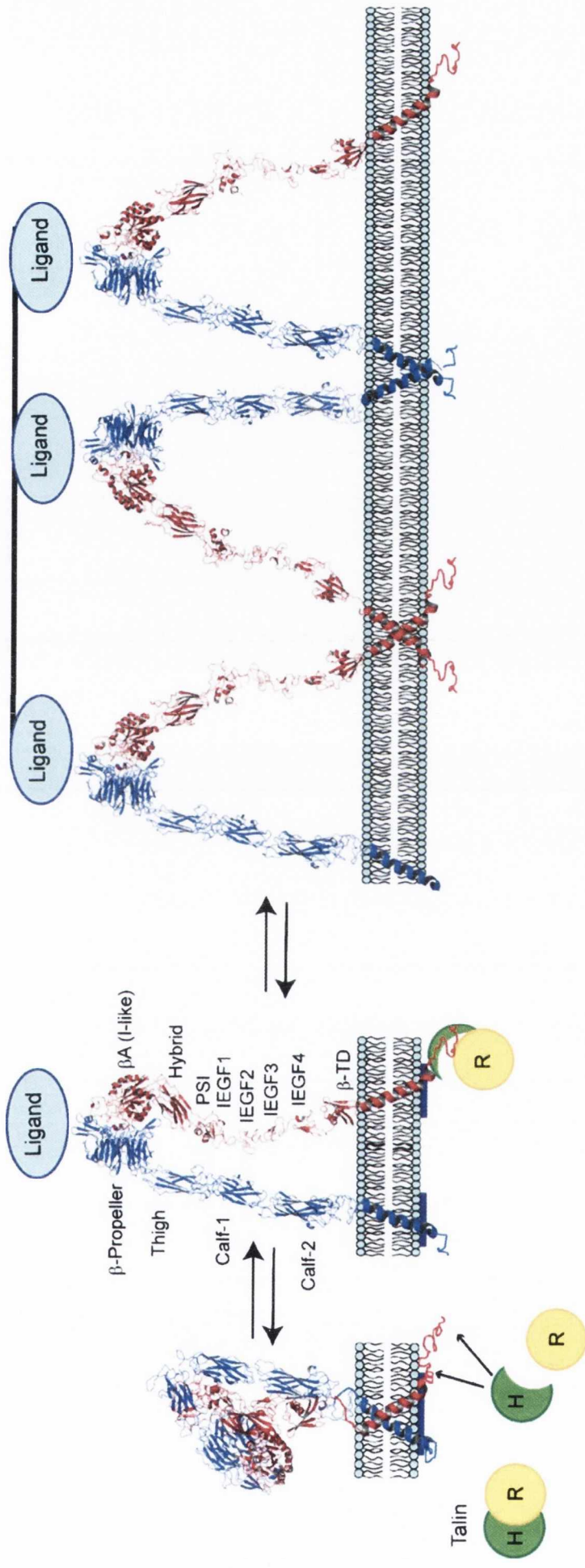


Figure 1.12 Model of $\alpha_{IIb}\beta_3$ crystal structure and its switchblade conformational changes associated with activation and ligand binding. Inside-out signaling ultimately results in the binding of the talin head (H) domain binding to the cytoplasmic domain of the β_3 subunit, resulting in subunit separation. This is transmitted through the transmembrane domains to the ectodomain where it results in extension of the α and β subunits and perhaps additional changes in the ligand binding region of β_3 . Ligand then binds, resulting in a swing-out motion of the β_3 hybrid and PSI domains that may initiate outside-in signaling. Additional post-ligand binding events may occur, including homo-oligomerization of integrin transmembrane domains, leading to receptor clustering. Taken from Collier & Shattil, 2008.

process by interacting with GPIb/IX/V or the collagen receptors GPVI or $\alpha_2\beta_1$. Intracellular signals stimulate the interaction of key regulatory ligands (such as talin) with integrin cytoplasmic domains (in this case the β_3 tail) (Fig. 1.12). This leads to conformational changes in the extracellular domain that result in increased affinity for adhesive ligands such as fibrinogen, von Willebrand factor and fibronectin (Ma *et al.*, 2007). On the other hand outside-in signalling requires extracellular ligand to occupy $\alpha_{IIb}\beta_3$. The generated signal is initially reversible but becomes progressively irreversible and promotes integrin clustering (receptor oligomerization) and further conformational changes that are transmitted to the cytoplasmic domains. This results in the recruitment and/or activation of enzymes, adaptors, and effectors to form integrin-based signalling complexes (Shattil *et al.*, 2004).

1.4.2 Interactions of *S. aureus* with platelets.

S. aureus is the most frequent etiological agent of infective endocarditis (IE) (Rasmussen *et al.*, 2011). Its interaction with platelets is well characterised and embraces many components that are either exposed on the bacterial surface or secreted. The components interact with platelet receptors either directly, or indirectly, *via* serum proteins. Stimulation upon bacterial adhesion to platelets triggers platelet activation which causes cross-bridging of platelets (platelet aggregation) and leads to formation of thrombi. Bacteria can also bind to platelets without activating them which leads to their engulfment (Youssefian *et al.*, 2002) where they can evade the immune response and survive in the bloodstream. Thus the interactions of bacteria with human platelets can have different outcomes, and this might depend on the strain of bacteria causing the infection.

The fibrinogen-binding surface protein ClfA is an important factor in promoting indirect adhesion to platelets employing a Fg bridge (Fig. 1.13A). The resting $\alpha_{IIb}\beta_3$ integrin has little or no affinity for soluble fibrinogen but can interact with fibrinogen bound to bacteria (Loughman *et al.*, 2005). ClfA-mediated adhesion to platelets leads to platelet aggregation. As measured by aggregometry, bacteria expressing high levels of ClfA cause platelet aggregation with short lag times (1-2 min) (Loughman *et al.*, 2005). *S. aureus*-mediated platelet aggregation can be induced by addition of fibrinogen and ClfA-specific immunoglobulin to the plasma-free system. The IgG crosslinks ClfA with the platelet receptor Fc γ RIIa, the clustering of which triggers a signal transduction cascade (inside-out signalling) resulting in platelet activation. Thus the rapid activation

depends on Fg and the presence of specific antibodies that are present in the sera of most individuals (Dryla *et al.*, 2005). ClfA is a virulence factor in the pathogenesis of experimental endocarditis (Moreillon *et al.*, 1995; O'Brien *et al.*, 2002a; Que *et al.*, 2005; Sullam *et al.*, 1996).

Exponentially growing *S. aureus* expresses FnBPA and FnBPB proteins which mediate rapid platelet activation using a similar mechanism to that of ClfA (Fig. 1.13A) (Fitzgerald *et al.*, 2006b). A fibrinogen bridge is formed between the FnBP A-domain and $\alpha_{IIb}\beta_3$ while specific IgG links the protein with the platelet Fc γ RIIa receptor. The fibronectin-binding region BCD of FnBPA can also activate platelets by binding to N-terminal domain of fibronectin (Fig. 1.8), which cross-links *S. aureus* to $\alpha_{IIb}\beta_3$ (Fig. 1.13) (Fitzgerald *et al.*, 2006b).

ClfB-mediated platelet aggregation occurs more slowly than that promoted by ClfA or FnBPs. This may be due to a lower affinity for Fg, different localization of the binding site on Fg α C region (Fig. 1.6) (Walsh *et al.*, 2008) or the density of ClfB on the bacterial surface. ClfB induces aggregation either *via* a fibrinogen bridge and anti-ClfB IgG or by the specific serum IgG and complement factors deposited on bacterial surface which interact with complement receptor on platelets (Fig. 1.13) (Miajlovic *et al.*, 2007).

The slow platelet activation (lag time of 8-10 min) is also characteristic of bacteria expressing SdrE and a non-fibrinogen binding mutant of ClfA (ClfA PY) (Fig. 1.13C). Both mechanisms involve IgG and complement fixation. SdrE, like ClfA and ClfB induce platelet aggregation when expressed in *L. lactis* (Loughman *et al.*, 2005; O'Brien *et al.*, 2002a).

Studies using a cone and plate viscometer (a device that shears cells at a given flow rate) have demonstrated that protein A, ClfA, SdrC, SdrD and SdrE are important in thrombus formation (George *et al.*, 2007; George *et al.*, 2006; Pawar *et al.*, 2004).

Studies of the influence of shear rates on the ability of *S. aureus* to induce platelet activation demonstrated that platelets perfused over immobilized *S. aureus* under high shear conditions (equivalent to arterial pressure) led to strong adhesion followed by rapid aggregate formation. This effect was dependent upon fibrinogen and IgG. A *S. aureus* *clfA* mutant could no longer promote platelet adhesion or aggregate formation indicating a primary role of ClfA in platelet activation under shear (Kerrigan *et al.*, 2008).

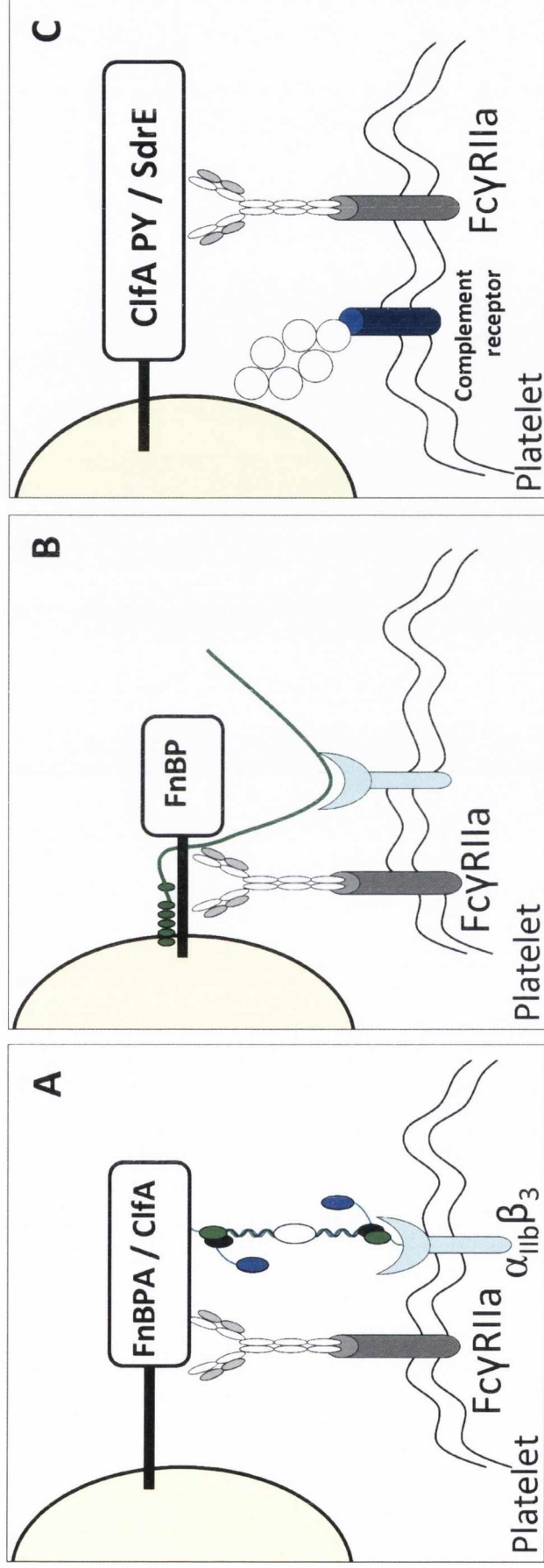


Figure 1.13 Mechanisms of bacterial interactions with platelets.

(A) Rapid platelet activation mediated by clumping factor A (FnBPA). Bacteria use these fibrinogen binding proteins to form a Fg-bridge with platelets. Platelet activation requires specific antibodies to crosslink bacteria with the platelet FcγRIIa receptor. (B) Rapid activation involving a fibrinogen bridge between FnBP and the platelet integrin $\alpha_{IIb}\beta_3$ and IgG specific for neo-epitopes created when FnBP binds fibrinogen, which engages FcγRIIa. (C) Complement-mediated platelet activation. This slow activation mechanism can be mediated by the deposition of complement proteins on the bacterial surface which cross-link bacteria to a complement receptor on platelets. Specific antibodies are required to engage platelet receptor FcγRIIa.

Protein A (Spa) also contributes to platelet activation under high shear conditions. Spa expression is thought to promote adherence to the platelet receptor GPIb through a vWF bridge (Hartleib *et al.*, 2000; Pawar *et al.*, 2004). Monoclonal antibodies against vWF and GPIb inhibited platelet activation and Spa has been shown to promote adhesion of *S. aureus* to immobilized collagen and vWF under flow conditions (Mascari *et al.*, 2003).

Proteins secreted by *S. aureus* were also shown to interact with human platelets. Cytolytic α -toxin triggers platelet activation and secretion platelets contents including procoagulant mediators (Arvand *et al.*, 1990). Recent studies revealed that α -toxin binding to the eukaryotic cell is mediated by disintegrin and metalloprotease 10 (ADAM10) (Wilke *et al.*, 2010), which might be the binding site for α -toxin on platelets. Extracellular fibrinogen binding (Efb) protein has been shown to bind to platelets, either indirectly *via* a fibrinogen molecule or directly by an unidentified receptor. The role of Efb in delaying wound healing by inhibiting platelet function was postulated since *S. aureus* *efb* mutants were less pathogenic in a rat wound infection model than the wild-type bacteria (Palma *et al.*, 2001; Shannon *et al.*, 2004).

1.5 *S. aureus* adhesion to and internalization by non-professional phagocytes.

Many pathogenic bacteria adhere to receptors on host cells, including integrins, cadherins and immunoglobulin-related cell adhesion molecules (IgCAM), leading to subsequent internalization. *Yersinia spp.* and *Helicobacter pylori* directly engage the host $\alpha_5\beta_1$ integrin (Isberg *et al.*, 1987; Tegtmeyer *et al.*, 2010). *S. aureus* and *Streptococcus pyogenes* indirectly bind to integrins by recruiting fibronectin (Fn) which provides a link to the host cells actin cytoskeleton triggering bacterial uptake (Hauck, 2002; Hauck *et al.*, 2006b).

Although *S. aureus* is not considered to be an intracellular pathogen its ability to enter and persist within cells that are not professionally phagocytic has been widely reported. *S. aureus* has been shown to invade mammalian endothelial and epithelial cells, as well as osteoblasts, fibroblasts and keratinocytes (Edwards *et al.*, 2010; Ellington *et al.*, 1999; Massey *et al.*, 2001; Sinha *et al.*, 1999; Sinha *et al.*, 2005; von Eiff *et al.*, 2001b). The most important mechanism of internalization of *S. aureus* into mammalian cells is that promoted by FnBPs. This mechanism involves extracellular fibronectin forming a bridge to the common host cells integrin $\alpha_5\beta_1$ (Fowler *et al.*, 2000; Sinha *et al.*, 1999). *S. aureus* cell wall proteins FnBPA and FnBPB both consist

of multiple motifs that bind the N-terminal domains of fibronectin (Chapter 1.2.3). The $\alpha_5\beta_1$ integrin recognizes an RGD motif located on the tenth type 2 repeat of the Fn (3Fn10) (Fig. 1.8). Several Fn molecules engage a single FnBP resulting in integrin clustering, signal transduction and actin rearrangement followed by bacterial uptake. The multiple Fn binding repeats are necessary for efficient binding to Fn and for triggering rapid bacterial uptake. A mutant comprising only low affinity repeats promoted binding to Fn but not invasion into endothelial cells. Similarly, only bacteria expressing FnBPs containing multiple Fn binding repeats were fully virulent in a murine model of sepsis (Edwards *et al.*, 2010; Massey *et al.*, 2001).

S. aureus binding to host cell integrins triggers an inside-out signalling and induces localized recruitment of focal adhesion kinase proteins (FAK). FAKs promote actin rearrangements guiding *S. aureus* uptake. The Src family of protein tyrosine kinases (PTKs) were also found to be essential in mediating the internalization (Agerer *et al.*, 2005).

Expression of FnBPA is sufficient for bacteria invasion and dissemination within animal tissues *in vivo*. Non-pathogenic *L. lactis* expressing FnBPA promoted heart valve colonization and invasion of surrounding endothelium in experimental rat endocarditis models (Que *et al.*, 2001; Que *et al.*, 2005). Bacterial expression of the FnBP B-domain alone was sufficient to trigger internalization of bacteria by endothelial cells. This correlated with the capacity of these fragments to induce expression of both ICAM-1 and VCAM-1 receptors on the endothelium and secretion of the CXCL8 chemokines and finally to induce a tissue factor-dependent endothelial coagulation response (Heying *et al.*, 2009).

It is likely that *S. aureus* has additional mechanisms for internalizing the host cells. Recently it has been shown that the autolysin (Atl) promotes *S. aureus* adhesion to and internalization by host cells (Heilmann *et al.*, 1997; Hirschhausen *et al.*, 2010). This protein is associated with the bacterial surface by ionic and hydrophobic interactions. It hydrolyses peptidoglycan supporting the process of cell division. Atl-mediated interaction with human cells involves direct binding to the host heat shock cognate protein (Hsc70) (Hirschhausen *et al.*, 2010).

Hiding within the host cells is considered to be a contributing factor in bacterial persistence. *S. aureus* can undergo phenotype-switching *in vivo* to a slow-growing, small colony variant (SCV) phenotype which is associated with persistence in host cells

and tissues. SCVs are a normal part of the *S. aureus* life-cycle and provide protection against exposure to antibiotics (Garzoni *et al.*, 2009; Tuchscher *et al.*, 2011).

Recent reports demonstrated that coagulase negative staphylococci, such as *S. epidermidis*, *S. saprophyticus* and *S. lugdunensis* can also be internalized (Khalil *et al.*, 2007; Pereira *et al.*, 2012; Szabados *et al.*, 2008; Szabados *et al.*, 2011). However the mechanisms involved have not been characterized.

1.6 Rationale for this study.

Iron regulated surface determinants (Isd) of *S. aureus* are up-regulated during infection to provide haem-iron and support bacterial survival under this iron - restrictive conditions (Allard *et al.*, 2006; Skaar *et al.*, 2004). The proteins are also multifunctional promoting bacterial adhesion and immune evasion and thus further supporting bacterial virulence (Clarke *et al.*, 2009; Visai *et al.*, 2009). Expression of the haemoglobin receptor IsdB has been shown to promote *S. aureus* adhesion to platelets and platelet aggregation. Studies using specific inhibitors localized the binding site as the platelet $\alpha_{IIb}\beta_3$ integrin (Miajlovic *et al.*, 2010).

At first, this study aimed to identify mechanical basis for the IsdB interaction with the $\alpha_{IIb}\beta_3$ integrin. Point mutations in genomic *isdB* were isolated to disrupt the IsdB-dependent adhesion to platelets and platelet aggregation. It was necessary to introduce the mutations into the genome due to previously reported failure in full complementation of the IsdB-dependent phenotype from a plasmid. Targeted amino acid motifs were either responsible for haemoglobin and haem binding or were derivatives of the known integrin-recognition motifs. In another approach to study this interaction recombinant IsdB and purified $\alpha_{IIb}\beta_3$ were used.

In the course of study an observation was made that IsdB expression promotes *S. aureus* adhesion to human cells, other than platelets. The adherence phenotype was studied with a range of *S. aureus* strains, a few human cell lines and inhibitors. Furthermore a hypothesis was formed that IsdB expression supports *S. aureus* internalization. To test that hypothesis *S. aureus* invasion into human cells was studied by gentamycin protection assays.

In the year of 2011 two newly sequenced genome of *S. lugdunensis* N920143 and HKU09-01 were reported to encode an *isd* locus (Heilbronner *et al.*, 2011). The *S. lugdunensis* Isd system is unique among the coagulase negative staphylococci and potentially an important characteristic of that poorly characterized staphylococcal

species. Initial characterization of the Isd system of *S. lugdunensis* N920143 was performed by comparative *in silico* studies with the Isd proteins of *S. aureus*. Subsequently the recombinant IsdB_{SL}, IsdC_{SL}, IsdJ and IsdK were purified and ligand identification was performed. Rabbit anti-Isd antibodies were generated in collaboration with Prof. Pietro Speziale allowing to study expression and cellular localization of the Isd proteins. Identified phenotypes were evaluated using generated *isd* deletion mutants in strain *S. lugdunensis* N920143.

Chapter 2

Materials and methods

2.1 Chemicals and reagents.

General laboratory chemicals, reagents and media were obtained from Sigma-Aldrich, unless otherwise stated. Antibodies used in this study are listed in Table 2.1.

2.2 Bacterial strains and growth conditions.

The bacterial strains used in this study are listed in Table 2.2. *Staphylococcus aureus* and *S. lugdunensis* strains were grown aerobically at 37°C in tryptic soya broth (TSB) with shaking at 200 r.p.m. or on tryptic soya agar (TSA) plates. TSB is commonly referred to as Soybean-Casein Digest Medium and was originally developed for use without blood in determining the effectiveness of sulfonamides against pneumococci and other organisms (McCullough, 1949). *Escherichia coli* strains were grown in similar manner in Luria-Bertani (LB) medium (Bertani, 1951). *L. lactis* was grown statically at 28°C in M17 medium supplemented with 0.5% (w/v) glucose (GM17) (Terzaghi *et al.*, 1975). The following antibiotics were added to the media as required: ampicillin (Amp) at 100 µg ml⁻¹, chloramphenicol (Chm) at 10 µg ml⁻¹, tetracycline (Tet) at 2 µg ml⁻¹, and erythromycin (Erm) at 10 µg ml⁻¹.

2.2.1 Growth of bacteria in iron limiting conditions.

To reflect growth conditions *in vivo*, bacterial strains were grown in iron-deficient medium - RPMI 1640 (Yuan *et al.*, 1995). RPMI 1640 was originally designed to culture human leukocytes (Moore *et al.*, 1967). It is a basal medium consisting of vitamins, amino acids, salts, glucose, glutathione and a pH indicator. It contains no proteins or growth promoting agents. Starter cultures were diluted in 20 ml of RPMI to an OD₆₀₀ 0.05 and grown at 37°C with shaking at 200 r.p.m. To eliminate contaminating iron bacteria were cultured in Falcon tubes or glassware which was first treated with 0.1 M HCl, followed by extensive washing with distilled H₂O and sterilization by autoclaving.

2.2.2 Bacterial growth on haemoglobin as a sole iron source.

The protocol by Pishchany *et al.* was used with minor modifications (Pishchany *et al.*, 2010). A single colony of *S. lugdunensis* was inoculated into RPMI and grown overnight. The concentration of cells was normalized to an OD₆₀₀ of 3.0, bacteria were sedimented (4000 × g, 10 min) and resuspended 1 : 50 in 1 ml NRPMI containing 0.25 mM of the divalent metal ion chelator ethylenediamine-di(o-hydroxyphenylacetic acid

(EDDHA; LGC Standards GmbH). NRPMI was prepared in advance by treatment of RPMI containing 1 % (w/v) casamino acids (BD Bacto) with Chelex 100 according to the manufacturer's recommendations and supplementing the resulting ion-deficient medium with 25 μ M ZnCl₂, 25 μ M MnCl₂, 100 μ M CaCl₂, and 1 mM MgCl₂. The resulting suspension of *S. lugdunensis* was subcultured (1: 50) into 5 ml NRPMI + 0.25 mM EDDHA with haemoglobin at 10 μ g ml⁻¹. The purified human and mouse haemoglobin were obtained from Dr Gleb Pishchany or Ponte Scientific. Cultures were incubated at 37°C in 15 ml conical tubes with shaking at 40 r.p.m. OD₆₀₀ measurements were taken at indicated time points.

2.2.3 Growth and induction of *S. aureus* strains carrying pRMC2 derivatives.

pRMC2 is a multicopy shuttle vector which allows anhydrotetracycline (aTc) - controlled expression of proteins in *S. aureus* (Corrigan *et al.*, 2009a). Addition of the inducer aTc activates expression of a protein encoded by the plasmid. Induction was performed by addition of aTc (at 500 ng ml⁻¹ or 1 μ g ml⁻¹) to exponentially growing cultures and further incubation for 4 h, 16 h or differently if stated, in the presence of the inducer.

2.2.4 Growth and induction of *L. lactis* strains carrying pNZ8048 derivatives.

Nisin is an antimicrobial peptide produced by *L. lactis* strains encoding the *nisA* gene, often as part of transposon Tn5276. Nisin is an autoregulator and through interaction with the two-component regulatory system NisKR can activate transcription of *nisA* (Kuipers, 1998). *L. lactis* strain NZ9000 is defective in nisin production so expression of genes cloned in-frame with the *nisA* start codon can be induced by addition of extracellular nisin (de Ruyter *et al.*, 1996). The nisin-controlled expression system (NICE) of *L. lactis* can be utilised to study staphylococcal proteins in isolation.

L. lactis strains carrying pNZ8048 derivatives were grown and induced with nisin as follows. Starter cultures of *L. lactis* strain NZ9000 (pNZ8048) were diluted 1: 100 into 5 ml of fresh GM17 medium containing 10 μ g ml⁻¹ Chm and grown to an OD₆₀₀ of 0.5. Nisin was added to exponential phase cultures at final concentrations that ranged between 100 and 300 ng ml⁻¹ to induce expression of the plasmid encoded protein and cultures were further incubated at 28 °C for 16 h.

Table 2.1 Antibodies.

Antibody	Relevant features	Working dilution/ concentration	Source
Rabbit anti-IsdB _{SA}	Raised against rIsdB _{SA} (45-480)	1:8000	(Miajlovic <i>et al.</i> , 2010)
Rabbit anti-IsdA	Raised against rIsdA	1:5000	- II -
Rabbit anti-IsdB _{SL}	Raised against rIsdB _{SL} (45-655)	1:5000	P. Speziale
Rabbit anti-IsdC _{SL}	Raised against rIsdC _{SL} (30-290)	1:5000	- II -
Rabbit anti-IsdJ	Raised against rIsdJ (45-610)	1:5000	- II -
Rabbit anti-IsdK	Raised against rIsdK (35-426)	1:5000	- II -
Rabbit anti-human platelet factor 4	Raised against recombinant human platelet factor 4	1:1000	GenWay
Rabbit anti-human haemoglobin	Raised against human haemoglobin ($\alpha_2\beta_2$)	1:1000	Dako
Rabbit anti-human haptoglobin	Raised against human haptoglobin	1:1000	Sigma-Aldrich
Monoclonal anti-human fibrinogen	HRP- conjugated anti-Fg IgG	1:5000	Dako
Goat anti-rabbit IgG	HRP-conjugated secondary IgG	1:2000	Dako
Monoclonal anti-polyhistidine	Unconjugated antibody used for ligand capture in surface plasmon resonance experiments	1:10000	R&D Systems

Table 2.2 Bacterial strains.

Strain	Relevant characteristics	Reference
<i>Staphylococcus spp.</i>		
<i>S. lugdunensis</i> N920143	Human breast abscess isolate	(Heilbronner <i>et al.</i> , 2011)
<i>S. lugdunensis</i> N910319	Human endocarditis isolate	(Vandenesch, unpublished)
<i>S. lugdunensis</i> N910320	Human endocarditis isolate	- II -
<i>S. lugdunensis</i> N930432	Human endocarditis isolate	- II -
<i>S. lugdunensis</i> N940025	Human endocarditis isolate	- II -
<i>S. lugdunensis</i> N920143 Δ <i>isdC</i>	<i>isdC</i> deletion	This study
<i>S. lugdunensis</i> N920143 Δ <i>isdK</i>	<i>isdK</i> deletion	- II -
<i>S. lugdunensis</i> N920143 Δ <i>isdB</i>	<i>isdB</i> deletion	(Heilbronner, personal communications)
<i>S. lugdunensis</i> N920143 Δ <i>isdJ</i>	<i>isdJ</i> deletion	- II -
<i>S. lugdunensis</i> N920143 Δ <i>isd</i>	<i>isd</i> locus deletion	- II -
<i>S. lugdunensis</i> N940025 Δ <i>isd</i>	<i>isd</i> locus deletion	- II -
<i>S. lugdunensis</i> N920143 Δ <i>fbl</i>	<i>fbl</i> deletion	- II -
<i>S. aureus</i> Newman	Widely used laboratory strain, defective in expression of FnBPA and FnBPB	(Duthie, 1953)
<i>S. aureus</i> Newman <i>isdB</i>	Frameshift mutation in <i>isdB</i>	(Miajlovic <i>et al.</i> , 2010)
<i>S. aureus</i> Newman <i>clfA</i> <i>clfB</i>	Frameshift mutation in <i>clfA5</i> , frameshift mutation in <i>clfA</i> , <i>clfB</i> :: <i>lacZ</i> Erm^R	- II -
<i>S. aureus</i> Newman <i>clfA</i> <i>clfB</i> <i>isdB</i> (Y165A/H166E/Y167A)	Strain expressing IsdB Y165A/H166E/Y167A	This study
<i>S. aureus</i> Newman <i>clfA</i> <i>clfB</i> <i>isdB</i> (G203A/D204A)	Strain expressing IsdB G203A/D204A	- II -
<i>S. aureus</i> Newman <i>clfA</i> <i>clfB</i> <i>isdB</i> (N229A)	Strain expressing IsdB N229A	- II -
<i>S. aureus</i> Newman <i>clfA</i> <i>clfB</i> <i>isdB</i> (N377A)	Strain expressing IsdB N377A	- II -

Table 2.2 Bacterial strains, continued.

Strain	Relevant characteristics	Reference
<i>Staphylococcus spp.</i>		
<i>S. aureus</i> Newman <i>clfA clfB isdB</i>	Frameshift mutation in <i>clfA5</i> <i>clfB::lacZ</i> Erm ^R , frameshift mutation in <i>isdB</i>	(Miajlovic <i>et al.</i> , 2010)
<i>S. aureus</i> Newman <i>isdA</i>	Newman <i>isdA::Tn917</i> Erm ^R	(Clarke <i>et al.</i> , 2007)
<i>S. aureus</i> Newman <i>isdA clfA clfB</i>	Frameshift mutation in <i>clfA</i> , <i>clfB::Tet^R</i> , <i>isdA</i>	(Miajlovic <i>et al.</i> , 2010)
<i>S. aureus</i> SH1000	Laboratory strain, <i>rsbU</i> ⁺ derivative of strain 8325-4	(Horsburgh <i>et al.</i> , 2002)
<i>S. aureus</i> SH1000 <i>isdB</i>	<i>isdB</i> deletion	This study
<i>S. aureus</i> SH1000 <i>clfA clfB</i> <i>fnbA fnbB</i>	<i>clfA::Tn917</i> , <i>clfB::Tet^R</i> , <i>fnbA::Tet^R</i> , <i>fnbB::Em^R</i>	(O'Neill <i>et al.</i> , 2009)
<i>S. aureus</i> SH1000 <i>clfA clfB</i> <i>fnbA fnbB isdB</i>	<i>isdB</i> deletion in SH1000 <i>clfA</i> <i>clfB fnbA fnbB</i>	This study
<i>S. aureus</i> SH1000 <i>clfA clfB</i> <i>fnbA fnbB isdB</i> (Y165A/H166E/Y167A)	Strain expressing IsdB Y165A/H166E/Y167A	- II -
<i>S. aureus</i> SH1000 <i>clfA clfB</i> <i>fnbA fnbB isdB</i> (G203A/D204A)	Strain expressing IsdB G203A/D204A	- II -
<i>S. aureus</i> SH1000 <i>clfA clfB</i> <i>fnbA fnbB isdB</i> (N229A)	Strain expressing IsdB N229A	- II -
<i>S. aureus</i> SH1000 <i>clfA clfB</i> <i>fnbA fnbB isdB</i> (N377A)	Strain expressing IsdB N377A	- II -
<i>S. aureus</i> COWAN	Laboratory strain	(Levinson <i>et al.</i> , 1983)
<i>S. carnosus</i>	Meat isolate	(Schleifer <i>et al.</i> , 1982)
<i>Lactococcus lactis</i>		
<i>L. lactis</i> NZ9000	<i>L. lactis</i> subsp. <i>Cremoris</i> MG1363 carrying NisRK two- component system	(Kuipers <i>et al.</i> , 1998)

Table 2.2 Bacterial strains, continued.

Strain	Relevant characteristics	Reference
<i>Escherichia coli</i>		
<i>E. coli</i> XL-1Blue	Δ recA1 <i>endA1 gyrA96 thi-1</i> <i>hsdR17 supE44 relA1 lac[F'</i> <i>proAB lacIqZ</i> Δ M15 <i>Tn10</i> , cloning host	Stratagene
<i>E. coli</i> DC10B	<i>dcm</i> deletion in strain DH10B strain: F- <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1</i> <i>endA1 araD139</i> Δ (<i>ara leu</i>) 7697 <i>galU galK rpsL nupG</i> λ -	(Monk <i>et al.</i> , 2012)
<i>E. coli</i> TOPP10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS</i> - <i>mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139</i> Δ (<i>ara</i> <i>leu</i>) 7697 <i>galU galK rpsL</i> (StrR) <i>endA1 nupG</i> , protein overexpression host	Invitrogen

2.3 Culturing mammalian cell lines.

Cell lines used in the study are listed in Table 2.3. The human epithelial cervical cancer (HeLa), the human embryo kidney 293T and the Chinese hamster ovary (CHO-K1) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS; Lonza). Media for cells stably expressing the $\alpha_{11b}\beta_3$ integrin variants were additionally supplemented with 1 mg ml⁻¹ of geneticin sulphate. The human breast adenocarcinoma (MCF-7) cell line was cultured in MEM/F12 (1:1) medium supplemented with 12.5 mM HEPES, 2 mM glutamine (Lonza), 5% (v/v) FBS, 1 µg ml⁻¹ insulin, 0.5 µg ml⁻¹ hydrocortisone and 50 ng ml⁻¹ epidermal growth factor. Cells were split once every 2-3 days using Trypsin-EDTA.

2.4 Plasmids.

The plasmids used in this study are listed in Table 2.4.

2.5 DNA manipulation.

Standard methods were used for DNA manipulations (Sambrook *et al.*, 2001). Restriction endonucleases were purchased from New England Biolabs and Roche and were used according to the manufacturers' instructions. T4 DNA ligase and alkaline phosphatase were purchased from Roche. DNA cloning into pKOR1 vector was performed using the BP Clonase II enzyme (Invitrogen) following manufacturer's protocol. Confirmatory DNA sequencing was carried out by Eurofins MWG Operon.

2.5.1 Preparation of plasmid and genomic DNA.

Plasmid DNA was isolated from bacteria using the Qiagen Plasmid Midi Kit or Wizard Plus™ Minipreps kit (Promega). Genomic DNA was purified using the Bacterial Genomic DNA purification kit (Edge BioSystems). Extraction of plasmid and genomic DNA from *S. aureus* and *S. lugdunensis* required the addition of 400 µg ml⁻¹ of lysostaphin to digest cell wall peptidoglycan. Lysozyme at 1 mg ml⁻¹ and mutanolysin at 1000 U ml⁻¹ were used to digest the cell wall of *L. lactis* prior the DNA purification from protoplasts.

2.5.2 Polymerase chain reaction (PCR).

PCR reactions were typically carried out in 50 µl volumes using 1 U Phusion™ Hot Start DNA polymerase in Phusion HF buffer (Finnzymes). 10 ng of plasmid DNA

Table 2.3 Cell lines.

Cell line	Relevant characteristic	Reference
293T cells	Permanent, established from primary embryonic human kidney	(Graham <i>et al.</i> , 1977)
HeLa	Permanent, derived from cervical cancer	(Nelson-Rees <i>et al.</i> , 1976)
MCF-7	Permanent, derived from breast adenocarcinoma	(Soule <i>et al.</i> , 1973)
CHO $\alpha_{IIb}\beta_3$	CHO-K1 stably expressing $\alpha_{IIb}\beta_3$	(Luo <i>et al.</i> , 2003)
CHO $\alpha_{IIb}\beta_3$ (N305T)	CHO-K1 stably expressing integrin mutant locked in the high affinity conformation by an N171 glycosylation site introduced into the interface between the β_3 A-like and hybrid 172 domains	(Luo <i>et al.</i> , 2003)

Table 2.4 Plasmids.

Plasmid	Relevant characteristics	Source
pQE30	<i>E. coli</i> cloning and expression vector, Amp ^R	Stratagene
pQE30 <i>isdB</i> _{SL}	pQE30 encoding residues 45 to 655 of IsdB	(Heilbronner, personal communications)
pQE30 <i>isdC</i> _{SL}	pQE30 encoding residues 30 to 290 of IsdC	- II -
pQE30 <i>isdJ</i> (45-610)	pQE30 encoding residues 45 to 610 of IsdJ	- II -
pQE30 <i>isdJ</i> (45-295)	pQE30 encoding residues 45 to 295 of IsdJ	This study
pQE30 <i>isdJ</i> (290-610)	pQE30 encoding residues 290 to 610 of IsdJ	- II -
pQE30 <i>isdK</i>	pQE30 encoding residues 35 to 426 of IsdK	(Heilbronner, personal communications)
pQE30 <i>isdA</i> (50-320)	pQE30 encoding residues 50 to 320 of IsdA	This study
pQE30 <i>isdB</i> _{SA} (45-480)	pQE30 encoding residues 45 to 480 of IsdB	- II -
pQE30 <i>isdB</i> _{SA} (45-290)	pQE30 encoding residues 45 to 290 of IsdB	- II -
pQE30 <i>isdB</i> _{SA} (270-480)	pQE30 encoding residues 270 to 480 of IsdB	- II -
pQE30 <i>isdB</i> _{SA} (45-480) (Y165A/H166E/Y167A)	Plasmid encoding IsdB (48-480) Y165A/H166E/Y167A mutant	- II -
pQE30 <i>isdB</i> _{SA} (45-480) (G203A/D204A)	Plasmid encoding IsdB (48-480) G203A/D104A mutant	- II -

Table 2.4 Plasmids, continued.

Plasmid	Relevant characteristics	Source
pQE30 <i>isdB</i> _{SA} (45-480) (N229A)	Plasmid encoding IsdB (48-480) N229A mutant	- II -
pQE30 <i>isdB</i> _{SA} (45-480) (N377A)	Plasmid encoding IsdB (48-480) N377A mutant	- II -
pGEX-4t-2	<i>E.coli</i> cloning and expression vector, Amp ^R	Amersham
pGEX-4t-2 <i>isdC</i>	pGEX4t-2 encoding residues 30- 190 of IsdC	This study
pRMC2	Chm ^R vector allowing aTc - inducible expression	(Corrigan <i>et al.</i> , 2009)
pRMC2 <i>isdA</i>	Chm ^R , plasmid for inducible expression of IsdA	This study
pRMC2 <i>isdJ</i>	Chm ^R , plasmid for inducible expression of IsdJ	- II -
pRMC2 <i>isdB</i> _{SA}	Chm ^R , plasmid for inducible expression of IsdB _{SL}	- II -
pNZ8048	<i>L. lactis</i> shuttle vector containing PnisA promoter and start codon in NcoI site, Chm ^R Allowing nisin- inducible expression of insert	(Kuipers <i>et al.</i> , 1998)
pNZ8048 <i>isdJ</i>	pNZ8048 encoding <i>isdJ</i> , for controlled expression in <i>L. lactis</i>	This study
pNZ8048 <i>isdA</i>	pNZ8048 encoding <i>isdA</i> insert, for controlled expression in <i>L. lactis</i>	- II -
pNZ8048 <i>isdB</i> _{SA}	<i>isdB</i> insert allows controlled expression of IsdB in <i>L. lactis</i> .	(Miajlovic, personal communications)
pKOR1	<i>E. coli</i> / <i>Staphylococcus</i> shuttle vector allowing allelic replacement in staphylococci	(Bae <i>et al.</i> , 2006)

Table 2.4 Plasmids, continued.

Plasmid	Relevant characteristics	Source
pKOR1 Δ <i>isdC</i> _{SL}	pKOR1 plasmid for in frame deletion of <i>isdC</i>	This study
pKOR1 Δ <i>isdK</i>	pKOR1 plasmid for in frame deletion of <i>isdK</i>	This study
pKOR1 Δ <i>isdB</i> _{SA}	For in-frame deletion of <i>isdB</i> for <i>S. aureus</i> SH1000	- II -
pKOR1 <i>isdB</i> _{SA} (Y165A/H166E/Y167A)	For introduction of Y165A/H166E/Y167A mutation into genomic <i>isdB</i>	- II -
pKOR1 <i>isdB</i> _{SA} (G203A/D204A)	Plasmid allowing introduction of G203A/D204A into genomic <i>isdB</i>	- II -
pKOR1 <i>isdB</i> _{SA} (N229A)	Plasmid allowing introduction of N229A point mutations into <i>isdB</i>	- II -
pKOR1 <i>isdB</i> _{SA} (N377A)	For introduction of N377A mutation into genomic <i>isdB</i>	- II -

Table 2.5 Primers.

Primer ^{a, b}	Sequence 5'-3'	Application ^{b, c}
1. Y165A F	ctgccgcatgataaaactgttgagttcc	IsdB _{SA} NEAT1 Y165A/H166E/Y167A
2. Y165A R	ctcaacagttttatcatgcggcaagttctg	
3. Y165A/H166E F	cagaacttgccgcttcataaaactgttgagttcc	
4. Y165A/H166E R	ggaaactcaacagttttatgaagcggcaagttctg	
5. Y165A/H166E/Y167A F	cagaacttgccgcttcggcaactgttgagttcc	
6. Y165A/H166E/Y167A R	ggaaactcaacagtttgcggaagcggcaagttctg	
7. G203A F	gcaacttttgcagcttcataaacttc	IsdB _{SA} NEAT1 G203A/D204A
8. G203A R	aagtttatgaagctgacaaaaagttgcc	
9. G203A/D204A F	gcaacttttggcagcttcataaacttc	
10. G203A/D204A R	aagtttatgaagctgccaaaaagttgcc	
11. Y440A F	gtattgtccatccgcatcaatcgtttttacg	IsdB _{SA} NEAT2 Y440A
12. Y440A R	gtaaaaacgattgatgcggatggacaatacc	
13. N377AF	accatatattttttccgcgcgacatacctgttttaatag	IsdB _{SA} NEAT2 N377A
14. N377AR	ctattaaaaacaggtatgctgcgcggcaaaaaatatatggtc	
15. N229AF	caattttaacagctttgttcccgtgatacagagaagcg	IsdB _{SA} NEAT1 N229A
16. N229AR	cgtctctctgtatcagcgggaacaaaagctgttaaaattg	
17. Y467AF	gatattgaccagacgagcctattgtggg	IsdB _{SL} NEAT2 Y467A
18. Y467AR	cccaacaataggctcgtctgtgtaataac	
19. Y191AF	ggagaaaattgtacttgccgcatgatagaactgaaggtcaccg	IsdB _{SL} NEAT1 Y189A/H190E/Y189A
20. Y191AR	cggtgaccttcagttctatcatgcggcaagtacaatttctcc	
21. Y191A/H190EF	ggagaaaattgtacttgccgcatggcggaactgaaggtcaccg	
22. Y191A/H190ER	cggtgaccttcagttcgcccatgcggcaagtacaatttctcc	
23. Y191A/H190E/Y189AF	ggagaaaattgtacttgccgcttcggcgaaactgaaggtcaccg	
24. Y191A/H190E/Y189AR	cggtgaccttcagttcgccgaagcggcaagtacaatttctcc	
25. <i>isdB</i> _{SA} 480 R	ggccatggatccgtaatacgaatgcaacgatgct	pQE30 <i>isdB</i> (45-480), (45-290), (270-480) (BamHI/PstI)
26. <i>isdB</i> _{SA} 45 F	ggccatggatccacaaatacagaagcacacacaaaa	
27. <i>isdB</i> _{SA} 290 R	ggccatcctgcagagtagcttccttcttagctga	
28. <i>isdB</i> _{SA} 270 F	ggccatggatccgcacaaccaatttataacag	
29. <i>isdJ</i> 45 F	ctgcaggatccgctgaagctaataccac	pQE30 <i>isdJ</i> (45-610) (BamHI/SacI)
30. <i>isdJ</i> 610 R	tccagtgtagctcatgaatgcatatgac	
31. <i>isdJ</i> 295 R	ctgcagagctcgttgtgtgtcttggc	pQE30 <i>isdJ</i> (45-295) (BamHI/SacI)

Table 2.5 Primers, continued.

Primer ^a	Sequence 5'-3'	Application ^{b, c}
32. <i>isdJ</i> 290 F	ctgcaggatccgccaaagcacaagcaa	pQE30 <i>isdJ</i> (290-610) (BamHI/SacI)
33. <i>isdC</i> 30 F	ggccatggatccggcagtcacttaatta	pGEX4t-2 <i>isdC</i>
34. <i>isdC</i> 190 R	ggccatgaattcttcaactgaagatcc	(BamHI/EcoRI)
35. Upstream F	ggggacaagtttgtacaaaaagcaggctccgtgttaacaatg catcattctgg	pKOR <i>IsdB</i> (in-frame deletion of <i>isdB</i> in <i>S. aureus</i> SH1000)
36. Upstream R	catgtttagaacaactcctaattg	
37. Downstream F	caattaggagttgttttacaacatgtaataaatcgtctttatattaa ttattaaattaac	
38. Downstream R	ggggaccactttgtacaagaaagctgggttagaggtgaagcca tatgaatggtcaatt	
39. R	ggggacaagtttgtacaaaaagcaggctacaatacagaagc acaacaaaa	pKOR <i>IsdB</i> (point mutations in <i>isdB</i> of <i>S. aureus</i> Newman)
40. F1	ggggaccactttgtacaagaaagctgggtagtagcttccttctta gctga	
41. F2	ggggaccactttgtacaagaaagctgggtgtaatacgaatgcaa cgatgct	
42. Upstream F	ggggacaagtttgtacaaaaagcaggcttggaggtgatgct tgtg	pKOR <i>ΔisdC</i> (in-frame deletion)
43. Upstream R	cacgcttatttgcctccttttc	
44. Downstream F	gaaaaggaggcaaaataagcgtgccactaataatgtctaagcg	
45. Downstream R	ggggaccactttgtacaagaaagctgggtgctgcttaacatgc gc	
46. Upstream F	ggggacaagtttgtacaaaaagcaggcttataactgtttaagca cgct	pKOR <i>ΔisdK</i> (in-frame deletion)
47. Upstream R	cattattagtggttaccttttc	
48. Downstream F	gaaaaggtaaccactaataatgtaacaaaatgaacatcagcaa	
49. Downstream R	ggggaccactttgtacaagaaagctgggtccaacaaccacagc aacatat	
50. <i>isdJF</i>	gcatggtaccgatacaggagacaaac	pRMC2 <i>isdJ</i>
51. <i>isdJR</i>	gcatggaattccgatattaacgttgttc	(KpnI/EcoRI)

Table 2.5 Primers, continued.

Primer^a	Sequence 5'-3'	Application^{b, c}
52. <i>isdA</i> F	gcatggtaccgtatccttaggagg	pRMC2 <i>isdA</i>
53. <i>isdA</i> R	gcatggaattccgagtgtgacgatg	(KpnI/EcoRI)
54. <i>isdB_{SA}</i> F	gcatggtaccgataatgattatcagttg	pRMC2 <i>isdB</i>
55. <i>isdB_{SA}</i> R	gcatgagctccgaatgcaacgatgc	(KpnI/SacI)
56. <i>isdJ</i> F	gccatggaaaaacatcaacgtatacaaaactaata	pNZ8048 <i>isdJ</i>
57. <i>isdJ</i> R	gcatgagctcgatattaacgttgttctttac	(NcoI/SacI)

^a (F) Forward and (R) Reverse primers are indicated, ^b Amino acid residues encoded by amplified DNA fragments are indicated, ^c Restriction enzymes used for cloning of amplified fragments are shown in brackets.

or 20 ng of genomic DNA were used as templates for PCR. Primers (IDT DNA Technologies) and dNTPs (Bioline) were used at final concentrations of 0.2 mM and 200 μ M, respectively. Primer sequences are listed in Table 2.5. Initial denaturation was carried out at 98 °C (30 sec) followed by 30 cycles of denaturation (10 sec) at 98 °C, 20 sec annealing (temperature dependent on primer used) and extension at 72 °C. When amplifying plasmid DNA, a 15 sec extension time per 1kb DNA was used. For high complexity genomic DNA a longer extension time was used (30 sec per kb). A final extension step was carried out at 72 °C for 5 min. PCR products were purified using Wizard SV gel and PCR clean-up system (Promega).

2.5.3 Site-directed mutagenesis.

Point mutations were introduced into pQE30*isdB*_{SA} and pQE30*isdB*_{SL} by sequential primer overlap mutagenesis with Phusion High-Fidelity Polymerase. Complementary forward and reverse primers incorporating the desired mutation were extended by thermal cycling to produce a mutated plasmid. Products were digested with *DpnI* for 1.5 h at 37°C to eliminate methylated, parental DNA and then transformed into competent *E. coli* strain XL-1 Blue or DC10B. Transformants were screened by restriction mapping and DNA sequencing.

2.6 Transformation.

2.6.1 Transformation of *E. coli*.

Plasmids were transformed into *E. coli* XL1-Blue, TOPP10 or DC10B cells which were made competent using the RbCl method (Hanahan, 1983). *E. coli* transformants were screened for the presence of recombinant plasmids using the rapid colony screening procedure developed by Le Gouill & Dery or the PCR colony method (Le Gouill *et al.*, 1991).

2.6.2 Electroporation of *S. aureus* and *S. lugdunensis*.

Plasmid DNA was purified from *E. coli* DC10B and electroporated into *S. aureus* or *S. lugdunensis* (Lofblom *et al.*, 2007). A starter culture was diluted in 50 ml of pre-warmed TSB to an OD₆₀₀ of 0.5 and grown for approximately 30 min at 37°C with shaking at 200 r.p.m. to reach an OD₆₀₀ of 0.8-0.9. The vessel was subsequently placed on ice. The cells were harvested by centrifugation (3900 × g for 10 min at 4°C) and washed four times with ice-cold dH₂O reducing the volume of dH₂O from 50 ml to

1 ml. After last harvest the pellet was resuspended with 250 μ l of sterile ice-cold 10% glycerol (v/v). 50 μ l aliquots were frozen and stored at -70 °C. Before electroporation aliquots were thawed on ice for 5 min and centrifuged at $5000 \times g$ for 1 min. The supernatant was discarded and the pellet resuspended in 50 μ l of transformation buffer (10% v/v glycerol, 500 mM sucrose). Up to 5 μ g of ethanol-precipitated plasmid was added to the cells. Electroporation was performed in 0.1 cm electroporation cuvettes (Biorad) with a pulse of 21 kV cm^{-1} , 100 Ω and 25 μ F. The cells were regenerated in 1 ml of TSB containing 500 mM sucrose at 37°C (pRMC2) or 28°C (pKOR1) with shaking for 1.5 h and then plated on TSA containing selective antibiotic(s). Transformants were screened by PCR, restriction mapping and Western immunoblotting.

2.6.3 Electroporation of *L. lactis*.

The protocol of Holo and Nes was adapted for transformation of *L. lactis* NZ9000 (Holo *et al.*, 1989). Overnight cultures of the NZ9000 in GM17 were diluted 1:100 into 5 ml of GM17 containing 500 mM sucrose and 2.5% glycine (w/v) (GS-GM17). This culture was inoculated into 50 ml of fresh GS-GM17 and grown overnight. The 50 ml culture was inoculated into 400 ml of fresh GS-GM17, grown to OD₆₀₀ of 0.3 and cells were subsequently harvested by centrifugation at $4000 \times g$ for 20 min at 4°C. The pellet was resuspended in 200 ml of ice-cold SGB (500 mM sucrose 10% w/v glucose, filter sterilized), centrifuged, resuspended in 100 ml SGB and left on ice for 15 min. The cells were centrifuged, resuspended in 50 ml SGB and left on ice for 15 min before a final centrifugation and re-suspension with 2 ml SGB. Cells were frozen at -70°C in 40 μ l aliquots. Cells were thawed on ice, mixed with 4 μ l of ethanol-precipitated ligated plasmid DNA and transferred to a 0.1 cm electroporation cuvette (Biorad). Cells were pulsed at 20 kV cm^{-1} , 200 Ω and 25 μ F, regenerated in 1 ml GM17 containing 2 mM CaCl₂ 20 mM MgCl₂ for 1.5 h and then plated onto GM17 agar containing 5 μ g ml⁻¹ Chm. Transformants were screened by plasmid restriction mapping and either dot immunoblotting or Western immunoblotting.

2.7 Allelic replacement mutagenesis.

Allelic exchange was performed by homologous recombination initiated by switching the bacterial growth temperature from that optimal for pKOR1 plasmid replication (28°C) to the non-permissive temperature of 42°C (Bae *et al.*, 2006). *S.*

aureus and *S. lugdunensis* strains containing pKOR1 plasmids (listed in Table 2.4) were grown at 28°C on TSA. Single colonies were streaked out on fresh TSA and grown with Chm at non-permissive temperature of 42°C. Only cells where plasmids integrated into the chromosome could replicate under this conditions. A big colony was inoculated into TSB and grown at 37°C to promote plasmid loss. The culture was platelet in dilutions on TSA and TSA containing 500 ng ml⁻¹ (for *S. aureus* SH1000) or 1 µg ml⁻¹ (for *S. aureus* Newman) of anhydrotetracycline (aTc). ATc induces production of antisense *secY* RNA which inhibits the growth of cells carrying the plasmid. TSA aTc plates were screened for DNA integration by colony PCR. Candidates were further validated by PCR amplification of the fragment using genomic DNA as a template following the fragment sequencing. Expression of proteins by generated mutants was investigated by Western immunoblotting.

2.7.1 Determination of haemolytic activity.

Qualitative evaluation of δ -haemolysin production was performed on sheep or horse blood agar as a surrogate for *agr* expression as proposed by Adhikari *et al.* (Adhikari *et al.*, 2007). Bacteria to be tested were streaked at a right angle to RN4220 and the plate incubated overnight. β -haemolysin forms a turbid zone of haemolysis surrounding RN4220. δ -haemolysin and β -haemolysin are synergistic resulting in a zone of clear hemolysis where they interact.

2.8 Cell fractionation.

Cultures of *S. lugdunensis* and *S. aureus* were harvested by centrifugation (4000 × g, 10 min), washed in phosphate buffer saline (PBS) and adjusted to an OD₆₀₀ of 20. 1 ml of the bacterial suspension was pelleted and resuspended in 200 µl of digestion buffer (50 mM Tris-HCl, 20 mM MgCl₂, 30 % (w/v) raffinose, pH 7.5) containing complete mini EDTA-free protease inhibitors (Roche). Cell wall proteins were solubilised by digestion with lysostaphin (400 µg ml⁻¹ for *S. lugdunensis* or 200 µg ml⁻¹ for *S. aureus*) at 37°C for either 15 min (*S. aureus*) or 30 min (*S. lugdunensis*). Protoplasts were harvested by centrifugation (5000 × g, 15 min) and the supernatant was retained as the cell wall fraction. Protoplast pellets were washed once in digestion buffer, sedimented (5.000 × g, 15 min) and resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5) containing protease inhibitors and DNase (80 µg ml⁻¹). Protoplasts were lysed on ice by vortexing. Complete lysis was confirmed by phase contrast

microscopy. The membrane fraction was obtained by centrifugation at $18500 \times g$ for 1 h at 4°C . The pellet (membrane fraction) was washed once and resuspended in ice-cold lysis buffer. Both fractions were centrifuged again under the same conditions, the pellets resuspended in 200 μl of lysis buffer and retained as the membrane fraction.

Fractionation of *L. lactis* was performed as described before, using lysozyme and mutanolysin to digest bacterial cell wall (Crow *et al.*, 1992; Smith *et al.*, 2012). Fractions were separated by SDS-PAGE and subjected to Western immunoblotting for detection of proteins.

2.9 SDS-PAGE.

Protein samples were diluted (1:1) in sample buffer (125 mM Tris-HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) β -mercaptoethanol and 0.002 % (w/v) bromophenol blue) and boiled for 5 min. 20 μl volumes of the diluted protein samples were loaded onto acrylamide gels (4.5 % stacking and 10 or 15 % separating gel) and separated by SDS-PAGE (Laemmli, 1970). Electrophoresis was carried out at 120 V after which proteins were visualised by Coomassie blue staining or electroblotted onto PVDF membranes (Roche) for Western immunoblotting.

2.9.1 Peroxidase staining.

TMBZ, a chromogen that turns blue in areas of haem-associated peroxidase activity, was used for staining of gels to detect haem containing proteins. The procedure was performed as described by Thomas *et al.* After electrophoresis, the gels were fixed in the dark for 1 h in a pre-chilled solution of 6:3:1 sodium acetate (0.25 M, pH 5.0) - methanol - H_2O . TMBZ was prepared fresh for each usage at a concentration of 6.3 mM in 20 ml of absolute methanol. The TMBZ solution was added to 70 ml of cold sodium acetate (0.25 M, pH 5.0) and 10 ml of H_2O . The gels were stained in this solution in a shaking ice bath for 35 min in darkness. Hydrogen peroxide was added to the staining solution to a final concentration of 30 mM and incubated for another 30 min to allow color development. Gels were washed in acetate-buffered isopropanol at a ratio of 8 : 2 and dried immediately (Thomas *et al.*, 1976). Digital pictures of stained gels were taken using ImageQuant LAS imager (GE Healthcare).

2.10 Western and ligand affinity immunoblotting.

Proteins or cellular fractions were separated by SDS-PAGE and electroblotted onto PVDF membranes (Roche) for 1 h at 100 V using a wet transfer cell (Bio-Rad). Membranes were incubated for 1 h at 37°C, or overnight at 4°C in TS buffer (10 mM Tris - HCl, pH 7.4, 150 mM NaCl) containing 10 % (w/v) skimmed milk (Marvel). For detection of proteins, primary antibodies (listed in Table 2.1), fibrinogen, biotinylated fibronectin or rIlsdB were diluted in 10% (w/v) Marvel TS and incubated with the membranes for 1 h at room temperature with shaking. Unbound reagent was removed by three 15 min washes with TS buffer. The HRP - conjugated secondary antibody/reagent was incubated with the membranes for 1 h at room temperature with shaking. Unbound secondary reagent was removed by washing three times with TS buffer and bound HRP developed with chemiluminescent substrate (LumiGlo; New England Biolabs) in ImageQuant LAS imager (GE Healthcare).

2.11 Whole-cell and protein dot immunoblotting.

Bacterial cells were washed twice in PBS and adjusted to an appropriate optical density (as indicated in figure legends). Each doubling dilution (5 µl) of the bacterial suspension or soluble protein (5 µl) was dotted onto a nitrocellulose membrane (Protran). The membranes were blocked for 1 h with Marvel TS buffer followed by blocking and detection performed as for Western immunoblotting described above.

2.12 In silico structural modelling.

Structural models were obtained using the PHYRE website (Kelley *et al.*, 2009) (Imperial College London, Structural Bioinformatics Group [<http://www.sbg.bio.ic.ac.uk/~phyre/>]) and analyzed using the molecular modelling UCSF Chimera software (University of California, Resource for Biocomputing Visualisation and Informatics [<http://www.cgl.ucsf.edu/chimera/>]).

2.13 Purification of recombinant proteins.

Recombinant proteins from *S. lugdunensis* or *S. aureus* were expressed from pQE30 with an N-terminal hexa-histidine (His₆) affinity tag to allow purification by nickel affinity chromatography or from pGEX4t-2 with a glutathione transferase (GST) fusion protein tag to allow purification by a glutathione affinity chromatography. The vectors are designed with sequences that encodes for 6 x His residues or GST protein

located 5' to the MCS. Both expression plasmids contain an IPTG-inducible promoter for controlled expression of recombinant proteins.

The pQE30 and pGEX4t-2 plasmids, listed in Table 2.4, were transformed into the protease-defective *E. coli* TOPP10 strain for large-scale expression and purification. Cultures were grown to an OD_{600 nm} of 0.6 - 0.8 and then induced with 1 mM IPTG for 3 h at 37°C. Cells were harvested by centrifugation and resuspended in 30 ml PBS containing EDTA protease inhibitors (Roche) prior to cell rupture in a French pressure cell. The lysate was centrifuged at 18000 × g for 20 min. DNase was added to the supernatant which was filtered through a 0.45 µm Sartorius filter before being subjected to affinity chromatography.

2.13.1 Nickel-affinity protein purification.

A HiTrapTM Chelating HP column (5 ml; Amersham) was equilibrated in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and then charged with 150 mM Ni²⁺. The filtered, cleared cell lysate was applied to the column. The column was washed with binding buffer until the A_{280 nm} of the eluate was < 0.001. Bound protein was eluted from the column with a continuous linear gradient of imadazole (5-100 mM; total volume of 100 ml) in 0.5 M NaCl and 20 mM Tris-HCl (pH 7.9). Eluted protein was collected in 3 ml fractions. Elution was monitored by measuring the A_{280 nm} of the eluate. Peak fractions were analysed by SDS-PAGE. Ten µl of each fraction was separated on 10 % (v/v) acrylamide gels and visualized by Coomassie blue staining. Fractions containing the purified recombinant protein were dialysed against PBS for 16 h at 4°C, concentrated by ultrafiltration (Amicon Ultra, EMD Millipore) and stored at -70°C.

2.13.2 GST-affinity protein purification.

Glutathione - affinity chromatography using 5 ml GSTrap FF columns (GE Healthcare) was performed as described previously (O'Connell *et al.*, 1998). The filtered, cleared cell lysate was applied to the column after its equilibration with 25 ml of PBS. The column was washed with 100 ml of PBS or until the A_{280 nm} of the eluate was < 0.001. GST tagged protein was eluted from the column with 50 ml of elution buffer (50 mM Tris-HCl, 10 mM glutathione, pH 8.0). Eluted protein was collected and dialysed against PBS for 16 h at 4°C concentrated by ultrafiltration (Amicon Ultra, EMD Millipore) and stored at -70°C.

2.14 Haem content.

The absorbance spectra of recombinant proteins were recorded on a Shimadzu UV-160PC spectrophotometer. The haem content of the proteins was estimated from the reduced pyridine haemochrome spectra (Smith, 1975). Protein concentrations were estimated by BCA Protein Assay Reagent (Thermo Scientific Pierce).

2.15 Haemin transfer from rIsdB to rIsdC.

Recombinant IsdB, purified from *E. coli* grown in RPMI, was gradually supplemented with porcine haemin dissolved in DMSO at 10 mM. Insoluble material was removed by centrifugation ($5000 \times g$, 5 min). The mixture was loaded onto calibrated Sephadex G-25 (GE Healthcare) and chromatography was performed to separate the saturated IsdB from unbound haemin. IsdB (haemin donor) and IsdC - GST (haemin acceptor) were mixed at 5 μ M and 20 μ M, respectively. After 4 h incubation at 22°C the mixture was loaded onto a GST column to separate GST-tagged IsdC from IsdB. Separation of the two proteins was monitored by SDS-PAGE. The spectra of the separated donor and acceptor were recorded to assess the haemin transfer.

2.16 Enzyme-linked immunosorbent assay (ELISA).

2.16.1 Recombinant Isd proteins binding to haemoproteins.

Haemoglobin-haptoglobin (Hb-Hpt) complexes were prepared by mixing human plasma haptoglobin (Molecular Innovations) with human haemoglobin (Pointe Scientific) at a 1 : 1 molar ratio for 1 h at 22 °C. Haemoprotein binding assays were performed in two ways.

(i) Microtitre plates (Nunc) were coated with recombinant Isd proteins at 2 μ M in 100 μ l of 50 mM sodium carbonate buffer, pH 9.5, overnight at 4°C. Coated wells were washed three times with PBS and blocked with 5% (w/v) Marvel in PBS for 2 h at 37°C. Following three washes with PBS, various concentrations of methaemoglobin (MetHb), haemoglobin (Hb), Hb-Hpt complexes or haptoglobin (Hpt) were added to the wells in 2% Marvel PBS and incubated for 2 h at 37°C. Wells were washed with PBS and bound proteins detected by incubation with rabbit anti-human Hb serum or rabbit anti – human Hpt serum in 2% (w/v) Marvel PBS for 1 h at 37°C.

(ii) Reciprocal binding experiments were performed by coating microtitre (Nunc) wells with MetHb, Hb, Hb-Hpt, Hpt or myoglobin at 1 μ M in 100 μ l of 50 mM

sodium carbonate buffer, pH 9.5 overnight at 4 °C. After blocking with 5% (w/v) Marvel PBS, the wells were incubated with increasing amounts of recombinant Isd proteins in 2% (w/v) skimmed milk in PBS for 2 h at 37 °C. Bound proteins were detected by incubation with appropriate polyclonal rabbit anti-Isd IgG in 2% (w/v) Marvel PBS for 1 h at 37°C and washed three times with PBS. To detect specific interactions in both types of assays, HRP-conjugated goat anti-rabbit IgG in 2% (w/v) Marvel PBS was added for 1 h at 37°C. After three washes, 100 µl of TMBZ at 0.1 mg ml⁻¹ prepared in 0.05 M phosphate citrate buffer containing 0.006% (v/v) hydrogen peroxide was added, and plates were developed for 5 min. The reaction was stopped by the addition of 50 µl of 2 M H₂SO₄ to each well. Plates were read at 450 nm using a Multiscan ELISA plate reader. Half-maximum binding values reported for all ELISA-type binding assays were predicted using GraphPad Prism software.

2.16.2 Binding of recombinant Isd proteins binding to fibrinogen, lorcrin and cytokeratin 10.

Human GST tagged - loricrin (1 µM), GST-cytokeratin 10 (1 µM) (Walsh *et al.*, 2004), GST (1 µM) and human fibrinogen (10 µg ml⁻¹) (Calbiochem) in 50 mM sodium carbonate buffer, pH 9.5 were coated onto microtiter wells (Nunc) overnight at 4°C. Plates were blocked with 5% (w/v) BSA in PBS for 2 h at 37 °C. Following three washes with PBS, various concentrations of recombinant IsdJ in 1% (w/v) Marvel PBS were added and incubated at 37°C for 2 h. After three washes with PBS, bound protein was detected by incubation with rabbit anti-IsdJ IgG in 1% (w/v) Marvel PBS at 37°C. After 1 h incubation the wells were washed three times with PBS followed by incubation with protein A- peroxidase conjugated to HRP in 1% (w/v) Marvel. After incubation for 1 h at 37 °C wells were washed three times with PBS, and bound HRP detected with TMBZ as described above. Purified human GST-loricrin and GST-cytokeratin 10 were kindly provided by Michelle Mulcahy.

2.16.3 Purified $\alpha_{IIb}\beta_3$ binding to recombinant IsdB variants.

Purified human $\alpha_{IIb}\beta_3$ (20 µg ml⁻¹; Enzyme Research Laboratories) in 50 mM sodium carbonate buffer, pH 9.5 was coated onto microtiter wells (Nunc) for 2 h at 37 °C. Plates were blocked with 5% BSA in PBS for 2 h at 37 °C. Following three washes with PBS, various concentrations of recombinant IsdB in 1% (w/v) Marvel PBS were added and incubated at 37 °C for 2 h. After three washes with PBS, bound IsdJ was

detected by incubation with rabbit anti-IsdB IgG in 1% (w/v) Marvel PBS at 37 °C. After 1 h incubation the wells were washed three times with PBS followed by incubation with protein A-peroxidase conjugated to HRP in 1% (w/v) Marvel. After incubation for 1 h at 37 °C wells were washed three times with PBS, and bound HRP detected with TMBZ as described above.

2.17 Surface Plasmon Resonance (SPR).

SPR analysis was used to study (i) rIsdB binding to haemoglobins and methaemoglobins and (ii) rIsdJ binding to loricrin and fibrinogen. The experiments were performed using the BIAcore X100 system (GE Healthcare). His-tagged recombinant IsdB and IsdJ proteins were captured and amine-coupled to monoclonal anti-His antibodies on a flow cell. The antibodies at 50 µg ml⁻¹ in sodium acetate buffer, pH 5.0 had been previously covalently immobilized on both flow cells of CM5 chip, using amine coupling. This was performed according to manufacturer's protocol, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, followed by N-hydroxysuccinimide (NHS) and ethanolamine. Increasing concentrations of analytes, such as methaemoglobins, haemoglobins, fibrinogen or loricrin in running buffer (PBS, pH 7.4, 0.005% v/v surfactant P₂₀) were flowed over immobilized IsdB and a reference flow cell (coated only with anti-His antibodies) at a rate of 5 µl min⁻¹. The sensorgram data presented were subtracted from the corresponding data on the reference flow cell and from the response generated by injection of buffer over the chip. Affinity analysis (BIAevaluation software 3.0) was used to determine the dissociation constants (K_D) for the interactions of IsdB with haemoglobins and methaemoglobins while kinetics analysis was performed to estimate the K_D of rIsdA interaction with fibrinogen and loricrin. Purified human and mouse haemoglobins were kindly provided by Dr Gleb Pishchany and Dr Eric Skaar.

The SPR analysis was also used to study purified $\alpha_{IIB}\beta_3$ binding to rIsdB. The $\alpha_{IIB}\beta_3$ (Enzyme Research Laboratories) at 100 µg ml⁻¹ in 10 mM sodium acetate, pH 4.5 supplemented with 1 mM MnCl₂, 1 mM MgCl₂ and 1 mM CaCl₂ was covalently immobilized on a flow cell of CM5 sensor chips using amine coupling at a flow rate of 30 µl min⁻¹ in HEPES-buffered saline achieving high level of integrin immobilized at 11000 RU. The second flow cell was treated the same way but without $\alpha_{IIB}\beta_3$ to provide an uncoated reference. Increasing concentrations of rIsdB were passed over the surface at a rate of 5 µl min⁻¹. The sensorgrams were subtracted from the corresponding data on

the reference flow cell and from the response generated by injection of buffer over the chip. Data were analysed by fitting the sensorgrams to a kinetics model of 1: 1 binding.

2.18 Bacterial adherence to immobilized ligands.

Microtiter plates were coated with doubling dilutions of fibrinogen, GST-cytokeratin 10 and or GST in PBS overnight at 4°C. Control wells contained PBS only. After washing in PBS, the plates were blocked for 2 h at 37 °C with 5% (w/v) BSA in PBS. After washing three times, 100 µl of a cell suspension (OD₆₀₀ of 1 in PBS) was added to each well, and the plates incubated for 2 h at 37°C. After washing three times, adherent cells were fixed by adding 100 µl of 25% (v/v) formaldehyde and incubating at room temperature for at least 20 min. The plates were then washed and stained with crystal violet for 2 min. After several washes, 100 µl of 5% (v/v) acetic acid was added to the wells to solubilise the crystal violet. Absorbances were read with a Multiscan ELISA plate reader at 570 nm.

2.19 Bacterial killing by linoleic acid.

Bacterial survival assays were performed as described previously (Clarke *et al.*, 2007). Bacteria were harvested by centrifugation and washed twice in sterile dH₂O. Cell suspensions were adjusted to 1×10^8 cfu ml⁻¹ in dH₂O and incubated at 37°C with linoleic acid at 10 µg ml⁻¹. The cfu was determined at 0, 30 and 60 minutes by viable counting.

2.20 Preparation of platelet rich plasma.

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

2.20.1 Preparation of washed platelets.

Blood was drawn into a syringe containing acid-citrate-dextrose (ACD, 25 mM citric acid, 75 mM Na citrate, 135 mM D-glucose). Following preparation of platelet

rich plasma (as described above) the pH of the platelets was adjusted to 6.5 using ACD. Prostaglandin E1 (at 1 μ M) was added to prevent activation of platelets during centrifugation. PRP was centrifuged at $720 \times g$ for 10 min. The supernatant was carefully removed and discarded. One ml of JNL buffer (6 mM dextrose, 130 mM NaCl, 9 mM NaCl₂, 10 mM Na-Citrate, 10 mM Tris, 3 mM KCL, 0.8 mM KH₂PO₄, and 0.9 mM MgCl₂, pH 7.4) was layered on top of the platelet pellet and removed carefully so as not to disturb the pellet. The platelet pellet was resuspended in 1 ml of JNL and then diluted to obtain a platelet count of 3×10^8 platelets ml⁻¹. Washed platelets were supplemented with 2 mM CaCl₂.

2.21 Platelet, 293T and HeLa cells adhesion assays.

Cultures of bacterial strains were harvested by centrifugation, washed in PBS and resuspended to an OD₆₀₀ nm of 1. Microtitre plates (Nunc) were coated with the bacterial suspensions (100 μ l per well) in triplicate and plates were incubated for 2 h at 37°C. Controls including 1% BSA (w/v), human fibrinogen and fibronectin (both at 50 μ g ml⁻¹) were also immobilized in the wells. Following incubation, microtitre wells were washed 3 times with PBS. 1% (w/v) BSA (100 μ l) was added to each well and plates were incubated for 90 min at 37 °C. Wells were three times with 100 μ l of JNL (platelets) or PBS (293T or HeLa cells) buffers. Washed platelets (50 μ l), 293T or HeLa cells (100 μ l), at 2×10^6 were added to wells and incubated at 37 °C for 60 min and subsequently washed three times with JNL (platelets) or PBS (293T or HeLa) buffers containing 1.8 mM CaCl₂. Adherent platelets were detected by using a lysis buffer containing a substrate for acid phosphatase (100 mM Na acetate, 0.1 % v/v Triton-X-100, 10 mM *p*-nitrophenol phosphate. Plates were incubated in the dark for 2 h at 37°C. Sodium hydroxide (20 μ l of 1 M) was added to each well to stop the reaction and absorbance at 405 nm was read in an ELISA plate reader (Labsystems).

2.22 Binding of fluorescently labelled recombinant proteins to platelets.

Fluorescein isothiocyanate (FITC) labelling of the recombinant proteins was performed using FluoReporter FITC Protein Labeling Kit (Invitrogen) according the manufacturer's instructions. To activate platelets, Thrombin Receptor Activating Peptide (TRAP; Sigma) at 10 μ M was added to a platelet suspension immediately before the experiment. 2×10^6 of resting or TRAP - activated platelets were incubated

with equimolar amounts of proteins (200 nM) for 30 minutes at 37°C. Bound IsdB was detected by flow cytometry.

2.23 Platelet aggregation.

S. aureus cells were grown to stationary phase in RPMI medium. Cells were washed twice with PBS and resuspended to an OD₆₀₀ nm of 1.6. Bacterial cells (25 µl) were added to 225 µl of PRP in siliconized flat-bottom glass cuvettes (BioData). PRP and bacteria were incubated with stirring (900 r.p.m) in an aggregometer (Bio-Data) at 37° C. Light transmission was monitored for 25 min.

2.23.1 Luciferase assay.

Luciferase catalyzes luciferin oxidation using ATP-Mg²⁺ as a co-substrate causing light emission. Luciferin was added to samples of platelet rich plasma to determine the levels of ATP released from dense granules by activated platelets. Changes in luminescence were measured by a SpectraMax Luminescence plate reader. Obtained values were compared with generated controls either untreated or treated with Thrombin Receptor Activating Peptide, which induces rapid platelet activation.

2.24 Gentamicin/lysostaphin protection assay.

HeLa or 293T cells were seeded into 6-well invasion plates at 1.5×10^6 cells per well in DMEM with 10% (v/v) FBS 24 h prior to the experiment and incubated at 37 °C in 5% (v/v) CO₂. Confluent monolayers that formed overnight were washed twice before the experiment with 2 ml of RPMI with or without 10% (v/v) FBS serum (as stated). Bacteria were grown for 18 h in RPMI or differently if stated, harvested by centrifugation (4000 × g, 10 min), washed with RPMI and diluted in RPMI to the concentration of 6×10^7 cfu ml⁻¹. The infection step was performed by adding 1 ml of this bacterial suspension to each well and incubating at 37 °C in 5% CO₂ (v/v) for 2 h. After 2 h infection bacterial suspensions were removed from each well of the invasion plate and 2 ml of killing medium (DMEM with 10% v/v FBS supplemented with 100 µg ml⁻¹ gentamicin and 20 µg ml⁻¹ of lysostaphin) was added to each well and incubated for 1 h at 37 °C in 5% (v/v) CO₂. After 1 h the killing medium was removed and each well was washed with 2 ml of PBS. 1 ml of lysing solution (1% TritonX-100 v/v, 0.1% w/v SDS in PBS) was added into each well and pipetted up and down for 10 min before being collected and plated on agar to estimate the viable count of intracellular bacteria.

The percentage internalization represents the ratio of intracellular bacteria compared to the inoculum. The inoculum was also incubated at 37 °C in 5% (v/v) CO₂ for 2 h before being plated on agar.

2.25 Real-time cell adhesion and spreading analysis.

The xCELLigence system was used according to manufacturer's instructions. The following system components were used: a Real-Time Cell Analyzer (RTCA) computer with integrated software, a station for the RTCA plates and disposable E-plates. E-plates were inserted into RTCA stations and remained in a standard tissue culture incubator. The E-plates are microtiter plates with glass bottoms containing capillary gold electrodes covering each well. They allow sensitive detection of the attachment of cells with relatively uniform distribution of the electric field. The voltage applied on electrodes during RTCA measurements was approximately 20 mV. The impedance was measured at three different frequencies (10, 25 or 50 kHz). The baseline was measured after only buffer was added to wells in the absence of cells. Subsequently cells are added to the wells and may attach causing an increase in the electrical impedance. The proliferation or spreading of the cells results in further increases of the impedance. The xCELLigence calculates these changes as dimensionless values called Cell Index (CI).

Cell attachment and spreading on immobilized ligands was performed as follows. 100 µl of soluble ligands in PBS were immobilized in E-plate wells at 37 °C for 2 h. The wells were washed three times with PBS and blocked with 100 µl of 3 % (w/v) BSA PBS solution for 2 h at 37 °C. Wells were washed three times with PBS and 100 µl of PBS (CHO-K1 cells) or medium (MCF-7 cells) was added to each well and the baseline impedance was measured. (Various cell lines were being prepared in the meantime). Detached cells were washed in PBS and adjusted to 2×10^5 cells ml⁻¹ in PBS with 2 mM CaCl₂ (CHO-K1 cells) or medium supplemented with recombinant proteins, IsdB at 5 µM or RGD 20 µM or BSA at 3% (w/v). After the baseline was measured, 100 µl of cell suspensions were added to wells and measurement continued over time. The xCELLigence software was used to obtain an average of measurements performed in triplicate, the traces of impedance versus time and the slopes changes (slope/h).

2.26 *In silico* structural modelling.

Structural models were obtained using the PHYRE website (Kelley *et al.*, 2009) (Imperial College London, Structural Bioinformatics Group [<http://www.sbg.bio.ic.ac.uk/~phyre/>]) and analyzed using the molecular modelling UCSF Chimera software (University of California, Resource for Biocomputing Visualisation and Informatics [<http://www.cgl.ucsf.edu/chimera/>]).

2.27 Statistical analysis.

The data presented in this study are the mean of three experiments $\pm$ SD, unless otherwise stated. Statistical analysis was performed using Prism software. The unpaired, two-tailed t-test was used to determine the significance of differences in adhesion of human cells, internalization of the bacteria by human cells or in bacterial growth on haemoglobin as the source of iron, with significance defined as * $P < 0.05$ or ** $P < 0.03$.

Chapter 3

Interactions of iron regulated surface determinant B of *S. aureus* with platelets

3.1 Introduction.

Platelets are small anucleate cells which in their ordinary (resting) state circulate in the blood vessels. Upon being stimulated platelets undergo intracellular signaling events that lead to simultaneous conformational changes of their integrin receptors and release of intracellular granules the contents of which mediate further transition of platelets from the resting to the activated state. Activated platelets use their active integrins to interact with each other *via* plasma proteins, such as fibrinogen, von Willebrand factor (vWf), fibronectin, vitronectin and thrombospondin forming an effective plug at site of injury which is reinforced by conversion of fibrinogen to fibrin through coagulation cascade.

Platelets play a key role in bloodstream infections by bacteria as demonstrated by experimental and clinical studies (Sjoberg *et al.*, 2002; Sullam *et al.*, 1996). The ability of *S. aureus* and other bacteria to adhere to and induce platelet aggregation is thought to contribute importantly to the development of endovascular infections such as the infective endocarditis (IE) (Fitzgerald *et al.*, 2006a). IE is characterised by the build-up of vegetative bodies on heart valve surfaces which contain clusters of bacteria, fibrin and aggregated platelets (Moreillon *et al.*, 2004). This form of vegetation facilitates colonization and isolates the bacterium from the immune system, since neutrophils do not penetrate thrombi (Fitzgerald *et al.*, 2006a). *Staphylococcus aureus* is among the leading causes of community-acquired as well as healthcare-associated and hospital-acquired endocarditis (Baddour *et al.*, 2005; Kern, 2010).

The interactions of *S. aureus* with platelets involve multiple factors. Several surface-expressed proteins of *S. aureus* have been shown to mediate adhesion to platelets resulting in their activation and aggregation. These factors include: the fibrinogen binding proteins ClfA and ClfB, the bi-functional fibronectin-fibrinogen binding proteins FnBPA and FnBPB (Fitzgerald *et al.*, 2006a; Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005; Miajlovic *et al.*, 2007; O'Brien *et al.*, 2002a). *S. aureus* utilizes these proteins to attach to the abundant platelet integrin $\alpha_{IIb}\beta_3$ *via* fibrinogen or fibronectin bridges. Rapid platelet activation and aggregation also requires specific serum IgG to crosslink the bacterial molecule with the platelet Fc γ RIIa. It is thought that clustering of Fc γ RIIa initiates the signal transduction cascade that causes the subsequent activation and aggregation of platelets. Activation therefore depends on the presence of specific antibodies that are present in the sera of most of the population

(Dryla *et al.*, 2005). Slower, alternative platelet activation involves IgG engaging FcγRIIa and complement being recognized by complement receptor (CR) on platelets.

Lactococcus lactis has been used as a surrogate host to express surface proteins of *S. aureus* and study them individually. When expressed on the surface of *L. lactis*, ClfA, FnBPA and FnBPB were all capable of inducing rapid platelet aggregation with lag times of 1-2 min. *L. lactis* expressing ClfB caused platelet aggregation with the average lag time of 7 min. SdrE was also able to cause aggregation with an extended lag time (13.6 min). Although protein A was not capable of inducing platelet aggregation when expressed from *L. lactis*, it enhanced aggregation mediated by *S. aureus* (O'Brien *et al.*, 2002a). The studies which characterised these interactions were carried out using aggregometry. More recently under high shear rates mimicking the *in vivo* conditions in arterioles Spa and ClfA were shown to induce platelet aggregation (Kerrigan *et al.*, 2008).

The platelet glycoprotein $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) is a major inducible receptor for adhesive proteins with about 80,000 copies per cell. It is one of the best characterized members of the large family of eukaryotic heterodimeric surface receptors called integrins. On the surface of resting platelets the integrin exists in a non-adhesive state. As a consequence of platelet activation triggered by platelet agonists such as thrombin that are produced from the activated coagulation cascade upon vascular injury, integrin $\alpha_{IIb}\beta_3$ rapidly switches from a low-affinity to a high-affinity ligand- binding conformation. The active integrin binds dimeric fibrinogen molecules on adjacent platelets forming aggregates. Following receptor occupancy, the binding information is transduced across the plasma membrane triggering $\alpha_{IIb}\beta_3$ clustering (Fig. 1.12) (Hantgan *et al.*, 2003). Transmembrane domain-mediated oligomerization of the integrin $\alpha_{IIb}\beta_3$ promotes and stabilizes the integrin - mediated association between extracellular matrix proteins and the tightly structured intracellular membrane skeleton. This is called avidity regulation and provides the physical link for the retraction of the fibrin clot and the arrest of bleeding.

The interaction of the iron regulated surface determinant B (IsdB) with platelets was discovered by Miajlovic *et al.* (Miajlovic *et al.*, 2010). *S. aureus* Newman has a nonsense mutation in the *fnbA* and *fnbB* genes and does not express FnA and FnB (Grundmeier *et al.*, 2004). A mutant deficient in the proaggregatory factors ClfA and ClfB could still adhere to platelets and cause platelet aggregation when grown in iron-limited medium (Miajlovic *et al.*, 2010). IsdB was identified as the factor mediating the

interaction with platelets as a Newman *clfA clfB isdB* mutant could not bind to platelets or cause their aggregation (Miajlovic *et al.*, 2010).

Expression of IsdB from a plasmid introduced into Newman *clfA clfB isdB* resulted in partial restoration of the ability to adhere to platelets. However platelet aggregation could not be restored (Miajlovic *et al.*, 2010). Studies using heated plasma or gel filtered platelets (GFP) supplemented with purified fibrinogen demonstrated that the IsdB-mediated platelet aggregation does not require complement factors. Strain Newman *clfA clfB isdB* was unable to cause activation of GFP supplemented with fibrinogen alone or fibrinogen and IgG together (Miajlovic *et al.*, 2010).

Various antagonist and monoclonal antibodies against platelet receptors FcR11a, GP1b or $\alpha_{IIb}\beta_3$ were tested for inhibition of adhesion of washed platelets to immobilized iron-starved Newman *clfA clfB*. Inhibition was observed with monoclonal anti - $\alpha_{IIb}\beta_3$ antibody (abciximab), synthetic RGD peptide and tirofiban, a specific inhibitor of the integrin which suggested a direct interaction between IsdB and the platelet integrin (Miajlovic *et al.*, 2010).

This study investigated IsdB-mediated adhesion of *S. aureus* to platelets. Point mutations were introduced within the *isdB* gene to allow expression of IsdB mutants on the bacterial cell in attempt to identify the integrin binding site. Recombinant IsdB proteins were used to study the interaction with the purified platelet integrin $\alpha_{IIb}\beta_3$. Recombinant IsdB was tested for binding to resting and activated washed platelets as well as CHO cells expressing platelet $\alpha_{IIb}\beta_3$ variants. In order to identify the integrin conformation required for binding.

3.2 Results.

3.2.1 Site-directed mutagenesis on the *isdB* gene of strains *S. aureus* Newman *clfA clfB* for use in platelet adherence and aggregation studies.

In order to determine if residues that are known to be important in binding to haemoglobin and haem are also involved in binding to platelet integrin, site directed mutagenesis of the chromosomal *isdB* gene was employed. The Protein Homology/analogY Recognition Engine (PHYRE) was used to obtain three dimensional models of IsdB NEAT1 and IsdB NEAT2 domains, based on their homology to available crystal structures of the IsdH NEAT1 and the IsdA NEAT domains respectively. Chimera molecular modelling software was used to visualise the 3D crystal structures (Fig. 3.1).

Residues considered for substitution were either conserved amino acids previously shown to be critical for ligand binding by *S. aureus* Isd proteins or motifs which were similar to those important for interactions with integrins. They all were localized in loops exposed on the surfaces of the models of NEAT1 and NEAT2. The residues that were changed were: (i) the (Y165A/H166E/Y167A) within the loop β 1b- β 2 on NEAT1 domain. These hydrophobic residues are critical for haemoglobin binding by the IsdH NEAT domain (Pilpa *et al.*, 2006; Visai *et al.*, 2009), (ii) (G203A/D204A) within loop β 4- β 5 on the NEAT1 domain being a variant of the RGD motif on fibrinogen, fibronectin or von Willebrand factor recognized by the ‘RGD integrins’, like $\alpha_{IIb}\beta_3$ on platelets, (iii) (N229A) within the loop β 6- β 7 on the NEAT1 domain as well as (iv) (N377A) within loop β 2- β 3 exposed on the surface of the NEAT2 domain. These are part of the NG(R) motif predicted to be important for fibrinogen binding to $\alpha_{IIb}\beta_3$ (Kerrigan S.W., personal communications) and (v) (Y440A) within the loop β 2- β 3. This Tyr co-ordinates haem by forming a hydrogen bond with Tyr450 located within loop β 3- β 4 and holds the porphyrin ring within the pocket (Grigg *et al.*, 2007). Altered residues are shown in Figure 3.1.

The mutagenesis procedure used to introduce altered employed PCR-based protocol described in the Chapter 2.5.3 and primers listed in Table 2.5. The template for mutagenic PCR was plasmid pQE30*isdB* (45-480). Mutated plasmids were cloned into *E. coli* and the presence of mutations was identified by DNA digestion and confirmed by DNA sequencing. To improve screening of recombinant strains, novel restriction sites (BglI, XhoI and Acil) were introduced within the DNA sequence in the region of the mutation (Fig. 3.2). Digestion of amplified *isdB* from the mutated plasmids with those enzymes resulted in specific restriction pattern (Fig. 3.2A, data not shown).

In order to introduce the point mutations into the *S. aureus* chromosome, DNA fragments containing changed sequences and flanking regions of at least 400 bp upstream and downstream from the altered nucleotides were amplified and cloned into pKOR1 vector, which was then electroporated into Newman *clfA clfB* and SH1000 *fnbA fnbB clfA clfB*. Allelic replacement was performed as described in Chapter 2.7. It was necessary to introduce the mutations into the chromosomal *isdB* gene since attempts to complement the *isdB* mutant from the multi-copy plasmid pCUI*isdB* were unsuccessful (Miajlovic H., unpublished). Expression of the mutant proteins was confirmed using Western immunoblotting (Fig. 3.2B, data not shown) The amino acid substitution mutants had similar levels of δ -toxin expression as the mother strain Newman *clfA clfB*

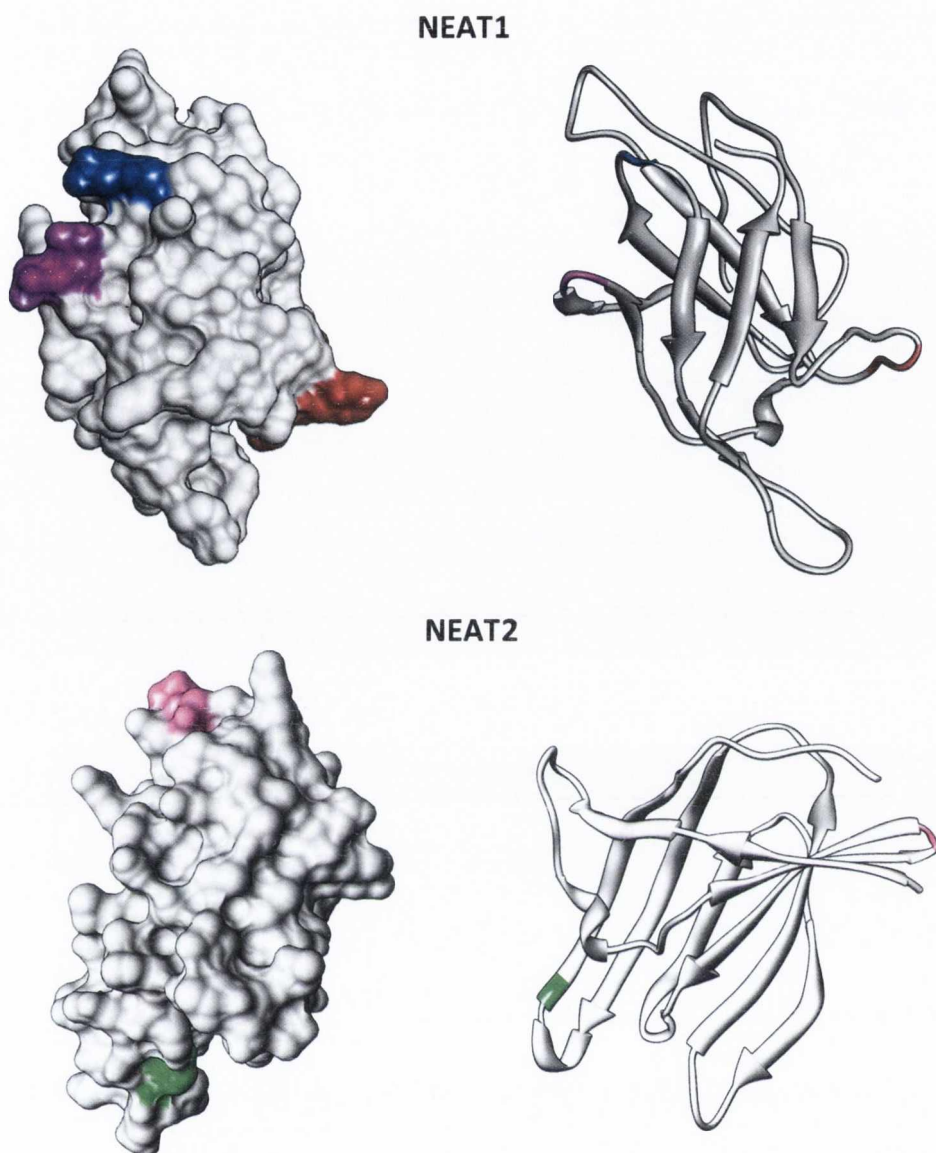


Figure 3.1 3D models of IsdB NEAT1 and NEAT2 domains.

Models were predicted using the Protein Homology/analogY Recognition Engine (PHYRE) based on their amino acids homology to the crystallized IsdH NEAT1 (PDB: 2H3K) and IsdA NEAT motifs (PBD: 2ITE). Altered residues are highlighted in colours: (red) Y165A/H166E/Y167A within the loop β 1b- β 2 on NEAT 1 domain, (magenta) G203A/D204A within loop β 4- β 5 on NEAT1, (blue) N229A within the loop β 6- β 7 on NEAT1 domain, (pink) N377A within loop β 2- β 3 exposed on the surface of the NEAT2 domain and (green) Y440A on β 3.

indicating that no secondary mutations occurred in the global regulator Agr (Fig. 3.2 and data not shown).

3.2.2 Platelet adhesion to *S. aureus* *isdB* mutants.

S. aureus Newman *clfA clfB* and SH1000 *clfA clfB fnbA fnbB* expressing mutant IsdB proteins were tested for binding to washed platelets (WP). Bacteria were grown in iron-limiting medium (RPMI) to induce expression of IsdB, washed and immobilized in microtitre plates. WP adhered directly to microtitre wells coated with Newman *clfA clfB* but not to the *isdB* deficient mutant, which is consistent with the earlier report (Fig. 3.3) (Miajlovic *et al.*, 2010). Similarly, WP adhered to iron- starved SH1000 *clfA clfB fnbA fnbB* but not to the Δ *isdB* mutant (Fig. 3.3). The adherence of WP to all IsdB substitution mutants of both Newman *clfA clfB* and SH1000 *clfA clfB fnbA fnbB* was found to occur at similar levels (Fig. 3.3), suggesting that the substitutions did not obstruct IsdB-mediated interactions with platelets.

3.2.3 Platelet aggregation by *S. aureus* *isdB* mutants.

Platelet aggregation by *S. aureus* Newman *clfA clfB* and SH1000 *clfA clfB fnbA fnbB* strains expressing IsdB mutants was measured using aggregometry. This method allows measurement of changes in optical density reflected by clumping of the plasma components. RPMI-grown bacteria were added to platelet-rich-plasma and aggregometry traces were analysed. SH1000 *clfA cflB fnbA fnbB* did not cause platelet aggregation suggesting that IsdB expression by this strain is not sufficient to activate this process. In contrast, Newman *clfA clfB* triggered platelet aggregation at a level of approximately 75% after a lag time of 5 ± 1 min. There was no platelet aggregation triggered by *S. aureus* Newman *clfA clfB isdB* agreeing with Miajlovic *et al.* (Fig. 3.4) (Miajlovic *et al.*, 2010). All the mutants expressing IsdB with amino-acid substitutions triggered platelet aggregation at similar levels to Newman *clfA clfB* (Fig. 3.4). The strain expressing IsdB (G203A/D204A) had an extended lag time of 6.8 ± 1.8 min, whereas the strain expressing IsdB (N377A) caused more rapid aggregation after only 2.6 ± 1.55 minutes. However, due to considerable variations in lag times between donors, these differences were not statistically significant (Fig. 3.4). This results suggest that introduced mutations did not alter the binding site on IsdB required to trigger platelet aggregation.

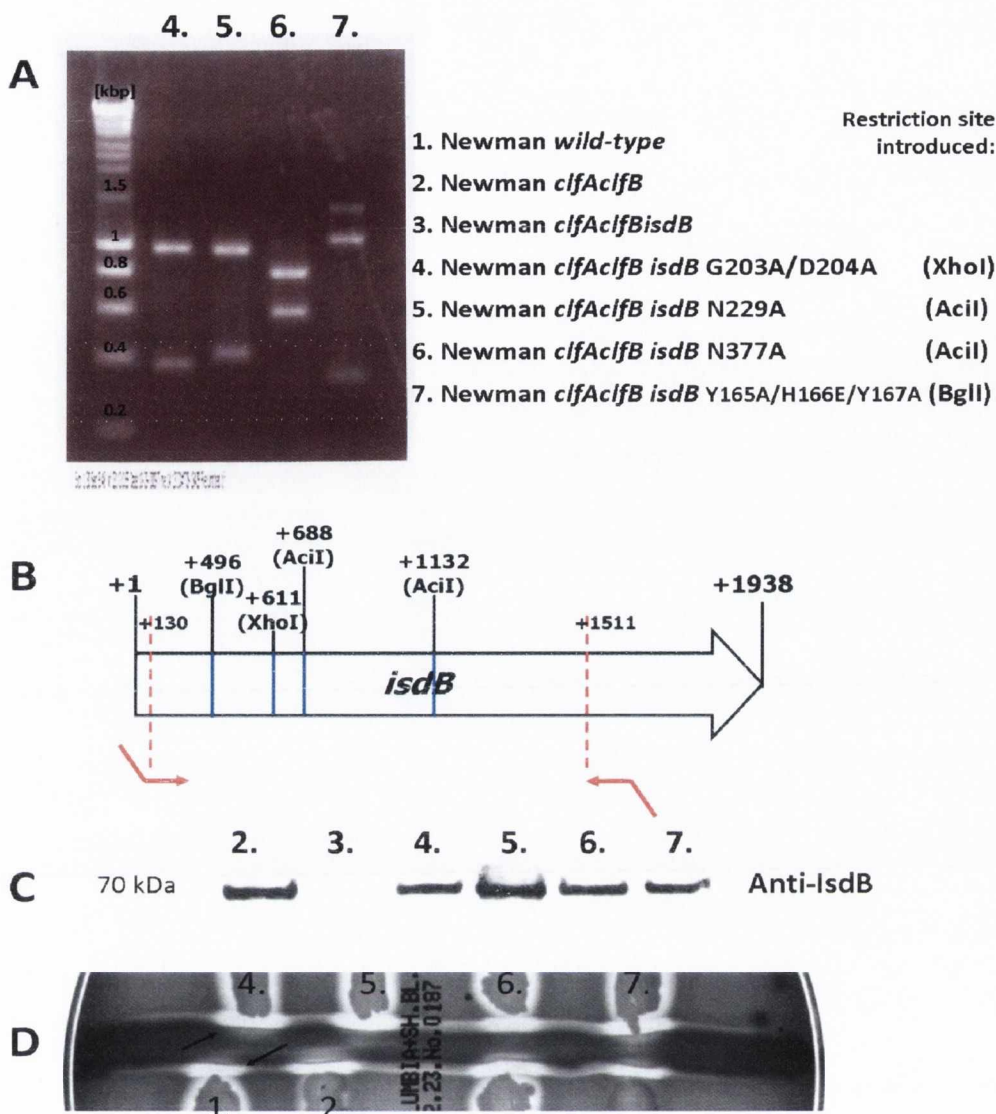


Figure 3.2 Validation of the generated *S. aureus* Newman *clfA clfB isdB* mutants.

(A) Restriction mapping of generated mutants of *isdB* gene (as indicated). PCR amplified *isdB* fragments (1.4 kbp) were subjected to restriction with indicated endonucleases. Obtained products were separated by DNA electrophoresis. (B) *isdB* gene map illustrating introduced restriction sites and their nucleotide positions within the gene. (C) Western immunoblotting of solubilised cell wall fractions of *S. aureus* Newman variants probed with rabbit-anti IsdB followed by HRP-conjugated protein A. (D) Haemolytic patterns of mutants on sheep blood agar cross-streaked with *S. aureus* RN4220. The clear arc is due to δ -toxin causing complete lysis of erythrocytes damaged by β -toxin.

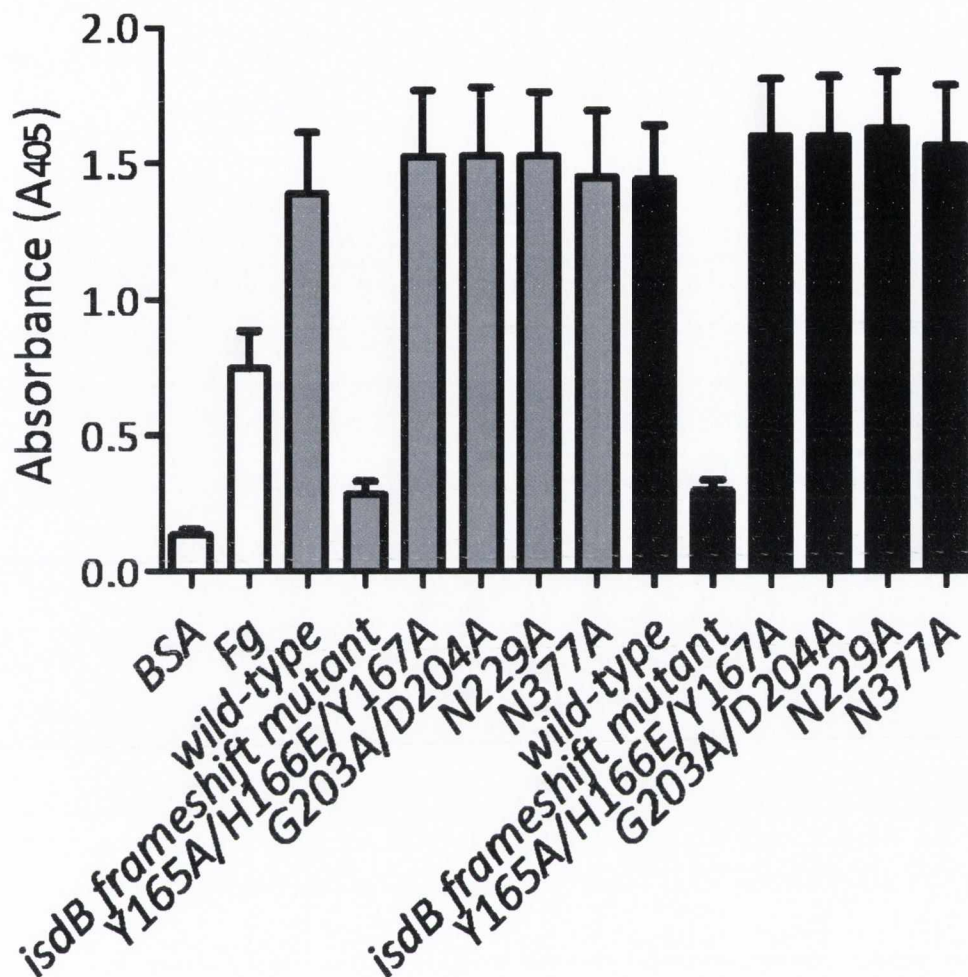


Figure 3.3 Adhesion of washed platelets to *S. aureus* expressing IsdB mutants. Bacteria were grown in iron limiting medium (RPMI) to induce expression of IsdB. Bovine serum albumin (BSA), fibrinogen (Fg) as well as iron-starved (grey) Newman *clfA clfB* and (black) SH1000 *clfA clfB fnbA fnbB* strains were immobilized in microtiter wells. Washed platelets were added and adherent platelet and the substrate for intracellular acid phosphatase was added. Absorbance at 405nm was measured. Results are means of three independent experiments $\pm$ SD.

3.2.4 Production and functionality of recombinant IsdB proteins.

The wild-type *isdB* gene was amplified from genomic DNA of *S. aureus* Newman and cloned into the expression vector pQE30. pQE30*isdB* (45-480) and mutants (see section 3.2.1) were used to overexpress the proteins in *E. coli* TOPP10. Purification of the proteins was performed using nickel affinity chromatography. The purity and integrity of recombinant IsdB variants was verified by SDS-PAGE (Fig. 3.5 and data not shown).

IsdB contains two functional NEAT domains (Fig. 3.5A), which bind to haemoglobin (NEAT1) or haem (NEAT2). To verify if purified recombinant IsdB variants are functional their ability to bind to human haemoglobin (Hb) was tested using an ELISA-based assay. The proteins were immobilized in microtiter dishes and various concentrations of haemoglobin were added to the wells. Hb bound to rIsdB (45-480) with a half maximum of ca. 50 nM (Fig. 3.5D). No binding occurred to rIsdB (45-480) (Y165A/H166E/Y167A) due to mutation in residues known to be critical for Hb coordination (Fig. 3.5D). The (G203A/D204A), (N229A), (N377A) and (Y440A) mutants bound to Hb with the same affinity as the wild-type (data not shown).

To test if the C-terminal NEAT2 of the mutant proteins could bind to haem, spectrophotometric analysis of the proteins purified from *E. coli* grown in LB broth were performed. There was a Soret peak at 406 nM for IsdB wild-type and variants implying they could bind haem (Fig. 3.5E and data not shown). Only the IsdB (Y440A) mutant did not bind haem, as the substituted Tyr is known to play a critical role in capturing of the porphyrin ring of haem (Fig. 3.5E).

Recombinant His-tagged IsdA and GST-tagged IsdH were expressed in *E. coli* from plasmids pQE30*isdA* (50-320) and pGEX4t-2*isdH* (41-321). Both proteins migrated as single bands when separated by SDS-PAGE (data not shown).

3.2.5 Purified $\alpha_{IIb}\beta_3$ binding to IsdB variants.

The $\alpha_{IIb}\beta_3$ is a heterodimeric membrane glycoprotein consisting of an α and a β subunit linked by a single disulfide bond. The α_{IIb} subunit (130 kDa) undergoes posttranslational proteolytic cleavage, producing a 105-kDa extracellular fragment and a 23-kDa fragment containing the single transmembrane spanning domain and a 26-residue cytoplasmic domain. In contrast, β_3 consists of a single polypeptide chain (95 kDa), including a single transmembrane spanning domain and a 45-residue cytoplasmic domain.

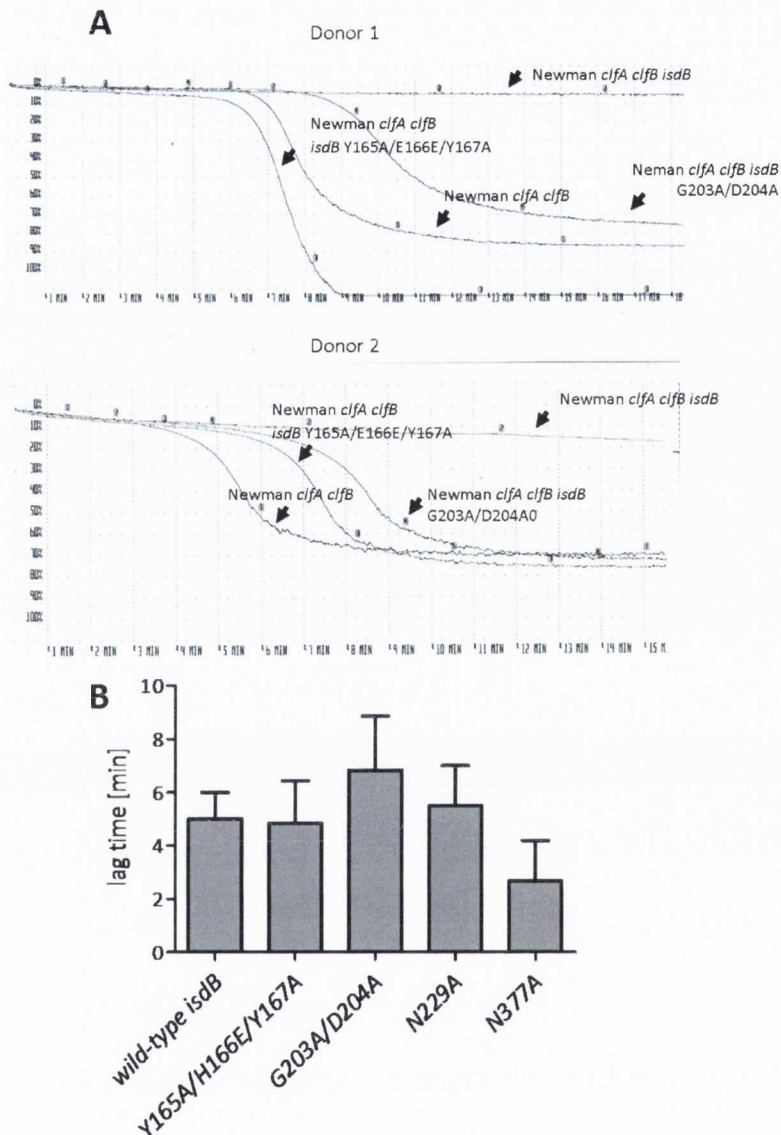


Figure 3.4 Platelet aggregation by *S. aureus* Newman *clfA* *clfB* expressing IsdB mutants.

Iron-starved bacteria expressing IsdB variants were washed and added to platelet rich plasma (PRP) and platelet aggregation was measured using aggregometry. **(A)** Representative traces of PRP aggregation upon addition of *S. aureus* are presented. **(B)** Mean lag times of IsdB – mediated platelet aggregation. The lag phase is the time [min] from the addition of the bacteria until the rapid change in light transmission measured by the aggregometer. Results are mean of three independent experiments $\pm$ SD.

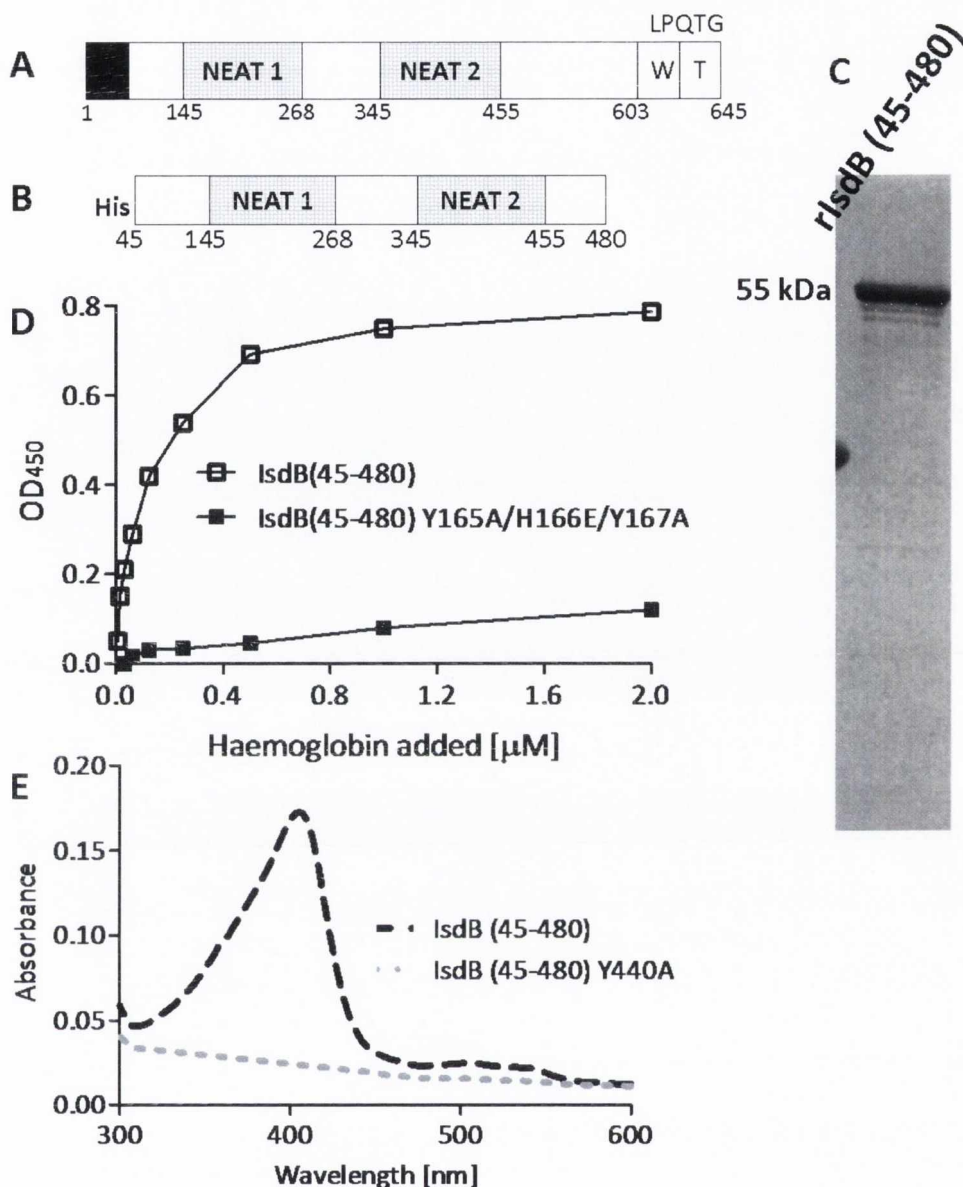


Figure 3.5 Binding of recombinant IsdB proteins to haemoglobin and haem. Schematic representations of (A) the full length IsdB and (B) recombinant IsdB (45-480). (C) SDS-PAGE of purified recombinant IsdB (45-480). (D) Recombinant IsdB binding to haemoglobin (Hb). Recombinant IsdB (45-480) wild-type and the (Y165A/H166E/Y167A) mutant were immobilized in microtiter wells. A range of concentrations of Hb was added to the wells and binding was detected by rabbit anti-human Hb followed by HRP-conjugated goat anti-rabbit IgG. (E) UV-visible spectra of purified IsdB (45-480) wild-type and (Y440A) substitution mutant.

Integrin purified from plasma was obtained commercially. The integrins purity and integrity was confirmed by SDS-PAGE followed by Coomassie staining (Fig. 3.6). An ELISA-based assay and surface plasmon resonance (SPR) were used to study the interaction between IsdB and $\alpha_{IIb}\beta_3$. In the microtiter assay the purified integrin was immobilized in wells and a range of rIsdB concentrations were added. A dose-dependent interaction between IsdB and $\alpha_{IIb}\beta_3$ could be detected. To study the interaction under flow conditions SPR was used. Purified $\alpha_{IIb}\beta_3$ was immobilized on a dextran chip by amine coupling. Increasing concentrations of recombinant IsdB (45-480) were flowed over the immobilized integrin. The sensorgrams indicated a specific, dose-dependent interaction (Fig. 3.6C). The dissociation constant (K_D) of the interaction was determined to be 350 ± 80 nM (BIAevaluation software version 3.0). Each of the rIsdB mutants bound to the immobilized integrin with affinities in the same range (100 - 400 nM) (data not shown).

Western immunoblotting analysis revealed contamination of the $\alpha_{IIb}\beta_3$ batch. A new uncontaminated batch of the integrin was obtained to validate the SPR results. However no binding of IsdB to the integrin could be detected. There was also no binding by fibrinogen or the RGD peptide which was used as controls, suggesting that the experimental model is not valid and cannot be used to study this interaction, perhaps due to conformational dependence of the interaction with the ligands, fibrinogen or RGD.

3.2.6 Analysis of IsdB binding to human platelet factor 4.

Far Western blotting of the purified resting $\alpha_{IIb}\beta_3$ integrin was performed. The integrin was separated under reducing conditions and probed with recombinant IsdB followed by rabbit anti-IsdB and HRP-conjugated protein A. A band of 28 kDa was detected (Fig. 3.7) indicating that IsdB could interact with an integrin degradation product or a co-purified plasma molecule. However, this band could not be detected by Coomassie staining (Fig. 3.6).

As indicated by the manufacturer (personal communication), the purified integrin could be contaminated with human platelet factor 4 (HPF4). To confirm that the contaminant is HPF4 rabbit anti- HPF4 serum was used to probe the purified $\alpha_{IIb}\beta_3$. A band of the same size as that recognized by rIsdB was detected by the antibodies. Purified HPF4 (obtained commercially) was used as a control and could also be detected by the antibodies, indicating that the contaminant is HPF4. Confusingly rIsdB

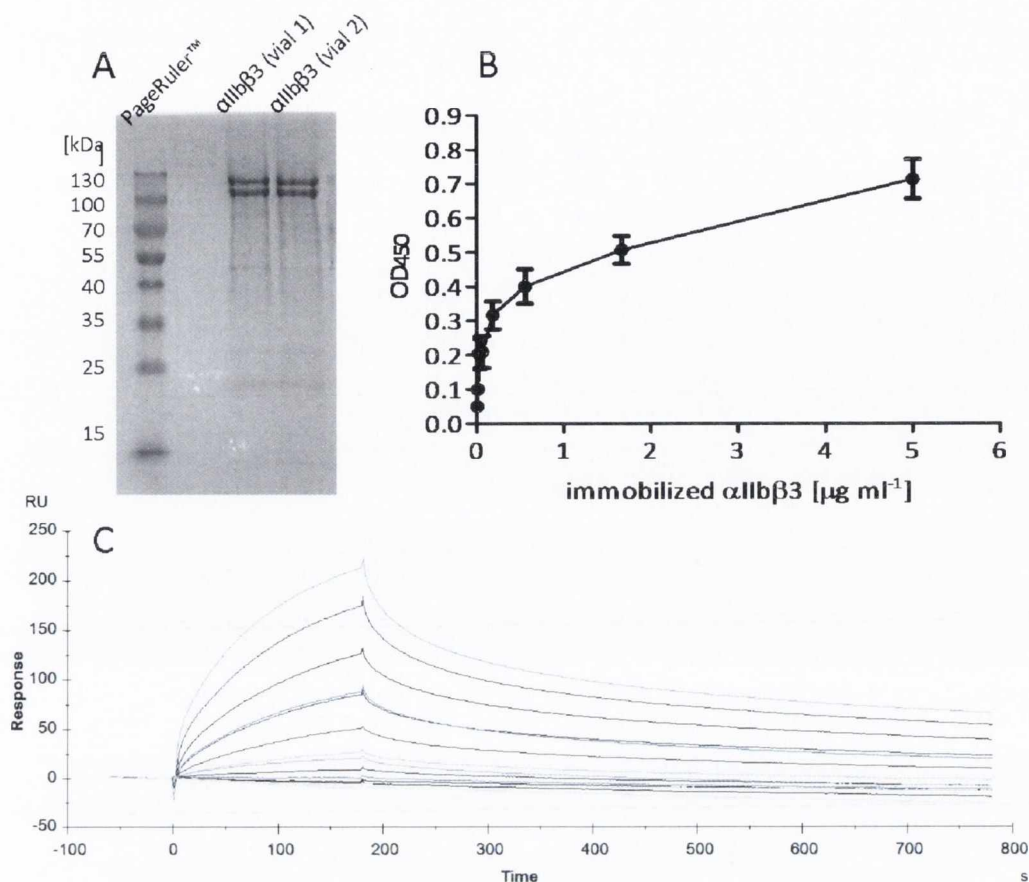


Figure 3.6 Recombinant IsdB binding to purified $\alpha_{IIb}\beta_3$.

(A) SDS-PAGE analysis of purified $\alpha_{IIb}\beta_3$, under reducing conditions. (B) The purified integrin was immobilized in microtiter wells. Recombinant IsdB (45-480) was added to the wells and binding was detected with rabbit anti-IsdB antibodies followed by HRP-conjugated protein A. (C) Surface plasmon resonance analysis of recombinant IsdB (45-480) binding to $\alpha_{IIb}\beta_3$. The $\alpha_{IIb}\beta_3$ was immobilized on the surface of a CM5 sensor chip by amine coupling followed by passing increasing concentrations of rIsdB over the surface. Injections began at 0 s and ended at 180 s. The data are presented as real-time graphs of response units (RU) against time

did not bind purified HPF4 suggesting that differences occurred between the $\alpha_{IIb}\beta_3$ contaminant and purified HPF4. These results suggest that rIsdB does not bind to resting, plasma derived $\alpha_{IIb}\beta_3$ *in vitro*.

3.2.7 Flow cytometric analysis of recombinant IsdB binding to resting and activated platelets.

In order to determine if recombinant IsdB could bind to resting and/or activated platelets the protein was labeled with fluorescein isothiocyanate (FITC) and incubated with washed platelets (WP) prior to and after activation with Thrombin Receptor Activating Peptide, which converts the $\alpha_{IIb}\beta_3$ integrin into the high affinity conformation (WP TRAP) (Coutinho *et al.*, 2007). As analysed by flow cytometry, FITC-labelled IsdB bound at a two-fold higher level to the WP TRAP than to the untreated WP, indicating its preference for the high affinity conformation of the integrin (Fig. 3.8AB). There was no binding of WP or WP TRAP to fluorescently labelled recombinant SasG, a surface protein of *S. aureus* which was used as a control (Fig. 3.8).

3.2.8 Recombinant IsdB does not induce dense granule release by platelets.

Stimulation of platelets with strong agonists results in centralization of cytoplasmic organelles and secretion of granules which contain ATP, ADP, serotonin and Ca^{2+} . This process activates platelets leading to platelet aggregation. To investigate whether recombinant IsdB can stimulate dense granule release either soluble recombinant IsdB or the positive control – TRAP was added to platelet rich plasma and platelet aggregation was monitored in an aggregometer. Upon platelet aggregation (initiated by TRAP) samples were taken from both cuvettes and the ATP content in both samples was measured by a luciferin - luciferase assay (see Chapter 2.23.1). Luminescence of both samples was measured. No increase in the dense granule release (ATP levels) was detected in samples treated with rIsdB compared to TRAP, indicating that soluble recombinant IsdB does not induce dense granule release from platelets.

3.2.9 Real-time impedance measurement of the attachment of CHO-K1 $\alpha_{IIb}\beta_3$ cells to IsdB.

To confirm that IsdB interacts with the high affinity conformation of the platelet integrin $\alpha_{IIb}\beta_3$ CHO-K1 cell line stably expressing human wild-type $\alpha_{IIb}\beta_3$ and the $\alpha_{IIb}\beta_3$ (N305T) mutant locked in the high-affinity conformation were employed (Luo *et al.*,

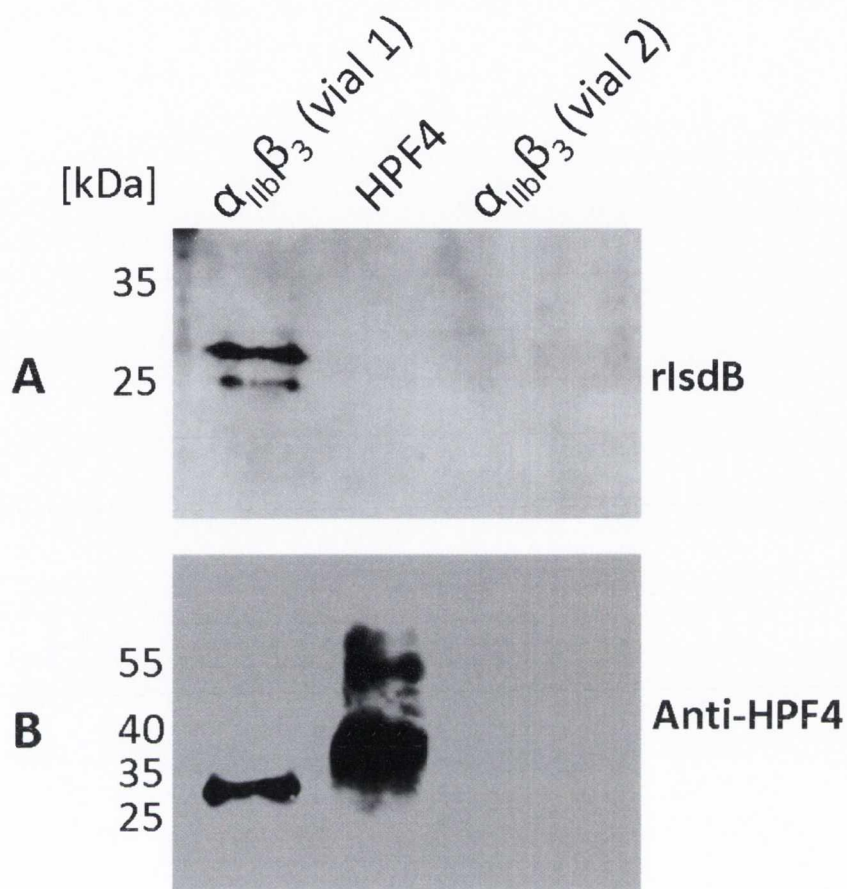


Figure 3.7 Western immunoblotting analysis of purified $\alpha_{IIb}\beta_3$ and human platelet factor 4. (A) Far Western affinity blot of purified $\alpha_{IIb}\beta_3$ and human platelet factor 4 (HPF4) probed with recombinant IsdB (45-480) followed by rabbit anti-IsdB and HRP-conjugated protein A. (B) Western immunoblot of $\alpha_{IIb}\beta_3$ and HPF4 probed with rabbit anti-HPF4 followed by HRP - conjugated goat anti-rabbit IgG.

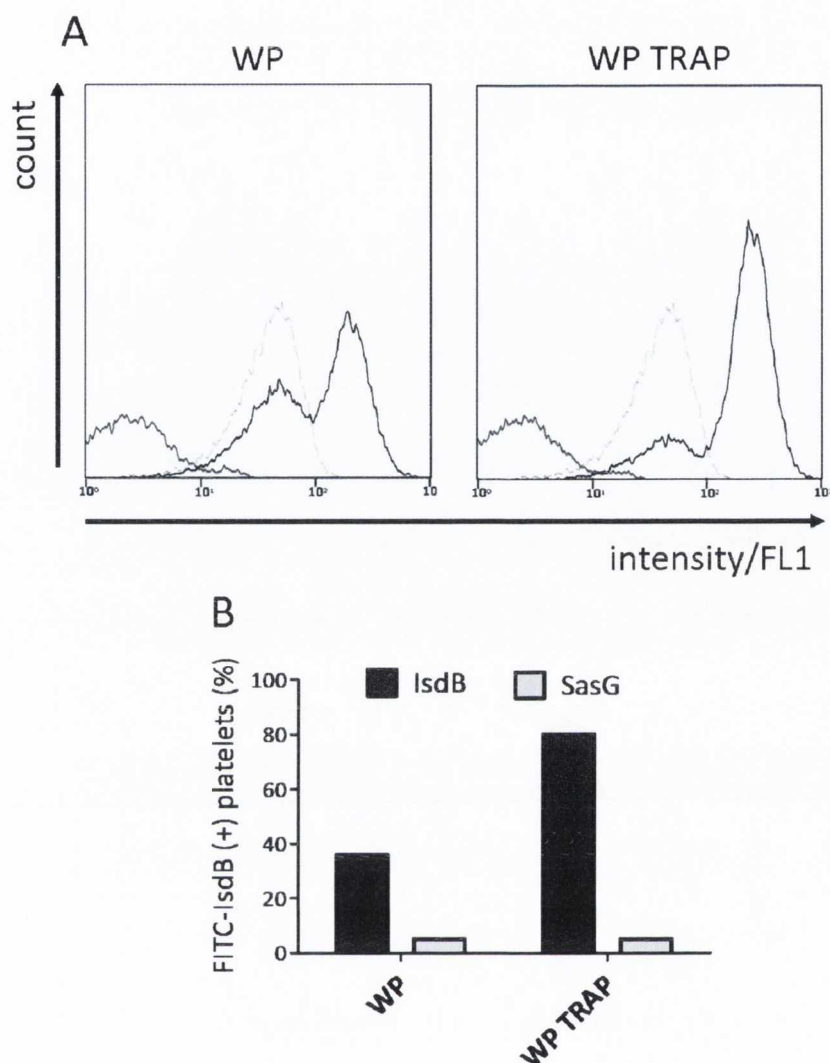


Figure 3.8 Flow cytometric analysis of IsdB protein binding to platelets.

(A) Representative flow cytometry traces of recombinant fluorescein isothiocyanate (FITC) labelled IsdB and SasG proteins binding to isolated and washed platelets (WP) or WP activated by TRAP (WP TRAP). Traces: (dark grey) platelets, (light grey, broken line) FITC-SasG incubated with platelets and (black) FITC-IsdB bound platelets. **(B)** Percentage of IsdB and SasG bound to WP and WP TRAP. Binding assays were performed three times with similar results.

2003; Luo *et al.*, 2004). Fibrinogen, IsdB and BSA were immobilized in microtiter dishes and cell attachment to and spreading on immobilized ligands was analysed using the xCELLigence system. This instrument performs quantitative real-time measurements of impedance in microtiter wells containing electrodes. The baseline impedance is measured in the absence of the cells. When cells stick to the electrode surface they act as isolators inducing an increase in the electrical impedance. If more cells attach or if cells spread there will be an increase in the impedance which corresponds to higher Cell Index (CI) values. Cells expressing the wild-type $\alpha_{Iib}\beta_3$ integrin in the presence of 2 mM Ca^{2+} , which stabilizes the low affinity conformation of the integrin, did not attach to or spread on surfaces coated with IsdB, fibrinogen or BSA (Fig. 3.9). Cells expressing the high affinity form of $\alpha_{Iib}\beta_3$ (N305T) attached to fibrinogen and IsdB but not BSA (Fig. 3.9). Consistent with previous observations, this result indicates that IsdB interacts with the high affinity form of $\alpha_{Iib}\beta_3$. The attachment and spreading of the high affinity mutant was stronger on surfaces coated with fibrinogen than with IsdB suggesting a higher affinity of the integrin for the plasma protein than for IsdB (Fig. 3.9). Microscopic analysis confirmed that the cells spread on Fg and IsdB but not on BSA (Fig. 3.10).

The attachment of the CHO-K1 $\alpha_{Iib}\beta_3$ (N305T) mutant to recombinant IsdH and IsdA proteins was also compared. The cells attached strongly to rIsdB, weakly to rIsdA and there was no binding to rIsdH (Fig. 3.11). The attachment to and spreading on rIsdB was more than 2.5 fold greater than on rIsdA. This is consistent with IsdB located on the bacterial cell surface providing more specific attachment for the high affinity $\alpha_{Iib}\beta_3$ than other Isd proteins.

Variants of recombinant IsdB carrying alanine-substitutions were compared in their ability to interact with the high affinity $\alpha_{Iib}\beta_3$. A subtle decrease in binding was observed for mutants carrying substitutions in Asp377 (N377A) (Fig. 3.12), suggesting that this mutation affects the binding site on IsdB.

3.3 Discussion.

Surface-associated proteins of *S. aureus* indirectly adhere to platelets. The components can stimulate platelet aggregation *via* host IgG which specifically recognizes the proteins and crosslinks the bacterium and platelet Fc γ RIIa receptor. Previous studies of interaction of *S. aureus* surface proteins with platelets were carried out using bacteria grown in rich laboratory media containing iron. The identification of

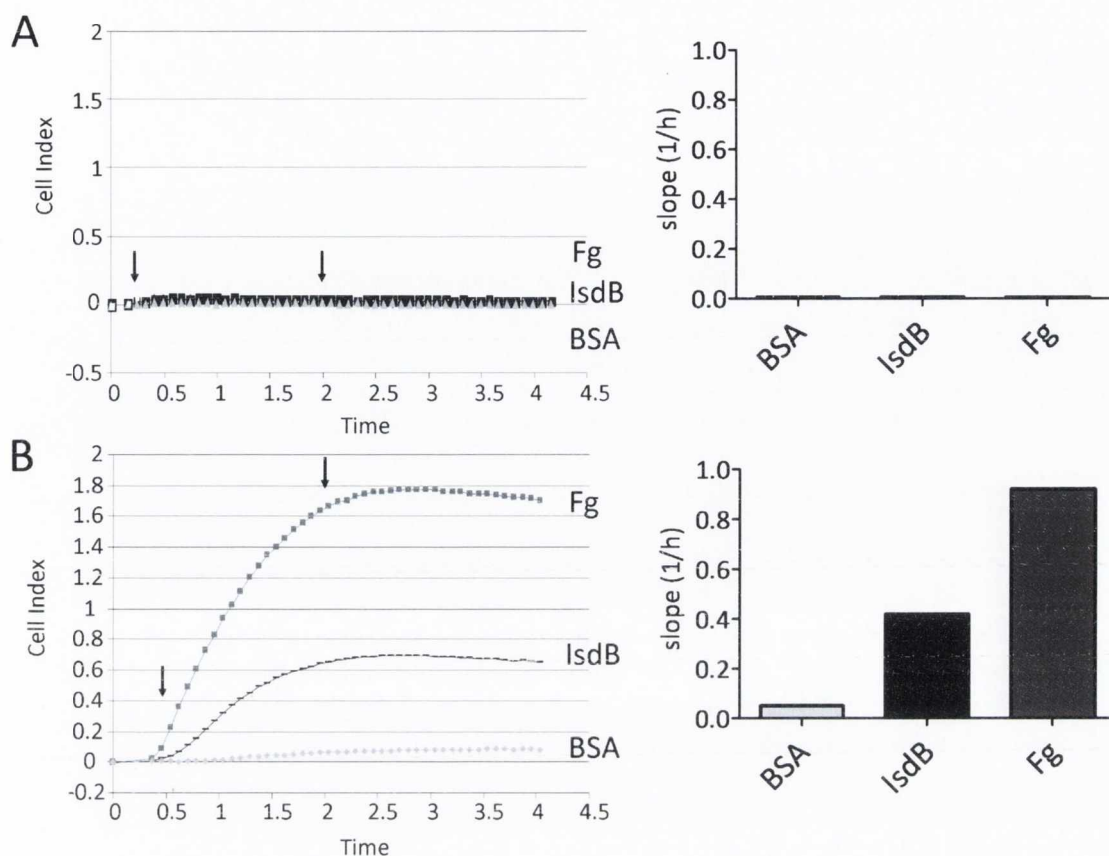


Figure 3.9 Real-time measurement of attachment and spreading of CHO-K1 cells expressing human $\alpha_{IIb}\beta_3$ variants on immobilized ligands.

(A and B) Bovine serum albumin (BSA), recombinant IsdB and fibrinogen (Fg) were immobilized in microtiter wells, blocked with BSA and incubated with equal numbers of CHO-K1 cells expressing $\alpha_{IIb}\beta_3$ (A) wild-type or (B) (N305T) high affinity mutant. XCELLigence was used to measure impedance of the cells added to those wells. Attachment and spreading of cells led to increased impedance in the wells which corresponded to higher cell index (CI) values. (Left) Traces representing the CI changes. (Right) Slope changes (slope/h) for CI traces within time points indicated by arrows. Slope changes were calculated by RTCA analyzer software.

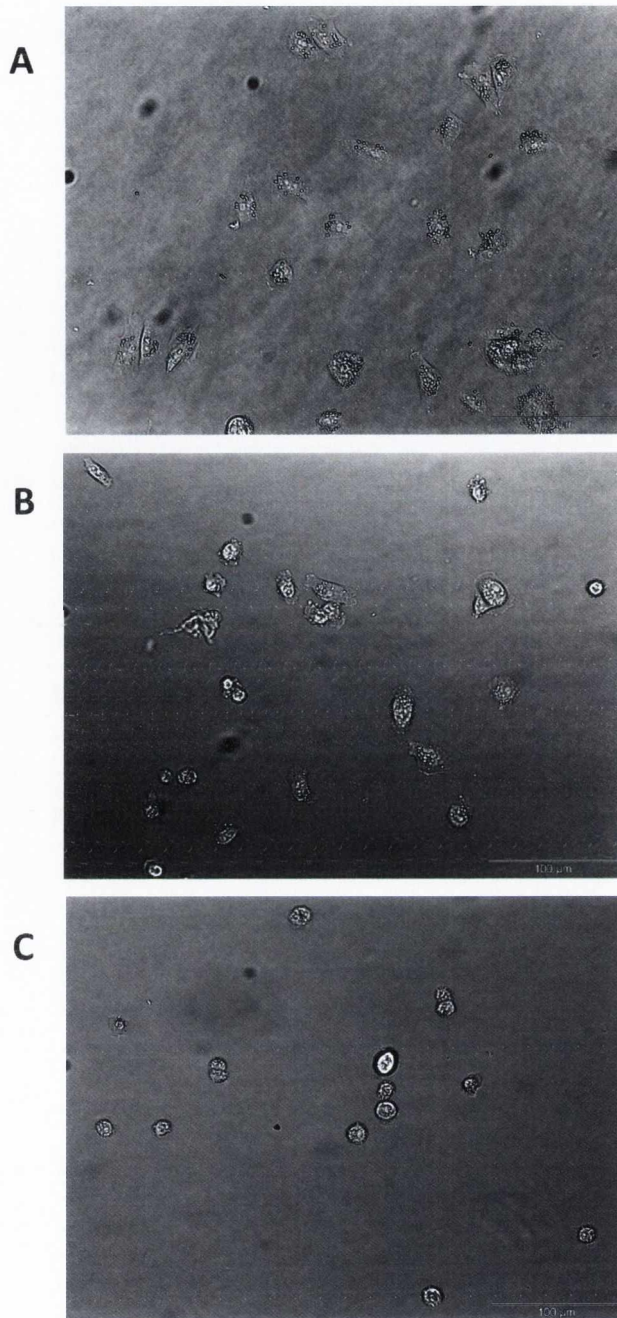


Figure 3.10 Micrographs showing spreading of CHO-K1 $\alpha_{IIb}\beta_3$ (N305T) on immobilized ligands.

The following ligands were immobilized in wells for 2 h at 37°C: (A) fibrinogen, (B) rIsdB (45-480) and (C) BSA. Wells were blocked with BSA and cells were added to the wells. Pictures were taken after 30 min of incubation at 37°C.

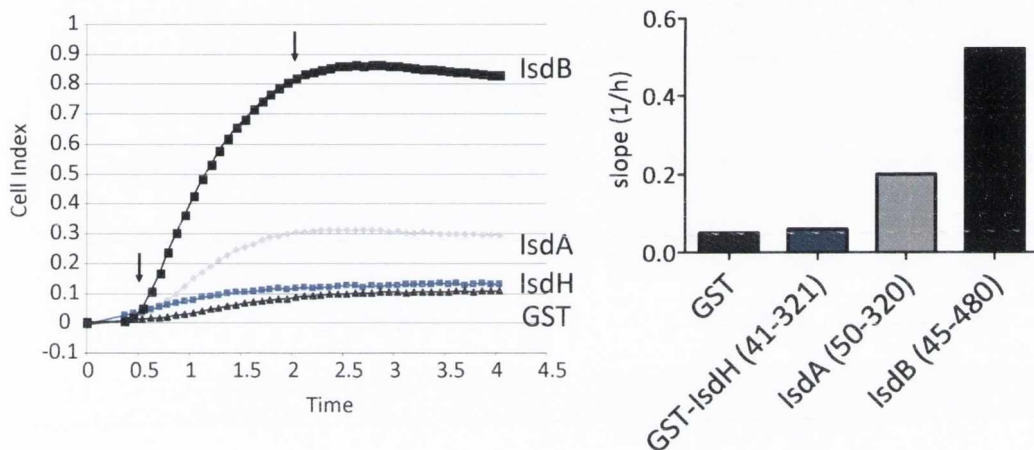


Figure 3.11 Real-time measurement of attachment and spreading of CHO-K1 $\alpha_{IIb}\beta_3$ (N305T) mutant on rIsdA, rIsdB and rIsdH.

(Left) Traces representing cell index (CI) changes over-time upon addition of the cells to wells coated with immobilized ligands. (Right) Slope changes (slope/h) for CI traces within time points indicated by arrows. Slope changes were calculated by RTCA analyzer software. The assays were performed three times with similar results.

IsdB interaction with platelets was discovered by testing bacteria that had been grown in iron restricted medium (Miajlovic *et al.*, 2010). IsdB - mediated adherence to platelets is unique as the protein directly recognizes platelet $\alpha_{IIb}\beta_3$ and do not engage plasma Fg (Miajlovic *et al.*, 2010). IsdB-dependent platelet aggregation is also novel as it does not require IgG (Miajlovic *et al.*, 2010). Suprisingly it was observed that the SH1000 *clfA clfB fnbA fnbB* strain lacking proaggregatory factors could not trigger platelet aggregation despite being able to adhere to platelets in an IsdB-dependent manner. This is consistent with the inability of soluble IsdB to cause dense granule release from platelets, This implies that IsdB may only contribute to platelet aggregation by adherence but requires additional factors. This factors may be exposed on *S. aureus* surface or secreted by the bacterium as *L. lactis* expressing IsdB could not adhere to platelets or induce platelet aggregation.

The interaction of IsdB with integrin $\alpha_{IIb}\beta_3$ requires the high affinity conformation of the receptor. Recombinant IsdB bound at a higher level to TRAP-activated platelets where the surface-located integrin has been converted to the high affinity state compared to resting platelets carrying conformationally bent integrins. Furthermore, CHO cells expressing a mutant of $\alpha_{IIb}\beta_3$ (N305T) which is locked in the high affinity conformation (Luo *et al.*, 2003) bound to immobilized rIsdB and to fibrinogen, whereas cells expressing wild-type $\alpha_{IIb}\beta_3$ did not adhere.

Adhesion of washed platelets to immobilized *S. aureus* expressing IsdB (Miajlovic *et al.*, 2010) seemed to occur at a higher level than to immobilized fibrinogen. However this method does not differentiate between the affinity of interacting components and their avidity and therefore may misrepresent the 'strength' of the interaction. To analyze binding in more detail we performed the quantitative real-time measurements by xCELLigence and compared the strength of binding of the cells to fibrinogen and IsdB, which indicated a possibility of higher affinity for the plasma fibrinogen than to IsdB.

In the course of the study a contaminant of the $\alpha_{IIb}\beta_3$ integrin was found which bound to rIsdB. This molecule was detected by rabbit anti-human platelet factor 4 antibodies (HPF4). HPF4 is a chemokine stored within α -granules released by activated platelets. HPF4 binds heparin forming a highly immunogenic complex. The antibodies bind to PF4/heparin at the platelet surface and the resulting immune complexes induce the Fc γ RIIIa receptor triggering platelet activation and enhanced thrombin generation. Heparin treatment can induce the spontaneous and dangerous most frequent immune-

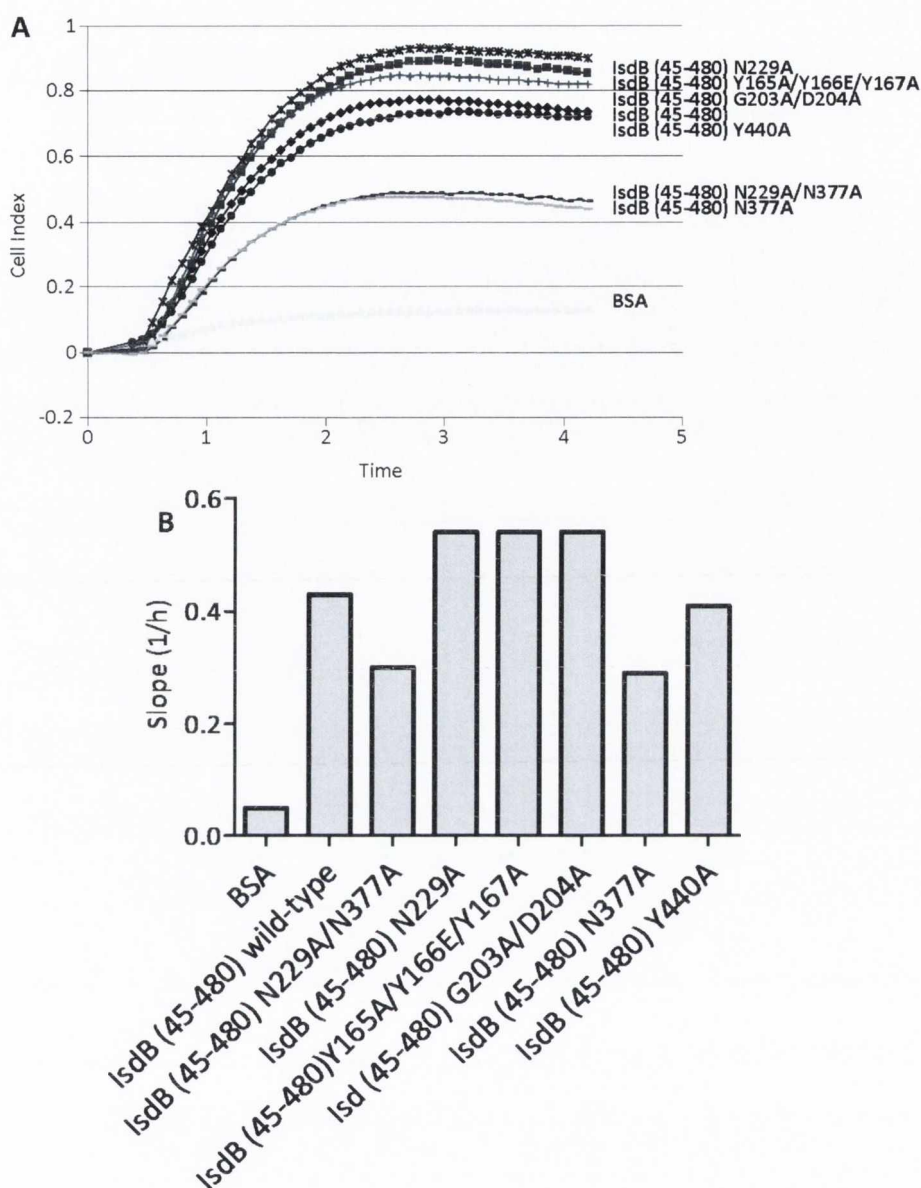


Figure 3.12 Real-time measurement of attachment and spreading of CHO-K1 $\alpha_{IIb}\beta_3$ (N305T) on rIsdB variants.

(A) Traces representing cell index (CI) changes over-time upon addition of the cells to wells coated with immobilized ligands. (B) Slope changes (slope/h) for CI traces within time points indicated by arrows. Slope changes were calculated by RTCA analyzer software.

mediated adverse drug reaction, heparin-induced thrombocytopenia (HIT). Krauel *et al.* demonstrated that PF4 binds to bacteria such as *E. coli* and *S. aureus* giving rise to IgM and IgG and proposed that HIT was a misdirected antibacterial host defence mechanism (Krauel *et al.*, 2011). It is possible that PF4 may contribute to the MSCRAMMs-mediated platelet aggregation as this requires host IgG. Even though the IsdB-dependent platelet aggregation by *S. aureus* strain Newman did not require serum there was an increase in the level of aggregation upon addition of plasma suggesting a possible contribution of the IsdB/PF4 IgG (Miajlovic *et al.*, 2010).

Recombinant IsdB bound to HPF4 contaminant but not to HPF4 purified from plasma, suggesting that differences must exist between them. It was observed that the purified HPF4 was highly unstable and degraded from the tetrameric to dimeric and monomeric forms implying that the IsdB recognizes only the tetrameric form of HPF4.

The study attempted to identify residues involved in the interaction of IsdB with $\alpha_{IIb}\beta_3$. Alanine substitutions were introduced into genomic *isdB* and recombinant IsdB. Neither studies on bacterial adhesion and aggregation by the mutants nor $\alpha_{IIb}\beta_3$ (N305T) attachment and spreading to immobilized recombinant IsdB variants identified residues involved in mediating binding to the integrin. A reduction in attachment was observed for the rIsdB (45-480) (N377A) and (N229/N377A) suggesting the NGR motif may be involved in the binding. But the same mutation in the genomic *isdB* resulted in a strain which adhered equally well to platelets and even slightly more quickly induced platelet aggregation and therefore no conclusions can be drawn.

Chapter 4

Interactions of the iron-regulated surface determinant B of *S. aureus* with human cells

4.1 Introduction.

S. aureus can express on its surface a range of proteins that promote binding to components of the extracellular matrix, fibrinogen (Fg), fibronectin (Fn), elastin and collagen (Hauck *et al.*, 2006a; Speziale *et al.*, 2009). Fibrinogen and fibronectin binding proteins can also promote interactions with host cells by using the plasma proteins to provide bridges to host cell integrin receptors ($\alpha_5\beta_1$ – Fn ; $\alpha_{IIb}\beta_3$, Fn, Fg). The integrins are heterodimers formed by non-covalent association of α and β subunits. They bind to components of the extracellular matrix and in some cases to counter-receptors on adjacent cells. They are essential for cell adhesion in tissue maintenance, embryonic development and haemostasis (Harburger *et al.*, 2009).

Until recently *S. aureus* was regarded as an extracellular pathogen. However it is now clear that the bacterium can adhere to and become internalized by a range of host cells that are not normally phagocytic. This has been demonstrated in cell culture with endothelial cells, epithelial cells, osteoblasts, fibroblasts and keratinocytes (Edwards *et al.*, 2010; Ellington *et al.*, 1999; Massey *et al.*, 2001; Sinha *et al.*, 1999; Sinha *et al.*, 2005; von Eiff *et al.*, 2001b). The fibronectin binding proteins (FnBPs) were the first bacterial proteins to be shown to promote internalization. The FnBP bind to the N-terminal domain of Fn. The host protein acts as a bridge between the bacterial cell and the $\alpha_5\beta_1$ integrin which occurs on a wide range of host cell types. The integrin also binds to the RGD containing domain of Fn that is located towards the C-terminus. A single FnBP protein has tandemly-arrayed ligand binding domains which attracts several molecules of Fn. This results in clustering of the integrin which in turn causes intracellular signalling that results in local actin rearrangements and bacterial invasion into a phagocytic vesicle (Edwards *et al.*, 2010; Schroder *et al.*, 2006; Sinha *et al.*, 1999; Sinha *et al.*, 2005).

Other mechanisms of internalization are available to *S. aureus* because mutants that lack FnBPs are often not completely deficient in invasion. One such “back up” mechanism is provided by the autolysins Atl which can bind directly to the heat shock cognate protein Hsc70 and indirectly to host integrins to promote uptake (Hirschhausen *et al.*, 2010).

Studies on *S. aureus* adhesion and internalization have been performed with bacterial cells grown in rich laboratory media containing iron. The objective of the current study was to investigate the contribution of IsdB to adherence of *S. aureus* to

human cells other than platelets and to investigate if IsdB could bind to other integrins and perhaps promote bacterial internalization.

4.2 Results.

4.2.1 Generation of an *isdB* deletion mutation in in *S. aureus* SH1000 wild-type and SH1000 *clfA clfB fnbA fnbB*.

To generate a deletion of *isdB* in *S. aureus* SH1000 sequences extending 500 bps upstream and downstream of the *isdB* gene of SH1000 were amplified to create a DNA fragment where start and stop codons of the gene were paired. This fragment was cloned into pKOR1. The pKOR1*isdB* plasmid was electroporated into *S. aureus* SH1000 and *S. aureus* SH1000 *clfA clfB fnbA fnbB* following an allelic replacement procedure to isolate the deletion mutants. The mutations were validated by PCR followed by the PCR product sequencing. The isolated mutants were analysed for δ -toxin production by growth on horse blood agar to determine if a secondary mutations occurred during the mutagenesis which could affect the global gene regulator Agr. Frameshift mutation in *isdB* gene of strains Newman and Newman *clfA clfB* were previously isolated by Miajlovic *et al.* (Miajlovic *et al.*, 2010 and Miajlovic, unpublished). Cell wall anchored proteins of *S. aureus* SH1000 *isdB*, SH1000 *clfA clfB fnbA fnbB isdB*, Newman and Newman *clfA clfB* were solubilised with lysostaphin and analysed by Western blotting probing with anti-IsdB serum. This showed that the protein was absent in the *isdB* mutants. The SH1000 and Newman strains expressed IsdB at similar levels and IsdA was expressed at the same level in all strains (Fig. 4.1).

It should be pointed out that pKOR1*isdB* may not be used for generating a deletion of *isdB* in other *S. aureus* strains as differences exist in the *isdB* downstream genomic regions (Fig. 4.1B). One example is *S. aureus* Newman strain which, unlike the SH1000, contains a prophage that is integrated very close to *isdB* (Fig. 4.1B).

4.2.2 IsdB - mediated adhesion to human cells other than platelets.

It was hypothesized that IsdB might promote interactions of *S. aureus* with other human cells as well as platelets. Newman *clfA clfB* and Newman *clfA clfB isdB* were grown in RPMI and immobilized in microtitre dishes. Washed human platelets, human kidney embryo 293T cells and human epithelial carcinoma HeLa cells were added to the wells and adherent cells were estimated by measuring intracellular acid phosphatase. BSA and the extracellular matrix proteins fibronectin (Fn) and fibrinogen

(Fg) served as controls. Platelets bound to Fg but not to Fn-coated wells. In contrast HeLa and 293T cells bound to Fn-coated wells, but not to those coated with Fg (Fig. 4.2), most likely employing Fn-binding integrins. Consistent with report by Miajlovic *et al.* and with results from Chapter 3.2.2 platelets adhered to the *S. aureus* Newman *clfA clfB* in an IsdB-dependent manner (Fig. 4.2). The 293T and HeLa cells, like platelets, bound to Newman *clfA clfB* but not to the *isdB* mutant, suggesting that IsdB can interact with these cells in similar manner, perhaps also involving integrins.

Studies showing that IsdB expressed on the surface of *S. aureus* promotes platelet adhesion and aggregation were carried out with Newman and SH1000 mutants lacking the Fg binding proteins ClfA and ClfB (section 3.2.2) both of which can interact indirectly with the platelet integrin $\alpha_{IIb}\beta_3$ by forming Fg-bridges. Strain Newman has stop codons in the 3' ends of the *fnbA* and *fnbB* genes and therefore, like the SH1000 *clfA clfB fnbA fnbB* mutant, it does not express FnBP adhesins, which also can interact with host cellular receptors *via* Fn bridges. To extend the study on IsdB, strain SH1000 which expresses each of the aforementioned surface proteins was used. Expression of fibronectin binding protein was validated by Fn affinity blotting with biotinylated Fn which showed they were expressed at similar levels by SH1000 and SH1000 *isdB* mutants (Fig. 4.1). Adherence to immobilized Fn and Fg by bacterial strains used in this study was evaluated (Fig. 4.3). Both Newman and SH1000 adhered dose-dependently and saturably with similar profiles to immobilized Fg while mutants lacking the Fg binding adhesins (Newman *clfAclfB*; SH1000 *clfAclfBfnbAfnbB*) did not (Fig. 4.3). SH1000 and SH1000 *isdB* adhered in a similar fashion to immobilized Fn but Newman (like SH1000 *clfAclfBfnbAfnbB*) did not adhere because of the defects in its *fnb* genes (Fig. 4.3). RPMI-grown Newman *isdB* and SH1000 *isdB* bound to Fn and Fg at levels similar to those of the wild-type strains demonstrating that IsdB expression does not promote binding to these ligands (Fig. 4.3).

Further adherence assays revealed that both *S. aureus* Newman and SH1000 bound to the 293T and HeLa cells (Fig. 4.4). Binding of these human cells to the *isdB* mutants was significantly reduced (Fig. 4.4). These experiments were performed in the absence of serum, and hence, Fn which would otherwise promote binding *via* FnBPs.

Pre-incubation of 293T and HeLa cells with the RGD peptide reduced the level of their binding to *S. aureus* Newman to the same level as the *isdB* mutant (Fig. 4.5) implying that IsdB can interact with "RGD" integrins, such as $\alpha_5\beta_1$.

Studies with the high-affinity integrin mutant (section 3.2.9) suggested that IsdA may be involved in adherence to the receptors. The possible IsdA contribution to *S. aureus* adherence to human cells was evaluated. Iron-starved *S. aureus* Newman *isdA* cells were tested for binding to platelets, 293T and HeLa cells. All cell types bound to both Newman and Newman *isdA* mutant at similar levels (data not shown), indicating that the contribution of IsdA to adhesion is not significant.

4.2.3 IsdB promotes *S. aureus* invasion.

The adherence of *S. aureus* to host cells is a prerequisite for invasion. *S. aureus* uses fibronectin-binding proteins A (FnBA) and B (FnBB) to bind to fibronectin which attaches to epithelial and endothelial cells by the β_1 integrins, such as $\alpha_5\beta_1$. This bridging of bacteria and host cell integrins triggers bacterial uptake by host cell driven processes involving actin remodelling, focal adhesion kinase and Src family kinases (Agerer *et al.*, 2005; Sinha *et al.*, 2005).

As shown above, iron-starved *S. aureus* Newman can adhere to human cells in an IsdB-dependent fashion (Fig. 4.4). To evaluate internalization of *S. aureus* Newman into human host cells gentamicin/lysostaphin protection assays were performed. Initially, RPMI-grown Newman wild-type and Newman *isdB* were added to 293T or HeLa cell monolayers. The invasion was performed in an iron-restricted medium lacking serum for 2 h. Approximately 15% of the *S. aureus* Newman inoculum was able to invade the cells within this time (Fig. 4.6) whereas only 7% of Newman *isdB* was recovered (Fig. 4.6). This indicates that IsdB promotes invasion of *S. aureus* into cells in the absence of Fn.

Electron microscopy showed that *S. aureus* Newman and SH1000 *clfA clfB fnbA fnbB* which lack the FnBPs had successfully invaded the 293T cells (Fig. 4.7AB). No disruption of the kidney embryo cells was seen upon the bacterial uptake. Internalized bacteria resided in the cytoplasm, sometimes surrounded by lysosomal membranes. Dividing cells were seen suggesting active intracellular growth (Fig. 4.7AB).

However, most if not all *S. aureus* strains express FnBPs which provide a potent mechanism for promoting invasion when serum (and hence Fn) is present (Edwards *et al.*, 2010). Thus we sought to determine if IsdB contributes to invasion of wild-type FnBP-expressing bacteria in the presence of Fn. Firstly, in the absence of serum both SH1000 and SH1000 *clfA clfB fnbA fnbB* expressing IsdB were found to invade at similar levels (43% and 40% of input inocula, respectively) while SH1000 *isdB* and

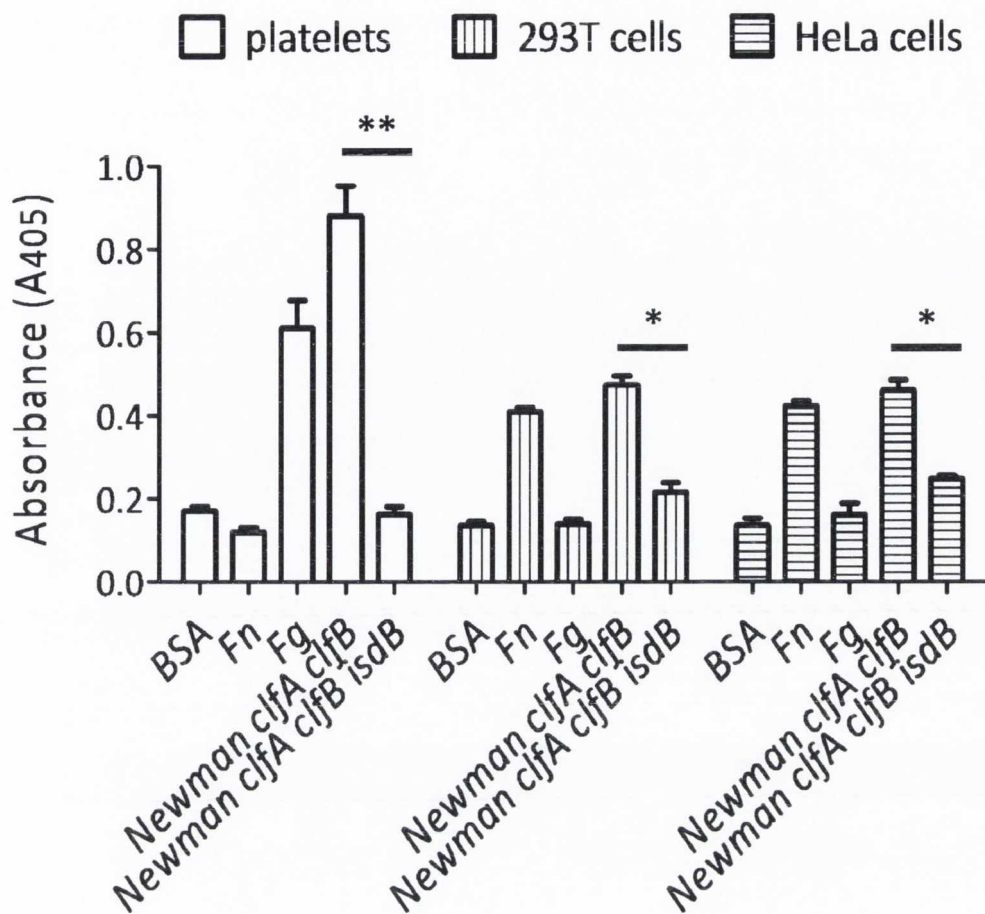


Figure 4.2 Adhesion of human platelets, 293T and HeLa cells to *S. aureus* Newman *clfA clfB* and Newman *clfA clfB isdB*.

Bacteria were grown in RPMI and immobilized in microtiter plates. BSA, fibronectin (Fn) and fibrinogen (Fg) were added to control wells. Platelets (white field), 293T (vertical lines) and HeLa (horizontal lines) cells were washed and added to microtitre wells and incubated for 1 h at 37°C. Adherent cells were lysed and intracellular acid phosphatase measured. Experiments were performed three times. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05, **P<0.03).

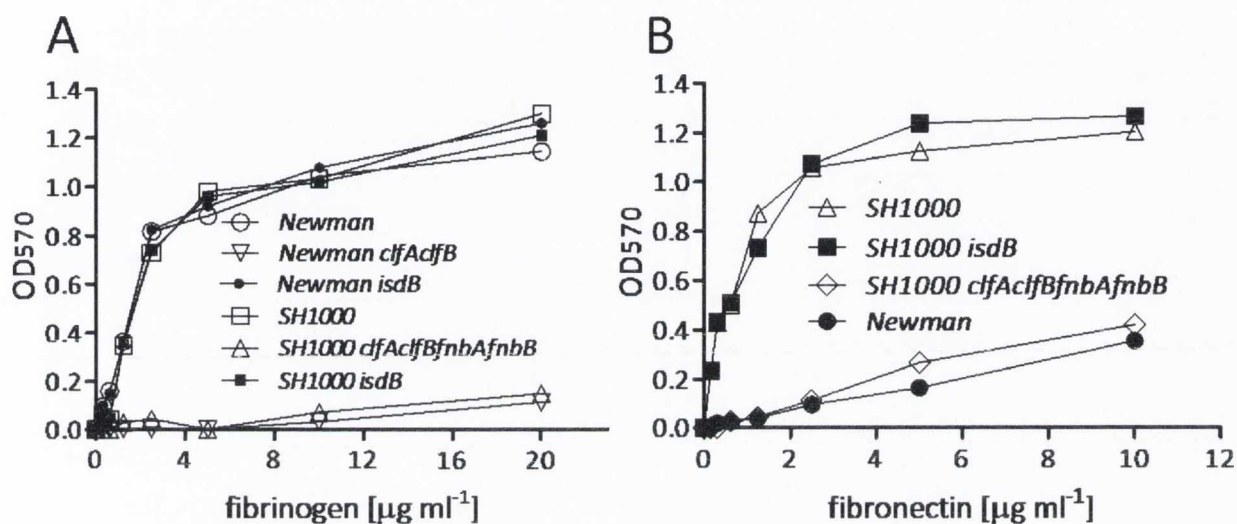


Figure 4.3 Binding to fibrinogen and fibronectin by *S. aureus* Newman and SH1000 and mutants.

Bacterial adherence was performed in microtiter plates coated with various concentrations of either (A) fibrinogen or (B) fibronectin and incubated with RPMI-grow bacterial strains. After binding and fixing the cells were stained with crystal violet and the optical density measured at 570nm. Values represent the means of triplicate wells. Binding assays were preformed three times with similar results.

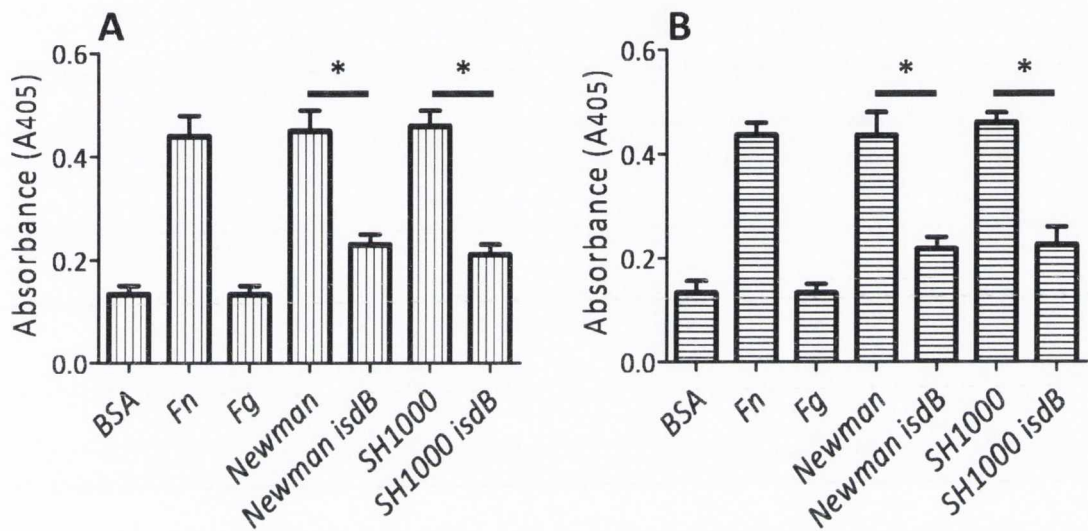


Figure 4.4 Adhesion of 293T and HeLa cells to *S. aureus* Newman and SH1000.

Wild-type Newman and SH1000, along with their *isdB* mutants were grown in RPMI and immobilized in microtiter plates. BSA, fibronectin (Fn) and fibrinogen (Fg) were added to control wells. **(A)** 293T cells and **(B)** HeLa cells were washed and added to microtitre wells and incubated for 1 h at 37°C. Adherent cells were lysed and intracellular acid phosphatase measured. Experiments were performed three times. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05).

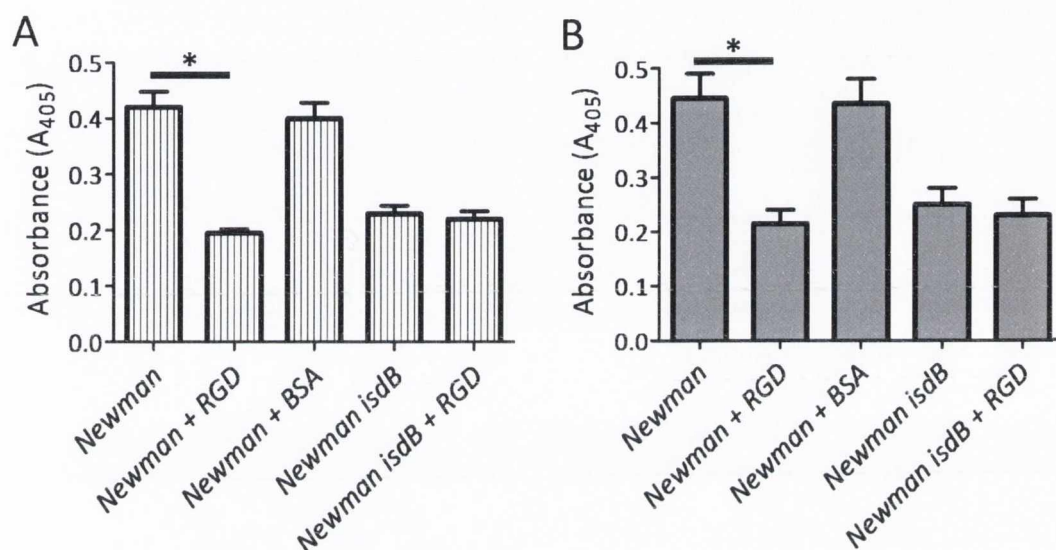


Figure 4.5 Inhibition of 293T and HeLa cells adhesion to *S. aureus*.

Newman strains were grown in RPMI to stationary phase and immobilized in microtiter wells. 293T and HeLa cells were washed and incubated with RGD peptide at 20 μ M, BSA or PBS for 10 min at 37 °C before being added to the wells. Adherent cells were lysed and intracellular acid phosphatase measured. Experiments were performed three times. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05).

SH1000 *clfA clfB fnbA fnbB isdB* were significantly less efficient at approximately 24% of the input inocula (Fig. 4.8). Secondly, in the presence of serum 93% of the SH1000 wild-type inoculum was taken up whereas the SH1000 *clfA clfB fnbA fnbB* mutant was much less effective at ca. 46% which was similar to SH1000 wild-type in the absence of Fn. This demonstrates that FnBPs and Fn are required for the highest levels of invasion. Comparing SH1000 to SH1000 *isdB* indicated a small but statistically significant reduction in invasion when IsdB was missing (80% uptake compared to 93%). SH1000 *fnbA fnbB isdB* was reduced to 27% compared to SH1000 *fnbA fnbB* (46%) (Fig. 4.8). These results show that IsdB promotes invasion by SH1000 both in the absence of and the presence of functional FnBPs and serum fibronectin.

Invasion was also performed with staphylococcal strains that had been grown in iron replete TSB, conditions that repress expression of Isd proteins. Invasion of human cells by *S. aureus* Cowan and *S. carnosus* has been studied previously (Hirschhausen *et al.*, 2010) and these strains were used as controls. Bacteria were incubated with monolayers of 293T cells that had been grown in DMEM supplemented with serum. One hundred % of the *S. aureus* Cowan input inoculum became internalized whereas *S. carnosus* did not invade detectably (Fig. 4.9). *S. aureus* SH1000 wild type and SH1000 *isdB* invaded at very similar levels (ca. 80% of input inocula) (Fig. 4.9) indicating that invasion attributed to IsdB expression reported above is indeed dependent on iron starvation and hence induction of IsdB.

4.2.4 Complementation of SH1000 *isdB*.

In order to complement the *ΔisdB* mutation in SH1000, the wild-type *isdB* gene was amplified and cloned into the anhydrotetracycline (aTc)-inducible expression vector pRMC2 (Corrigan *et al.*, 2009a). Both the pRMC2*isdB* and the empty vector were transformed into SH1000 *isdB*. Expression of IsdB was verified by Western immunoblotting of proteins solubilised from the cell wall of bacterial cells that had been grown in aTc (Fig. 4.10A).

To determine if IsdB - mediated invasion could be restored in the *isdB* mutant expression of IsdB was induced in *S. aureus* SH1000 *isdB* (pRMC2*isdB*) and the ability of bacteria to become internalized by 293T cells was measured and compared to SH1000 wild-type, SH1000 *isdB* and SH1000 *isdB* (pRMC2). *S. aureus* wild-type was internalized at 9% of the inoculum, in contrast to SH1000 *isdB* where ca. 3.5 % was taken up. The presence of pRMC2*isdB* restored the level of invasion of *S. aureus isdB*

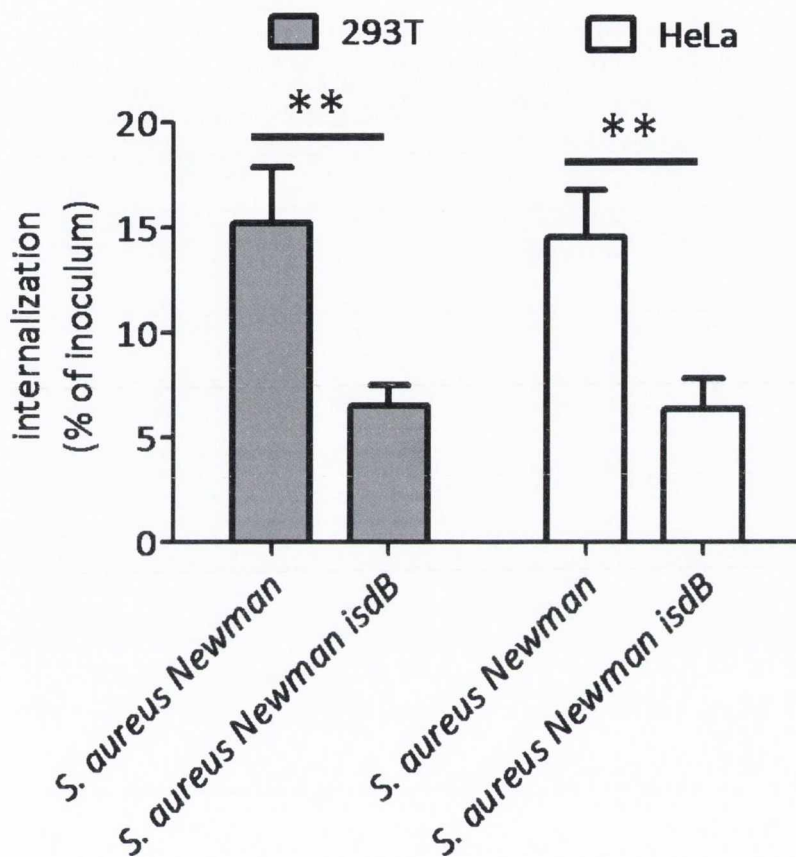


Figure 4.6 *S. aureus* Newman invasion into 293T and HeLa cells.

Gentamicin / lysostaphin protection assays to quantify the internalization of *S. aureus* Newman wild-type and Newman *isdB* mutant into the human cells. Infection was performed in RPMI for 2 h at 37°C. Inocula and internalized bacteria were quantified by viable counting. The experiment was performed three times. Differences that are statistically significant are indicated (Student's two tailed t test, **P<0.03).

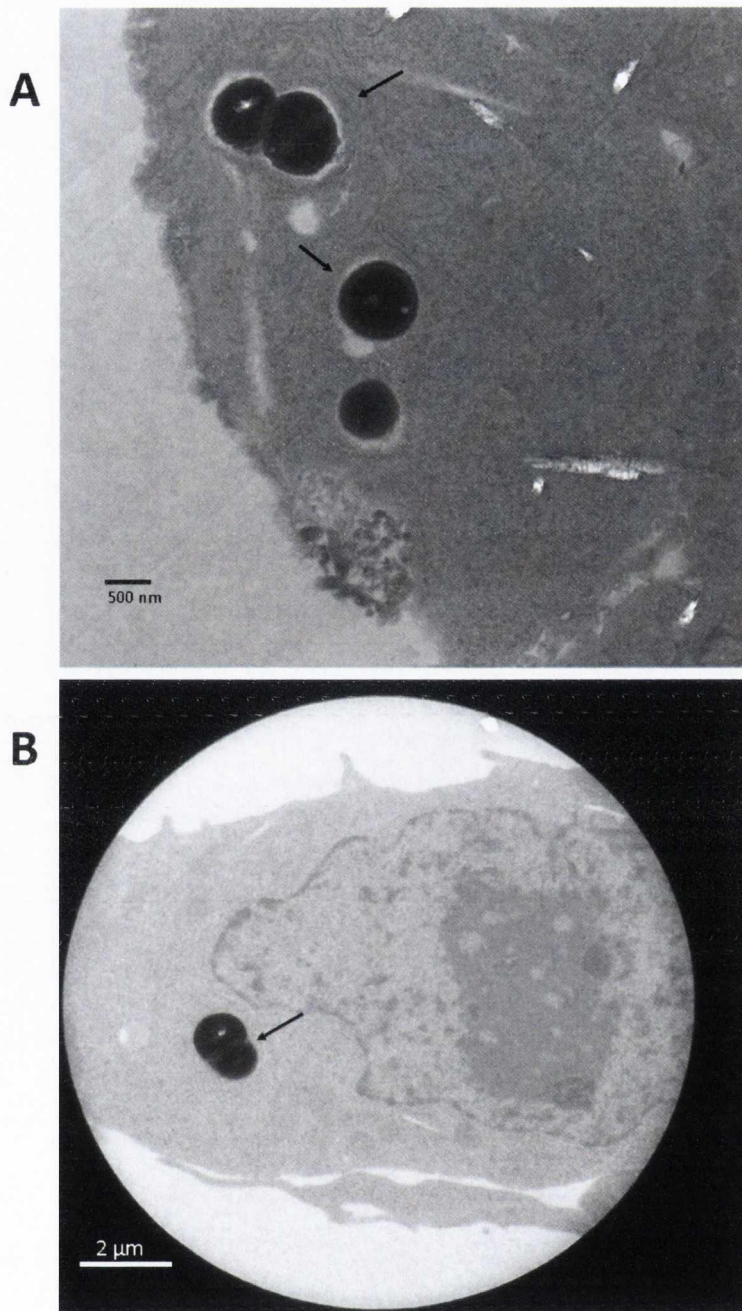


Figure 4.7 Transmission electron micrographs (TEM) representing 293T cells internalized by *S. aureus*.

Infection of 293T cells with **(A)** *S. aureus* Newman and **(B)** SH1000 *clfA clfB fnbA fnbB* was performed for 2 h, cells were washed and fixed with gluteraldehyde and processed for electron microscopy. Scale bars: **(A)** 500 nm and **(B)** 2 μm are shown.

to that of the wild-type while in *S. aureus* SH1000 (pRMC2) invasion occurred at approximately 3% of the input inoculum, a similar level as the *isdB* mutant (Fig. 4.10B). It should be noted that the lower levels of invasion compared to those in Figure 4.8 are the result of anhydrotetracycline present in the infection medium. The results demonstrated that IsdB - mediated invasion can be restored in *S. aureus* $\Delta*isdB* by expression of IsdB from the inducible plasmid.$

We then investigated if expression of IsdB in a heterologous host is sufficient to enable internalization. IsdB was expressed from the nisin-inducible plasmid pNZ8048 in *L. lactis* NZ9000 (Fig. 4.11). *L. lactis* NZ9000 carrying the empty vector pNZ8048 and the plasmid-free *L. lactis* NZ9000 host strain were used as controls. No invasion was observed by any of the strains (data not shown) indicating that expression of IsdB alone is not sufficient to promote bacterial internalization.

4.2.5 IsdB residues involved in binding to haemoglobin.

IsdB is the major haemoglobin (Hb) receptor of *S. aureus*. It uses a conserved mechanism to capture Hb that involves the aromatic residues in the loop 1 (β 1- β 2) on the N-terminal NEAT domain (Krishna Kumar *et al.*, 2011). As demonstrated in section 3.2.4 recombinant IsdB (Y165A/H166E/Y167A) does not bind to human Hb compared to the wild-type protein (Fig. 3.5D).

The Newman *clfA clfB isdB* (Y165A/H166E/Y167A) mutant was tested for adherence to and internalization by 293T cells. Adhesion and invasion by the mutant reached the same level as the parental (IsdB wild-type) Newman *clfA clfB* strain (Fig. 4.12AB) indicating that residues involved in Hb binding are not involved in adherence and invasion of the non-professional phagocytes.

4.2.6 IsdB promotes adhesion of MCF-7 cells to Fn and Vn.

The attachment of breast cancer MCF-7 cells to immobilized integrin ligands (fibronectin or vitronectin) or BSA was monitored after addition of recombinant IsdB, RGD peptide or BSA. The real-time measurements were performed using the xCELLigence. Microtiter wells were coated with ligands, blocked with BSA and equal numbers of MCF-7 cells (2×10^4) were added to each well. The MCF-7 cells bound strongly and spread on fibronectin and vitronectin as indicated by the increasing cell index (CI) values (Fig. 4.13AB). In contrast only small CI increase was seen in wells coated with BSA (Fig. 4.13C). Addition of soluble BSA did not influence the binding

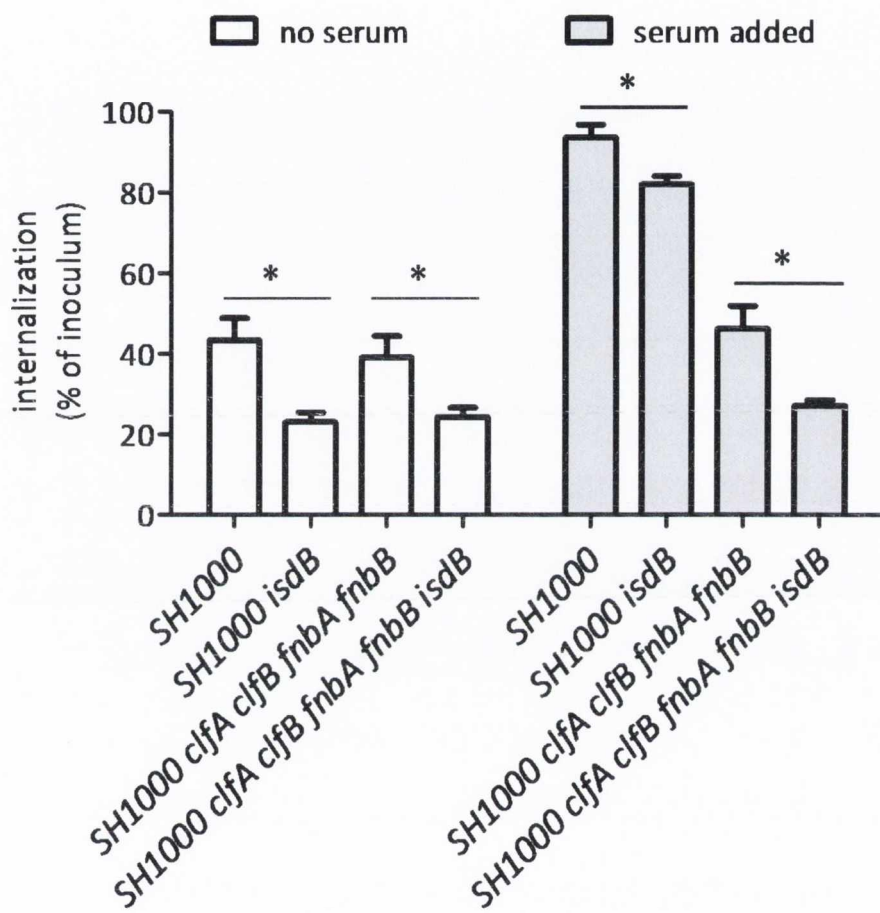


Figure 4.8 Invasion into 293T cells by *S. aureus* SH1000 and variants.

RPMI-grown *S. aureus* strains were used to infect 293T cells in iron restricted medium with or without added fetal bovine serum. The inocula and internalized bacteria were quantified by viable counting. The experiments were performed three times with similar results. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05).

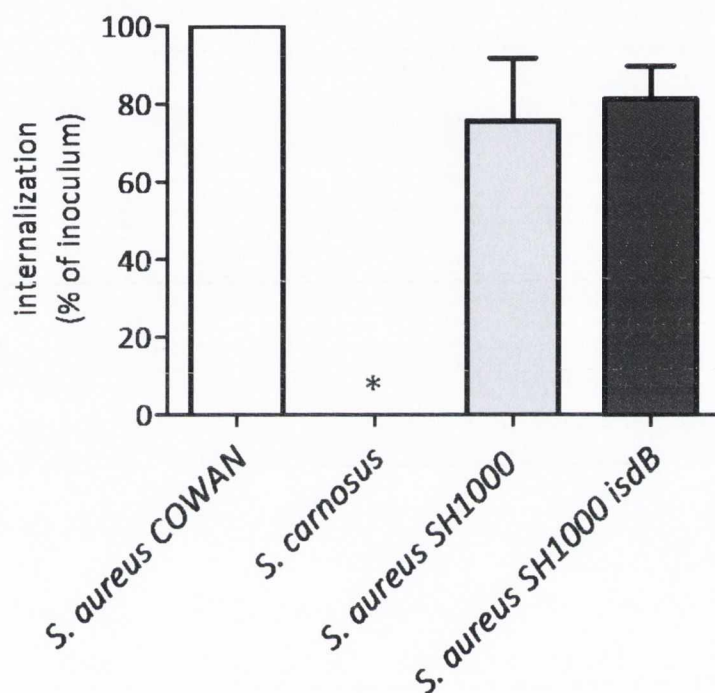


Figure 4.9 Internalization of *S. aureus* and *S. carnosus* by 293T cells.

Bacterial strains were grown in tryptic soya broth (TSB) for 16 h and added to 293T cells in monolayers. The experiment was performed in DMEM supplemented with 10% serum for 2 h at 37°C. The inocula and internalized bacteria were quantified by viable counting. The experiment was repeated three times with similar results.

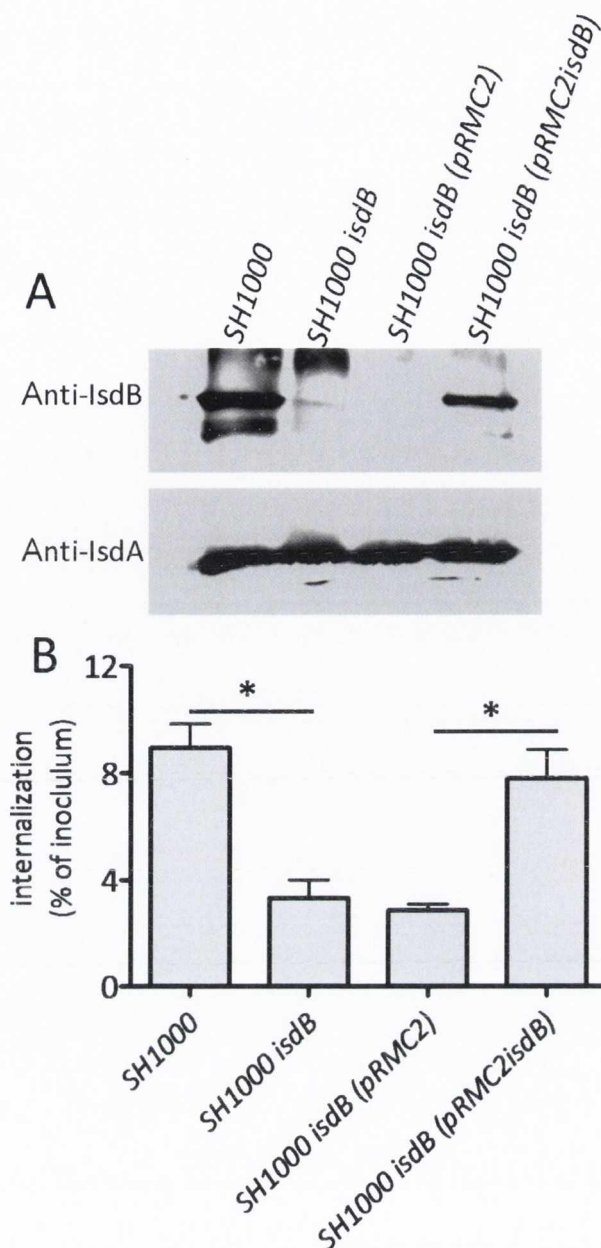


Figure 4.10 Complementation of *S. aureus* Δ isdB.

(A) Western immunoblotting analysis of IsdB expression by *S. aureus* SH1000 variants. Bacteria were grown in RPMI to exponential phase and supplemented with anhydrotetracycline (aTc). Cultures were harvested and cell walls were solubilised by lysostaphin digestion and proteins separated by SDS-PAGE. Western immunoblotting was performed using rabbit anti-IsdB and HRP - protein A.

(B) Gentamicin protection assay to quantify the levels of internalization. Strains were grown as described above, harvested and incubated with 293T cells. Infection was performed in RPMI supplemented with 300 ng ml⁻¹ of aTc for 2 h at 37°C. The inocula and internalized bacteria were quantified by viable counting. The experiments were performed three times and statistically significant differences are indicated (Student's two tailed t test, *P<0.05).

of MCF-7 cell to immobilized ligands (Fig. 4.13ABC). Upon addition of the RGD peptide inhibition of attachment to vitronectin and fibronectin was observed (Fig. 4.13AB) indicating that the peptide bound to integrins and obstructed the binding of the cells onto the immobilized integrin ligands. In contrast, when recombinant IsdB was added, an increase in adhesion and spreading of the cells on the immobilized fibronectin and vitronectin was observed (Fig. 4.13AB). This effect was not seen in wells coated with BSA (Fig. 4.13C), suggesting that IsdB specifically modulated the activity of integrins that bind to fibronectin and vitronectin. Neither IsdA nor IsdH had any effect on the spreading of MCF-7 cells (Fig. 4.14). These results indicate that IsdB promotes the binding of cells to ligands by potentiating the activity of integrins.

4.3 Discussion

Studies using various cell culture models demonstrated that *Staphylococcus aureus* can successfully invade cells that are not normally phagocytic and persist within the cells without causing their damage (Garzoni *et al.*, 2009). A subpopulation of cells which emerges and grows as small colonies variants (SCVs) when initially plated onto agar seem to be well adapted to the intracellular environment (Garzoni *et al.*, 2011; Proctor *et al.*, 2006; Vaudaux *et al.*, 2002). Intracellular persistence has been proposed to contribute to the pathogenesis of chronic osteomyelitis, which is difficult to treat. Relapses often occur despite extensive surgical cleansing and prolonged antimicrobial treatment. The intracellular dormant state may contribute to treatment difficulties in these and other infections because bacteria are inaccessible both to antibiotics and host immune defences (Ellington *et al.*, 2003).

The first mechanism of *S. aureus* invasion to be described involves the use of fibronectin-binding proteins and plasma fibronectin to provide a bridge to host cell integrins. This mechanism is sufficient to trigger bacterial uptake as demonstrated *in vitro* and *in vivo* by *L. lactis* expressing FnBPA which invades endothelial cells both in culture and in the endothelium lining heart valves during endocarditis infection (Que *et al.*, 2005). However this mechanism is clearly not the sole means by which *S. aureus* invades host cells. For instance in the mouse mastitis model an *S. aureus fnbA fnbB* mutant was still able to invade mammary gland epithelial cells, indicating that uptake could be mediated by other factors (Brouillette *et al.*, 2003) possibly Eap or Atl (Haggar *et al.*, 2003; Hirschhausen *et al.*, 2010).

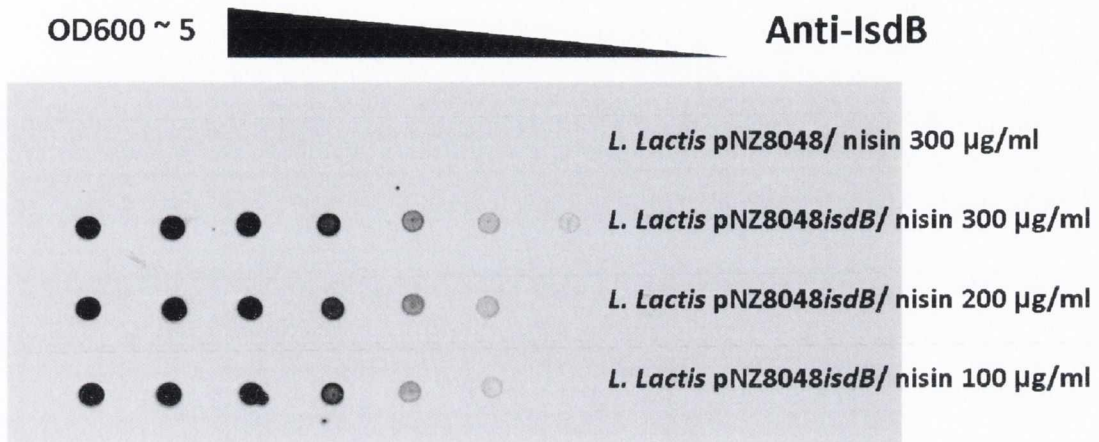


Figure 4.11 Inducible expression of IsdB by *L. lactis*.

Whole-cell immunoblot to detect surface expression of IsdB by *L. lactis* NZ9000. *L. lactis* pNZ8048*isdB* and *L. lactis* pNZ8048 were grown to exponential phase and induced with nisin. After 16 h induction the bacteria were harvested, dotted on cellulose membranes (at indicted concentrations) and subjected to immunoblotting. Detection was performed using rabbit anti-IsdB IgG followed by HRP-conjugated goat anti-rabbit IgG.

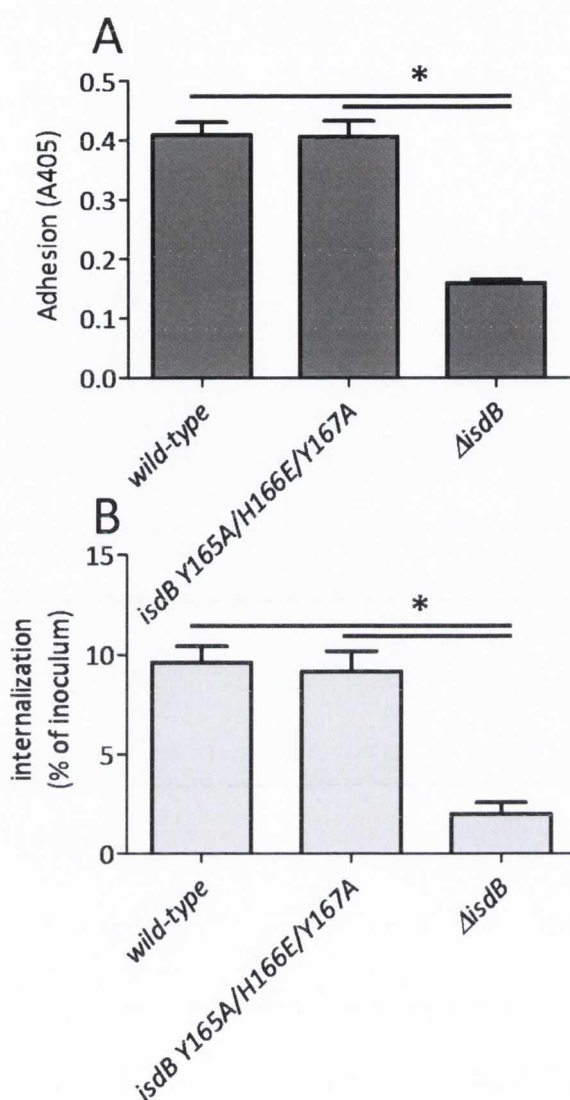


Figure 4.12 *S. aureus* IsdB Y165A/H166E/Y167A-mediated adherence to and internalization by 293T.

(A) Adhesion of cells to immobilized *S. aureus* was performed as described in Figure 1. The experiments were performed three times with similar results. Statistically significant differences are indicated (Student's two tailed t test, * $P < 0.03$). (B) Bacteria were grown as described above and used to infect 293T cells. Infection was performed in RPMI for 2 h at 37°C. Inocula and internalized bacteria were quantified by viable count. The experiment was performed three times.

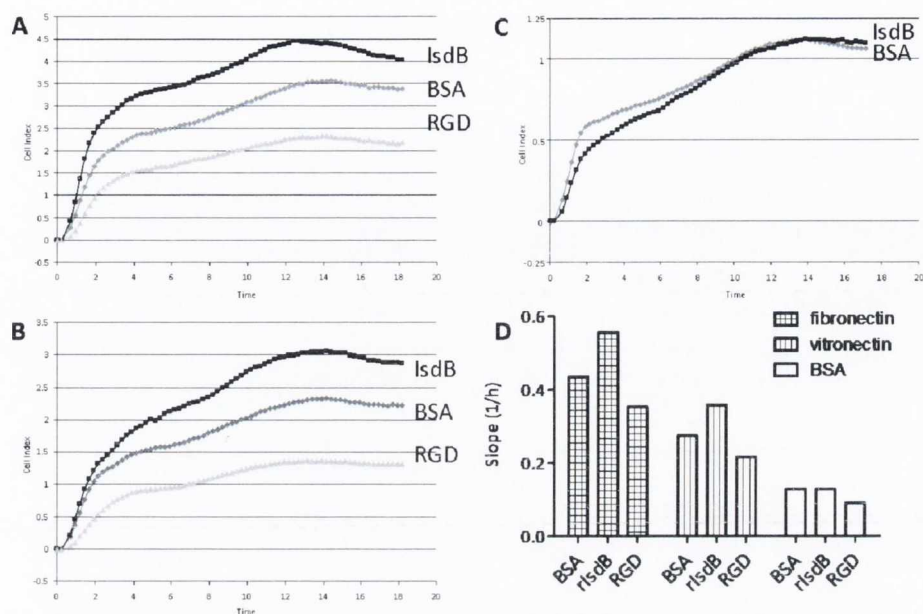


Figure 4.13 Real-time measurement of attachment and spreading of epithelial cells MCF-7 in the presence of soluble IsdB.

(A) Fibronectin, (B) vitronectin or (C) bovine serum albumin (BSA) were immobilized in microtiter wells, blocked with BSA and incubated with equal numbers of MCF-7 cells supplemented with BSA, IsdB or RGD peptide. XCELLigence was used to measure impedance in those wells. Cells attachment and spreading led to increased impedance which corresponded to higher cell index (CI) values. (A, B and C) Traces representing CI changes over-time. (D) Slope changes (slope/h) were calculated for CI traces within time points from 30 min to 4 h. the experiments were performed three times with similar results.

Here a role for the *S. aureus* IsdB protein in promoting adhesion to and internalization by non-professional phagocytes was identified. IsdB is a member of a group of *S. aureus* proteins that are up-regulated under iron-restricted conditions that pertain during growth *in vivo* and which may contribute to the ability of this pathogen to infect mammalian hosts (Allard *et al.*, 2006).

Expression of IsdB promotes adhesion of *S. aureus* to the human kidney embryo 293T cells and breast cancer epithelial HeLa cells. These cells adhered to iron-starved *S. aureus* Newman and SH1000 better than to the *isdB* deficient mutants. Initially, adhesion was studied in the absence of fibrinogen and fibronectin which eliminated any contribution from FnBPs or Clf proteins, both of which require host proteins to act as bridging ligands. Adhesion could be inhibited by the RGD peptide which binds at the interface between the α and β subunits of 'RGD integrins' suggesting that IsdB could interact with integrins other than $\alpha_{IIb}\beta_3$. This group of integrins are promiscuous receptors, with the β_3 subunit involved in binding to a large number of extracellular matrix and soluble ligands in plasma. The family comprises five α_v integrins, two β_1 integrins (α_5 , α_8) and the platelet integrin $\alpha_{IIb}\beta_3$. They have some ligands in common but differ in affinity due to differences in the ability of the RGD domain to bind to the different α - β pockets (Harburger *et al.*, 2009).

The expression of IsdB also promotes invasion of *S. aureus* into 293T and HeLa cells. Enhanced levels of invasion by *S. aureus* Newman and SH1000 grown under iron limited conditions occurred both in the presence of and absence of FnBPs or plasma fibronectin, suggesting that IsdB may provide a back-up mechanism of uptake. It is noteworthy that IsdB contributed significantly to *S. aureus* internalization even when the fibronectin and FnBPs were present. The contribution of IsdB to invasion is dependent on the conditions of bacterial growth and infection, because when cultured in iron-containing media *S. aureus* SH1000 and SH1000 *isdB* were internalized at equal levels.

IsdB and IsdH are both receptors for haemoglobin. Purified recombinant IsdB and IsdH proteins bind to human haemoglobin with similar affinities in low nanomolar range (Dryla *et al.*, 2007; Pishchany *et al.*, 2010). However, IsdB was shown to be more important in binding to human haemoglobin by *S. aureus* cells and is therefore considered to be the major haemoglobin receptor (Torres *et al.*, 2006). IsdB was also found to bind preferentially to human haemoglobin rather than mouse haemoglobin (Pishchany *et al.*, 2010). Despite this specificity towards human haemoglobin, several

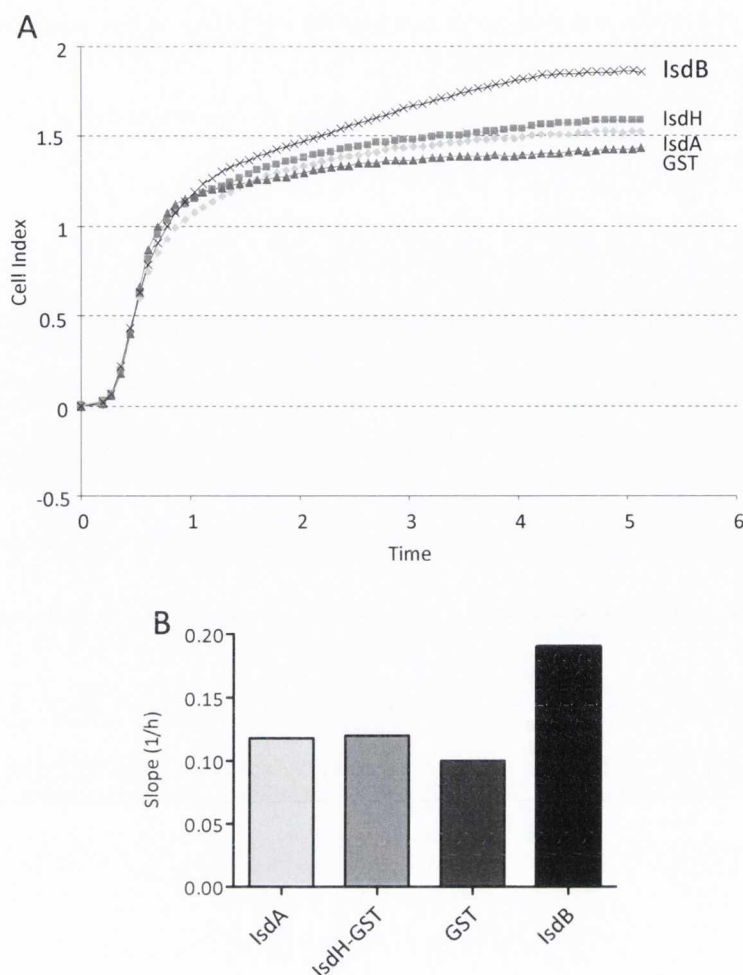


Fig. 4.14 Real-time measurement of attachment and spreading of epithelial cells MCF-7 in the presence of soluble IsdB variants.

(A) Fibronectin (Fn) was immobilized in microtiter wells, blocked with BSA and incubated with equal numbers of MCF-7 cells supplemented with either IsdA, IsdH-GST, GST or IsdB. XCELLigence was used to measure the real-time impedance in those wells. Cells attachment and spreading led to increased impedance which corresponded to higher cell index (CI) values. (A) Traces of CI changes representing MCF-7 spreading on Fn in the presence of IsdA (light grey), IsdH-GST (middle grey), GST (dark grey) or IsdB (black) over-time are shown. (B) Slope changes (slope/h) of CI values representing MCF-7 cells attachment to fibronectin upon incubation with different proteins (as indicated). Slope changes were calculated for CI traces within time points of 30 min to 4 h. The experiments were performed three times with similar results.

studies have demonstrated that IsdB is an important virulence factor that promotes sepsis and abscess formation in murine models of infection (Cheng *et al.*, 2009; Kim *et al.*, 2010; Torres *et al.*, 2006). In contrast, IsdH did not contribute to virulence in the same models (Kim *et al.*, 2010). Perhaps the role of IsdB in virulence could be indicative of another role for the protein. The ability to bind to haemoglobin and to mediate bacterial adherence to and invasion of cells do not seem related. *S. aureus* Newman *clfA clfB* expressing a mutant form of IsdB (Y165A/H166E/Y167A) that is unable to bind to haemoglobin could still adhere to and be internalized by the 293T cells to the same extent as the isogenic strain expressing wild-type IsdB. This alteration to IsdB might be able to differentiate between these two functions of the protein during *in vivo* studies to analyze its contribution to *S. aureus* virulence.

Interestingly, when added to a suspension of human epithelial cells, recombinant IsdB actually promoted binding of these cells to the integrin ligands fibronectin and vitronectin. This suggests that soluble IsdB may interact with the integrins to enhance or stabilize their binding with RGD-containing ligands. There may be a connection between this phenomenon and the ability of surface-bound IsdB to promote fibronectin-dependent invasion by *S. aureus*, particularly because expression of IsdB was shown to increase *S. aureus* invasion in the presence of FnBPs and fibronectin. The conformation of the receptors changes to the active form during seeding and, upon their contact of the cells with their RGD ligands. IsdB has a lower affinity for integrins than their natural ligands and only binds to the active form. We propose that soluble rIsdB binds to and stabilizes the active conformation of integrins. The interaction is weak and the integrins remain in the active conformation transiently allowing them to adhere more efficiently to the immobilized ligands. However, multiple copies of the low affinity IsdB protein attached to the bacterial cell surface can cooperate to promote adhesion to and invasion of bacteria into host cells.

IsdB-mediated invasion was restored in the SH1000 *isdB* mutant when IsdB was expressed from an inducible plasmid which confirms that the phenotype is IsdB-dependent. In contrast, expression of IsdB in the surrogate host *L. lactis* did not promote internalization by 293T cells suggesting that the IsdB is not sufficient and requires other factors. It is possible that other factors expressed by *S. aureus* could trigger a conformation change in the integrin or IsdB may be part of a protein complex on the cell surface that enhances integrin binding.

In summary this study demonstrated that IsdB expression promotes *S. aureus* adherence to human cells and internalization by these non-professional phagocytes. The IsdB-mediated adhesion to those human cells was demonstrated to be independent of haemoglobin binding. These studies help explain the important contribution of IsdB to virulence of *S. aureus* and underpin its use as a vaccine candidate antigen.

Chapter 5

**Characterization of the iron regulated surface determinants
of *S. lugdunensis* and their role in haemoglobin and haem-iron acquisition**

5.1 Introduction.

S. lugdunensis is part of the normal flora of human skin. It colonizes several distinct niches mainly in the lower part of the body (Bieber *et al.*, 2010). Standard identification procedures used in clinical microbiology laboratories can allow misidentification of *S. lugdunensis* as *S. aureus* (Schnitzler *et al.*, 1998). The bacteria have similar colony morphology and many strains of *S. lugdunensis* are haemolytic and express clumping factor activity (Lambe *et al.*, 1990). *S. lugdunensis* can cause infections similar to those caused by *S. aureus* ranging from localized to systemic, including skin and soft tissue infections (SSTI), breast abscesses, osteomyelitis, prosthetic joint infections, pneumonia and meningitis (Asnis *et al.*, 2003; Bocher *et al.*, 2009; Kaabia *et al.*, 2002; Klotchko *et al.*, 2011; Weightman *et al.*, 2000). Most notably *S. lugdunensis* can cause acute infective endocarditis with a high mortality rate (Anguera *et al.*, 2005; Liu *et al.*, 2010; Vandenesch *et al.*, 1993). *S. lugdunensis* infections occur at a much lower frequency than those caused by *S. aureus*, although the numbers may be underestimated (Arias *et al.*, 2010; Choi *et al.*, 2010; Wu *et al.*, 2011; Zinkernagel *et al.*, 2008). Understanding of this emerging pathogen requires in depth studies of molecular determinants of its virulence.

The complete genome sequences of *S. lugdunensis* strains HKU09-01 and N920143, as well as partial genome sequences of strains VCU139 and M23590, have been published in recent years (Heilbronner *et al.*, 2011; Tse *et al.*, 2010) allowing studies of the molecular determinants potentially responsible for virulence of this human pathogen. The precisely annotated N920143 genome revealed 2447 coding sequences, 77.8 % of which have reciprocal matches with *S. aureus* MRSA252, 74.7 % with *S. epidermidis* RP62a and 95.4 % with HKU09-01 (Heilbronner *et al.*, 2011).

Uniquely for CoNS, *S. lugdunensis* possesses a cluster of genes with similarity, both in terms of sequence and organization, to the iron regulated surface determinant (*isd*) locus of *S. aureus* (Fig. 5.1A). The *isd* locus occurs in both sequenced genomes of *S. lugdunensis* and is tandemly duplicated in HKU09-01 (Heilbronner *et al.*, 2011). In *S. aureus* the *isd* genes are induced by iron limitation with promoters that are controlled by the ferric uptake regulator (Fur). The *S. aureus* Isd system is up-regulated *in vivo* during infection due to sequestration of iron by the host (Allard *et al.*, 2006; Baker *et al.*, 2003; Haley *et al.*, 2012). The system comprises nine proteins (IsdA-IsdI) whose main task is to bind haemoglobin and extract haem which is transported into the cytoplasm to provide a source of iron. Four of the proteins are anchored to cell wall

peptidoglycan by either sortase A or sortase B. The IsdA, IsdB and IsdH each contain the sortase A recognition sequences (LPXTG) (Fig. 1B). IsdC has a distinct recognition sequence NPQTN and is sorted by a type B sortase encoded by *srtB* which is itself part of the *isd* regulon (Mazmanian *et al.*, 2003). These proteins each contain one or more near iron transporter (NEAT) motifs which bind to haemoglobin or haem. Two receptors, IsdB and IsdH, participate in recognition and capturing of the most abundant haemoproteins within human host, haemoglobin and haemoglobin-haptoglobin complexes (Fig. 1.11). The N-terminal NEAT motifs of IsdH (NEAT1 and NEAT2) and IsdB (NEAT1) bind to the haptoglobin-haemoglobin complex and to haemoglobin but cannot bind haem (Dryla *et al.*, 2007; Pishchany *et al.*, 2010; Torres *et al.*, 2006). They share 47-65% identity and bind to a site on the α -chain of human Hb using conserved aromatic residues within loop 1 (β 1- β 2) (Fig. 1.11) (Krishna Kumar *et al.*, 2011). Haem is then removed from Hb and transported to the haem-binding NEAT domains that coordinate haem *via* a conserved Tyr in β 8 (Grigg *et al.*, 2011). The haem binding NEAT domains of IsdH NEAT3 and IsdB NEAT2 transfer haem to those of IsdA and IsdC and then to the membrane located haem transporter (IsdEF) (Grigg *et al.*, 2010b; Liu *et al.*, 2008; Muryoi *et al.*, 2008; Zhu *et al.*, 2008). In the cytoplasm the porphyrin ring is broken by haem oxygenases (IsdG and IsdI) and free iron is released (Reniere *et al.*, 2007).

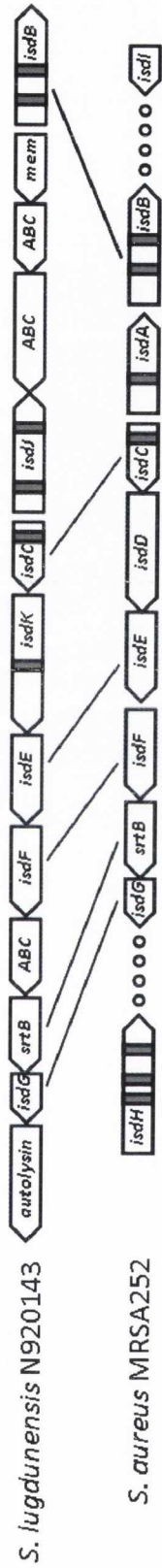
The objective of this study was to investigate four putative cell wall-anchored Isd proteins of *S. lugdunensis*, IsdB, IsdC, IsdJ and IsdK with respect to their expression, localization, haemoglobin and haem binding capabilities and their role in bacterial growth under iron limitation.

5.2 Results.

5.2.1 Sequence analysis of *S. lugdunensis* proteins with NEAT domains.

The predicted amino acid sequences of putative cell wall-anchored Isd proteins of *S. lugdunensis* N920143 and HKU01-09 were compared to Isd proteins of *S. aureus*. The IsdB and IsdJ proteins of *S. lugdunensis* are each predicted to have two NEAT domains, while IsdC and IsdK have one (Fig. 5.1B). IsdB and IsdJ have sortase A recognition sequences (LPATG and LPNTG, respectively) while IsdC and IsdK have sortase B-like recognition sequences (NPQTS and NKQPN, respectively) (Fig. 5.1B). *S. lugdunensis* IsdB and IsdC proteins are orthologues of *S. aureus* proteins with 37% and 57% identities, respectively. The identities occur mostly within the NEAT domains

A



B

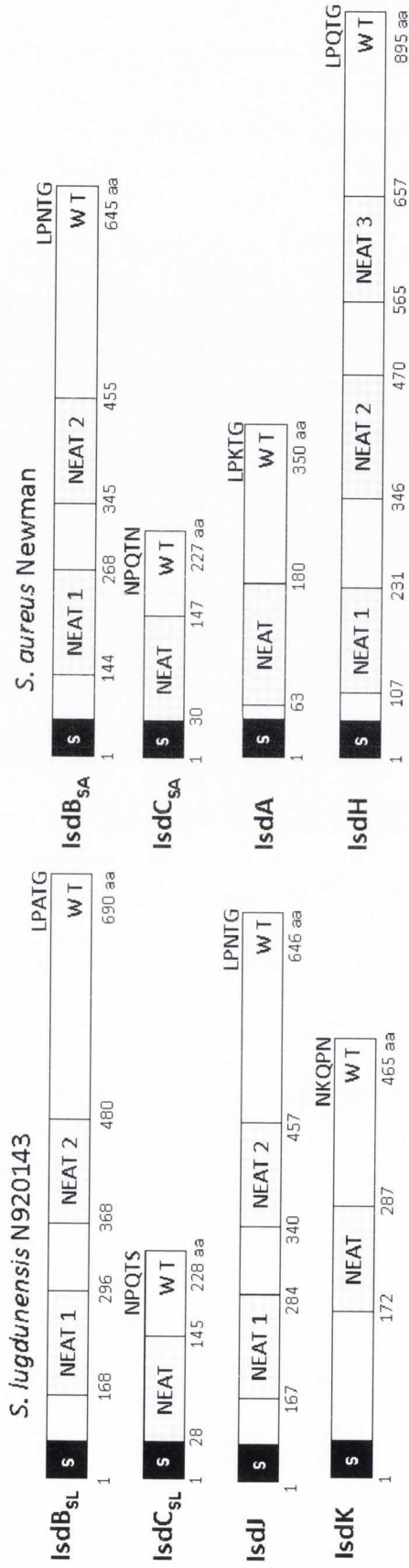


Figure 5.1 Schematic representations of the *isd* locus and Isd proteins of *S. lugdunensis* and *S. aureus*.

(A) Comparison of the *isd* loci of *S. aureus* and *S. lugdunensis*. Black lines connect orthologous genes. NEAT domains are indicated by grey boxes. The *isdH* and *isdI* genes of *S. aureus* are located outside the locus as indicated by circles. (B) Schematic representation of the putative cell wall anchored proteins IsdB, IsdC, IsdJ and IsdK of *S. lugdunensis* and IsdA, IsdB, IsdC and IsdH of *S. aureus*. The positions of the NEAT domains were determined from their predicted structures based on homology to available crystal structures of Isd proteins of *S. aureus*. Signal sequences (S) and putative cell wall anchoring motifs (W T) are indicated.

(Fig. 5.2B). The IsdJ has segments with similarities to IsdA of *S. aureus* (Fig. 5.2A). The NEAT1 domain of IsdJ has 48% identity to the single NEAT domain of IsdA while IsdJ NEAT2 and C-terminal residues 457-646 have 39% identity to IsdA (Fig. 5.2).

The NEAT domains of *S. lugdunensis* Isd proteins were modelled in 3D based on known X-ray crystal structures of the highly conserved NEAT domains of *S. aureus*. IsdA structure (PDB ID: 2ITE) was used to model the IsdJ NEAT1 and NEAT2 domains, and the IsdK NEAT domain (Grigg *et al.*, 2007). *S. aureus* IsdH NEAT3 (PDB ID: 2E7D) and IsdB NEAT2 (PDB ID: 3RTL) were used to model *S. lugdunensis* IsdB NEAT2 (Gaudin *et al.*, 2011; Watanabe *et al.*, 2008). The NEAT domain of IsdC was modelled on its orthologue from *S. aureus* (PDB ID: 2O6P) (Sharp *et al.*, 2007; Villareal *et al.*, 2008).

Each of the putative haem-binding NEAT domains was predicted to be composed of an IgG-like β -sandwich fold with 8 anti-parallel β -strands. Each had conserved residues within the predicted hydrophobic haem-binding pocket (Fig. 5.3A). A key metal coordinating residue Tyr is conserved in β 8. Conserved Lys, Ser, and Met residues which have been shown to form hydrogen bonds with the propionate groups of bound haem occur in a predicted helix linking β 1b and β 2. Hydrophobic residues involved in capturing the porphyrin also occur in β 1- β 2 (Tyr) and β 7 (Val, Ile, Val or similar) (Fig. 5.3A). IsdC is unique in using Tyr and Ile in β 4 to make van der Waal's contacts with haem. These residues are present in the *S. lugdunensis* orthologue (Fig. 5.3A) along with a 5 residue insertion in loop β 7- β 8 that is identical to that in IsdC of *S. aureus* (Fig. 5.3A). *S. aureus* IsdB NEAT2 domain coordinates haem in a different way using Met362 resulting in a Tyr444-Tyr440-Ser361-Met362 hexacoordinate haem-iron state (Grigg *et al.*, 2010b). This residue is, however, substituted by a Thr in the *S. lugdunensis* IsdB NEAT2 sequence (Fig. 5.3A).

The NEAT1 domain of IsdB from *S. lugdunensis* is 56% identical to the IsdH NEAT1 domain of *S. aureus* (Fig. 5.2B). Therefore the available crystal structures of IsdH NEAT1 (PDB ID: 3SZK, 3OVU and 2H3K) were used to model the domain allowing the prediction that it binds to haemoglobin (Krishna Kumar *et al.*, 2011; Pilpa *et al.*, 2006). The NEAT1 domain of IsdB from *S. lugdunensis* is 60% identical to the NEAT1 domain of *S. aureus* IsdB and 59% identical to the NEAT 2 of IsdH (Fig. 5.2B). The structural model presented a 9 stranded distorted β -barrel with an immunoglobulin-like fold. A characteristic helix-containing loop occurs between β 1

and $\beta 2$ which contains a string of aromatic residues which are known to be crucial for binding to α -chain of haemoglobin (Fig. 5.3B).

5.2.2 Production of recombinant IsdB_{SL}, IsdC_{SL}, IsdJ, IsdK and their variants.

Site-directed mutagenesis of recombinant IsdB_{SL}.

DNA fragments encoding Isd proteins of *S. lugdunensis* were amplified by PCR and cloned into the IPTG-inducible expression vectors pQE30 or pGEX4t-2. Recombinant plasmids (listed in Table 2.4) were used to overexpress the proteins in *E. coli* following their purification by either nickel - affinity chromatography or glutathione - affinity chromatography, respectively. The following recombinant Isd variants were expressed and purified to homogeneity: His-tagged: IsdB_{SL} (45-655), IsdC_{SL} (30-290), IsdJ (45-610), IsdJ (45-295), IsdJ (290-610), IsdK (35-426) and GST-tagged IsdC (30-290) (Fig. 5.4, data not shown). The IsdJ (45-610) protein was found unstable, with characteristic degradation pattern (Fig. 5.4).

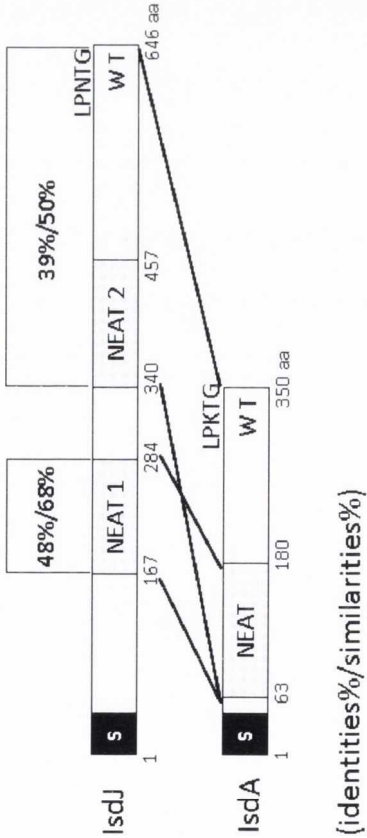
Recombinant IsdB substitution mutants (Y191A/H190E/Y189A) and (Y467A) for studies on haemoglobin and haem binding were created by sequential primer overlap mutagenesis on pQE30*isdB*_{SL} (45-655) used as a template and primers listed in Table 2.5. This PCR based procedure was performed using Phusion High-Fidelity Polymerase as described in the methods section.

5.2.3 Expression and cellular localization of Isd proteins of *S. lugdunensis* strains.

Recombinant IsdB_{SL} (45-655), IsdC_{SL} (30-290), IsdJ (45-610) and IsdK (35-426) proteins purified with N-terminal hexa-histidine affinity tags were used to immunize rabbits in order to obtain specific anti-sera (Table 2.1). The potency and specificity of the sera was determined by ELISA. Antisera were used to probe whole cells by dot immunoblotting and cell fractions by Western immunoblotting.

Absence of IgG-binding proteins, like protein A facilitated specific detection of proteins exposed on *S. lugdunensis* surface by whole cells dot immunoblotting. *S. lugdunensis* N920143 was grown to stationary phase in TSB containing iron (Moreira Lde *et al.*, 2003) or iron-restricted RPMI with or without added FeCl₃. Whole cells were dotted onto membranes beginning at an OD₆₀₀ of 1 for probing with anti-IsdC and anti-IsdB sera or an OD₆₀₀ of 50 for IsdK and IsdJ. IsdB, IsdC and IsdK were detected on the surface of cells grown in RPMI but not on TSB-grown cells indicating that they were only expressed in iron limited conditions (Fig. 5.5ABC). Addition of increasing

A



B

S. lugdunensis N920143

% identities/ % similarities	IsdB _{SL} NEAT1	IsdB _{SL} NEAT2	IsdC _{SL} NEAT	IsdJ NEAT1	IsdJ NEAT2	IsdK NEAT1
IsdB _{SA} NEAT1	<u>60/71</u>	-	-	30/50	28/48	-
IsdH NEAT1	<u>56/73</u>	-	-	34/50	-	25/43
IsdH NEAT2	<u>59/73</u>	-	-	28/45	-	-
IsdB _{SA} NEAT2	-	<u>56/72</u>	27/44	27/42	29/47	28/46
IsdH NEAT3	-	<u>57/69</u>	-	-	26/46	-
IsdA NEAT	30/49	-	25/45	<u>48/68</u>	<u>55/73</u>	<u>36/61</u>
IsdC _{SA} NEAT	-	-	<u>84/91</u>	26/43	-	27/43

S. aureus Newman

Figure 5.2 Homology of the Isd proteins of *S. lugdunensis* and *S. aureus*.

(A) Scheme representing conservation within specified regions of *S. aureus* IsdA and *S. lugdunensis* IsdJ. (B) Table comparison of the amino acid conservation between NEAT domains of (vertical) *S. aureus* and (horizontal) *S. lugdunensis* Isd proteins (as indicated). Fields highlighted in grey represent Hb-binding NEAT domains while white fields show haem-binding NEAT domains. (-) Represents homology lower than 25% of identity between the domain's amino acid chains. Most significant levels of homology are underlined.

concentrations of FeCl₃ to RPMI resulted in decreasing levels of the proteins being expressed. 1 mM FeCl₃ completely abolished expression of IsdC and IsdK whereas 2 mM FeCl₃ inhibited production of IsdB (Fig. 5.5ABC).

Unexpectedly IsdJ was not detected on the cell surface. In order to determine if the antisera recognized the N-terminal region (likely to be surface-exposed) rIsdJ (45-295) and rIsdJ (295-610) truncates were expressed and purified. Dot immunoblotting and ELISA indicated that both regions were equally immunogenic (Fig. 5.5D) so it can be concluded that the N-terminal region is not exposed on the surface. Since the anti-IsdK serum was as potent in recognizing the recombinant protein as anti-IsdB and anti-IsdC serum (data not shown) it can also be concluded that IsdK is poorly expressed on the surface whereas IsdB is well exposed (Fig. 5.5).

Cells grown in TSB and RPMI were suspended in a buffer containing 30% raffinose and protoplasts were formed by treatment with lysostaphin. The protoplasts were removed by centrifugation leaving solubilised cell wall proteins in the supernatant (wall fraction). The protoplasts were lysed and the membranes separated by centrifugation to yield the membrane fraction. Wall and membrane fractions were analyzed by Western immunoblotting (Fig. 5.6). Each protein was expressed in RPMI with only a very slight expression of IsdB seen in TSB. IsdB and IsdC were predominantly found in the wall fraction but a significant amount was always found in the membrane fraction. Interestingly IsdC from the membrane fraction is larger than that from the wall fraction which indicates that a proportion of IsdC has not been sorted and is retained in the membrane with its hydrophobic and positively charged C-terminus (Fig. 5.6). Surprisingly, IsdJ and IsdK were only detected in the membrane fraction and appeared not to be sorted at all (Fig. 5.6 and 5.7). The same pattern of expression of Isd proteins was seen in four other isolates of *S. lugdunensis* N910319, N910320, N930432, N940025 (Fig. 5.7). This suggests that sortases are defective in anchoring the IsdJ and IsdK, perhaps due to their inability to recognize the proteins' sorting signals.

A possibility that IsdJ and IsdK are secreted by *S. lugdunensis* was investigated. Wild-type N920143 and the *isdK* and *isdJ* mutants were grown in RPMI, culture supernatants were concentrated and proportional amounts of each fraction were probed with anti-Isd sera. Similar amounts of IsdJ and IsdK were detected in the membrane and supernatant fractions and were missing from cultures of appropriate mutants (Fig. 5.8). However, the cell wall anchored IsdB protein was also detected in the supernatants

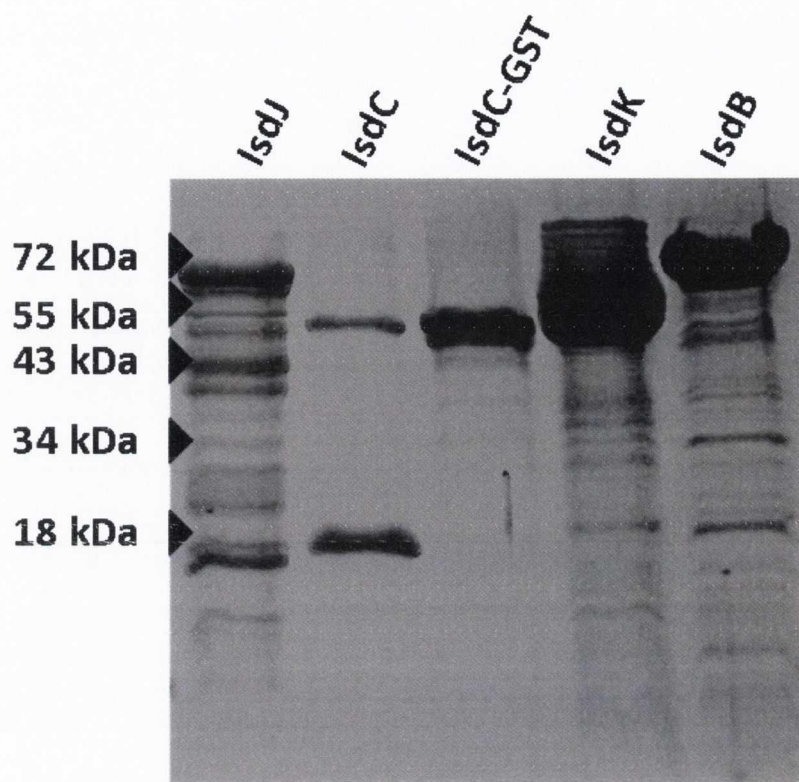


Figure 5.4 Purified recombinant Isd proteins of *S. lugdunensis*.

Coomassie blue – stained SDS-PAGE gel of the recombinant hexahistidine-tagged IsdJ, IsdC_{SL}, IsdK, IsdB_{SL} and GST-tagged IsdC_{SL}. Molecular weights are indicated.

possibly resulting from autolysis during growth. It is therefore difficult to determine if IsdJ and IsdK occur in the supernatant due to direct secretion because all three Isd proteins found in the supernatants were smaller in size suggesting that proteolytic cleavage had occurred (Fig. 5.8).

5.2.4 Isolation of *ΔisdC* and *ΔisdK* mutants by allelic replacement.

To study expression, cellular localization and roles of individual Isd proteins deletion mutants were generated in *S. lugdunensis* N920143. To generate null deletions of the genes, DNA sequences extending 500-1000 bps upstream and downstream of each gene were amplified to create fragments where the start and stop codons of each gene were paired forming a deletion. These fragments were cloned into pKOR1 vector. Obtained pKOR1*isdC* and pKOR1*isdK* plasmids were electroporated into *S. lugdunensis* N920143. Allelic exchange by homologous recombination was initiated by switching the bacterial growth temperature from that optimal for plasmid replication (28°C) to the non-permissive temperature of 42°C (pKOR1) as described in chapter 2.

Other deletion mutants used in this study, including *S. lugdunensis* N920143 *ΔisdB*, *ΔisdJ*, *Δisd* locus or *Δfbl* were isolated previously (Table 2.2) (Heilbronner S., personal communication) using the pIMAY vector.

The *ΔisdC*, *ΔisdB* and *Δisd* locus mutants were grown in RPMI and expression of the surface-exposed IsdB and IsdC proteins was measured by whole cell dot immunoblotting with appropriate anti-Isd sera. The data shows that the proteins were expressed on the surface at the same level as wild-type and were absent from the appropriate mutants (Fig. 5.9A). All Isd mutants were analyzed by cellular fractionation and Western immunoblotting which revealed the same pattern of expression of the Isd proteins as in Figure 5.6, with IsdB and IsdC being partially sorted to the wall with IsdJ and IsdK being found only in the membrane fraction (Fig. 5.9B). The level of expression of IsdB was the same in the wild-type and in the *ΔisdC*, *ΔisdJ* and *ΔisdK* mutants and was missing in the *isdB* mutant and the complete *isd* locus deletion. Similar patterns were obtained with IsdC, IsdJ and IsdK indicating an absence of polarity (Fig. 5.9B and 5.8).

5.2.5 Cellular localization of IsdJ expressed by *S. aureus* and *L. lactis*.

In order to investigate expression of IsdJ in other Gram-positive bacterial hosts the *isdJ* gene was cloned into the nisin-inducible *Lactococcus lactis* expression vector

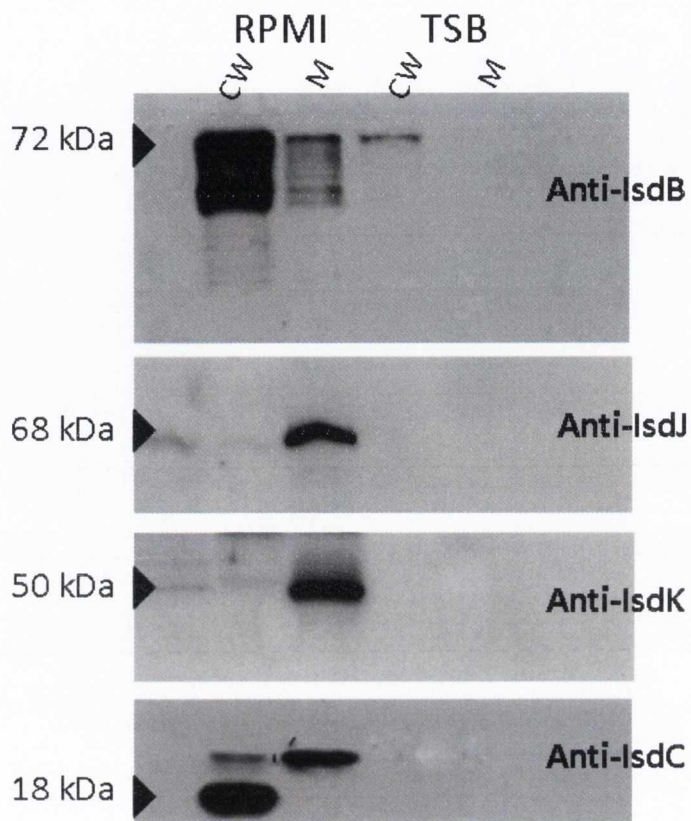


Figure 5.6 Cellular localization of the IsdB_{SL}, IsdC_{SL}, IsdJ and IsdK proteins. Western immunoblot performed to detect expression of Isd proteins in cellular fractions of *S. lugdunensis* N920143 grown in either TSB or RPMI. Lanes marked as CW contain solubilised cell wall fraction. Lanes marked with M contain membrane fractions. Immunoblotting was performed using rabbit serum recognizing IsdB (anti-IsdB), IsdC (anti-IsdC), IsdJ (anti-IsdJ) or IsdK (anti-IsdK). Bound IgG was detected by HRP-conjugated protein A.

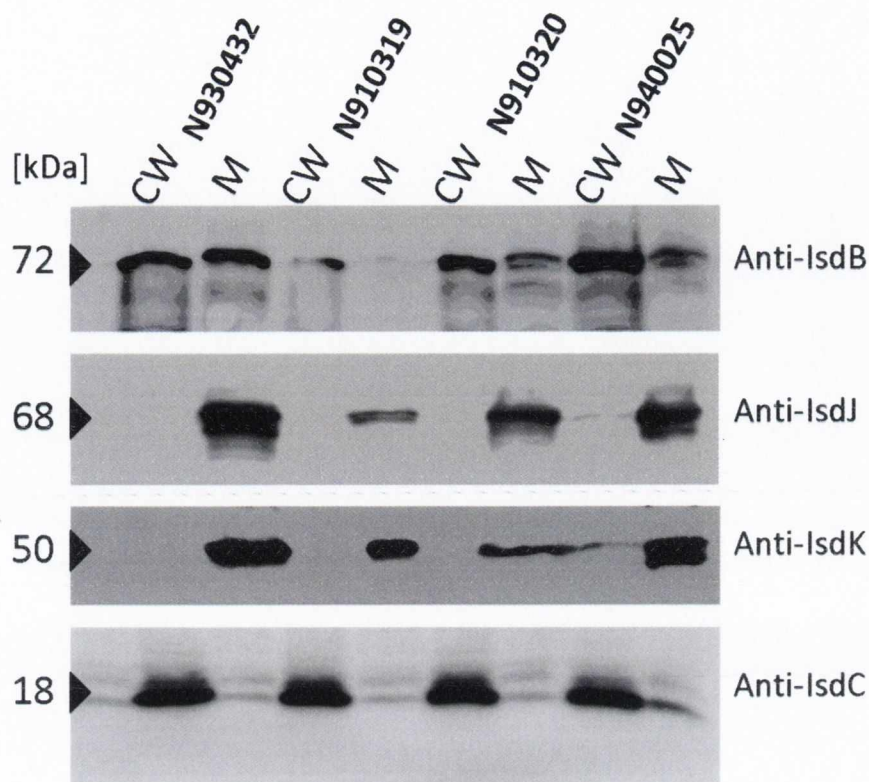


Figure 5.7 Expression and cellular localization of IsdB, IsdJ, IsdK and IsdC proteins in different *S. lugdunensis* strains.

Western immunoblot was performed to detect expression of Isd proteins in the cell wall (CW) and the membrane (M) fractions of *S. lugdunensis* clinical isolates (as indicated). Immunoblotting was performed using rabbit-anti-Isd antibodies (as indicated) followed by HRP-conjugated protein A to detect bound IgG.

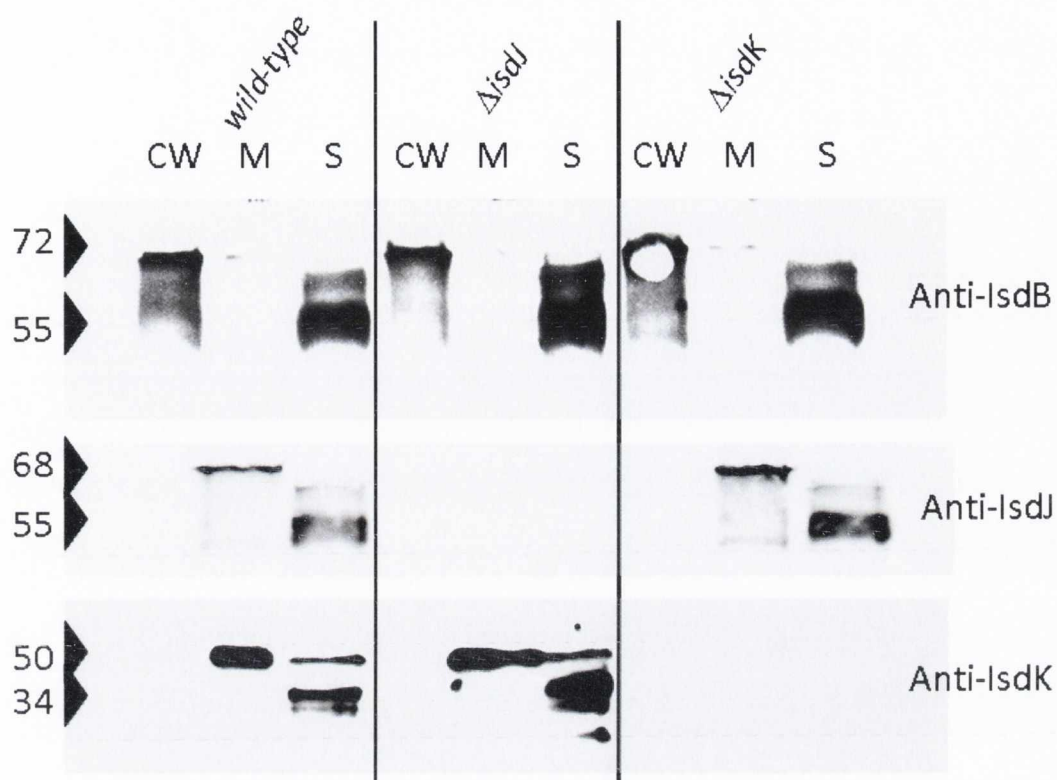


Figure 5.8 Secretion of the IsdJ and IsdK proteins.

Cell wall (CW), membrane (M) and supernatant (S) fractions of RPMI-grown *S. lugdunensis* wild-type, $\Delta isdJ$ and $\Delta isdK$ (as indicated) were subjected to Western immunoblotting. Immunodetection was performed using rabbit-anti-Isd antibodies (as indicated) followed by HRP-conjugated protein A to detect bound IgG. Experiments were performed three times with similar results.

pNZ8048 and into the anhydrotetracycline (aTc) - inducible staphylococcal expression vector pRMC2 to form pNZ8048*isdJ* and pRMC2*isdJ* (Table 2.4). Plasmids were cloned into *L. lactis* NZ9000 and *S. aureus* Newman *spa*, respectively. Following induction, expression of IsdJ was detected on the surface of *L. lactis* and on the surface of *S. aureus* Newman *spa* by whole cell dot immunoblotting (Fig. 5.10A). Interestingly the IsdJ protein was found only in the cell wall fractions of *L. lactis* and *S. aureus* and not in the membrane fraction, indicating that it has been sorted in the heterologous host (Fig. 5.10B). This further suggests that sortase A is not properly functional in *S. lugdunensis*.

5.2.6 Isd binding to haemoproteins: haemoglobin, haemoglobin-haptoglobin, haptoglobin, myoglobin.

Recombinant Isd proteins were tested for binding to methaemoglobin (MetHb; occurs mostly as an $\alpha\beta$ dimer with haem groups that contain oxidated ferric iron that are unable to bind oxygen), human haemoglobin (hHb, an $\alpha_2\beta_2$ tetramer), the haemoglobin-haptoglobin complexes (Hb-Hpt), human haptoglobin (Hpt) and human myoglobin (hMb). IsdB is the only Isd protein of *S. lugdunensis* that bound to soluble haemoproteins (Fig. 5.11AB). Human MetHb, Hb and Hp-Hpt bound to immobilized IsdB dose-dependently and saturably with half maxima at 8 ± 1.6 nM, 12 ± 2 nM and 2 ± 0.5 nM, respectively (Fig. 5.11, data not shown). Binding to haptoglobin alone or myoglobin was not detected (data not shown). Aromatic residues in the NEAT1 and NEAT2 domains of IsdH ($\beta 1$ - $\beta 2$ loop) were previously shown to be critical for binding to α -chains of hHb (Krishna Kumar *et al.*, 2011; Pilpa *et al.*, 2006). A mutant of *S. lugdunensis* recombinant rIsdB (Y189A/H190E/Y191A) was generated and found not to bind to hHb (Fig. 5.11C) or to Hb-Hpt complexes (data not shown). This demonstrates a conserved mechanism of binding of haemoglobin by the Isd haemoglobin receptors IsdB of *S. lugdunensis* and of *S. aureus*.

Solid phase binding assays were used to compare the ability of rIsdB_{SL} to bind to methaemoglobins of different animal origins. Recombinant IsdB bound to equine, bovine and porcine MetHb (Fig. 5.11D). Higher half-maximal binding of IsdB to animal MetHb (at 240 nM for porcine MetHb, 234 nM for equine MetHb and 250 nM for bovine MetHb) suggests lower affinities of the protein to those ligands compared to the human MetHb (at 10 nM) (Fig. 5.11D).

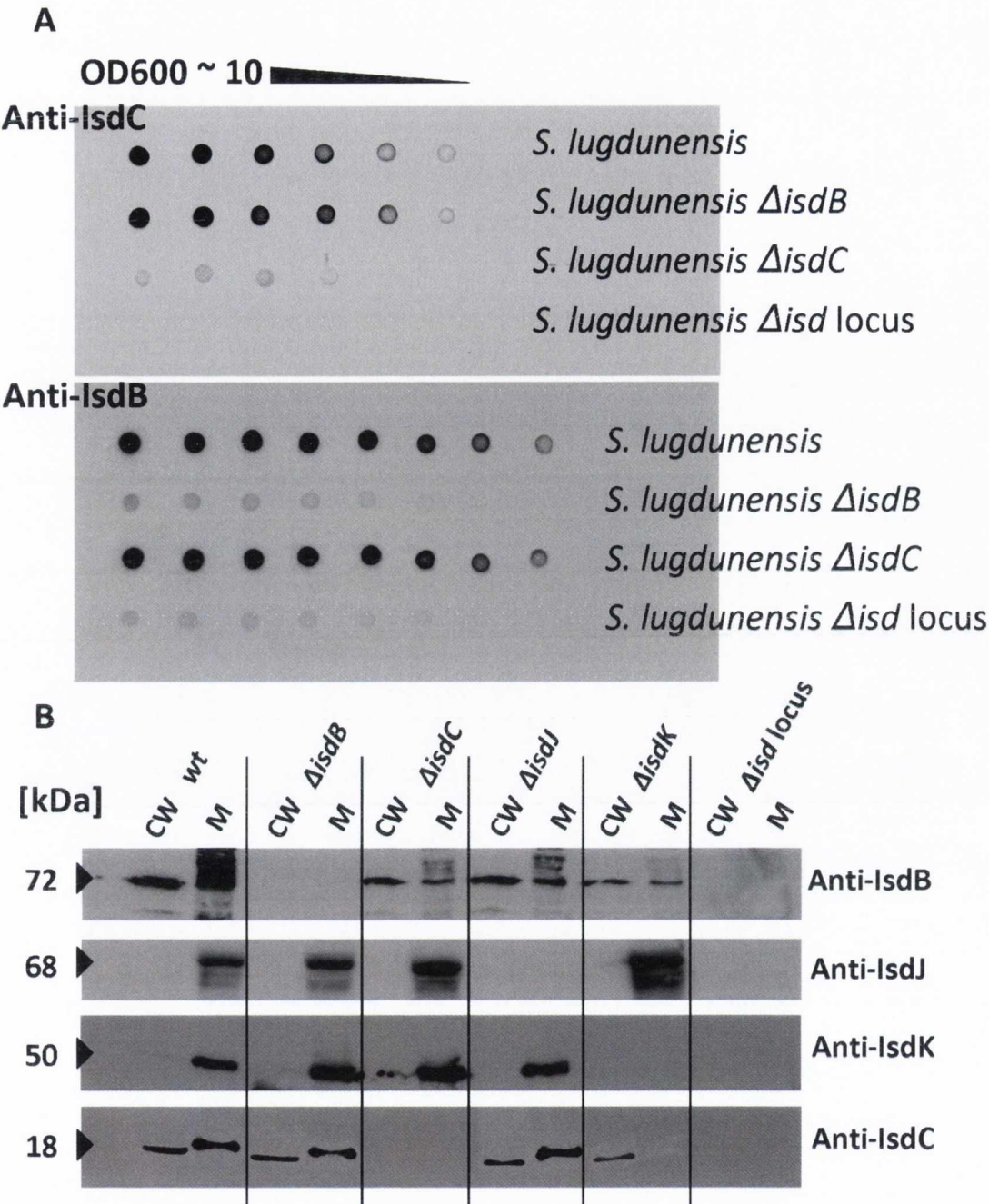


Figure 5.9 Validation of protein expression by *S. lugdunensis* isd deletion mutants.

(A) Expression of surface-exposed IsdB and IsdC by *S. lugdunensis* deletion mutants demonstrated by whole-cell dot immunoblotting of RPMI grown cells. (B) Western immunoblot to detect expression of Isd proteins in the cell wall (CW) and the membrane (M) fractions of *S. lugdunensis* N920143 mutants grown in RPMI. Immunoblotting was performed using rabbit anti-Isd IgG (as indicated) followed by HRP-conjugated protein A.

5.2.7 Surface plasmon resonance analysis of IsdB_{SL} binding to haemoglobins and methaemoglobins of different origins.

Solid phase assays measured specific binding of rIsdB_{SL} to hHb and MetHb with half-maximal binding concentrations in the low nanomolar range. Surface plasmon resonance (SPR), which allows real-time binding analysis under flow conditions, was applied to measure the affinities more accurately. Recombinant IsdB_{SL} was immobilized onto a dextran chip using monoclonal anti-polyhistidine antibodies and the human MetHb and human Hb were passed over the surface in concentrations ranging from 19.5 nM to 10 μ M and 11.7 nM to 6 μ M, respectively (Fig. 5.12AB). Human Hb and MetHb immediately associated to recombinant IsdB_{SL} reaching an equilibrium response for every concentration (Fig. 5.12, 5.13, data not shown). Due to predicted differences in stoichiometry of IsdB interaction with the ligands distinct evaluation models were adopted. IsdB, like IsdH can recognize two sites (α -chains) on tetrameric Hb and only one α -chain on the dimeric MetHb. Therefore the steady-state responses against concentrations were fitted with a two-binding-site affinity model for hHb and 1:1 binding model for MetHb. An accurate fitting was judged from the low χ^2 values of 9 and 10 giving an affinity K_D of 23.13 ± 4 nM for human Hb and 18.83 ± 2 nM for MetHb.

As demonstrated by Pishchany *et al.* *S. aureus* IsdB has a higher affinity for hHb than mouse haemoglobin (mHb) (Pishchany *et al.*, 2010). We measured *S. lugdunensis* IsdB binding to mHb using SPR. Purified mHb was passed over the surface coated with rIsdB_{SL} in concentrations ranging from 156.25 nM to 40 μ M. In great contrast to hHb, the mouse Hb binding levels were too low to be evaluated for an accurate affinity K_D as the response levels did not reach the required steady state equilibrium (Fig. 5.12B). This demonstrated a low affinity of *S. lugdunensis* IsdB for mHb with a theoretical K_D predicted at approximately 177 μ M.

As suggested by the results obtained by solid phase assays, IsdB_{SL} bound to the animal MetHb approximately 25-fold less effectively than to the human ligand. These results were validated using the SPR analysis. Soluble porcine MetHb, bovine MetHb and equine MetHb were passed over immobilized rIsdB_{SL} in concentrations ranging from 156.25 nM to 40 μ M. Despite the high concentrations being applied only low levels of binding responses could be detected by SPR for the ligands. An approximate affinity K_D for equine MetHb was evaluated at 31 ± 6.8 μ M (Fig. 5.13), about 1000-fold lower than the K_D for human MetHb. Porcine and bovine MetHb binding

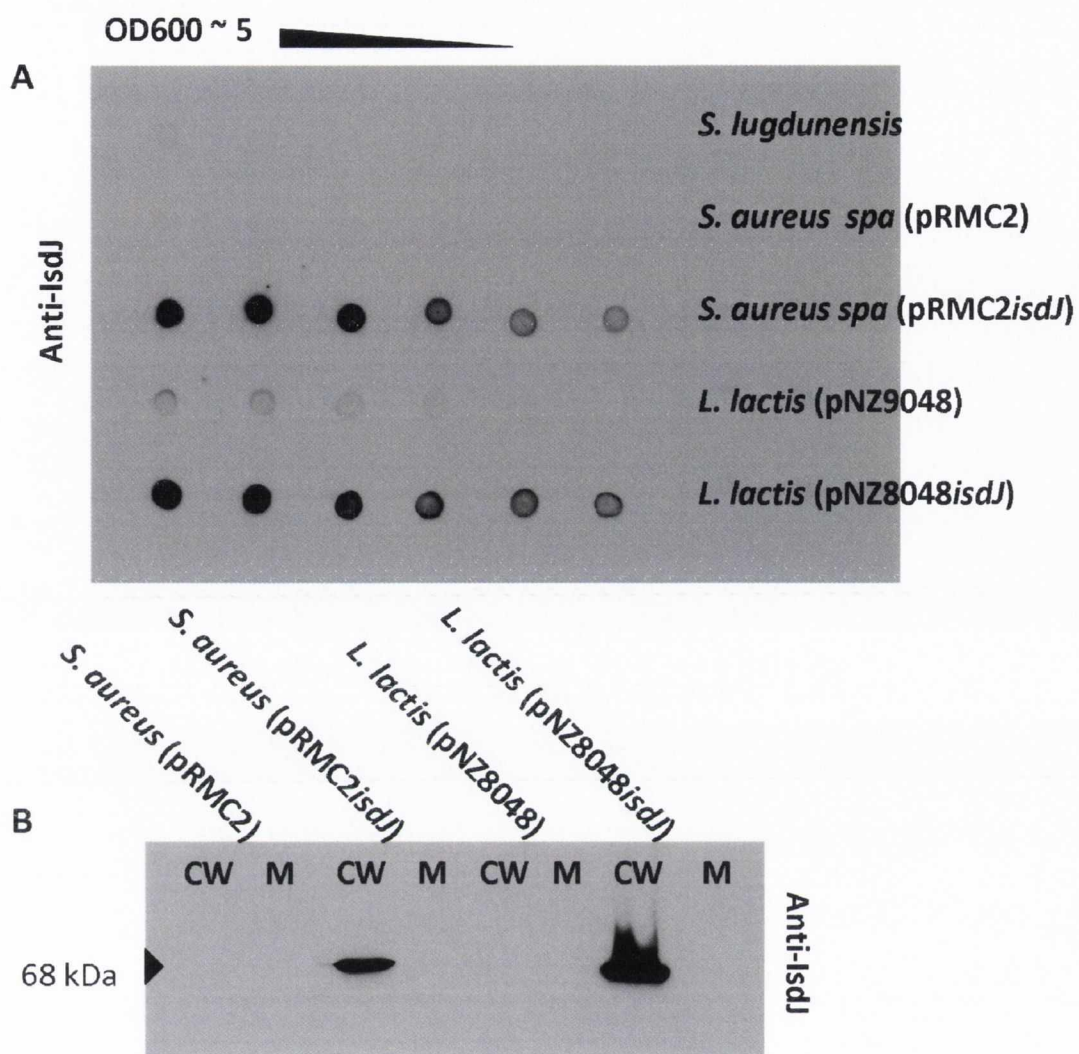


Figure 5.10 Cellular localization of IsdJ expressed in *S. aureus* and *L. lactis* from inducible plasmids pRMC2isdJ and pNZ8048isdJ, respectively.

(A) Whole-cell immunoblot to detect expression of surface-exposed IsdJ by *S. aureus* Newman *spa* and *L. lactis* NZ9000 and *S. lugdunensis* N920143. (B) Western immunoblot identifying cellular localization of IsdJ expressed by *S. aureus* and *L. lactis*. Cell wall (CW) and membrane (M) fractions were separated by SDS-PAGE. Immunoblotting was performed using rabbit anti-IsdJ serum followed by HRP-conjugated goat anti-rabbit IgG.

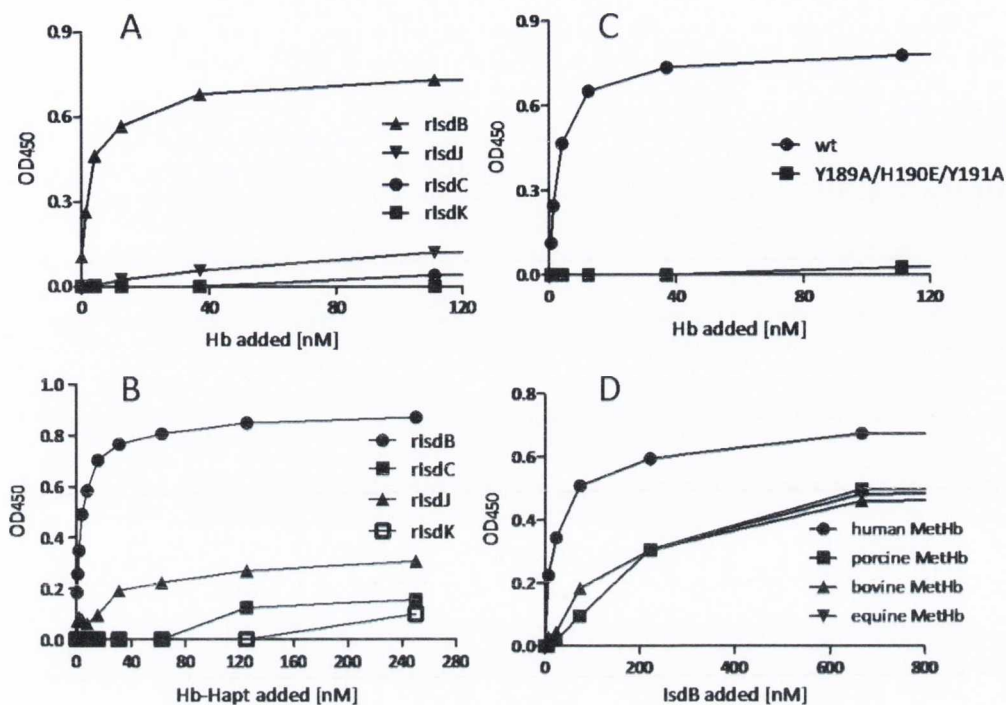


Figure 5.11 IsdB_{SL} binding to human haemoglobin (Hb), haemoglobin-haptoglobin (Hb-Hpt) complexes and methaemoglobins (MetHb) of different origins.

96-well plates were coated with (A and B) recombinant IsdB, IsdC, IsdJ and IsdK or (C) recombinant IsdB_{SL}, IsdB_{SL} Y189A/H190E/Y191A and IsdB_{SL} Y467A or (D) animal MetHb and incubated with various concentrations of (A and C) hHb, (B) Hb-Hpt complexes or (D) rIsdB_{SL}. Bound proteins were detected with (A, B and C) polyclonal rabbit anti - human haemoglobin serum or (D) rabbit anti-IsdB serum followed by HRP-conjugated goat anti - rabbit IgG. Values represent the mean of triplicate wells. Binding assays was preformed three with similar results.

responses to immobilized IsdB_{SL} have not reached the required steady state equilibrium and therefore the affinity could not be determined. Binding responses of the porcine and bovine MetHb to IsdB_{SL} suggested their affinities to be lower than the equine K_D.

5.2.8 Growth of iron-starved *S. lugdunensis* is enhanced by haemoglobin.

To test the hypothesis that the Isd system of *S. lugdunensis* is important for iron acquisition from haemoglobin (Hb) and that IsdB_{SL} in particular has a crucial role, we studied a nutritional contribution of Hb towards the bacterial growth. *S. lugdunensis* growth was significantly impaired when cultured in iron-restricted medium (RPMI) by comparison with rich medium (tryptic soya broth; TSB) (Fig. 5.14). To measure contribution of the human Hb (hHb) the bacteria were cultured in a modified RPMI medium pre-treated with Chelex 100 and containing a specific divalent metal chelator (EDDHA) to eliminate any iron residues. Preparation of the medium was described in the methods section. Growth of *S. lugdunensis* in this modified RPMI medium was further impaired (Fig. 5.15). Addition of haemoglobin resulted in significant growth enhancement starting at 16 h post inoculation and continuing until 48 h post inoculation (Fig. 5.15A). Wild-type *S. lugdunensis* grew to an OD_{600nm} of 0.86 in 48 h in modified RPMI containing human haemoglobin while in the absence of hHb the OD_{600 nm} was less than 0.4 (Fig. 5.15D). Mutants of *S. lugdunensis* lacking *isdB* or the entire *isd* locus grew to an OD_{600 nm} of ca 0.6 in the presence of hHb and to less than 0.4 in its absence (Fig. 5.15BCD). This demonstrates that growth of *S. lugdunensis* is enhanced by hHb in an IsdB-dependent fashion. Nevertheless the presence of hHb did support growth to a limited extent, perhaps due to unspecific binding of bacteria to haemoglobin which was observed before (Torres *et al.*, 2006).

To confirm the specificity of IsdB_{SL} for hHb, wild-type *S. lugdunensis* was grown in modified RPMI supplemented with mouse Hb (mHb), which in *in vitro* studies was shown to bind very weakly to IsdB_{SL} (Fig. 5.12). Wild-type *S. lugdunensis* grew better when supplemented with hHb than mHb, demonstrating the IsdB_{SL}-dependent preference towards the human haemoprotein (Fig. 5.16). However, addition of mHb resulted in enhanced growth compared to growth in the medium without added Hb. This growth enhancement initiated by mHb resulted in similar optical densities to those of *isdB* mutant supplemented with hHb, suggesting it is caused by the same IsdB_{SL}-independent mechanism (Fig. 5.16). This indicates a role for IsdB_{SL} in binding haemoglobin to support bacterial growth when iron is strictly limited. It also shows that

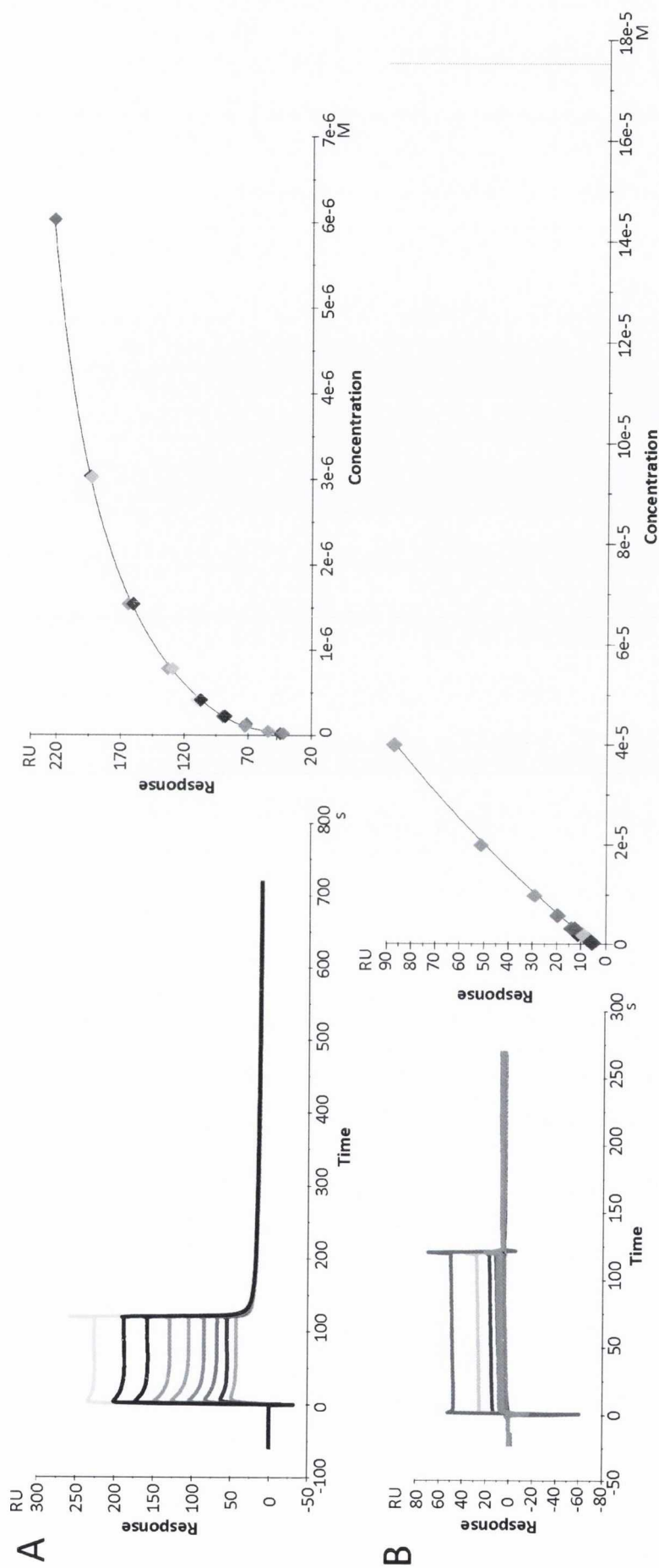


Figure 5.12 Surface plasmon resonance analysis of human Hb and mouse Hb binding to recombinant IsdB_{SL}.

Data were generated using Biacore X-100. IsdB_{SL} was immobilized on the surface of a CM5 sensor chip, followed by passing increasing concentrations of (A) human Hb or (B) mouse Hb over the surface. Injections began at 0 s and ended at 180 s. The data are presented as real-time graphs of (left) response units (RU) against time and (right) as SPR signals against ligands concentration in the late dissociation phase.

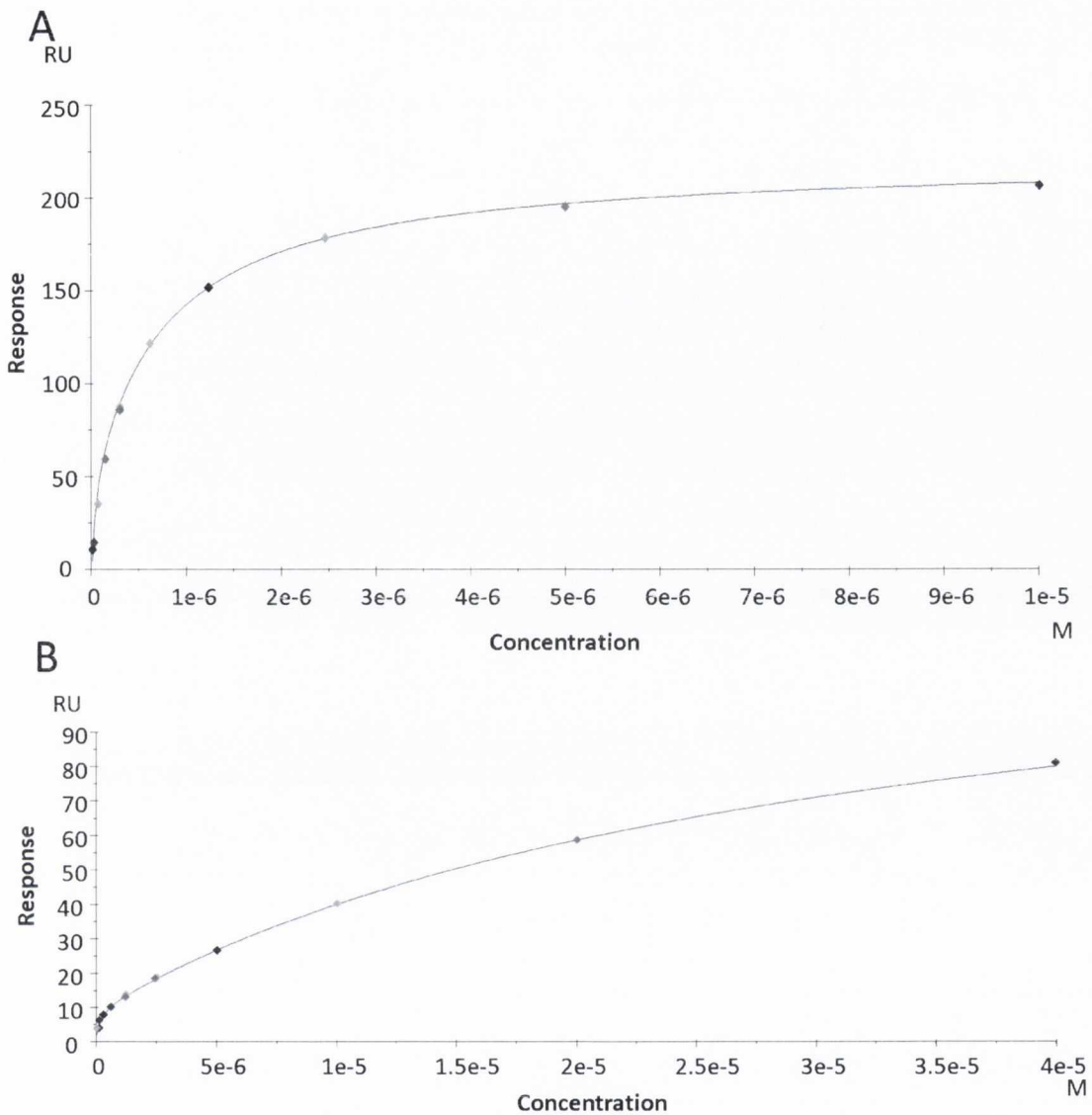


Figure 5.13 Surface plasmon resonance analysis of human and equine MetHbs binding to rIsdB_{SL}.

Data were generated using Biacore X-100. rIsdB_{SL} was immobilized on the surface of a CM5 sensor chip, followed by passing increasing concentrations of **(A)** human or **(B)** equine MetHb over the surface. Injections began at 0 s and ended at 180 s. The data are presented as real-time graphs of response units (RU) ligand concentration in the late dissociation phase.

the *S. lugdunensis* IsdB receptor like the one from *S. aureus* preferentially binds hHb over mHb.

5.2.9 Absorption spectra of the recombinant Isd proteins and haem transfer from IsdB_{SL} to IsdC_{SL}.

Recombinant Isd proteins purified from *E. coli* which was grown in LB were red in colour indicating their haem content. When the absorption spectra of each protein were measured Soret peaks at 400 nm (IsdB), 406 nm (IsdC), 404 nm (IsdK) and 403 nm (IsdJ) were detected (Fig. 5.17A). Recombinant IsdJ truncations containing either NEAT domain bound haem with each having Soret peaks at 403 nm (data not shown). It was determined from the spectra of pyridine haemochromes that the occupancy level was 10-30%. In contrast, proteins purified from *E. coli* cells grown in RPMI were not red in colour and had only 1-3% haem occupancy.

A conserved Tyr in $\beta 8$ of the NEAT motif of haem-binding Isd proteins is essential for ligand binding. An alanine substitution was introduced at (Y467) in NEAT2 of rIsdB_{SL}, which when purified from *E. coli* grown in LB resulted in a protein that lacked a Soret peak (Fig. 5.17B) and did not gain it after incubation with porcine haemin (data not shown). This strongly suggests that IsdB uses the same conserved haem binding mechanism as *S. aureus*. In *S. aureus* haem is stripped from haemoglobin bound to NEAT1 and NEAT2 of IsdH and/or NEAT1 of IsdB and transferred (*via* IsdA) to IsdC and then to the membrane transporters (Liu *et al.*, 2008; Muryoi *et al.*, 2008; Zhu *et al.*, 2008). *S. lugdunensis* lacks an orthologue of IsdH so IsdB NEAT1 is the only putative haemoglobin-binding NEAT domain among its Isd proteins. As IsdB_{SL} and IsdC_{SL} are the only cell wall-anchored and surface-exposed Isd proteins, we propose that IsdB NEAT1 captures haemoglobin and the stripes-out haem which is then transferred from NEAT2 to IsdC. To test part of this hypothesis we grew *E. coli* pQE30isdB_{SL} in RPMI, purified the apo- form of the protein and saturated it with haemin. This was then incubated with GST-tagged IsdC. The latter was separated from the mixture by glutathione sepharose chromatography. The eluted GST-IsdC had an enhanced Soret peak at 406 nm indicating that the protein had accepted haem transferred from IsdB (Fig. 5.17C). Equal amounts of the proteins were separated by SDS-PAGE and stained either with Coomassie blue or TMBZ peroxidase, a reagent that turns blue from the haemin-generated peroxides (Fig. 5.17D). This results indicate that IsdB_{SL} can transfer

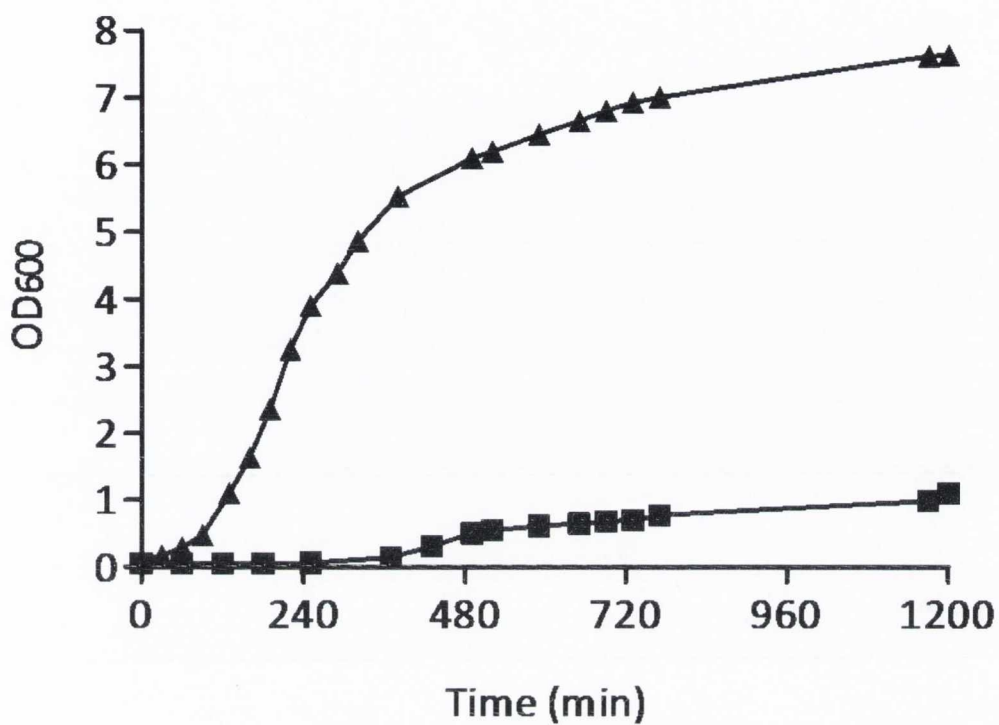


Figure 5.14 Growth of *S. lugdunensis* N920143 in tryptic soya broth or RPMI. Wild-type *S. lugdunensis* was grown in (triangles) tryptic soya broth or in (squares) RPMI. Measurements were taken over time based on the optical densities at 600 nm (OD₆₀₀). Experiment was repeated with similar results.

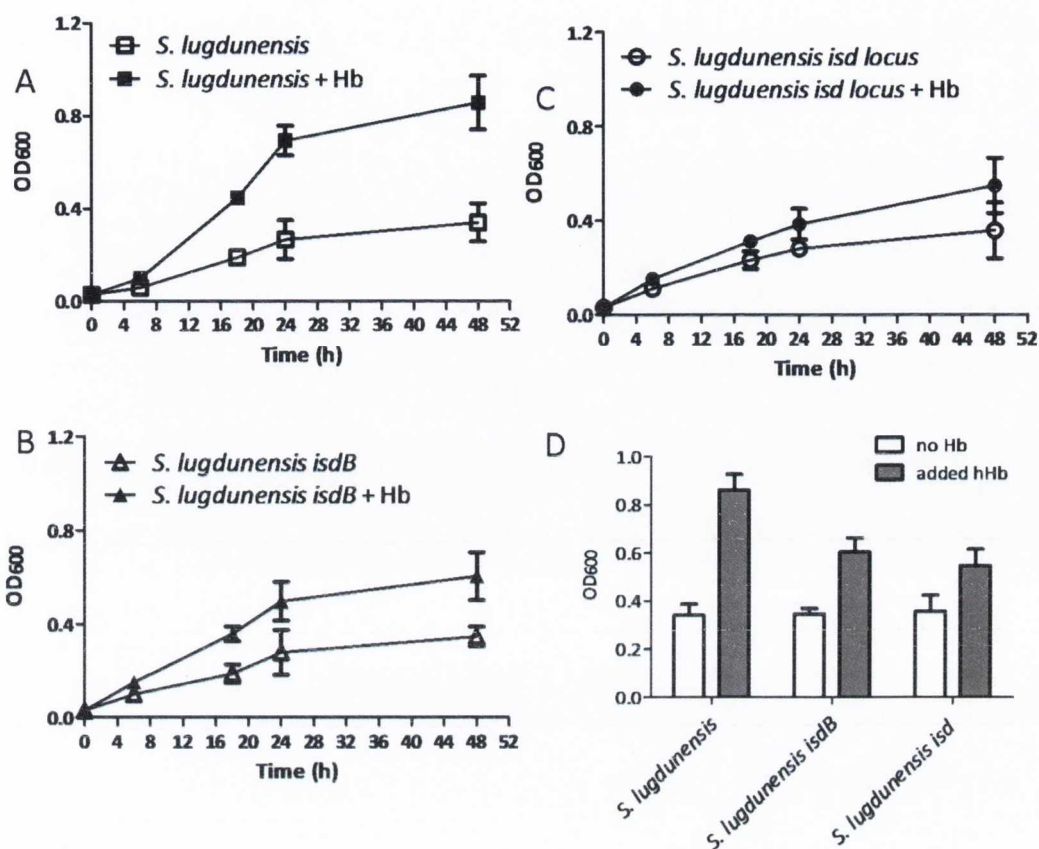


Figure 5.15 Growth of *S. lugdunensis* with or without human Hb as an iron source.

S. lugdunensis (A) wild-type, (B) Δ isdB and (C) Δ isd locus were grown in iron-depleted medium with or without human Hb (at $10 \mu\text{g ml}^{-1}$). Measurements were performed over 48 h based on optical densities at 600 nm (OD₆₀₀). (D) The means of OD₆₀₀ after 48 h post inoculation are presented. Experiment was performed three times. Error bars represent standard deviation of the OD₆₀₀. Differences are statistically significant (Student's two tailed t test, $P < 0.05$).

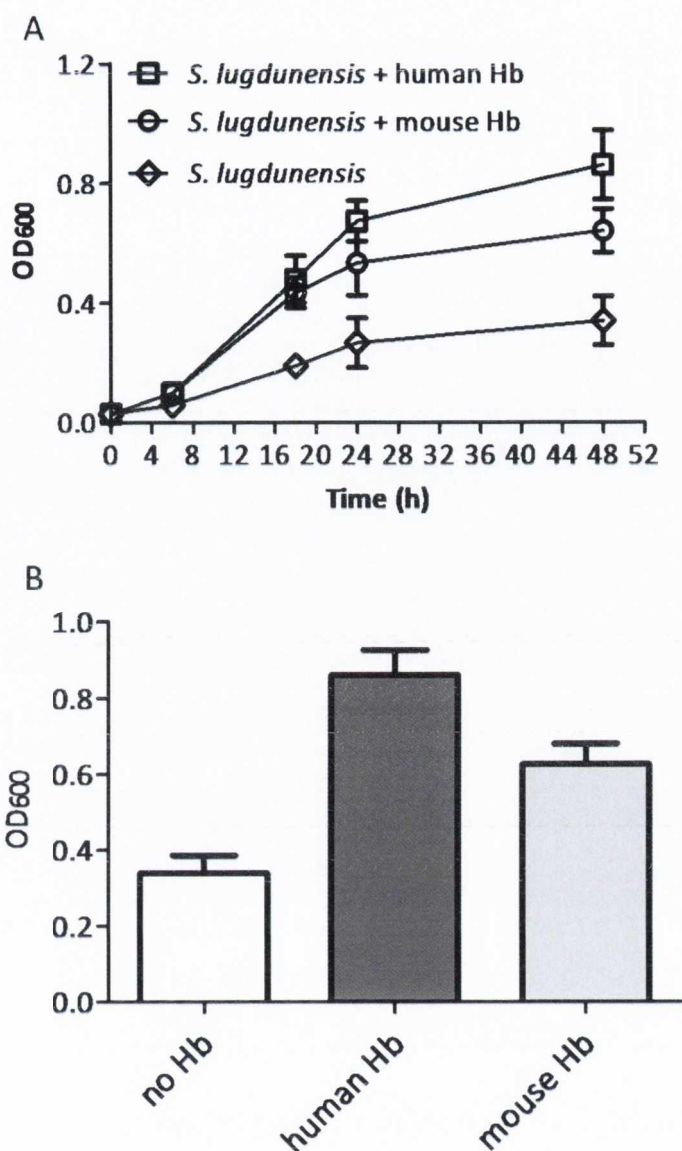


Figure 5.16 Growth of *S. lugdunensis* with human or mouse Hb as an iron source.

S. lugdunensis wild-type was grown in iron-depleted medium with or without human Hb or mouse Hb at $10 \mu\text{g ml}^{-1}$. **(A)** Measurements were performed over 48 h based on optical densities at 600 nm (OD₆₀₀). **(B)** The means of OD₆₀₀ after 48 h post inoculation are presented. Experiment was performed three times. Error bars represent standard deviation of the OD₆₀₀. Differences are statistically significant (Student's two tailed t test, $P < 0.05$).

haem directly to IsdC_{SL} and perhaps these two proteins form a two-component haemoglobin-haem acquisition system.

5.3 Discussion.

Uncharacteristically for CoNS *S. lugdunensis* is capable of causing serious systemic diseases in healthy adults. To do so it needs to overcome several challenges, among which is nutrient accessibility. Iron is a critical component for bacterial growth which is sequestered in the host as haem, mostly within haemoglobin (Otto *et al.*, 1992a; Otto *et al.*, 1992b). Additional iron-sequestration mechanisms are employed by the human host during infection to counteract the ability of pathogens to obtain iron (Baker *et al.*, 2003; Haley *et al.*, 2012; Hentze *et al.*, 2004; Legrand *et al.*, 2005). The inevitable competition with the host has led to bacterial pathogens developing several distinct mechanisms for accessing iron (Wilks *et al.*, 2007).

Four proteins from an Isd system of the emerging pathogen *S. lugdunensis* were characterized here to identify their potential in iron acquisition. The bacterium is the second species of *Staphylococcus* which produces the Isd proteins. It is also the sole example of a CoNS utilizing the Isd system for iron acquisition. The Isd proteins share significant structural and functional homology with the Isd receptors of *S. aureus*. Both systems are expressed under low-iron conditions. The *S. lugdunensis* proteins IsdB, IsdC, IsdJ and IsdK were induced in cultures grown in iron-restricted medium. This is consistent with report by Haley *et al.* which studied the role of *S. lugdunensis* haem monooxygenase (IsdG) (Haley *et al.*, 2011). These findings suggest that transcription of the *isd* locus in *S. lugdunensis*, like *S. aureus*, is regulated by the iron-dependent repressor Fur. Indeed Fur boxes were identified upstream from the start codons of *isdB*, *isdC* and *isdJ* genes (Fig. 5.1). Interestingly, the *isd* locus is duplicated in *S. lugdunensis* HKU09-01 which may increase the levels of transcription of the *isd* genes in that strain (Heilbronner *et al.*, 2011).

In both systems the IsdB and IsdC orthologues are localized in the cell wall of iron-starved bacteria. IsdB_{SL} is likely to be processed by sortase A (SrtA), while IsdC_{SL} linkage to peptidoglycan is most probably catalyzed by sortase B (SrtB) (Fig. 5.18). We observed that the *S. lugdunensis* IsdB and IsdC proteins seem only to be partially wall-anchored, as a portion of those proteins could be found in the membrane fraction. IsdK and IsdJ were detected only in the membrane fraction and were hardly detectable on the surface of the bacterium (IsdK) or could not be detected on the surface (IsdJ). IsdJ and

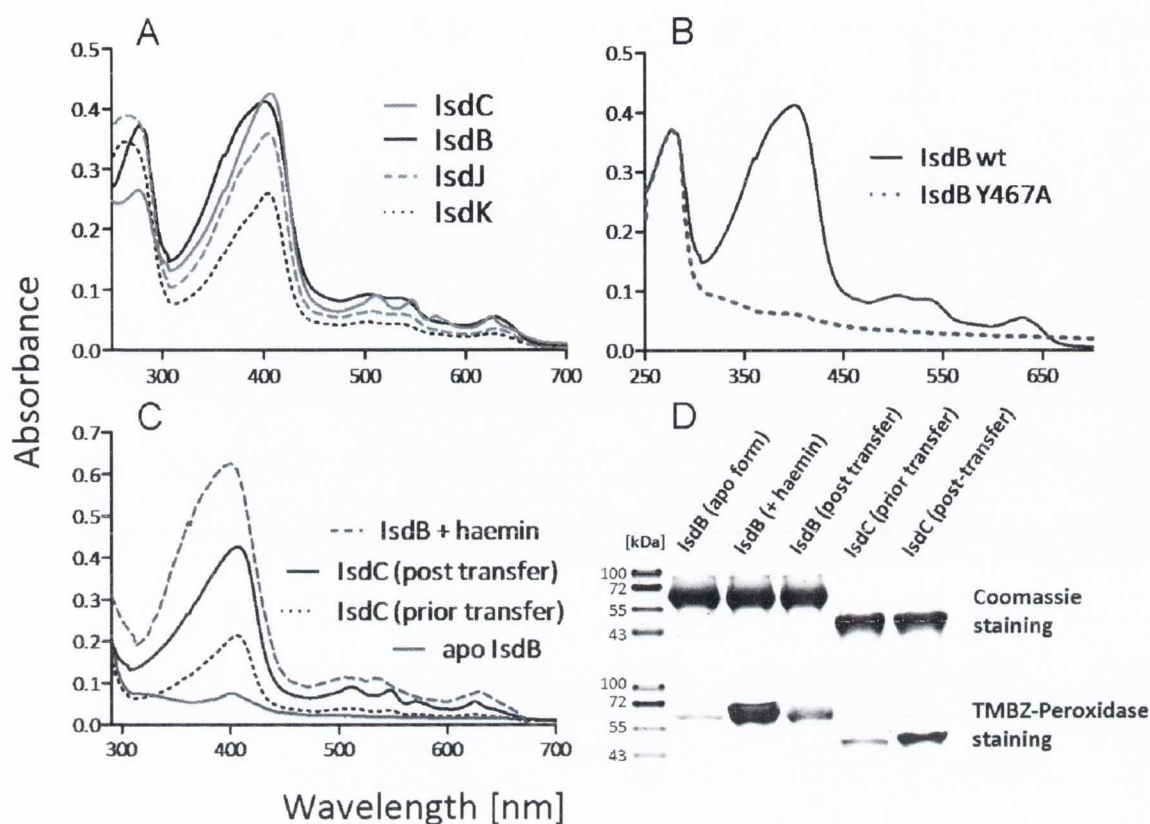


Figure 5.17 The absorption spectra of Isd proteins and spectral shifts following co-incubation of haemin-saturated IsdB with GST - IsdC.

(A) Recombinant His-tagged IsdB_{SL}, IsdC_{SL}, IsdJ, and IsdK proteins (at 10 μ M) purified from *E. coli* grown in LB broth. (B) Recombinant IsdB_{SL} and the Y467A mutant purified from *E. coli* grown in LB broth. (C) Coomassie and TMBZ stained SDS-PAGE gels with equal amounts of apo- and holo-IsdB_{SL} and apo- and holo-IsdC_{SL} proteins. (D) Visible spectra of His-tagged IsdB_{SL} and GST-tagged IsdC_{SL} (at 5 μ M) purified from *E. coli* grown in RPMI and their spectral shifts following saturation with haemin and haemin transfer to GST-IsdC_{SL}. These are representatives of three independent experiments.

IsdK contain C-terminal sorting signals composed of a sortase recognition motif (LPXTG or NPQTN, respectively) followed by a hydrophobic domain and a tail of positively charged residues and were therefore expected to be anchored to the cell wall and exposed on the cell surface. Despite SrtA and SrtB being capable of anchoring IsdB_{SL} and IsdC_{SL}, it is unclear why IsdJ and IsdK remain in the membrane. The ability of IsdJ to be sorted to the cell wall was demonstrated following inducible expression of IsdJ in *S. aureus* and *L. lactis*. IsdJ was sorted to cell wall of these heterologous indicating that differences in anchoring surface proteins to the cell wall must exist in *S. lugdunensis*.

The IsdJ and IsdK proteins were detected in *S. lugdunensis* culture supernatants suggesting an additional role in iron acquisition. Haem acquisition by secreted proteins containing NEAT-like domains has been well characterized in *Bacillus anthracis* (Ekworomadu *et al.*, 2012; Honsa *et al.*, 2011; Tarlovsky *et al.*, 2010). The bacterium secretes IsdX1 and IsdX2 haemophores (acquiring haem from host haemoglobin), which deliver haem to IsdC exposed on bacterial surface, allowing transfer of the iron-porphyrin into the cytoplasm. IsdX1 contains a single NEAT domain, whereas IsdX2 has five. NEAT1, 3, 4, and 5 bind haem while all motifs associate with haemoglobin. NEAT1 and NEAT5 extract haem which is then transferred by NEAT1, NEAT3 and NEAT4 to the cell wall-anchored IsdC protein. *S. lugdunensis* IsdJ and IsdK cannot bind Hb but perhaps could acquire haem directly from the extracellular environment during infection or from IsdB which occurs abundantly in the external milieu. Perhaps two parallel haemoglobin-haem acquisition pathways exist in *S. lugdunensis*, one utilizing the cell wall-anchored IsdB and IsdC and the second employing secreted proteins which acquire haem from the environment (IsdB, IsdJ and IsdK) and pass it onto IsdC before transferring the porphyrin ring into the cytoplasm (Fig. 5.18). However more studies are required to reveal the role of IsdJ and IsdK in the haem acquisition mechanism.

The specific binding of haemoproteins by *S. lugdunensis* is restricted to a single IsdB receptor (Fig. 5.18) which contrasts with *S. aureus* where IsdH and IsdB express three haemoglobin binding NEAT domains (Dryla *et al.*, 2007; Torres *et al.*, 2006). In *S. lugdunensis* the IsdB protein uses its NEAT1 domain to bind to haemoglobin and haemoglobin-haptoglobin complexes but has no affinity for myoglobin or haptoglobin. Even though haemoglobin binding has been reported for a number of bacterial proteins containing NEAT-like domains, the characteristic aromatic loop co-ordinating

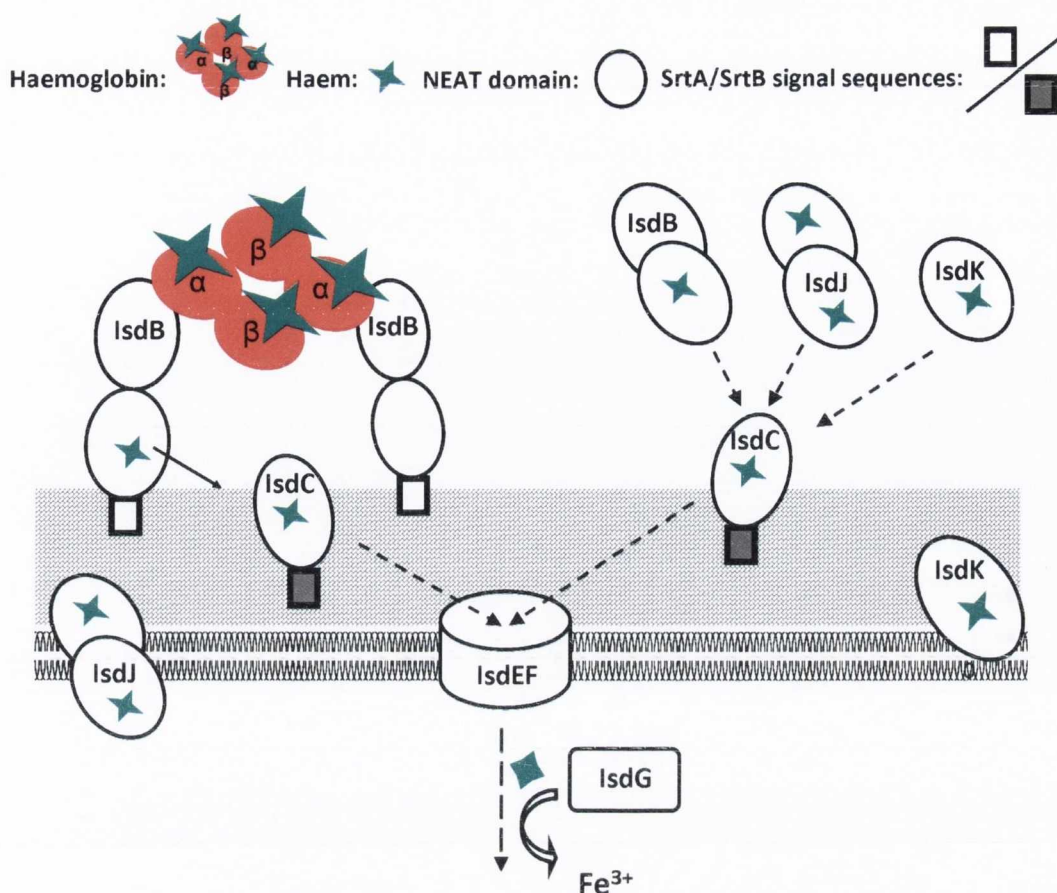


Figure 5.18 Model of haem transfer by the Isd proteins of *S. lugdunensis*.

IsdB and IsdC, which have SrtA and SrtB recognition signals, respectively, are anchored to the cell wall peptidoglycan. IsdB NEAT1 domain binds human haemoglobin using a conserved mechanism which allows recognition of the α-chains of haemoglobin α₂β₂ heterodimer. Haem can be transferred from the haem-binding IsdB NEAT2 to IsdC NEAT domain and is thought to be transported through the membrane *via* IsdEF encoded within the *isd* locus. IsdJ and IsdK are present in the membrane despite carrying the putative sorting sequences (SrtA and SrtB, respectively). Neither protein is well-exposed on the bacterial surface. IsdJ, IsdK and IsdB occur in the external milieu and bind haem which can be transferred on IsdC providing a putative parallel acquisition pathway. In cytoplasm haem monooxygenase IsdG binds and degrades haem releasing free iron and producing chromophore staphyloglobin.

haemoglobin is unique to *S. lugdunensis* and *S. aureus*. Furthermore both species show selectivity in the source of haemoglobin. The *S. lugdunensis* IsdB was shown to bind strongly to human Hb (K_D of 23 nM) and has weak affinity for mouse Hb. It was also demonstrated that the haemoglobin receptor preferentially binds to human MetHb than to porcine, bovine or equine MetHb, suggesting an adaptation to colonization of the human host. This correlates with the bacterial preference for utilizing human Hb over mouse Hb to support growth in iron-restricted media. Therefore it could be difficult to evaluate the importance of the Isd system in mouse models of colonization or infection. Since nutrient availability may have a critical effect on bacterial pathogenesis, the humanized animal model (mouse expressing human haemoglobin) should be strongly considered to study invasiveness and virulence of *S. lugdunensis* (Pishchany *et al.*, 2010).

The *S. lugdunensis* IsdB receptor was shown to be capable of binding haem by a conserved Tyr (IsdB Y467) probably in a pentacoordinate complex (a hydrogen bond is formed with the phenolate of another conserved Tyr in $\beta 8$). This interaction is absolutely conserved among haem-binding NEAT domains (Grigg *et al.*, 2011). The *S. aureus* haem uptake pathway IsdH-IsdB-(IsdA)-IsdC-IsdEF transfers haem from the surface exposed haemoprotein receptors (IsdH and IsdB) through the cell wall-bound IsdA or directly onto the IsdC (buried in the cell wall), which passes it onto the membrane transporter (IsdDEF) (Liu *et al.*, 2008; Muryoi *et al.*, 2008; Zhu *et al.*, 2008). The *S. lugdunensis* Isd system may represent a more modest set of receptors, considering that IsdJ and IsdK may be redundant due to their cellular localization. We observed direct transfer of haemin from recombinant holo-IsdB to apo-IsdC suggesting that these two cell wall receptors may be sufficient for the efficient transfer of haem onto the putative membrane transporter system encoded by *isdE* and *isdF* (Fig. 5.18). Another restriction occurring in the *S. lugdunensis* Isd system is a single haem oxygenase, unlike two of these enzymes being expressed by *S. aureus*.

This modestly equipped haem-uptake system is proportional to a lower haemolytic capacity of *S. lugdunensis*, which has been shown only to produce the delta toxin-like haemolysin called SLUSH (Frank *et al.*, 2008; Vandenesch *et al.*, 1993). In contrast *S. aureus* expresses a range of haemolysins (α , β , γ , δ) which lyse the erythrocytes and provide a rapid access to a pool of haemoglobin.

Chapter 6

Other functions of the iron regulated surface determinants of *S. lugdunensis*

6.1 Introduction.

Since discovery of the iron regulated surface determinant (Isd) proteins of *S. aureus* ten years ago (Mazmanian *et al.*, 2003), their roles in binding to host haemoproteins and haem-iron acquisition during infection has been extensively studied. Upon understanding of their primary roles, alternative functions of these factors have been identified. The IsdA, IsdB and IsdH are abundantly displayed on the surface of iron-starved *S. aureus* and have been shown to play important roles in colonisation, survival in the host and pathogenesis.

The IsdA protein binds to an array of human proteins contributing to *S. aureus* colonization, survival and invasion of the host. As well as transporting haem it promotes bacterial adhesion to desquamated nasal epithelial cells, most likely by binding to involucrin, loricrin and cytokeratin 10, which all occur in the cornified envelopes of squames (Clarke *et al.*, 2009; Corrigan *et al.*, 2009b). The role of IsdA in promoting *S. aureus* nasal colonisation has been demonstrated in the cotton rat model (Clarke *et al.*, 2009). Inside human host IsdA mediates binding to the extracellular matrix proteins such as fibrinogen and fibronectin (Clarke *et al.*, 2004). The mechanism of binding to each of these discussed above ligands involves the protein's NEAT domain. Another function of the IsdA is conferring resistance to hydrophobic bactericidal lipids and host antimicrobial peptides in sebum (Clarke *et al.*, 2007). The protein achieves that by its hydrophilic C-terminal domain which decreases cellular hydrophobicity. This mechanism is required for survival of *S. aureus* on live human skin (Clarke *et al.*, 2007). The IsdA also binds to lactoferrin, conferring resistance to killing by this most abundant anti-staphylococcal polypeptide in human nasal secretions (Clarke *et al.*, 2008).

IsdH, on the other hand, was shown to facilitate enhanced conversion of complement opsonin protein C3b to iC3b and C3d enabling *S. aureus* to avoid neutrophil uptake. This was manifested by promoting the ability of the bacterium to survive in the blood (Visai *et al.*, 2009; Smith *et al.*, 2011).

The IsdB protein was demonstrated to promote *S. aureus* adhesion to platelets resulting in platelet aggregation. This phenotype was a result of the protein's ability to bind to the platelet integrin $\alpha_{IIb}\beta_3$ (Miajlovic *et al.*, 2010).

This study analyzed the potential of the Isd proteins of *S. lugdunensis* to possess additional functions. The IsdJ and IsdK proteins, which have similarities to IsdA, were tested for their ability to bind to fibrinogen, cytokeratin 10 and loricrin, and to confer

resistance to killing by the bactericidal lipid linoleic acid. The ability of *S. lugdunensis* to adhere to platelets and other human cells together with its ability to internalize into non-professional phagocytes was studied to determine if the IsdB ortholog promotes these activities.

6.2 Results.

6.2.1 Recombinant IsdJ binds to fibrinogen, cytokeratin and loricrin.

The IsdA protein of *S. aureus* uses its NEAT domain to bind to a broad spectrum of host ligands. The IsdA NEAT motif has specificity towards the extracellular matrix (ECM) proteins fibrinogen and fibronectin as well as proteins present in the cornified envelopes of human skin, including loricrin and cytokeratin 10 (Clarke *et al.*, 2009; Clarke *et al.*, 2004). Significant similarities exist between IsdA and the *S. lugdunensis* proteins IsdJ and IsdK (Fig. 5.2). This occurs mostly within the NEAT domains. Both IsdJ NEAT domains are similar to conserved with the single NEAT motif of IsdA. The NEAT1 domain of IsdJ has 48% identity to the single NEAT domain of IsdA while IsdJ NEAT2 and C-terminal residues 457-646 have 39% identity to IsdA (Fig. 5.2). IsdK NEAT2 and C-terminal sequences 287-356 also have a high level of identity to IsdA at 39%.

ELISA-based assays were performed to test the ability of purified IsdJ and IsdK binding to the ECM ligand – fibrinogen as well as loricrin and cytokeratin 10 from squames. A range of concentrations of the ligands were immobilized in microtiter wells and varying concentrations of recombinant IsdJ (45-610) and IsdK (35-426) were added. Recombinant IsdJ bound to each ligand with half maxima of approximately 200 nM for loricrin, 250 nm for cytokeratin 10 and 350 nM for fibrinogen (Fig. 6.1). Binding of recombinant IsdK to any of these ligands could not be detected (data not shown).

Ligand affinity blotting was performed with full length rIsdJ (45-610), and the N- and C-terminal truncates, rIsdJ (45-295) and IsdJ (290-610) to identify the domain(s) of IsdJ involved in ligand binding. Probing was performed with soluble fibrinogen followed by HRP-conjugated mouse anti-human Fg IgG (Fig. 6.2). The N-terminal NEAT-domain construct was found to support fibrinogen binding.

6.2.2 Surface plasmon resonance analysis of the IsdJ interactions with fibrinogen and loricrin.

Surface plasmon resonance analysis was used to measure more accurately the interactions of rIsdJ with soluble fibrinogen (Fg) and loricrin. Recombinant IsdJ (45-610) was immobilized using monoclonal anti-polyhistidine antibodies which were firstly amine-coupled to the dextran surface of a CM5 chip. Increasing concentrations of the soluble ligands (from 150 nM to 20 μ M) were passed over the flow cell coated with rIsdJ and the control flow cell coated only with antibodies. Representative sensorgrams are shown in Fig. 6.3.

Association of the soluble Fg to rIsdJ occurred in two stages. A short immediate association was followed by a slower binding phase indicating a two-step interaction. The dissociation step was quick. Biacore software was used to evaluate the sensorgrams by fitting them to a kinetics model. The dissociation constant K_D of Fg binding to rIsdJ was estimated at 256 ± 29 nM within the same range as results obtained using ELISA-based assays.

Soluble human loricrin associated slowly to immobilized rIsdJ and slowly dissociated from the rIsdJ-coated surface. The sensorgrams were evaluated using a 1:1 kinetics model and the interaction K_D was estimated at 4.8 ± 0.8 μ M, a 25 - fold weaker interaction than was suggested by ELISA assays. Accurate fittings for both experiments were judged from the low χ^2 values ($\ll 10$).

6.2.3 Generation of *S. aureus* Newman *clfA clfB isdA* (pRMC2*isdA*) and Newman *clfA clfB isdA* (pRMC2*isdJ*).

To study and compare the role of IsdJ of *S. lugdunensis* and IsdA of *S. aureus* in adherence to human ligands *isdA* gene were amplified using specific primers (Table 2.4) and cloned into the anhydrotetracycline (aTc)-inducible staphylococcal expression vector pRMC2 (Table 2.4). Cloning of pRMC2*isdJ* was described in Chapter 5.2.5. Plasmids pRMC2*isdA* and pRMC2*isdJ* were electroporated into *S. aureus* the Newman *clfA clfB isdA* mutant. This host strain is unable to bind to the ligands of interest due to mutations in ClfA, ClfB and IsdA (Table 2.2). Expression of IsdA and IsdJ was induced by addition of aTc at 0.5-1 μ g ml⁻¹ to culture media and was validated by Western immunoblotting of solubilised bacterial cell walls and membrane fractions (Fig. 6.4). Both, IsdA and IsdJ were detected only in the cell wall fraction in Newman *clfA clfB isdA* which produced the proteins from either plasmid. Iron starved *S. lugdunensis*

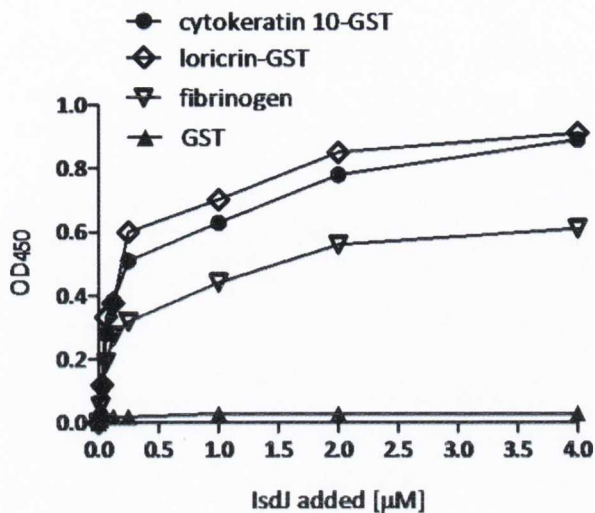
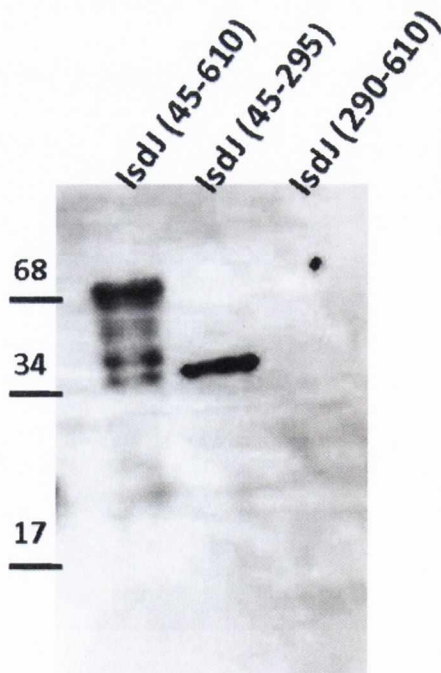


Figure 6.1 Recombinant IsdJ binding to loricrin, cytokeratin 10 and fibrinogen. 96-well plates were coated with GST-loricrin, GST-cytokeratin 10, GST or fibrinogen and incubated with increasing concentrations of recombinant IsdJ (45-610). Bound IsdJ was detected with rabbit anti-IsdJ serum followed by HRP-conjugated protein A. Results are the means of triplicate wells. Experiment was performed three times with similar results.

Figure 6.2 Ligand affinity blot to detect the fibrinogen - binding domain of IsdJ.

Lanes contained equal amounts of recombinant IsdJ variants. The IsdJ truncations were separated by SDS-PAGE and subjected to affinity blotting. Bound Fg was detected with HRP-conjugated monoclonal anti-human Fg IgG.



expressed only IsdJ which was detected in the membrane fraction while *S. aureus* Newman produced IsdA which was detected in the bacterial cell wall.

6.2.4 Generation of *L. lactis* (pNZ8048*isdA*).

In order to investigate and compare roles of IsdJ and IsdA in other Gram-positive bacterial hosts the *isdA* gene was amplified using primers listed in Table 2.3. and cloned into the pNZ8048 vector in *Lactococcus lactis* NZ9000. The obtained plasmids allowed nisin-inducible expression of the proteins in *L. lactis* host (Table 2.2.). Cloning of *L. lactis* (pNZ8048*isdJ*) was described in chapter 5.2.5. Expression of IsdA and IsdJ was validated by whole cell immunoblot (Fig. 6.5) Both, IsdA and IsdJ were detected on the surface of *L. lactis* upon addition of nisin at 100-300 ng ml⁻¹.

6.2.5 The role of IsdJ in bacterial adherence to human ligands.

In order to determine if IsdJ could support bacterial adherence to immobilized ligands the ability of the iron-starved wild-type *S. lugdunensis* to bind to fibrinogen and cytokeratin 10 was compared to the *fbl* and *isdJ* mutants. *S. aureus* Newman wild-type and *isdA* mutant were used as controls. There was no difference in adherence to immobilized ligands by *S. lugdunensis* and the isogenic *isdJ* mutant. Moreover the *fbl* mutant could not bind to fibrinogen, indicating that IsdJ does not contribute to bacterial adherence to fibrinogen in the presence and absence of Fbl (Fig. 6.6). Unexpectedly there was no difference in the levels of binding of *S. aureus* Newman and Newman *isdA*, even though a role for IsdA in promoting bacterial adherence has been described before (Clarke *et al.*, 2004; Clarke *et al.*, 2009) (Fig. 6.6).

It was demonstrated in Chapter 5.2.5 that heterologous expression of IsdJ in *S. aureus* or *L. lactis* results in the protein being cell wall-anchored and exposed on the cell surface, unlike in *S. lugdunensis* where IsdJ is present in the membrane and is not surface exposed. To test if the heterologous expression of IsdJ could support adhesion to immobilized ligands, the IsdJ protein was expressed in *S. aureus* Newman *clfA clfB isdA*, a mutant that is defective in all fibrinogen and keratin 10 adhesins, and in *L. lactis* which cannot bind these ligands. Similarly IsdA was expressed from the same inducible plasmid in *S. aureus* Newman *clfA clfB isdA* and *L. lactis* NZ9000. In neither case could adhesion be detected (Fig. 6.7AB, data not shown) implying that both proteins are insufficient to support bacterial adhesion to immobilized ligands.

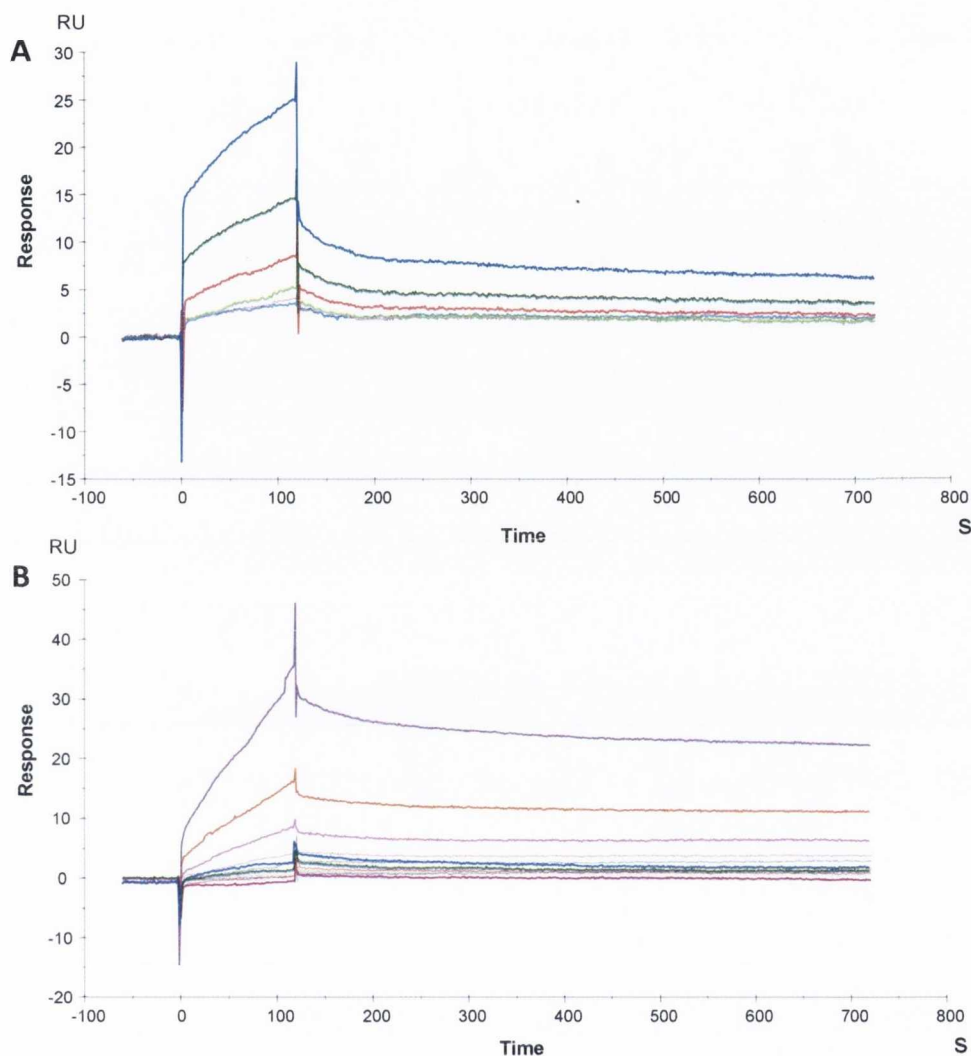
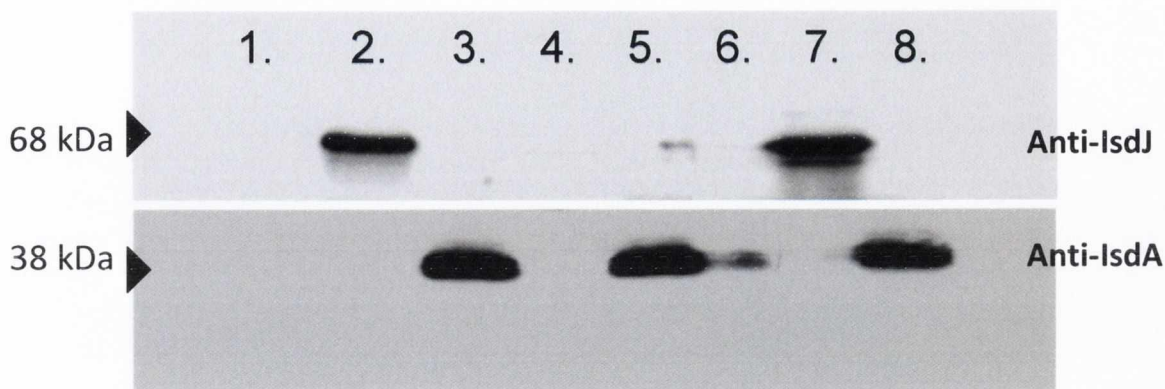


Figure 6.3 Surface plasmon resonance binding analysis of IsdJ binding to human fibrinogen and loricrin.

Data were generated using Biacore X-100. IsdB was immobilized on the surface of a CM5 sensor chip, followed by passing increasing concentrations (150 nM to 20 μ M) of the (A) Fg or (B) loricrin over the surface. Injections began at 0 s and ended at 180 s. The data are presented as real-time graphs of response units (RU) against time. Experiments were repeated three times with similar results.



1. *S. lugdunensis* N920143 (CW)
2. *S. lugdunensis* N920143 (M)
3. *S. aureus* Newman (CW)
4. *S. aureus* Newman *isdA* (CW)
5. *S. aureus* Newman *clfAclfB* (CW)
6. *S. aureus* Newman *clfAclfB isdA* (pRMC2) (CW)
7. *S. aureus* Newman *clfAclfB isdA* (pRMC2*isdJ*) (CW)
8. *S. aureus* Newman *clfAclfB isdA* (pRMC2*isdA*) (CW)

Figure 6.4 Expression of the IsdA or IsdJ by *S. lugdunensis* N920143, *S. aureus* Newman and Newman *clfA clfB* carrying pRMC2 constructs.

Solubilised cell wall (CW) and membrane (M) fractions of *S. aureus* or *S. lugdunensis* are indicated. SDS-PAGE and Western immunoblotting were performed to detect expression of the IsdA and IsdJ proteins. The immunoblotting was performed using rabbit anti-Isd IgG followed by HRP-conjugated goat anti-rabbit IgG.

6.2.6 IsdJ - mediated resistance to killing by linoleic acid.

The multifunctional IsdA protein of *S. aureus* confers resistance to killing by bactericidal lipids that occur in sebum due to the C-terminal region that confers decreased hydrophobicity on the bacterial cell (Clarke *et al.*, 2007). Since the C-terminus of IsdJ has similarity to IsdA it was decided to investigate if the *S. lugdunensis* protein conferred the same property. Mutants of *S. aureus* and *S. lugdunensis* that were defective in IsdA and IsdJ, respectively, were compared with the parental strains for survival in a buffer containing linoleic acid for 30 min. The *isdA* mutant survived poorly compared to *S. aureus* wild-type, while there was a trend towards the same effect that was not statistically significant for *S. lugdunensis isdJ* (Fig. 6.8A).

To test if less effective protection by IsdJ could be caused by its cellular localization IsdJ and IsdA were expressed on the surface of *S. aureus* Newman *isdA* to compare the roles of both proteins being anchored to the cell wall and test if the cell wall anchored IsdJ could complement the *isdA* mutation in the killing assay. Expression of IsdA and IsdJ was induced from pRMC2 constructs and bacterial survival was measured after 30 min. Expression of IsdA and IsdJ both resulted in increased protection compared to the *isdA* mutant or the mutant carrying the empty pRMC2 vector reaching survival levels of Newman wild-type (Fig. 6.8B). This results suggested that the cellular localization of IsdJ may determine its functionality.

Furthermore expression of surface exposed IsdJ in *L. lactis* promoted enhanced survival at levels comparable to IsdA-mediated survival (Fig. 6.9). Thus IsdJ appears to confer resistance to linoleic acid and may help promote survival of *S. lugdunensis* in its natural habitat on the human skin.

6.2.7 Adherence of *S. lugdunensis* *Δisd* locus mutants to fibrinogen.

In order to verify if Isd proteins contribute to bacterial adherence to human ligands, RPMI-grown *S. lugdunensis* adherence to immobilized fibrinogen was studied using the wild-type strains N920143, N940025 and their isogenic *Δisd* loci mutants. Strain *S. lugdunensis* N920143 was previously shown to bind to immobilized fibrinogen (Fg) promoted by the surface protein Fbl (Fig. 6.6A) (Mitchell *et al.*, 2004).

There was no decrease in binding of the *S. lugdunensis* *Δisd* mutants compared to the parental strains indicating that the Isd proteins do not contribute to bacterial adherence to Fg (Fig. 6.10). Unexpectedly the *Δisd* mutants of both *S. lugdunensis* strains bound stronger to the immobilized fibrinogen than wild-type strains. The

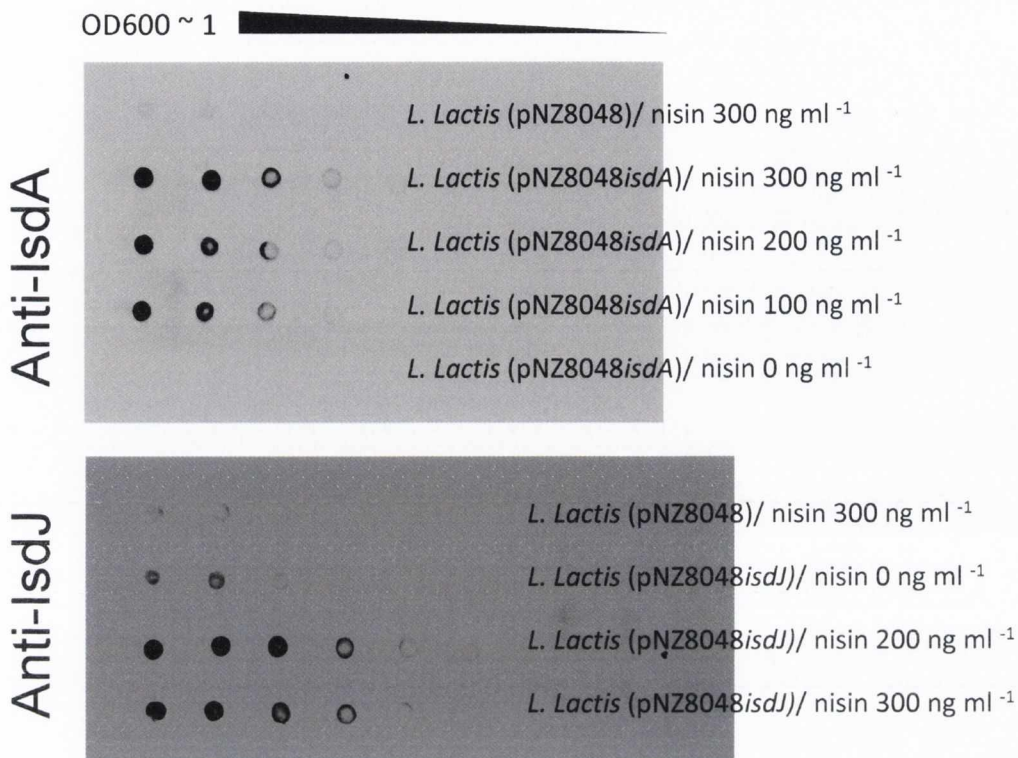


Figure 6.5 Expression of IsdA and IsdJ on the surface of *L. lactis* NZ9000 from inducible plasmids pNZ8048isdA and pNZ8048isdJ.

Whole-cell immunoblot was performed using the rabbit-anti IsdA or rabbit anti-IsdJ IgG followed by HRP-conjugated protein A.

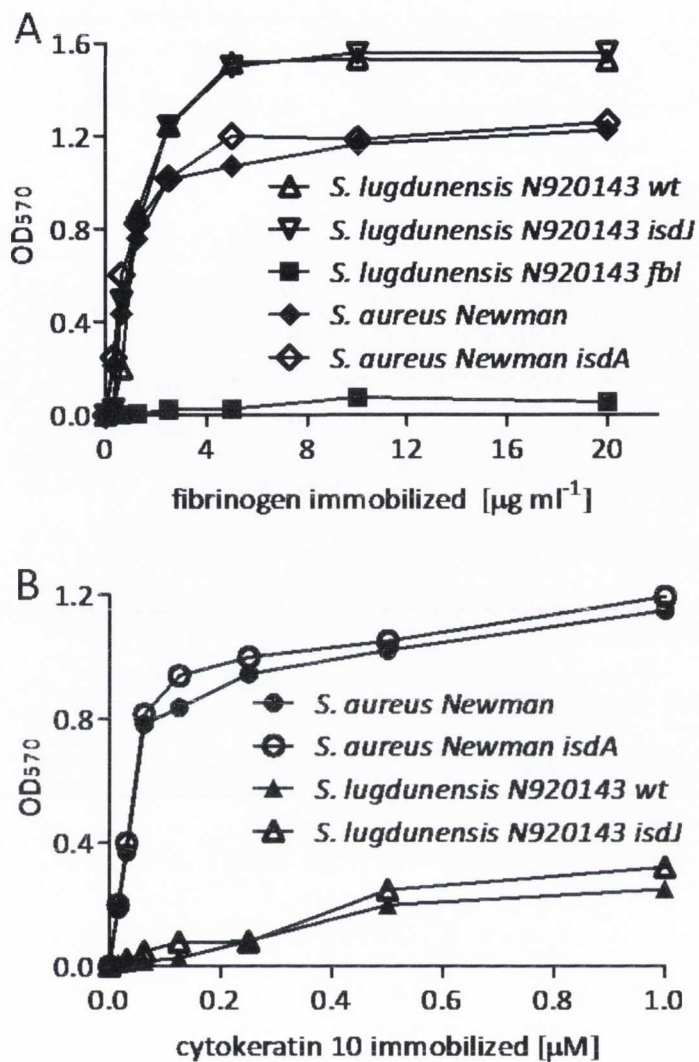


Figure 6.6 Adherence of *S. aureus* and *S. lugdunensis* to immobilized fibrinogen and cytokeratin 10.

96-wells plates were coated with various concentrations of either (A) fibrinogen or (B) cytokeratin 10 and incubated with RPMI - grown *S. lugdunensis* N920143 or *S. aureus* Newman variants. After binding and fixing, the cells were stained with crystal violet and the optical densities were measured at 570 nm (OD_{570}). Values represent the means of triplicate wells. Binding assays were performed three times with similar results.

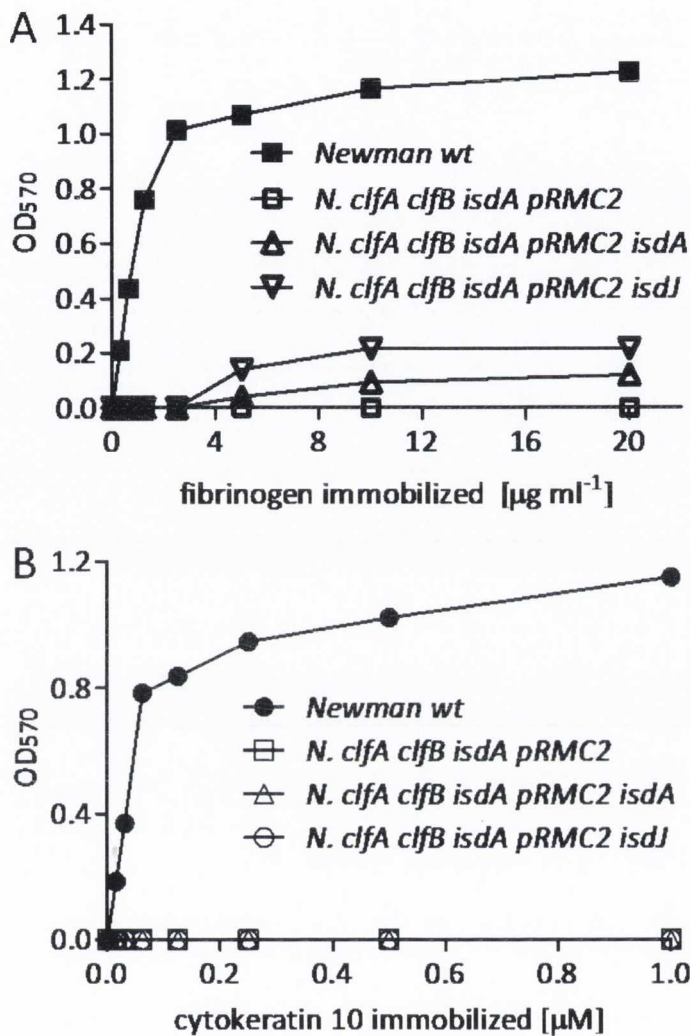


Figure 6.7 The role of IsdA and IsdJ in mediating bacterial adherence to immobilized fibrinogen and cytokeratin 10 .

96-wells plates were coated with various concentrations of either (A) fibrinogen or (B) cytokeratin 10 and incubated with *S. aureus* Newman wild-type or Newman *clfA clfB isdA* either carrying an empty pRMC2 or expressing IsdJ or IsdA. The bacterial suspensions in PBS were adjusted to an optical density (OD₅₇₀) of 1 prior being added to the wells. After binding and fixing the cells were stained with crystal violet and the OD₅₇₀ were measured. Values represent the means of triplicate wells. Binding assays were performed three times with similar results.

increase in binding was seen for strain N920143 which binds to Fg at lower level and for strain N940025 which does not bind to immobilized Fg when expressing the Isd locus proteins. Understanding of the mechanism responsible for higher ability to bind Fg requires further studies.

6.2.8 *S. lugdunensis* does not adhere to or invade human cells.

Recent studies demonstrated that *S. lugdunensis* has the ability to adhere to and invade into cells which aren't professionally phagocytic (Pereira *et al.*, 2012; Szabados *et al.*, 2011). *S. lugdunensis* N920143 does not encode fibronectin binding proteins, which on *S. aureus* provide the most important mechanisms of invasion into human cells, but expresses IsdB protein which could promote adhesion and invasion like its ortholog from *S. aureus* (see Chapter 4). The IsdB_{SA} and IsdB_{SL} are 37% identical with conservation occurring mostly within NEAT1, NEAT2 and the fragment in-between the domains (Fig. 6.11).

S. lugdunensis N920143 ability to adhere to and to be internalized by non-professional phagocytes was studied. *S. lugdunensis* was grown in RPMI and immobilized in microtiter wells. Washed platelets and kidney embryo 293T cells were added to the wells but no binding could be detected (data not shown). There was no platelet aggregation upon addition of the *S. lugdunensis* to platelet rich plasma (data not shown), implying inability of this bacterium to cause platelet aggregation. Gentamicin protection assays were performed to determine if the RPMI-grown *S. lugdunensis* can be internalized by the non-professional phagocytic 293T cells. No significant invasion by the bacterium could be detected (data not shown).

6.3 Discussion.

Several functions in addition to haem-iron acquisition have been identified for the surface-exposed Isd proteins of *S. aureus*. The multifunctional IsdA protein utilizes its NEAT domain to bind to a broad spectrum of host ligands other than haem whereas the C-terminal tail is responsible for providing resistance to antimicrobial fatty acids and peptides in human sebum.

There is no IsdA ortholog in the Isd system of *S. lugdunensis* but its IsdK and IsdJ proteins have domains with similarity to IsdA and therefore were identified as candidates to study ligand specificity. ELISA-based assays, fibrinogen-affinity Western blots and surface plasmon resonance analysis demonstrated that recombinant IsdJ binds

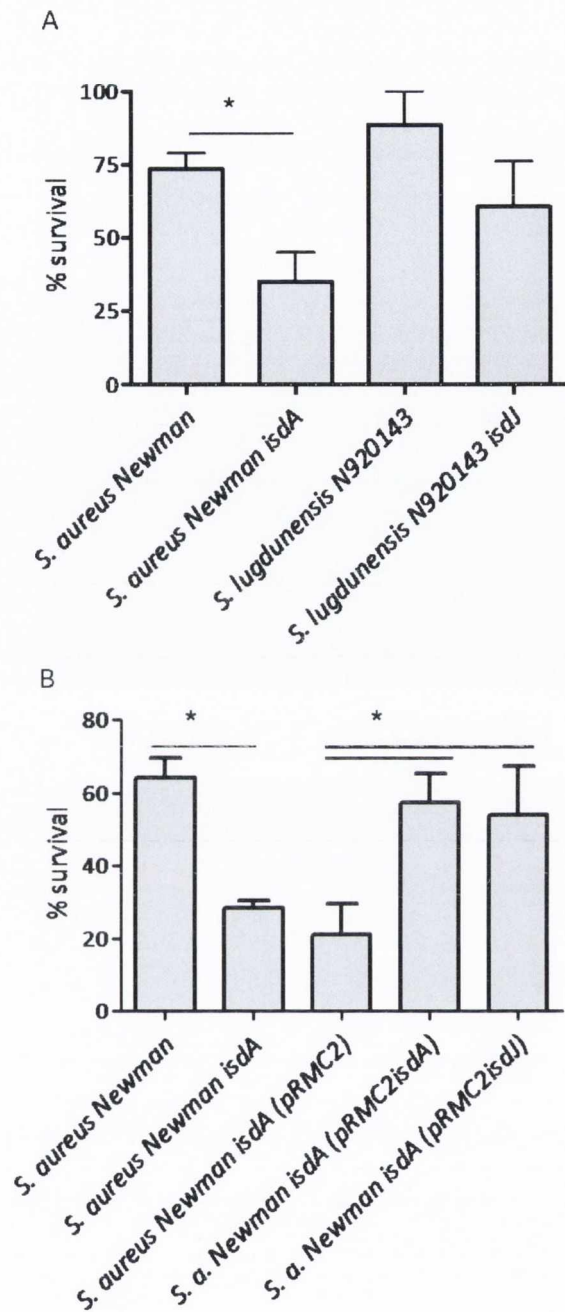


Figure 6.8 Resistance of *S. aureus* and *S. lugdunensis* to killing by linoleic acid.

(A) *S. lugdunensis* N920143 wild type and the $\Delta isdJ$ mutant, *S. aureus* Newman wild-type and $\Delta isdA$ were grown in RPMI for 16 h. (B) *S. aureus* strains were grown in RPMI to an optical density (OD₆₀₀) of 0.6 and supplemented with anhydrotetracycline at 1 $\mu\text{g ml}^{-1}$ to induce IsdA and IsdJ proteins expression (AB) Bacteria were harvested, washed and resuspended at 1×10^8 cfu ml^{-1} in distilled H₂O were treated with 10 $\mu\text{g ml}^{-1}$ of linoleic acid for 30 minutes. Survival was measured by viable counting. Values are the means of three independent experiments.

□ 0 minutes ■ 30 minutes ▒ 60 minutes

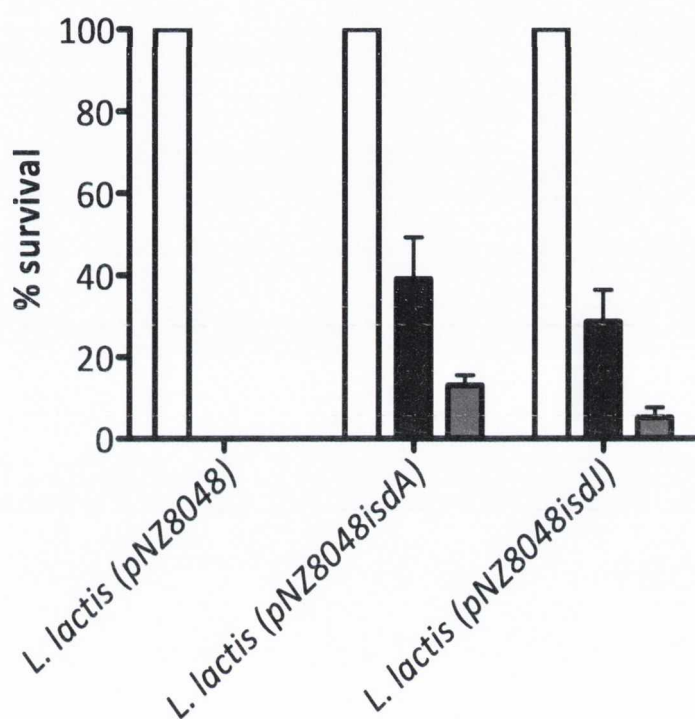


Figure 6.9 The roles of IsdA and IsdJ in providing bacterial resistance to killing by linoleic acid.

L. lactis NZ9000 (pNZ8048), (pNZ8048isdA) and (pNZ8048isdJ) were grown in GM17 to mid-exponential phase and induced for 4 h with nisin at 300 ng ml⁻¹. Cells were harvested, washed and adjusted to a concentration of 1 x 10⁸ cfu ml⁻¹ in distilled H₂O. The bacteria were treated with 10 µg ml⁻¹ of linoleic acid for 30 or 60 minutes. Survival was measured by viable counting. Values are the means of three independent experiments.

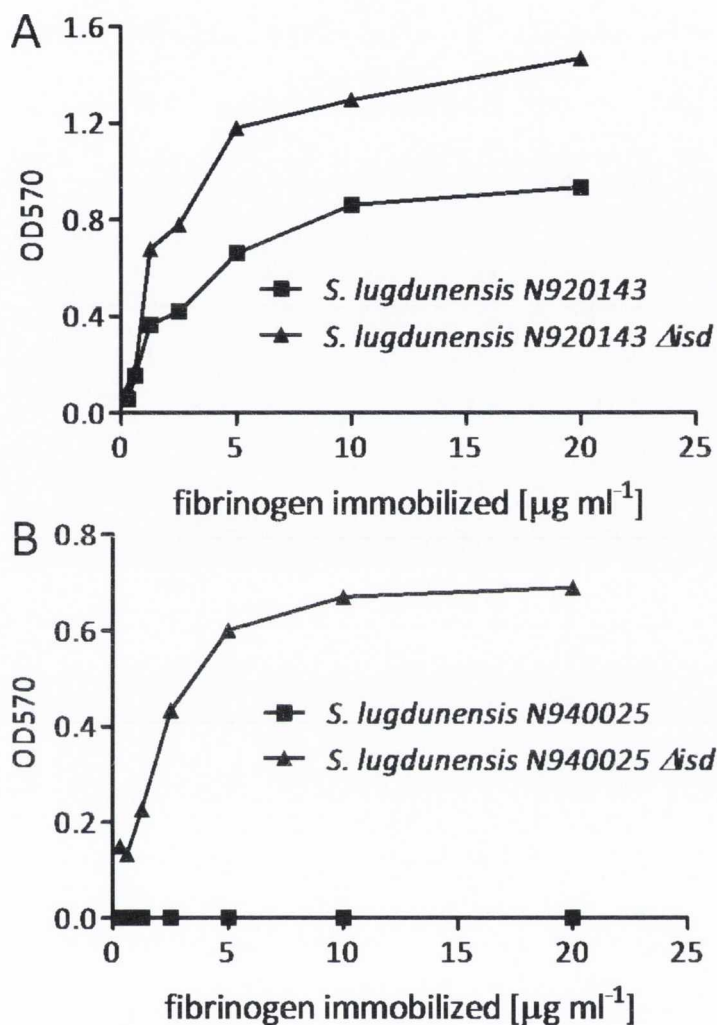


Figure 6.10 The role of *isd* locus in *S. lugdunensis* adherence to immobilized fibrinogen.

Wild-type *S. lugdunensis* strains (A) N920143 and (B) N940025 and their *isd* locus mutants were tested for binding to immobilized Fg. 96-wells plates were coated with various concentrations of fibrinogen and incubated with the RPMI-grown *S. lugdunensis* strains at an optical density (OD₅₇₀) of 1. After binding and fixing, the cells were stained with crystal violet and the optical densities were measured at OD₅₇₀. Values represent the means of triplicated wells. Binding assays were performed three times with similar results.

fibrinogen, cytokeratin 10 and loricrin and identified the N-terminal domain of IsdJ as the active domain. The interactions of IsdJ with the other ligands bound by IsdA, such as fibronectin and lactoferrin were not investigated. However it seems likely that IsdJ can recognize these ligands.

Despite affinities for fibrinogen in nanomolar range, IsdJ expression by bacterial cells did not promote bacterial adherence to the immobilized protein. Iron starved *S. lugdunensis* adhered strongly to fibrinogen, which was not due to IsdJ expression, as there was no difference in adherence by the wild-type and *isdJ* deficient mutant. Also iron-starved *S. lugdunensis fbl* did not adhere to fibrinogen suggesting that Fbl is the major fibrinogen receptor on *S. lugdunensis*. *S. aureus* and *L. lactis* strains expressing IsdJ on their surfaces also failed to adhere to cytokeratin 10, fibrinogen and loricrin, which demonstrates that the protein is not sufficient to promote bacterial adherence to those ligands.

Contribution to bacterial fibrinogen binding by other Isd proteins could not be detected. There was no defect in adhesion to this ligand by the fibrinogen binding *S. lugdunensis Δisd* locus mutant. Quite opposite observation was made, the mutant lacking the whole *Δisd* locus bound stronger to fibrinogen than the wild-type strain suggesting that expression of the Isd components may have a masking effect for other surface proteins recognizing adhesive molecules.

IsdJ hydrophilic C-terminal domain seemed to confer some protection of *S. lugdunensis* from killing by linoleic acid, albeit not as efficiently as IsdA in *S. aureus*. However when expressed on the bacterial surface from a plasmid, IsdJ protected *S. aureus* and *L. lactis* as well as IsdA suggesting that cellular localization determines its lower functionality in *S. lugdunensis*.

Unlike *S. aureus* IsdB, the *S. lugdunensis* ortholog did not seem to promote bacterial binding to human cells or internalization by the non-professional phagocytes, as the studied *S. lugdunensis* strain N920143 did not adhere to platelets nor invade kidney embryo cells. Thus the differences between IsdB_{SA} and IsdB_{SL} may be critical for the interaction or other factors required for the IsdB-dependent adhesion and invasion are not present in *S. lugdunensis*.

<i>S. aureus</i> 12	YSIRKSSLGVASVAISTLLLLMSNGEAQAAAEETGGTNTAQPKTEAVASPTTTSEKAPE	71
	+SIRK S+G AS+ + T L L N E QA A E +P AVAS T +	
<i>S. lugdun.</i> 16	FSIRKFSIGAASITLGTCLFL--NAEHQAQAENA-----VEPAHSAVASENNTPA---Q	65
<i>S. aureus</i> 72	TKPVANAVSVSNKEVEAPTSETKEAKEVKEVKAPKETKE---VKPAAKATNNTYP-----	123
	+KPVA V +N++ A ++ A K+ + + V PA +N T P	
<i>S. lugdun.</i> 66	SKPVAPTVE-TNEQPNADHTDKTVATTTTNDKSRQAQNDKQTVTPATNVSNNTTQPKAAPA	124
<i>S. aureus</i> 124	-----ILNQELREAIKNPAIKDKDHS <u>AFNSRPIDFEMKKKDG</u>	160
	++Q +++A+K+ +K H NSRP+ FEMK KDG	
<i>S. lugdun.</i> 125	STALTNAVNPKSSSTAVSNPAKQAVDQSIKDAVKDKTYTNKPHD <u>TSNSRPVSFEMKNKDG</u>	184
<i>S. aureus</i> 161	<u>TQQFYHYASSVKPARVIFTD--SKPEIELGLQSGQFWRKFEVYEGDKKLPIKLVSYDTVK</u>	218
	QFYHYAS++ PA V F +K +ELGL++ W+ +VYE +KKLPI+LV+Y+	
<i>S. lugdun.</i> 185	<u>DLQFYHYASTISPANVYFNKDKNKTTVELGLKTASTWKNLKVYENNNKLPIELVTYNPET</u>	244
<i>S. aureus</i> 219	<u>DYAIRFVSNGTKAVKIVSSTH-FNNKEEKYDYLMEFAQPIYNSADKFKTEEDYKAEK</u>	277
	D AYIRF VSNGTK + I SST +N+ E YDYT M F QPIYN KFKT ED++ E	
<i>S. lugdun.</i> 245	<u>DDAIRFDVSNGTKELVIKSSSTQIYNDPETNYDYTKMVFEQPIYNDESFKFTRDFETEA</u>	304
<i>S. aureus</i> 278	LLAPYKKAKTLERQVYELNKIQDKLPEKLKAEYKKKLEDTKKALDEQVKSAITFQN---	334
	LLA Y KAKTLER++YELN+++D+LPEKL+A+Y +KLE KK LD++V SA EF++	
<i>S. lugdun.</i> 305	LLADYNKAKTLERKIYELNRVKDQLPEKLRAQYSQKLEQLKKDLDQEVASAKVEFEHAPV	364
<i>S. aureus</i> 335	-- <u>VQPTNEKMTDLQDTKYVVYESVENNESMMDTFVKHPIKTGMLNGKKYVMVMTTNDYIW</u>	392
	VQ TN K D+ V +S +++ S MD FV+ P + ++GKKY+ + TT D W	
<i>S. lugdun.</i> 365	<u>KKVQITNSKSLDIS-----VLQSDDDLSTMDGFVEKPFELVEVDGKKYIKLTTTIDSIW</u>	419
<i>S. aureus</i> 393	<u>KDFMVEGQRVRTISKDAKNRTRTIIFPYVEGKTLYDAIVKVHVKTIDYDGQYHVRIVDKE</u>	452
	KDF VEGQRV TI KD N+ RT++FPYVEGK +Y++IVKV TI Y GQYHVRI	
<i>S. lugdun.</i> 420	<u>KDFQVEGQRVMTIDKDTVNDKRTVLFPPYVEGKEIYNSIVKVDFFTIGYSGQYHVRI</u> ----	475
<i>S. aureus</i> 453	<u>AFTKANTDKSNKKEQQDNSAKKEATPATPSKPTSPVEKESQKQDSQKDDNKQLPSVEKE</u>	512
	++N+ K+A TP+ P +KE++ P + +	
<i>S. lugdun.</i> 476	-----KNNAISKKADAETPTTPNN---KKEAK-----PDINQA	505
<i>S. aureus</i> 513	NDASSESGDKTPTATKPTKGEVSSSTPTKVVSTTQNV-----KPTTA-----SSKTT	562
	+ A+S + K P KP + T + VV+TT A KPT + + T	
<i>S. lugdun.</i> 506	DTATSNNKADKPIVKPADTTTTHKKVTKS-VVTTTHAPANHKQMNKPTVSVASNTGHTK	564
<i>S. aureus</i> 563	KDVVQTSAGSSEAKDSAPLQKANIK-----NTNDGHTQ-----	595
	+ VVQ +A + + Q + K N HTQ	
<i>S. lugdun.</i> 565	QTVVQQTAAHNHTSNTSHQNVSTKTQISTLNVAKHSVHNQVQHNNQRSHTTQQQDVTKP	624
<i>S. aureus</i> 596	---SQNNKNTQENKA-----KSLPQTGEESNKDMTFLMALLALSSIVAFV	638
	+QNN Q + +LP TG+ +N TL + L I+ +	
<i>S. lugdun.</i> 625	LSHAQNNHTVQSQISHMHKTAAPSPVAQHTLPATGQTTNH-ATLWASLVAILGGIMFLI	683
<i>S. aureus</i> 639	LPRKR 643	
	P+K+	
<i>S. lugdun.</i> 684	RPKKK 688	

Figure 6.11 Amino acid sequences alignment of IsdB proteins from *S. aureus* and *S. lugdunensis*. Alignment was obtained using the Basic Local Alignment Search Tool (BLAST®). NEAT1 domains are indicated in red whereas NEAT2 domains are shown in blue. Identical and similar residues are indicated.

Chapter 7
Discussion

7. 1 Discussion

S. aureus is the most virulent species of *Staphylococcus*. It is a versatile pathogen that is capable of causing a wide range of human diseases. The armamentarium of virulence factors of *S. aureus* is extensive, with both cell wall-anchored and secreted proteins playing a role during infection. Many factors have several functions in pathogenesis and multiple virulence factors may perform the same function (Gordon and Lowy, 2008; Speziale *et al.*, 2008). Different strains have different constellations of these factors making them predisposed to causing particular kinds of infections (Patti *et al.*, 1994; Foster, 1998).

S. lugdunensis is an emerging pathogen which, despite being classified as a less virulent CoNS species, paradoxically is often reported to cause severe diseases alike those caused by *S. aureus*, such as infective endocarditis (IE). Reported mortality rates for the *S. lugdunensis* IE seem much higher than those caused by *S. aureus*. However its occurrence is contrastingly lower than that caused by *S. aureus*. Similarities between the species exist also in their morphology and ability to clump blood plasma.

The limited studies performed previously to identify molecular determinants of *S. lugdunensis* virulence focused on features which are conserved in *S. aureus*. The fibrinogen-binding protein from *S. lugdunensis* (Fbl) was identified as an orthologue of ClfA. Their ligand-binding domains share 60% identity and bind to fibrinogen with identical affinities (Geoghegan *et al.*, 2010). However, unlike ClfA, Fbl expression is not sufficient to initiate platelet aggregation by *S. lugdunensis*. This could be due to lack of specific antibodies in donors serum required to trigger this response or due to low level of Fbl expression *in vitro*. Thus despite similarities in the proteins' structures and their conserved ligand-binding mechanism, expression of Fbl by *S. lugdunensis* may not result in similar phenotype *in vivo* leading to formation of thrombus.

A group of secreted virulence factors was also identified in *S. lugdunensis* based on their similarities to those expressed by *S. aureus*, *S. epidermidis* and *S. capitis*. Three phenol-soluble modulin (PSM) peptides (SLUSH-A, SLUSH-B, and SLUSH-C), like their homologs, were shown to interact with the formyl-peptide receptor 2 (FPR2) on neutrophils to stimulate, attract and disrupt the cells and thus are thought to contribute to evasion of host's immune response (Rautenberg *et al.*, 2011).

Studies on the iron regulated surface determinant G (IsdG_{SL}), an ortholog of the haem monooxygenase from *S. aureus* demonstrated that haem degradation by IsdG_{SL}

results in the release of free iron and the production of the chromophore staphylobilin. IsdG_{SL} utilizes host haem after its transfer into bacterial cytoplasm (Haley *et al.*, 2011).

This study continued to characterize the iron regulated determinants (Isd) of *S. lugdunensis* that are conserved with those of *S. aureus*. Initially, an *in silico* analysis was performed to compare the amino acid sequences of IsdB_{SL}, IsdC_{SL}, IsdJ and IsdK proteins with the homologues IsdB_{SA}, IsdC_{SA}, IsdA and IsdH. This allowed predictions about their structures and ligand-binding potential to be made, which are evaluated by *in vitro* experiments using recombinant proteins. The Isd_{SL} proteins like Isd_{SA} specifically recognized human haemoglobin and bound haem. The NEAT domains responsible for haem and haemoglobin coordination were shown to exploit the same strictly conserved mechanism in both staphylococcal species implying a strong adaptation to these iron sources. Haem transfer from the most surface-exposed proteins IsdB_{SL} to IsdC_{SL} was demonstrated. Due to the unusual localization of IsdJ and IsdK in the membrane and also extracellularly, the IsdB_{SL} and IsdC_{SL} proteins were proposed to form the core of the system.

IsdB expression by *S. lugdunensis* promoted bacterial growth on human haemoglobin as a source of iron in an otherwise iron-depleted medium. Thus the Isd_{SL} system may potentially determine the ability of *S. lugdunensis* to replicate upon an entry into the hosts bloodstream or tissues. Even though structurally similar NEAT domains coordinate haemoglobin binding and haem uptake in *Clostridium*, *Listeria*, *Bacillus* and *Streptococcus*, *S. lugdunensis* like *S. aureus* remains unique in its preference for human over animal haemoglobins (Pishchany *et al.*, 2011). This adaptation to survive in the human host creates a challenge to evaluate accurately the virulence of *S. lugdunensis in vivo*. A mouse model where animals express human haemoglobin has been developed (Pishchany *et al.*, 2011). This humanized animal model could be used to evaluate the role of Isd proteins *in vivo*. Another approach to evaluate virulence potential of *S. lugdunensis* in an animal model involves increasing the susceptibility of an animal to *S. lugdunensis* infection by development of an IsdB mutant with increased specificity to animal Hb. Bacterium expressing such IsdB mutant should have increased ability to survival in an animal host allowing to examine *S. lugdunensis* pathogenesis.

Homology based predictions also highlighted the multifunctional potential of the Isd_{SL} which was then evaluated by *in vitro* studies. Although IsdJ acted like IsdA in binding to host ligands such as fibrinogen and cytokeratin, expression of IsdJ was

insufficient to mediate bacterial adherence to these proteins. Similarly, despite high levels of identity between the IsdB orthologs, no similar phenotype associated with the protein expression occurred in *S. lugdunensis* as the bacterium could not adhere to human cells nor invaded them. The recently published genome sequences of *S. lugdunensis* strains facilitated this study and may encourage further efforts to understand the specificity of the bacterium through comparison to *S. aureus*.

Discovering virulence factors of pathogenic bacteria is a key in understanding pathogenesis. One way to identify those factors is by screening for similarity with well-characterized from other species. Such an approach was used in the studies discussed above. However, high levels of identity between factors produced by different species may not necessarily be associated with the same phenotype, as it seems to be in the case of *S. lugdunensis* Fbl or IsdB. Therefore this 'abstract' approach remains only partially effective in revealing the pathogenic potential of the bacterium. Identification of unique molecular mechanisms driving a virulence requires disguising the real conditions of the pathogen's ecological niche. Indeed *S. lugdunensis* comprises a niche that is distinct from *S. aureus*. It is an integral part of the normal skin flora, predominant at the abdomen, groin, and lower extremities (especially the nail bed of the first toe) and is only rarely obtained from cultures of nasal swabs - a pattern of distribution distinct from *S. aureus* (Bieber *et al.*, 2010). Thus studies on *S. lugdunensis* interactions with the host could be initiated by approaches leading to isolation of mutants challenged in survival in this comfortable environment for instance by a transposon mutagenesis followed by a negative selection of candidates.

Global gene expression profiles may provide valuable information on transcriptional modulation in bacteria during infection. One such study by Allard *et al.* demonstrated up-regulation of the *isd* locus during *S. aureus* infection in a tissue cage model. Previously performed *in vitro* studies used bacteria cultured in rich laboratory media and thus did not consider Isd_{SA} - associated phenotypes which turned out to be of importance in multiple *S. aureus* interactions with the host. Numerous functions of IsdA, IsdB_{SA} and IsdH were identified *in vitro* (Clarke *et al.*, 2004; Clarke *et al.*, 2007, Clarke *et al.*, 2009; Corrigan *et al.*, 2009; Visai *et al.*, 2009; Miajlovic *et al.*, 2010).

Another role for IsdB_{SA} was identified in this study. IsdB expression promoted *S. aureus* adhesion to human 293T or HeLa cells. The interaction was inhibited by an RGD peptide suggesting that it occurs in the same manner as the IsdB-mediated adherence to platelets by direct binding to 'RGD integrins' (Miajlovic *et al.*, 2010). A

novel approach, called xCELLigence (Roche) was applied to quantify the attachment of cells expressing $\alpha_{IIb}\beta_3$ integrin mutants to immobilized IsdB_{SA}. These experiments confirmed the previous observation that IsdB_{SA} binds to the high-affinity conformation of the integrin. The IsdB-dependent increase in *S. aureus* adherence correlated with an increase in internalization of the bacterium by human cells. This novel function for IsdB_{SA} may provide a back-up mechanisms supporting *S. aureus* spreading within host tissues. The direct interaction of IsdB with human cells receptors does not require plasma components and was most prominent in medium without plasma. Addition of serum initiates the dominant mechanism which is mediated by the fibronectin-binding proteins but contribution of the IsdB is still significant.

Complementation of the *isdB* deficient mutant resulted in recovery of the ability of *S. aureus* to invade, validating the IsdB_{SA}-dependent phenotype. However, expression of IsdB_{SA} by *L. lactis* did not trigger invasion into human cells indicating that the protein may not be sufficient in mediating that interaction and may require other factors produced by *S. aureus*. This could perhaps explain the lack of a similar phenotype associated with the IsdB_{SL} ortholog. Since IsdB_{SA} interacts with the high-affinity conformation of integrin the putative additional factors could be involved in activation of the conformational form of the integrins. To determine if the factors necessary for IsdB-mediated invasion are anchored to the cell wall peptidoglycan by SrtA or SrtB initial invasion experiments could be performed with a *srtAB*-deficient strain expressing IsdB from a plasmid and an empty plasmid carrying strain acting as a negative control. Invasion by the IsdB-expressing strain would indicate that the additional components may not be sorted to the cell wall. But if neither strain invaded human cells additional studies should be performed to confirm that more cell wall anchored elements is required for the IsdB-dependent mechanism. Such components could also be secreted therefore future studies could examine bacterial culture supernatants ability to stimulate the high-affinity conformation of integrins. This could be studied by supplementing the IsdB -expressing *L. lactis* with *S. aureus* supernatant prior infection of human cells, like the 293T or HeLa cells.

Iron regulated surface determinants occur solely in *S. aureus* and *S. lugdunensis* supporting their iron uptake mechanisms in the harsh environment of biological host where all free iron is carefully sequestered. A number of additional functions for those Isd proteins has been characterized. These roles were shown to further support bacterial interactions with the host but seemed to differ between *S. aureus* and *S. lugdunensis*

systems. Functionality of the Isd proteins depends on their NEAT domains which despite their structural conservation may provide mechanism supporting wide range of interactions. These IgG-like folds seem to be evolutionally malleable and used as tools required in specific environmental niches of these staphylococci.

Performed here mutagenesis of the *isdB* gene did not allow to reveal the binding site responsible for engaging the high-affinity integrin. A future study could apply a high throughput assay to identify the recognition site. For example, a transposon library could be generated following invasion studies using quick and convenient flow cytometry analysis rather than slower and more time consuming viable counting. Identification of the binding site could be also accomplished by crystallography studies of soluble IsdB and extracellular “headpiece” domain of $\alpha_{\text{IIb}}\beta_3$ which most probably are involved in the interaction.

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