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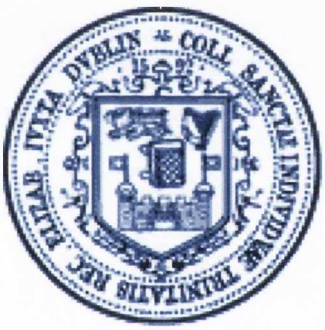
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Characterisation of the Second Binding protein of Immunoglobulin (Sbi) in *Staphylococcus aureus*

A thesis submitted for the degree of Doctor in Philosophy

by

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Summary

The second binding protein for immunoglobulin (Sbi) of *Staphylococcus aureus* comprises two IgG binding domains (D1 and D2) that share 30% sequence homology with the IgG binding domains of protein A (Spa) and two domains (D3 and D4) that bind to and trigger the consumption of complement factor C3. Sbi is assumed to be expressed on the cell surface but its C-terminal domain 'Y' lacks motifs found in proteins that are bound to the cell wall or membrane of Gram-positive bacteria.

The cellular localisation of Sbi was investigated by constructing a *sbi* mutant in *S. aureus* strain Newman and combining this mutation with a previously isolated mutation in Spa. Immunoblotting analysis of cell envelope fractions and the culture supernatant of wild type *S. aureus* and the mutants lacking Sbi and/or protein A demonstrated that Sbi occurs both extracellularly and in the cytoplasmic membrane fraction. Cell surface expression of Sbi was analysed by whole cell dot immunoblotting. Whole cells of Newman *spa* reacted with HRP-labelled rabbit IgG whereas cells of Newman *spa sbi* (pRMC2-*sbi*Δ_{D1D2}) failed to react. Indicating that the IgG binding domains of Sbi are expressed on the bacterial cell surface. Antibodies raised against the D3D4 and C-terminal domains of Sbi reacted with whole cells of Newman *spa sbi* (pRMC2-*sbi*Δ_{D1D2}) however, no detectable levels of C3 binding were seen for Sbi expressed on the cell surface. Indicating that although detectable on the cell surface D3D4 of Sbi cannot bind C3 on the bacterial cell surface.

Analysis of *S.aureus* expressing truncates of Sbi with deletions in the 'Y' domain showed that the C-terminal residues 335-436 were required for anchoring of Sbi to the membrane. This was supported by measuring the ability of recombinant Sbi and truncates to bind to purified cytoplasmic membranes *in vitro*. Recombinant Sbi was shown to bind to purified lipoteichoic acid with a high affinity, an interaction that explains how Sbi partitions with the membrane in fractionation experiments yet is also partially exposed on the cell surface. Prior incubation of recombinant Sbi with soluble LTA inhibited Sbi binding to both purified cytoplasmic membrane material and LTA. Furthermore, soluble LTA was shown to displace Sbi from the cytoplasmic membrane of Newman *spa*. Finally, an LTA-defective mutant of *S. aureus* had reduced levels of Sbi in the cytoplasmic membrane.

It has been proposed that Sbi helps bacteria avoid the innate immune defences of the host. By comparing a mutant defective in Sbi with mutants defective in protein A, clumping factor A, iron regulated surface determinant H and capsular polysaccharide it was shown that Sbi is indeed an immune evasion factor that promotes bacterial survival in whole human blood and avoidance of neutrophil-mediated opsonophagocytosis. Sbi is present in the culture supernatant and is also associated with the cell envelope. *S. aureus* strains that expressed truncates of Sbi lacking N-terminal domains D1D2 or D3D4, or a C-terminal truncate that was no longer retained in the cell envelope were analysed. Both the secreted and envelope-associated forms of Sbi contributed to immune evasion. The IgG-binding domains only contributed when Sbi was attached to the cell presumably by binding the Fcγ region of IgG and blocking the binding site for the neutrophil Fc receptor. The C3 binding domains were biologically active only when secreted. It has been proposed that the extracellular form of Sbi contributes to immune evasion by catalyzing futile consumption of C3 only in the fluid phase. The precise mechanism by which Sbi interferes with the host's immune response in the fluid phase remains to be answered.

Publications

Smith, E.J., Visai, L., Kerrigan, S.W., Speziale, P. and Foster, T.J. (2011). The Sbi Protein Is a Multifunctional Immune Evasion Factor of *Staphylococcus aureus*. Infection and Immunity, 79(9):3801-9

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Contents

Declaration i

Acknowledgements ii

Summary iii

Publications iv

List of Tables xi

List of Figures xii

Chapter 1 Introduction 1

1.1 Biology of staphylococci 1

1.1.1 Classification and identification 1

1.1.2 Colonisation and disease 1

1.2 The *S. aureus* cell wall 3

1.3.2 The wall teichoic acid and lipoteichoic acid polymers of *S. aureus* 3

1.3 Anchoring of surface proteins in *S. aureus* 5

1.3.1 Proteins covalently linked to the cell wall.... 5

1.3.2 Proteins anchored by non-covalent interaction 6

1.3.3 Proteins anchored to the cell surface via lipid moieties 7

1.4 Innate immune evasion by *S. aureus* 8

1.4.1 Survival on the skin 8

1.4.2 Inhibition of neutrophil migration 9

1.4.3 Inactivation of complement 10

1.4.4 Resistance to phagocytosis 12

1.4.5 Toxins that kill leukocytes 13

1.4.6 Survival of *S. aureus* in neutrophil phagosomes 14

1.5 Surface components involved in innate immune evasion 15

1.5.1 Protein A 15

1.5.2 Clumping factor A 17

1.5.3 Iron regulated surface determinants 19

1.5.4 Sas proteins 21

1.5.5 Capsular polysaccharide 22

1.6 Secreted proteins involved in immune evasion 22

1.6.1 SCIN 22

1.6.2 CHIPS 24
1.6.3 Efb 24
1.6.4 Ehp 26
1.7 Rational for this study 27
1.7.1 Sbi 27
1.7.2 Aims and objectives 30

Chapter 2 Materials and Methods 31
2.1 Bacterial strains and growth conditions 31
2.1.1 Growth of bacteria in iron limiting conditions 31
2.1.2 Growth of bacteria in human plasma 31
2.1.3 Growth and induction of *S. aureus* strains carrying pRMC2 derivatives 31
2.2 Plasmids 32
2.3 DNA manipulations 32
2.3.1 Preparation of plasmid and genomic DNA 32
2.3.2 Polymerase chain reaction 32
2.4 Transformation 33
2.4.1 Preparation and electroporation of *S. aureus* 33
2.5 Phage transduction 33
2.6 Strain construction 34
2.6.1 Allelic replacement mutagenesis of *sbi* 34
2.6.2 Allelic replacement mutagenesis of *isdH* 35
2.6.3 Construction of strains by transduction 36
2.6.4 Complementation of the *sbi*::Em^R mutation 36
2.6.5 Construction of Sbi C-terminal truncates 36
2.6.6 Construction of Sbi N-terminal truncates 37
2.7 Isolation of *S. aureus* cell wall and cell envelope components 37
2.7.1 Preparation of staphylococcal whole cell lysates 37
2.7.2 Preparation of solubilised proteins associated with the staphylococcal cell wall 37
2.7.3 Preparation of staphylococcal protoplasts and cytoplasmic membrane fractions 38

2.7.3.1 Preparation of *L. monocytogenes* and *E. faecalis* cytoplasmic membrane fractions 38
2.7.3.2 Preparation of *E. coli* cytoplasmic membrane fractions 38
2.7.4 Preparation of extracellular proteins 39
2.7.5 Cell wall preparation 39
2.8 Electrophoresis 39
2.8.1 Agarose gel electrophoesis 39
2.8.2 SDS-PAGE 40
2.9 Immunoblotting 40
2.9.1 Western immunoblotting 40
2.9.2 Far Western blotting 40
2.9.3 Whole cell dot immunoblotting 41
2.10 Recombinant protein purification 41
2.10.1 Purification of GST-tagged recombinant proteins 41
2.10.2 Purification of MBP-tagged recombinant proteins 42
2.11 Enzyme linked immunosorbent assays (ELISA) 42
2.11.1 Solid phase binding assay 42
2.11.1.2 Inhibition studies 43
2.11.2 Bacterial cellular fraction ELISA 43
2.12 Fractionation of cells following incubation with MBP-Sbi 43
2.13 Displacement of Sbi from the cytoplasmic membrane by soluble LTA 44
2.14 Polymorphonuclear leukocyte isolation 44
2.15 Phagocytosis assay 45
2.16 Whole blood survival assay 45
2.17 Experimental arthritis and sepsis 45
2.18 Ethics statement 46
2.19 Determination of haemolytic activity 46
2.20 Densitometric analysis 47
2.21 Antibodies 47
2.22 Proteomics tools for the prediction of protein topology 47

Chapter 3 Genetic manipulation of *sbi* 48
3.1 Introduction 48
3.2 Results 53

3.2.1 Allelic replacement mutagenesis of *sbi* 53
3.2.2 Validation of the *sbi*::Em^R mutation 53
3.2.3 Construction and validation of combined mutations in S. aureus strain Newman 54
3.2.4 Complementation of the *sbi*::Em^R mutation 56
3.2.5 Construction and validation of *S. aureus* strain USA300 *sbi* 56
3.2.6 Construction of Sbi C-terminal 'Y' domain truncates 56
3.2.7 Construction of Sbi $\Delta_{335\text{-}368}$ 56
3.2.8 Construction of Sbi truncates lacking N-terminal domains 57
3.3 Discussion 58

Chapter 4 Analysis of the cellular localisation of Sbi 61
4.1 Introduction 61
4.2 Results 64
4.2.1 Cellular location of Sbi 64
4.2.2 *In silico* analysis of Sbi 65
4.2.3 Surface expression of Sbi ligand binding domains 66
4.2.4 Can Sbi domains D3D4 bind complement factor C3 on the cell surface? 67
4.2.5 Sbi binding to the cytoplasmic membrane 67
4.2.6 Recombinant Sbi binds to whole cells and fractionates with the cytoplasmic membrane 68
4.2.7 Sbi binds to lipoteichoic acid 69
4.2.8 Can Sbi bind to purified cell wall? 70
4.2.9 Displacement of Sbi from purified cytoplasmic membrane by soluble LTA 70
4.2.10 Detection of the Sbi-LTA interaction by Far Western blotting 70
4.2.11 An LTA-defective mutant has decreased levels of Sbi in the cytoplasmic membrane 71
4.2.12 Anchorage of Sbi to the membrane by the C-terminal Y domain 72
4.2.13 Why does Sbi occur extracellularly? 73
4.2.14 Can the Sbi Y domain support binding to the cytoplasmic membranes of other LTA type I expressing strains 73
4.3 Discussion 75

Chapter 5 Analysis of the immune evasion properties of Sbi 78
5.1 Introduction 78
5.2 Results 81
5.2.1 Expression of Sbi by clinical isolates 81
5.2.2 Expression of anti-phagocytic factors in iron limited conditions 81
5.2.3 Expression of S. aureus strain Newman anti-phagocytic determinants in mutant strains 81
5.2.4 Expression of Sbi inhibits killing by neutrophils in whole human blood.. 82
5.2.5 Phagocytosis by human neutrophils 83
5.2.6 Complementation of the *sbi* mutation 83
5.2.7 Expression of Sbi inhibits killing of USA300 by neutrophils in whole human blood 84
5.2.8 Inhibition of killing in human blood by truncates of Sbi 84
5.2.9 Sbi is not a virulence factor in the murine infection model for septic arthritis 85
5.3 Discussion 86

Chapter 6 Discussion 90
6.1 Discussion 90
6.1.1 Innate immune evasion mechanisms of Gram-positive bacteria 90

References 97

List of Tables

Following page

1.1 Functions of *S. aureus* WTA and LTA 4

2.1 Bacterial strains 32

2.2 Plasmids 32

2.3 Primers 32

2.4 Antibodies 32

List of Figures

Following page

1.1 Structure of *S. aureus* peptidoglycan 4
1.2 Structure of *S. aureus* teichoic acids 4
1.3 Surface protein anchoring in *S. aureus* 6
1.4 Inhibition of neutrophil migration by *S. aureus* 10
1.5 Complement fixation 12
1.6 Regulation of complement fixation 12
1.7 Schematic diagrams of Spa 16
1.8 Schematic representation of ClfA (A) and the dock, lock and latch-binding mechanism (B) 18
1.9 Putative pathway of haem transfer between Isd proteins of *S. aureus* 20
1.10 Structure of SCIN 24
1.11 Schematic diagrams of Spa and Sbi 28
1.12 Structural comparison of *S. aureus* innate immune evasion proteins 28
1.13 Structure of SbiD4 in complex with C3d 30
3.1 Suicide plasmid integration 49
3.2 Transcriptional fusion to *lacZ* 51
3.3 Schematic representation of plasmid integration and excision 51
3.4 Schematic representation (A) and validation (B) of the *sbi*::Em^R mutation 53
3.5 (A) Schematic representation and validation of the *cap*::Tc^R mutation, (B) Schematic representation and validation of the *isdH2* mutation (C)Validation of the *sbi*::Em^R mutation in Newman *spa clfA cap isdH sbi* (D) Haemolytic patterns..... 55
3.6 (A) Structural analysis of pRMC2-*sbi* (B) Restriction analysis of plasmid pRMC2-*sbi* 57
3.7 Validation of USA300 *sbi* 57
3.8 (A) Schematic representation of *sbi* deletions (B) PCR of amplified *sbi* C-terminal truncates (C) Restriction analysis of plasmids pRMC2-$sbi_{1\text{-}335}$, pRMC2-$sbi_{1\text{-}368}$ and pRMC2-$sbi_{1\text{-}403}$ (D) Restriction analysis of plasmid pRMC2-$sbi\Delta_{335\text{-}368}$..... 57
3.9 (A) Schematic diagram of sbi N-terminal in-frame deletions (B) Restriction analysis of plasmids 57
4.1 Biosynthesis of LTA 62
4.2 Cellular localisation of Sbi 64

4.3 Bioinformatic analysis of Sbi 66
4.4 Surface expression of Sbi 68
4.5 Complement factor C3 binding 68
4.6 Binding of MBP-Sbi_{41-436}, MBP-Sbi_{41-253}, MBP-$Sbi_{254-436}$ and MBP to purified cytoplasmic membrane 68
4.7 Binding of MBP-Sbi_{41-436}, MBP-Sbi_{41-253}, MBP-$Sbi_{254-436}$ and MBP to whole cells of Newman *spa sbi* 70
4.8 Interaction of MBP-Sbi with LTA 70
4.9 Binding of MBP-Sbi_{41-436}, MBP-Sbi_{41-253}, MBP-$Sbi_{254-436}$ and MBP with purified cell wall material 70
4.10 Analysis of the Sbi-LTA interaction 70
4.11 Sbi cellular location in LTA negative strains 72
4.12 Cellular localisation of Sbi C-terminal truncates 72
4.13 Binding of MBP-Sbi to whole cells and purified cytoplasmic membrane 74
4.14 Binding efficiency of MBP-Sbi303-436 to the cytoplasmic membranes of a range of Gram-positive bacteria 74
5.1 Expression of Sbi by clinical isolates of *S. aureus* 81
5.2 Expression of anti-phagocytic factors in iron limited conditions 81
5.3 Expression of S. aureus strain Newman anti-phagocytic determinants in mutant strains 81
5.4 Survival of *S. aureus* in whole human blood: 1 83
5.5 Resistance of *S. aureus* to phagocytosis by human neutrophils 83
5.6 Survival of *S. aureus* in human blood: 2 83
5.7 Expression of Sbi truncates and survival in blood 85
5.8 Expression of secreted Sbi truncates and survival in blood 85
5.9 Murine infection model 85
6.1 Proposed model for Sbi cellular localisation and surface expression 95

Chapter 1
Introduction

1.1 Biology of Staphylococci

1.1.1 Classification and identification

Bacteria of the genus *Staphylococcus* are non-motile, facultative aerobic, Gram positive cocci, approximately 1μm in diameter that characteristically divide in more than one plane to form grape like clusters. The name is from the Greek staphyle for 'bunch of grapes' and kokkus for 'berry'.

Molecular typing and genetic analysis have placed the staphylococci in the *Bacillus-Lactobacillus-Streptococcus* cluster of *Micrococcaceae* (Ludwig *et al.*, 1985, Stackebrandt & Teuber, 1988). Staphylococci are most closely related to *Enterococcus*, *Bacillus* and *Listeria* and their genomes contain DNA of a low G+C content (30-39%). The staphylococci are resistant to desiccation and are extremely halotolerant growing at up to 3.5M NaCl.

There are at present 36 staphylococcal species of which nine contain subdivisions with subspecies designations (Drancourt & Raoult, 2002). *Staphylococcus aureus* is distinguished from other staphylococci by the presence of a golden pigment, the ability to ferment mannitol and expression of extracellular coagulase, thermostable DNase and clumping factor (the cell wall-associated fibrinogen binding protein ClfA). Production of coagulase separates *S. aureus* from the other coagulase negative staphylococci (CoNS) which are considered to be less virulent. However some CoNS species, in particular *S. epidermidis*, *S. lugdunensis* and *S. haemolyticus* can cause serious infections in humans.

1.1.2 Colonisation and disease

Humans are the natural reservoir of *S. aureus*. Approximately one-fifth of the population are permanently colonised whilst the remainder are colonised intermittently (van Belkum *et al.*, 2009). The primary ecological habitat of *S. aureus* in humans is the moist squamous epithelium of the anterior nares. Colonisation of the nares depends on the ability of *S. aureus* to adhere to desquamated epithelial cells and to avoid the host immune response.

Several surface-associated proteins of *S. aureus* promote adhesion to desquamated nasal epithelial (squamous) cells *in vitro*. Clumping factor B (ClfB) is

believed to mediate adhesion to squamous cells through binding to cytokeratin 10 (O'Brien *et al.*, 2002b). Active immunisation of mice with recombinant ClfB protein and administration of a monoclonal function-blocking antibody directed against ClfB reduced nasal colonisation (Schaffer *et al.*, 2006). A *S. aureus* ClfB-deficient mutant was eliminated from the nares of humans faster than the wild type strain showing that the protein has a role in human nasal colonisation (Wertheim *et al.*, 2008). IsdA is a component of the haem-acquisition system which also promotes adhesion to squames (Clarke & Foster, 2008). IsdA can bind to loricrin, involucrin and cytokeratin 10, all of which are associated with cornified epithelial cells and may provide a ligand for IsdA mediated adhesion (Clarke *et al.*, 2009). Vaccination of cotton rats with IsdA reduced nasal colonisation (Clarke *et al.*, 2006). The serine-asperate repeat proteins, SdrC and SdrD and the *S. aureus* surface protein G (SasG) also promote adhesion of bacteria to squamous cells although their ligands are not yet known (Corrigan *et al.*, 2007, Corrigan *et al.*, 2009). A role for wall teichoic acid (WTA) in nasal colonisation has also been proposed. A WTA-defective mutant had decreased adherence to nasal cells and was unable to colonise the nares of cotton rats (Weidenmaier *et al.*, 2004). In addition, the WTA biosynthesis genes *tagO* and *tarK* are strongly up-regulated during colonisation of the nares of cotton rats 1-4 days post inoculation (Burian *et al.*, 2010). Indicating a vital role for WTA during the initiation of nasal colonisation.

When *S. aureus* colonises the anterior nares it must overcome both innate and adaptive immune responses controlled by nasal-associated lymphoid tissue (NALT). Host factors associated with immune responses are believed to play a role in determining carriage status. For example, increased rates of persistent carriage are seen in individuals with an interleukin 4 gene promoter polymorphism which is associated with lower Il-4 serum concentrations and reduced mucin (Emonts *et al.*, 2008). Nasal carriage is also associated with glucocorticoid receptor gene polymorphisms. Glucocorticoid receptors are associated with immune modulation. Individuals with haplotype 5, which is associated with immune suppression, have an 80% increased *S. aureus* carriage rate (van den Akker *et al.*, 2006), whereas individuals with haplotype 3, which is associated with enhanced immunity have lower persistent carriage rates.

Nasal colonisation is an established risk factor in the development of invasive infection with the majority of patients being infected with their own isolate. About 80%

of nosocomial *S. aureus* bacteraemia cases in persistent carriers were attributed to an endogenous source (von Eiff *et al.*, 2001). However, it has been noted that bacteraemia related death is significantly higher in infected non-carriers compared to persistent carriers, indicating that the latter could be partially protected from the strain that they carry (Wertheim *et al.*, 2004).

Staphylococcal infections are initiated when a breach of the skin or mucosal barrier allows staphylococci access to adjoining tissues or the bloodstream. The most common types of *S. aureus* infections are superficial skin lesions such as boils, impetigo and abscesses (Lowy, 1998). If the organism gains access to the bloodstream (bacteraemia), it can cause a wide variety of invasive infections such as osteomyelitis, pneumonia, septic arthritis and endocarditis (Lowy, 1998). Treatment of invasive *S. aureus* infections relies heavily on the use of antimicrobial agents, to which the organism is increasingly developing resistance.

1.2 The *S. aureus* cell wall

The cell wall acts as a physical barrier and provides a rigid exoskeleton which protects *S. aureus* from the surrounding environment. The main component of the cell wall is peptidoglycan (approximately 60%) with the remainder comprising wall teichoic acids, lipoteichoic acids and small amounts of protein. Peptidoglycan consists of glycan strands made of repeating disaccharide units, N-acetylglucosamine and N-acetylmuramic acid (GlcNAc-(β1-4)-MurNAc) (Ghuysen & Strominger, 1963). MurNAc moieties in the glycan chains are cross-linked by short tetrapeptides (L-Ala-D-Glu-L-Lys-D-Ala) to generate a rigid three-dimensional cell wall network (Figure 1.1; Ghuysen *et al.*, 1965, Strominger & Tipper, 1965). A characteristic feature of *S. aureus* peptidoglycan is the presence of pentaglycine interpeptide bridges that link the tetrapeptide units of neighbouring glycan chains. This feature renders *S. aureus* peptidoglycan susceptible to cleavage by the endopeptidase lysostaphin (Schleifer & Kandler, 1972).

1.2.1 The wall teichoic acid and lipoteichoic acid polymers of *S. aureus*

Teichoic acids (TA), of which there are two types, are a major component of the *S. aureus* cell wall. TAs are produced from separate biochemical pathways and are

either covalently linked to peptidoglycan in case of wall teichoic acids (WTA), or are linked to the cytoplasmic membrane in the case of lipoteichoic acids (LTA).

WTAs are made up of ribitol-phosphate polymers (poly-Rbo-P) substituted with N-acetylglucosamine and D-alanine residues (Ward, 1981, Endl *et al.*, 1983, Collins *et al.*, 2002). WTAs are covalently linked to the peptidoglycan by the 6-OH of MurNAc via a disaccharide consisting of GluNAc and N-acetylmannosamine (ManNAc), which is followed by two units of glycerol phosphate (Figure 1.2; Yokoyama *et al.*, 1989, Brown *et al.*, 2008). The WTA polymer is composed of 11-40 repeating units of Rbo-P in the majority of *S. aureus* strains.

LTA is a macroamphiphile composed of Gro-P polymers attached to the cytoplasmic membrane via a glycolipid anchor (Figure 1.2). In *S. aureus* and the majority of other staphylococcal species the glycolipid is composed of diglucosyl diacylglycerol (Wicken & Knox, 1975, Fischer, 1988). The poly-Gro-P backbone is attached to the C-6 of the non-reducing glucosyl of the glycolipid anchor (type I LTA) (Neuhaus & Baddiley, 2003). The LTA polymer is composed of 18-50 repeating units of Gro-P in the majority of staphylococci (Xia *et al.*, 2010). *S. aureus* LTA is substituted with a D-alanyl ester or α GluNAc (Fischer, 1988). With the exception of D-alanyl esters, the biosynthesis of WTA and LTA occurs via different biochemical pathways and precursor molecules. Even in organisms which have a poly-Gro-P backbone for both WTA and LTA such as *Bacillus subtilis* 168, the Gro-P units are derived from different sources (Neuhaus & Baddiley, 2003).

The functions of *S. aureus* TAs can be summarised under three main headings: (i) resistance of the bacteria to environmental stress and damaging molecules, (ii) modulation of enzyme activity and cation concentrations in the cell envelope, and (iii) interactions with receptors and surfaces (Xia *et al.*, 2010). Specific examples of some of these functions are described in Table 1.1. WTA has been shown not to be necessary for cell viability of *S. aureus* and *B. subtilis* (Weidenmaier et al., 2004, D'Elia *et al.*, 2006). However WTA is important in nasal colonisation and infection in not only *S. aureus* but also *L. monocytogenes* and *S. gordonii* (Weidenmaier et al., 2004, Weidenmaier *et al.*, 2005, Dubail *et al.*, 2006, Bizzini *et al.*, 2007, D'Elia *et al.*, 2009). On the other hand, LTA is vital for cell viability at temperatures above 30 °C. It would appear that WTA and LTA compensate for each other to some extent as it is impossible to delete genes

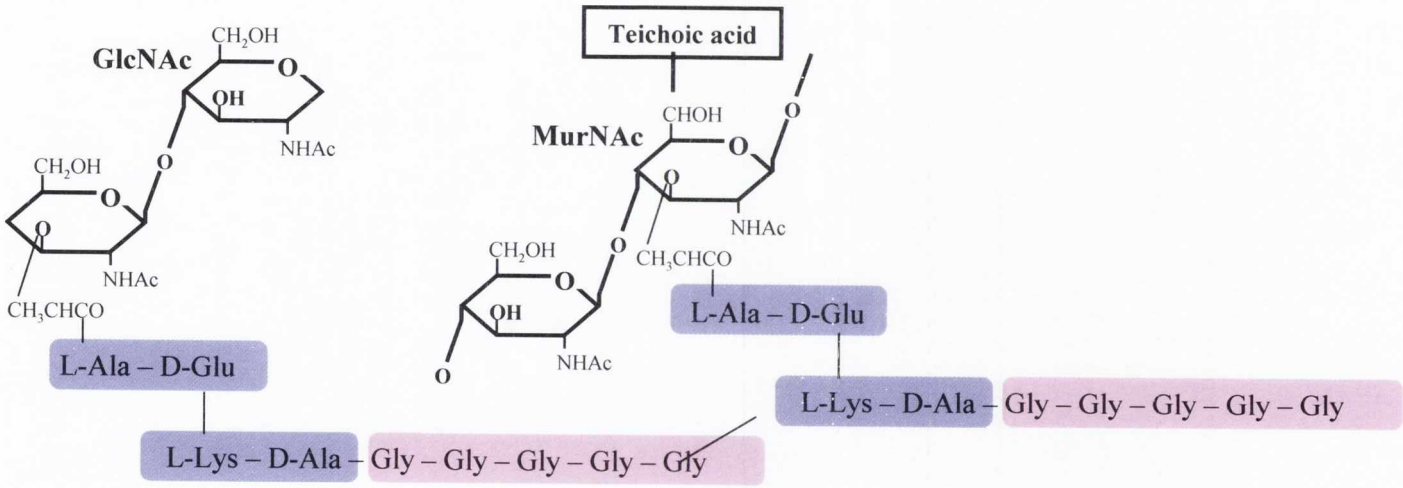


Figure 1.1 Structure of *S. aureus* peptidoglycan

β(1→4)-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) form the murein backbone. Cell wall tetrapeptides (purple) are amide-linked to MurNAc. Pentaglycine bridging peptides (pink) link the cell wall peptides of neighbouring chains. Teichoic acids are linked at C6 of MurNAc.

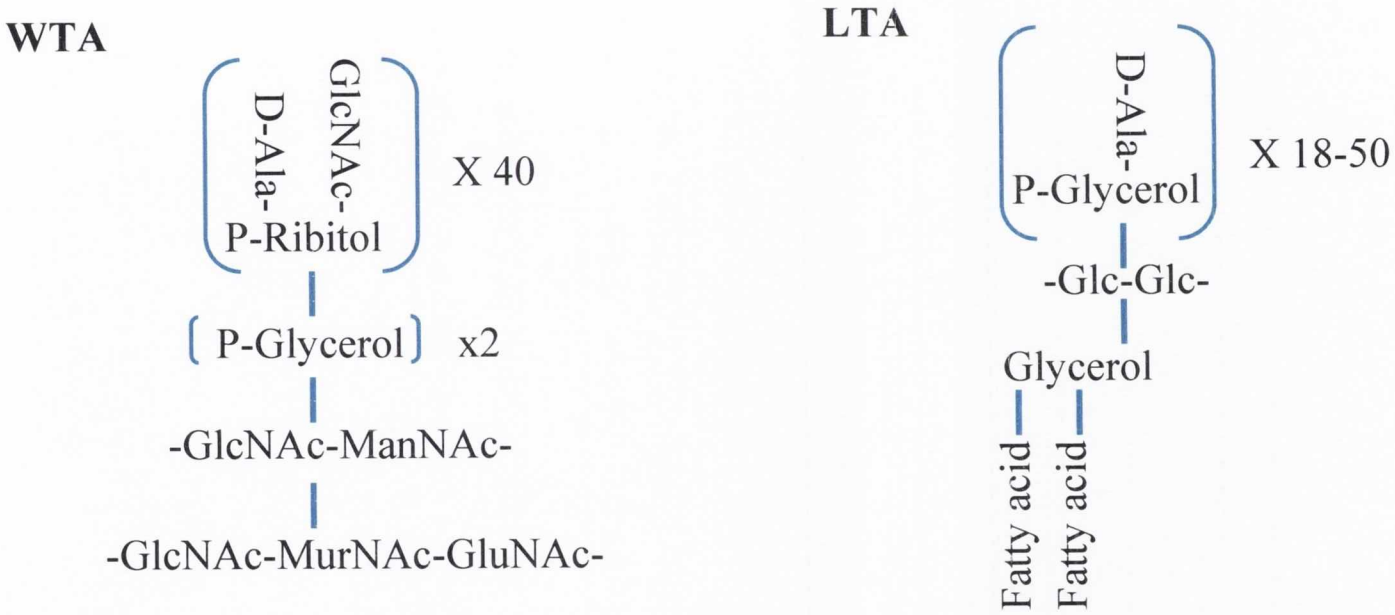


Figure 1.2 Structure of *S. aureus* teichoic acids

Structure of *S. aureus* wall teichoic acids (WTA) and lipoteichoic acid (LTA). P, phosphate; D-Ala, D-alanine; GlcNAc, N-acetylglucosamine; ManNAc, N-acetylmannosamine; MurNAc, N-acetylmuramic acid; Glc, glucose. Adapted from Xia *et al*, 2010.

Table 1.1 Functions of *S. aureus* WTA and LTA

Function	Teichoic Acid (TA)	References
Resistance to cationic antimicrobial peptides and antibiotics	D-ala of TAs	(Peschel *et al.*, 1999, Peschel *et al.*, 2000, Collins *et al.*, 2002)
Resistance to lysozyme	WTA	(Bera *et al.*, 2007)
Resistance to antimicrobial fatty acids	WTA	(Kohler *et al.*, 2009)
Resistance to low osmolarity	LTA	(Oku *et al.*, 2009)
Adherence to epithelial and endothelial cells	WTA	(Weidenmaier *et al.*, 2004, Weidenmaier *et al.*, 2005, Weidenmaier *et al.*, 2008)
Attachment to biomaterials and biofilm formation	LTA, WTA	(Gross *et al.*, 2001, Fedtke *et al.*, 2007)

encoding both simultaneously (Oku *et al.*, 2009, Schirner *et al.*, 2009). A recent study identified LTA-deficient suppressor strains that were capable of normal growth and cell division in the absence of LTA. These strains had acquired mutations in the *gdpP* gene which encodes a c-di-AMP phosphodiesterase. Disruption of the *gdpP* gene led to increased levels of intra-cellular c-di-AMP which supports the growth of these LTA-negative strains of *S. aureus* (Corrigan *et al.*, 2011).

1.3 Anchoring of surface proteins in *S. aureus*

The cell wall is a point of contact between Gram positive bacteria and their surrounding environment. It acts as a docking site for proteins that interact with the host during colonisation and infection. While major functions of surface-anchored proteins are to act as adhesins and invasins, it is also clear that several can help the bacteria evade innate immune responses (Foster, 2005). This is discussed in detail in section 1.4. Proteins can be anchored to the cell envelope of Gram positive bacteria by several mechanisms (i) Covalent linkage to cell wall peptidoglycan via carboxy terminal wall-anchoring domains, (ii) non-covalent linkage to peptidoglycan and teichoic acids and (iii) lipoproteins anchored to the outer face of the cytoplasmic membrane via N-terminal lipid moieties.

1.3.1 Proteins covalently linked to the cell wall

Covalent linkage of proteins to the cell wall of Gram positive bacteria requires a specific carboxy-terminal sorting signal, the LPXTG motif (Fischetti *et al.*, 1990, Navarre & Schneewind, 1999). The LPXTG motif is followed by a hydrophobic membrane-spanning domain of ~20 residues and a positively charged cytoplasmic tail. *S. aureus* has the potential to express up to 20 proteins on its surface that are covalently linked to cell wall peptidoglycan by a process called 'sorting' (Figure 1.3).

An N-terminal signal sequence of ~40 residues directs these proteins to the secretory (Sec) pathway. An 'AXA' motif at the end of the signal sequence is recognised and cleaved by the membrane-anchored signal peptidase enzymes SpsA and SpsB during translocation across the cytoplasmic membrane (Mazmanian *et al.*, 2001, Cregg *et al.*, 1996). Covalent attachment of surface proteins to the cell wall peptidoglycan is catalysed by the sortase (Srt) enzymes. *S. aureus* has two sortases, SrtA and SrtB (Mazmanian et al., 2001, Pallen *et al.*, 2001). SrtA anchors the majority

of *S. aureus* surface proteins and recognizes the LPXTG motif. SrtB anchors the iron-regulated surface protein C (IsdC) and recognises the NPQTN motif (Mazmanian *et al.*, 2002). SrtA is a membrane-anchored protein, with the catalytic domain embedded in the peptidoglycan layer (Mazmanian *et al.*, 2001). Recognition of LPXTG is highly stringent and substitutions at positions 1, 2, 4 and 5 are not tolerated (Kruger *et al.*, 2004).

The crystal structure of the N-terminal catalytic domain of SrtA in complex with an LPETG peptide has been solved (Zong *et al.*, 2004, Liew *et al.*, 2004). Proline and threonine residues of the LPXTG motif are held in position within the substrate binding pocket of SrtA by hydrophobic contacts near the sortase active site residues C184, R197 and H120 (Zong et al., 2004). Cleavage of the LPXTG motif by SrtA occurs between the threonine and glycine residues. SrtA covalently attaches the cleaved protein via threonine to glycine in the pentaglycine bridge of the nascent peptidoglycan subunits (Ton-That *et al.*, 2000). The C-terminal membrane-spanning domain and positively charged tail are then released and degraded. The peptidoglycan precursor with the attached protein is then linked to the peptidoglycan by transglycosylation (Ton-That *et al.*, 1997, Navarre *et al.*, 1998). *S. aureus* SrtA-deficient mutants, which fail to anchor LPXTG-containing surface proteins resulting in their secretion from the bacteria, are attenuated in animal infection modules of septic arthritis and endocarditis (Jonsson *et al.*, 2002, Weiss *et al.*, 2004).

1.3.2 Proteins anchored by non-covalent interactions

Internalin B (InlB) of *L. monocytogenes* is a surface protein that is required for invasion of mammalian cells (Ireton & Cossart, 1997). InlB activates phosphoinositide (PI)-3 kinase resulting in cytoskeletal rearrangements and engulfment of the bacteria (Ireton *et al.*, 1996, Ireton *et al.*, 1999). InlB is associated with the cytoplasmic membrane in cell fractionation experiments but also occurs extracellularly (Jonquieres *et al.*, 1999). The cell surface anchor of InlB consists of the last 232 carboxy terminal residues organised into three highly conserved repeats of ~80 residues containing the dipeptide Gly-Trp 'GW modules' (Braun *et al.*, 1997). These modules interact with lipoteichoic acids on the bacterial cell surface and also with glucosaminoglycans on mammalian cells (Jonquieres *et al.*, 1999, Jonquieres *et al.*, 2001). Increased numbers of GW modules leads to higher efficiency cell wall anchoring as seen with the *L.*

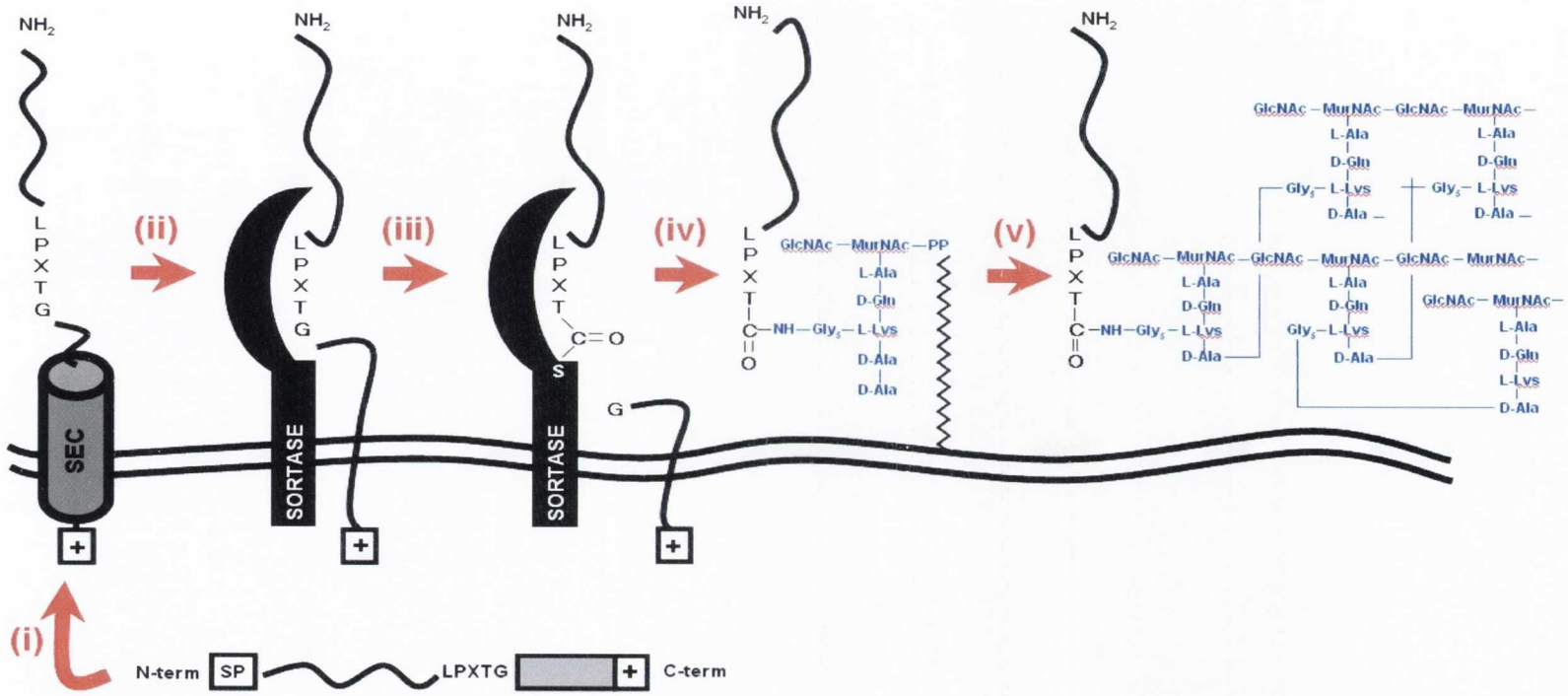


Figure 1.3 Surface protein anchoring in *Staphylococcus aureus*. (**i**) Export. Precursor proteins with an N-terminal signal peptide (SP) are initiated into the secretory (Sec) pathway and the signal peptide is removed. (**ii**) Retention. The C-terminal sorting signal retains polypeptides within the secretory pathway. (**iii**) Cleavage. Sortase cleaves between the threonine and the glycine of the LPXTG motif, resulting in the formation of a thioester enzyme intermediate. (**iv**) Linkage. Nucleophilic attack of the free amino group of lipid II 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 sulphydryl. (**v**) Cell wall incorporation. Lipid-linked surface protein is first incorporated into the cell wall via the transglycosylation reaction. The murein pentapeptide subunit with attached surface protein is then cross-linked to other cell wall peptides, generating the mature murein tetrapeptide.

monocytogenes surface associated protein Ami which contains eight GW modules (Braun et al., 1997). GW modules occur in the surface autolysins Atl from *S. aureus* (Oshida *et al.*, 1995), AtlC from *S. caprae* (Allignet *et al.*, 2001), AtlE from *S. epidermidis* (Heilmann *et al.*, 1997) and Aas from *S. saprophyticus* (Hell *et al.*, 1998). The GW modules of the staphylococcal autolysins have all been shown to be cell surface targeting repeats.

The surface-associated *S. aureus* autolysin Aaa contains three repeat domains that are highly homologous to the lysin motif (LysM), a consensus sequence found in peptidoglycan hydrolases (Heilmann *et al.*, 2005). The LysM region of the *L. lactis* autolysin AcmA binds peptidoglycan (Steen *et al.*, 2003). It has been proposed that the peptidoglycan-binding function of the LysM domain may account for the cell surface expression of Aaa. (Heilmann *et al.*, 2005)

The extracellular adherence protein (Eap) of *S. aureus* is non-covalently associated with the cell surface as well as being secreted (Harraghy *et al.*, 2003). Secreted Eap can bind back to the *S. aureus* cell surface by binding to both surface expressed Eap in a Eap-Eap interaction and/or directly to components of the cell surface. Binding of Eap to the cell surface is dependent on the D-alanylation of teichoic acids, as Eap does not bind to the cell surface of *dlt* deficient strains (Harraghy *et al.*, 2003). Most likely this is due to the disruption of electrostatic interactions.

1.3.3 Proteins anchored to the cell surface via lipid moieties

Bacterial lipoproteins are a subset of membrane proteins that are characterised by the presence of a conserved sequence at the C-terminus of the signal peptide. The conserved motif or lipobox, (L(A/S)(G/A)C), surrounds the signal peptide cleavage site (Kovacs-Simon *et al.*, 2011, Halegoua *et al.*, 1977). The lipobox is modified by the covalent linkage of a diacylglycerol molecule to the thiol (-SH) of the cysteine residue by the catalytic action of lipoprotein diacylglycerol transferase (Lgt) (Sankaran & Wu, 1994, Babu *et al.*, 2006). The signal sequence of the pro-lipoprotein is subsequently cleaved by lipoprotein signal peptidase (Lsp) resulting in the cysteine of the lipobox becoming the amino terminal residue of the mature lipoprotein (Tokunaga *et al.*, 1982). The mature lipoprotein is then anchored to the membrane long chain fatty acids via its lipid moiety.

Based on genome sequence analysis lipoproteins account for 2% of the staphylococcal proteome (Babu *et al.*, 2006). Lipoproteins primarily function in protein maturation and as substrate-binding proteins for ABC transporter uptake systems. For example the oligopeptide permease OppA aids protein folding whilst IsdE binds heme and transports it through the membrane (Stoll *et al.*, 2005, Grigg *et al.*, 2007). The generation of Lgt-deficient strains of *S. aureus* indicated a role for lipoproteins in survival within macrophages, invasion of non-phagocytic cells and in nutrient uptake, in particular iron acquisition (Stoll *et al.*, 2005, Schmaler *et al.*, 2010).

1.4 Innate immune evasion by *S. aureus*

The disease causing potential of *S. aureus* reflects its ability to express a repertoire of evasins classified according to their roles in innate immune evasion. They may be associated with the bacterial cell wall or secreted into the extracellular environment. *S. aureus* innate immune evasion is a complex phenotype with many evasins being pluripotent and capable of multiple interactions with host components. The purpose of this section is to review how staphylococci avoid innate immunity. Innate immune evasion mechanisms will be categorized according to survival on the skin, inhibition of neutrophil migration, inactivation of complement, resistance to phagocytosis and survival within professional phagocytic cells. Proteins introduced in this section will be discussed in detail in sections 1.5 and 1.6.

1.4.1 Survival on the skin

The outer layer of the human skin, the stratum corneum, provides a physical barrier to *S. aureus*. In order to survive the bacteria must overcome acidic conditions, antimicrobial peptides (AMPs) and fatty acids. Expression of IsdA makes the *S. aureus* cell surface hydrophilic due to the amino acid composition of its C-terminal domain. Consequently IsdA confers resistance to fatty acids and AMPs that bind to the bacterial cell surface by hydrophobic interactions (Clarke *et al.*, 2007). IsdA was also required for survival of *S. aureus* on the skin of healthy human volunteers. Modifications to *S. aureus* teichoic acids (TAs) and phosphatidylglycerol also protect the bacteria from AMPs on the surface of the skin and the nasal mucosa. The DltA proteins mediate D-alanine substitutions of TAs while MprF adds L-lysine residues to phosphatidylglycerol exposed on the outer surface of the cytoplasmic membrane (Collins *et al.*, 2002, Peschel

et al., 1999, Peschel *et al.*, 2001). These modifications increase the positive charge at the cell surface which repulses cationic antimicrobial peptides such as defensins.

Lysozyme is present in many body fluids and is an important component of the innate defences against bacterial infections. Resistance of *S. aureus* peptidoglycan to lysozyme degradation is due to membrane-bound O-acetyltransferase that modifies the C6 hydroxyl group of muramic acid (Bera *et al.*, 2005).

A unique feature of the CA-MRSA strain USA300 is the presence of an arginine catabolic mobile element (ACME) integrated directly adjacent to the type IV SCC*mec* cassette. ACME encodes enzymes of the arginine deiminase pathway that convert arginine into carbon monoxide, ATP and ammonia. It has been proposed that production of ammonia neutralises the acidic pH of the skin aiding bacterial survival which may in fact be a contributing factor to the rapid spread of CA-MRSA (Diep *et al.*, 2008).

1.4.2 Inhibition of neutrophil migration

Invading bacteria generate chemoattractants that stimulate the migration of neutrophils to the site of infection. Neutrophils are attracted by short formyl peptides cleaved from newly synthesised bacterial proteins and by chemoattractant molecules C3a and C5a released by cleavage of C3 and C5, respectively (Gasque, 2004). Each chemoattractant diffuses away from the bacteria creating a concentration gradient. To counter these attractants, *S. aureus* secretes proteins that specifically block neutrophil activation and migration (Figure 1.4). The secreted chemotaxis inhibitory protein of *S. aureus*, CHIPS, binds to the formyl peptide receptor (FPR) and the C5a receptor (C5aR). This blocks the cognate agonist from binding and results in potent inhibition of chemotaxis (de Haas *et al.*, 2004). A related *S. aureus* protein, FLIPr, binds to the formyl peptide receptor like-1 (FPRL-1) receptor on neutrophils and blocks FPR-like agonists (Prat *et al.*, 2006).

The cell adhesion molecule P-selectin is translocated to the endothelial cell surface during inflammation. The P-selectin receptor PSGL-1 on circulating neutrophils binds P-selectin and initiates the process of neutrophil rolling which mediates the migration of the phagocyte into adjoining tissues. Staphylococcal superantigen-like 5 (SSL5) is a secreted protein that binds PSGL-1 and inhibits binding to P-selectin *in*

vitro preventing neutrophil rolling on activated endothelial cells (Bestebroer *et al.*, 2007).

To transmigrate across blood vessels neutrophils must adhere securely to endothelial cells. This is mediated by the β2 integrins Mac-1 and leucocyte function associated antigen-1 (LFA-1) that interact with the counter-receptor intercellular adhesion molecule-1 (ICAM-1) on endothelial cells. The secreted extracellular adhesion protein (Eap) can bind to numerous ligands including ICAM-1 where it blocks the interaction with LFA-1 reducing neutrophil attachment to and transmigration through endothelial cells (Chavakis *et al.*, 2002).

1.4.3 Inactivation of complement

When *S. aureus* breaches the physical barriers of the skin and mucosal membranes it must overcome the innate and acquired immune system of the host. The complement system is a family of plasma proteins and proteolytic fragments derived from them that react with one another to opsonise pathogens and induce a series of inflammatory responses that help fight infection (Figure 1.5). There are three pathways that trigger complement activation and fixation. The alternative and lectin pathways are components of innate immunity whereas the classical pathway is triggered by specific interactions with antibodies bound to antigens on the surface of the bacteria. One of the purposes of complement fixation is opsonisation promoting phagocytosis by neutrophils and macrophages. The FcγR1 receptor on neutrophils specifically recognises the Fc region of IgG molecules whilst complement proteins bound to the bacterial cell surface are recognised by receptors CR1 and CR3. *S. aureus* exhibits several distinct mechanisms that interfere with complement by inactivation of or sequestering of key components.

Assembly of C3 convertases on the surface of *S. aureus* is a prerequisite for complement activation (Fujita, 2002). C4bC2a (classical and lectin pathways) and C3bBb (alternative pathway) cleave C3 resulting in the release of chemoattractant C3a and the attachment of the opsonin C3b to the bacterium. *S. aureus* secretes a small protein called staphylococcal complement inhibitor (SCIN) that binds to and stabilises both C4bC2a and C3bBb resulting in inhibition of further C3b formation (Rooijakkers *et al.*, 2005a). Recently SCIN was found to block phagocytosis of *S. aureus* by inducing

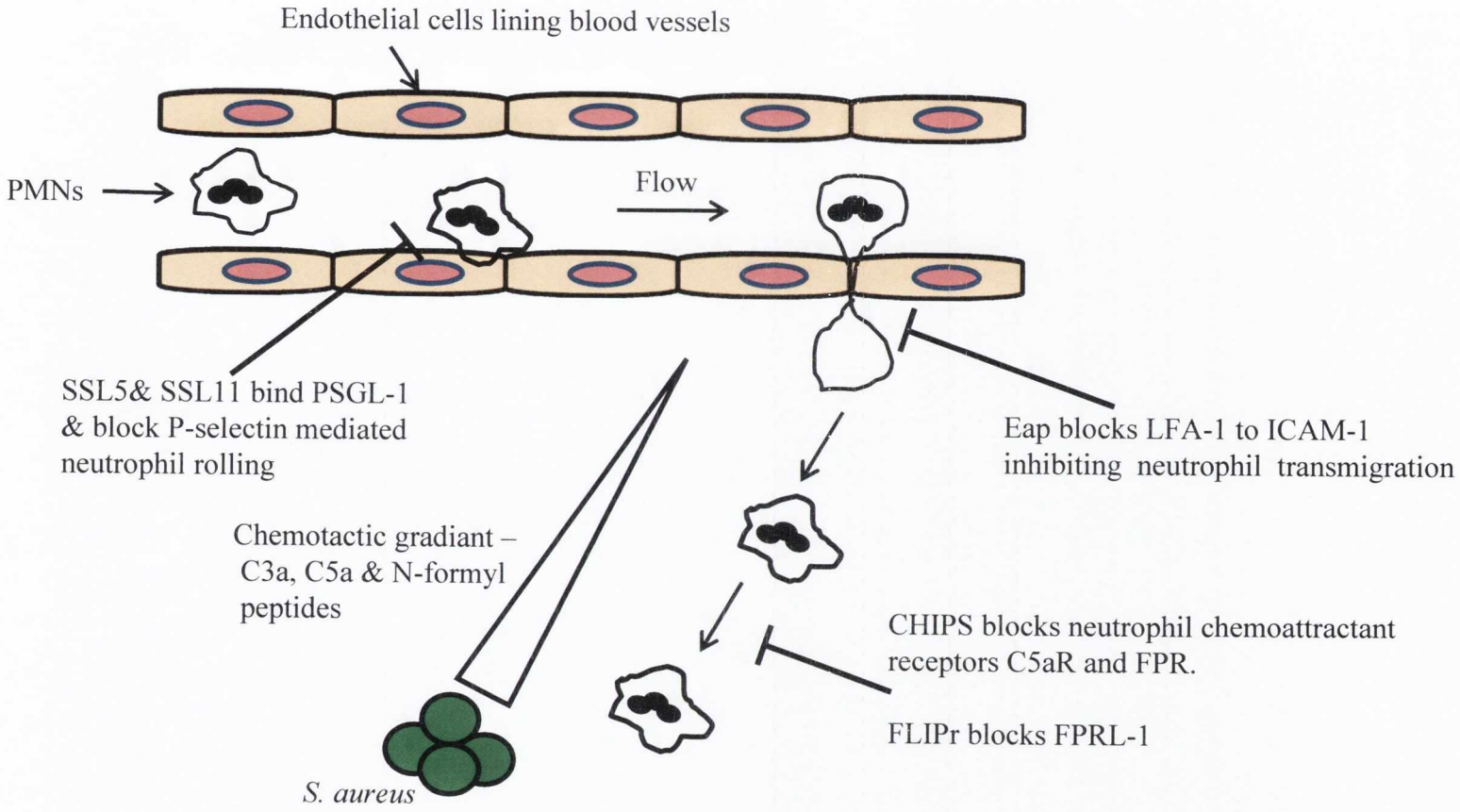


Figure 1.4 Inhibition of neutrophil migration by *S. aureus*

Invading bacteria generate the chemoattractants C3a, C5a and N-formyl peptides that stimulate the migration of neutrophils to the site of infection. Each chemoattractant diffuses away from the bacteria creating a concentration gradient along which neutrophils migrate, guiding them to the site of infection. To counter these attractants, *S. aureus* secretes proteins that specifically block (i) neutrophil rolling , SSL5 and SSL11 (ii) transmigration, Eap and (iii) migration in response to chemotactic signals, CHIPS and FLIPr.

dimerization of the C3 convertase C3bBb (Jongerius *et al.*, 2010). Normally C3 convertases are transiently active and dissociation leaves the bound C4b and C3b to act as catalysts for further cleavage of C2 and factor B, respectively. Stabilisation of the complexes by SCIN blocks the crucial amplification loop and is a potent mechanism for preventing complement activation. Preventing C3b formation ultimately inhibits C5 convertase activity, thus reducing the level of the neutrophil chemoattractant C5a. SCIN was shown to block phagocytosis and killing of *S. aureus* cells by human neutrophils (Rooijakkers *et al.*, 2005a). The secreted *S. aureus* protein Efb and its homologue Ehp also bind complement factor C3 and block convertases containing C3b (Lee *et al.*, 2004a, Jongerius *et al.*, 2007). These include the C5a convertases C4bC2aC3b and C3bBbC3b resulting in the reduction of the chemoattractant C5a and reduced neutrophil recruitment.

The *S. aureus* metalloprotease aureolysin blocks complement-mediated phagocytosis of the bacteria by preventing C3b deposition on the bacterial cell surface (Laarman *et al.*, 2011). Aureolysin was shown to inhibit both the alternative and classical complement pathways by cleaving C3. Aureolysin cleaves C3 in the fluid phase generating functional derivatives of C3a and C3b. In serum the C3b-like derivative is then naturally degraded by factor H and factor I (Laarman *et al.*, 2011). Aureolysin-mediated degradation of C3b results in inhibition of C5a formation as there is no C3b available to form the C5 convertases. Aureolysin was also shown to inhibit C3a-mediated neutrophil activation by cleaving C3a into an inactive derivative referred to as C3a' (Laarman *et al.*, 2011). *S. aureus* superantigen-like protein 7 (SSL7) binds directly to complement factor C5 inhibiting the generation of C5a by all three pathways of complement activation. SSL7 has been shown to abolish the C5a-driven influx of neutrophils in murine models of peritonitis (Bestebroer *et al.*, 2010).

Host plasminogen can attach to the surface of *S. aureus* where it is bound by the secreted plasminogen activator staphylokinase. Staphylokinase activates the serine protease activity of plasminogen on the bacterial cell surface which cleaves surface bound C3b and IgG resulting in reduced phagocytosis by neutrophils (Bokarewa *et al.*, 2006, Rooijakkers *et al.*, 2005c).

Complement factors I and H are down-regulators of complement fixation (Figure 1.6 Walport, 2001a, Walport, 2001b). Factor I cleaves C3b to iC3b in the

presence of factor H. While C3b and iC3b are recognised by specific receptors on neutrophils and thus promote phagocytosis, iC3b cannot activate the alternative pathway or the terminal cascade and is degraded to C3d by factor I. The *S. aureus* MSCRAMM ClfA captures and activates factor I on the bacterial cell surface independently of factor H, resulting in increased levels of iC3b and C3d on the cell surface coupled with a decrease in C3b and a reduction in phagocytosis (Cunnion *et al.*, 2004, Cunnion *et al.*, 2005, Hair *et al.*, 2008). The iron regulated surface determinant IsdH has also been shown to cause accelerated degradation of C3b to iC3b and C3d (Visai *et al.*, 2009).

1.4.4 Resistance to phagocytosis

Capsular polysaccharide is produced by the majority of *S. aureus* clinical isolates (Roghmann *et al.*, 2005, O'Riordan & Lee, 2004). Expression of capsular polysaccharide serotypes 5 and 8 reduces phagocytosis *in vitro* and is associated with increased virulence in animal infection models (Luong & Lee, 2002, Thakker *et al.*, 1998). Capsule inhibits binding of antibodies to *S. aureus* cell surface components which hinders opsonisation (Thakker *et al.*, 1998). It also obstructs access of phagocyte complement receptors to complement components assembled beneath the capsule layer (Cunnion *et al.*, 2003). Reduced O-acetylation of capsular polysaccharide decreases antiphagocytic activity due to increased antibody penetration and cell surface recognition (Bhasin *et al.*, 1998).

Binding of the Fc-region of IgG to protein A (Spa) of *S. aureus* results in the bacteria becoming coated with IgG molecules in the incorrect orientation to be recognized by neutrophil Fc-receptors and for activation of complement by the classical pathway. This could explain why Spa-deficient *S. aureus* strains are phagocytosed more efficiently *in vitro*, and why Spa promotes virulence in several animal infection models (Patel *et al.*, 1987, Gemmell, 1991, Palmqvist *et al.*, 2002). Staphylococcal superantigen-like protein 10 (SSL10) also binds the Fc region of IgG and inhibits the interaction between IgG and the complement component C1q, resulting is repression of the classical complement activation pathway (Itoh *et al.*, 2010). In addition to its interaction with complement protein C5, SSL7 also binds to IgA and blocks recognition by the neutrophil receptor FcαRI (Ramsland *et al.*, 2007, Wines *et al.*, 2006). If SSL7

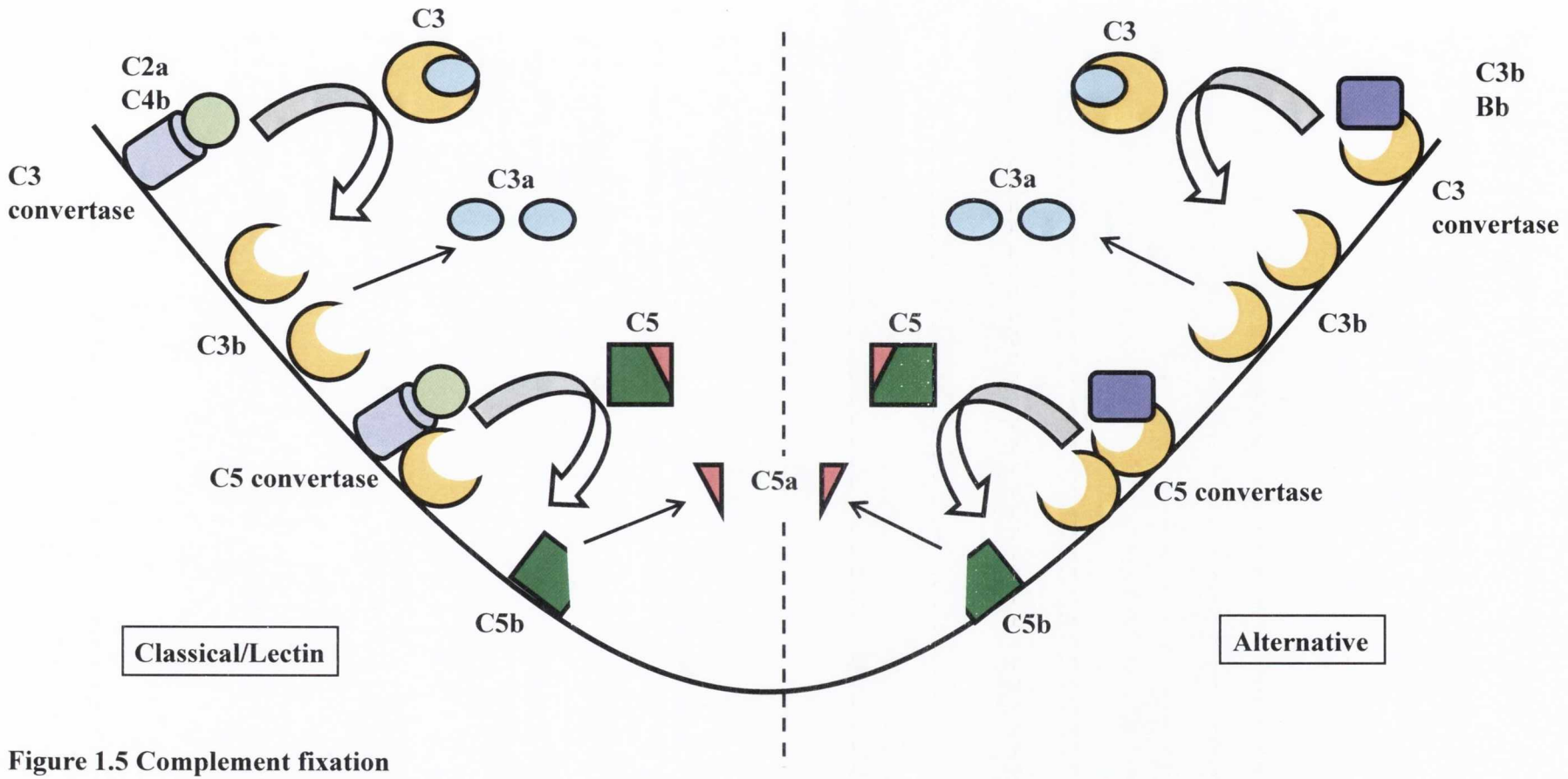


Figure 1.5 Complement fixation

The classical and lectin pathways activate the C3 convertase C4bC2a. C4bC2a cleaves C3 releasing C3a and resulting in the deposition of C3b on the bacterial cell surface. C3b then binds C4bC2a forming the C5 convertase C4bC2aC3b which cleaves C5 into C5b on the cell surface and releases the chemoattractant C5a. The alternative pathway C3 convertase results from spontaneous conversion of C3 to C3b which binds Bb to form the alternative pathway C3 convertase C3bBb. C3bBb then binds C3b to form the alternative pathway C5 convertase C3bBbC3b.

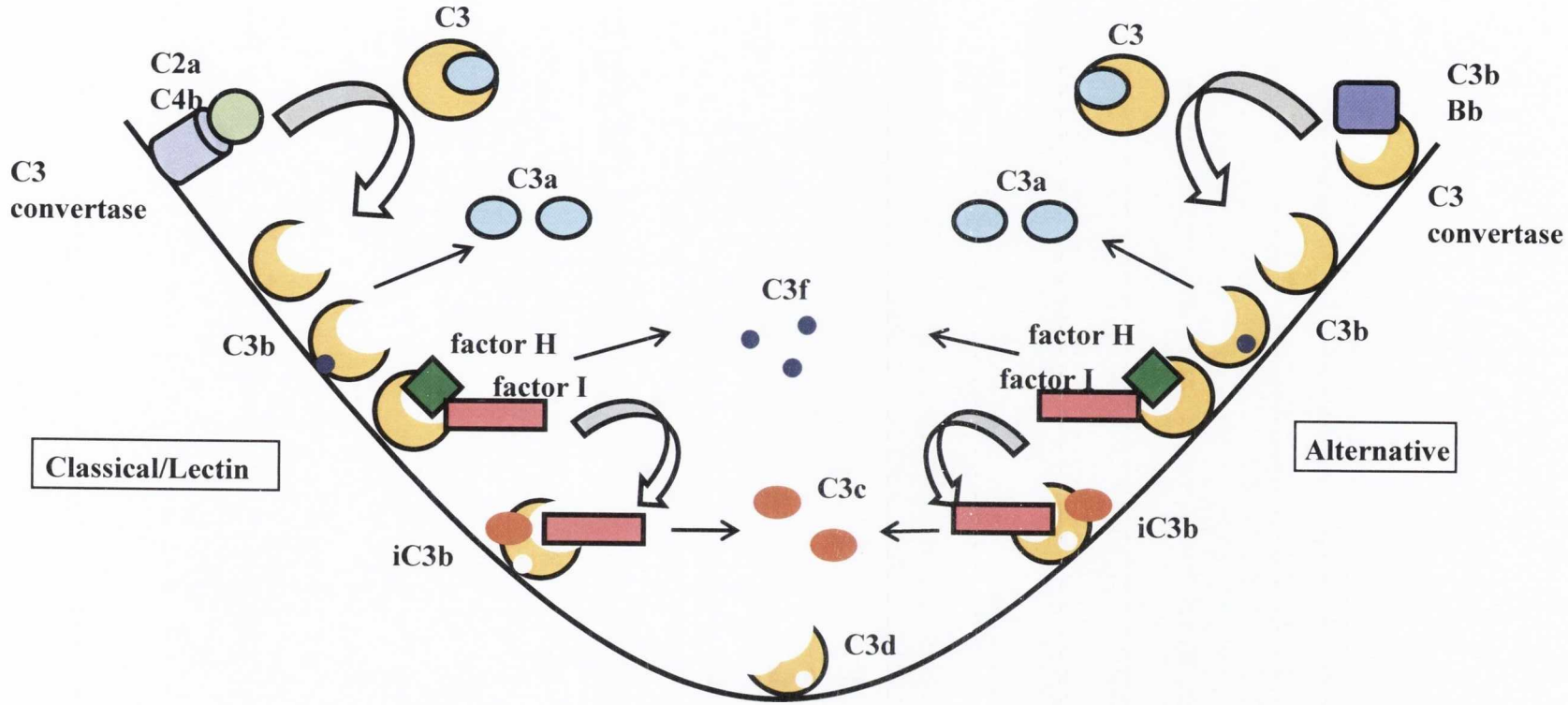


Figure 1.6 Regulation of complement fixation

Complement factors I and H are down-regulators of complement fixation and prevent complement activation on host cells. Factor I cleaves C3b to iC3b in the presence of factor H. This results in the release of C3f from C3b. While C3b and iC3b are recognised by specific receptors on neutrophils and thus promote phagocytosis, iC3b cannot activate the alternative pathway or the terminal cascade and is degraded to C3d by factor I with the release of C3c.

blocks IgA bound to antigens on the bacterial cell surface this will impede phagocytosis.

ClfA is another cell surface-associated protein that displays anti-phagocytic properties (Palmqvist *et al.*, 2004b). Bacteria expressing a non-fibrinogen binding ClfA mutant (ClfA PY) were phagocytosed more efficiently *in vitro* than bacteria expressing the wild-type protein. The coating of bacteria expressing ClfA with fibrinogen inhibited deposition of or access to opsonins (Higgins *et al.*, 2006). This may be important in the pathogenesis of invasive infections where *S. aureus* must survive in the blood stream as is the case in sepsis and septic arthritis. Other *S. aureus* fibrinogen-binding surface proteins (ClfB, FnBPA and FnBPB) may exhibit anti-phagocytic properties in a similar manner. The ability of ClfA to bind and activate complement factor I also contributes to the anti-phagocytic nature of the *S. aureus* protein.

1.4.5 Toxins that kill leukocytes

S. aureus produces several cytolytic toxins that contribute to the development of infection by killing neutrophils that are attempting to engulf and destroy invading organisms. The archetypal *S. aureus* toxin that forms β-barrel pores in the cytoplasmic membrane of leukocytes is α-toxin. It is secreted as a monomer which attaches to the membranes of cells. Monomers assemble into a heptamer in the membrane with β-strands from each monomer forming a 14-stranded β-barrel that spans the membrane creating a pore (Montoya & Gouaux, 2003). The membrane-associated metalloprotease 10 (ADAM 10) has been shown to mediate *S. aureus* α-toxin binding to eukaryotic cells (Wilke & Bubeck Wardenburg, 2010). ADAM 10 is necessary for α-toxin-mediated cytotoxicity at low concentrations of the toxin. ADAM 10 coordinates the formation of the β-barrel pore in the membrane of eukaryotic cells (Wilke & Bubeck Wardenburg, 2010). In addition, α-toxin upregulates ADAM 10 metalloprotease activity in alveolar epithelial cells resulting in cleavage of the paracellular junction protein E-cadherin (Inoshima *et al.*, 2011). Cleavage of E-cadherin is associated with disruption of the epithelial barrier. Transgenic mice with a conditional disruption of the Adam10 gene in their lung epithelium are resistant to *S. aureus*-mediated lethal pneumonia (Inoshima et al., 2011).

By contrast, the bi-componant leukotoxins comprise two subunits that are secreted separately and assemble into hexameric oligomers in the membrane of leukocytes. There are four types of bicomponent leukotoxin, the γ-toxin or γ-haemolysin, the Panton-Valentine leucocidin (PVL), leukocidin E/D and leukocidin M/F-PV-like toxins. γ-toxin lyses both erythrocytes and leukocytes while PVL is toxic only for leukocytes (Menestrina *et al.*, 2003).

The genes encoding PVL are present in a temperate bacteriophage which is found in only 1-2% of *S. aureus* strains. However, there is a strong association between PVL expression and strains that cause severe skin infections suggesting that PVL enhances virulence (Prevost *et al.*, 1995). In recent years, CA-MRSA strains have emerged that cause severe necrotising pneumonia and severe contagious skin infections in previously healthy individuals (Gillet *et al.*, 2002, Lina *et al.*, 1999). Not all CA-MRSA strains produce PVL and the role of PVL as a key virulence factor of CA-MRSA has been controversial (Moroney *et al.*, 2007, Voyich *et al.*, 2006, Brown *et al.*, 2009). PVL is highly specific for human polymorphonuclear leukocytes and fails to bind murine and primate PMNs. However, it does bind rabbit PMNs (Loffler *et al.*, 2010). Most recent studies of PVL showed that expression of the toxin enhanced the ability of the epidemic CA-MRSA strain, USA300, to cause lung inflammation and injury in a rabbit model of necrotising pneumonia. PVL contributed to the virulence of USA300 by recruiting and lysing polymorphonuclear leukocytes which damage the lung by releasing cytotoxic granule contents (Diep *et al.*, 2010).

1.4.6 Survival of *S. aureus* in neutrophil phagosomes

If *S. aureus* becomes phagocytosed it has numerous mechanisms to survive within the neutrophil. Resistance to cationic antimicrobial peptides found in the phagosome is mediated by modifications to the bacterial surface that reduce the net negative charge of the bacteria (see section 1.4.1). The secreted *S. aureus* proteins aureolysin and staphylokinase also neutralize cationic peptides (Jin *et al.*, 2004, Sieprawska-Lupa *et al.*, 2004). *S. aureus* is partially protected from oxygen free radicals by its yellow carotenoid pigment. A mutant defective in pigment biosynthesis showed increased phagocytosis by neutrophils *in vitro* and reduced virulence in a mouse subcutaneous abscess model (Liu *et al.*, 2005). *S. aureus* expresses two superoxide dismutases encoded by the *sodA* and *sodM* genes which remove O_2^-. Isogenic *sodA*,

sodM and *sodAsodM* mutants exhibited reduced virulence in a murine abscess model (Karavolos *et al.*, 2003). The cell wall-anchored enzyme adenosine synthase A (AdsA) contributes to the survival of *S. aureus* in human blood and to abscess formation in a murine renal abscess model (Thammavongsa *et al.*, 2009). AdsA catalyses the conversion of AMP to adenosine. Upon phagocytosis AdsA-deficient bacteria were killed within 30 mins where as the wild-type bacteria persisted within the neutrophil, indicating a role for AdsA in survival within the phagosome (Thammavongsa et al., 2009).

1.5 Surface components involved in innate immune evasion

1.5.1 Protein A

Protein A (Spa) is a surface-associated protein of *S. aureus* best known for its ability to bind to the Fc region of mammalian IgG in a non-immune manner (Uhlen *et al.*, 1984, Moks *et al.*, 1986). It is expressed on the surface of over 95% of *S. aureus* strains (Forsgren & Nordstrom, 1974) and is a prime example of the multi-functionality shown by many *S. aureus* surface proteins. Other ligands of Spa are the Fab heavy chain of V_H3 subclass immunoglobulins (Hillson *et al.*, 1993), von Willebrand factor (vWF) domains A1 and D1-D3 (Hartleib *et al.*, 2000, O'Seaghdha *et al.*, 2006) and tumour necrosis factor receptor-1 (TNFR-1) (Gomez *et al.*, 2004). The *spa* gene is transcribed during the mid-exponential phase of growth with repression of gene expression occurring as cultures enter stationary phase (Vandenesch *et al.*, 1991).

The surface exposed region of Spa is composed of five homologous repeat domains (EDABC) of ~58 residues linked to the cell surface by a short polymorphic repeat region (Xr) containing a variable number of eight residue repeats and a conserved region (Xc) (Figure 1.7, panel A; Uhlen et al., 1984, Moks et al., 1986). Structural analyses of a single Spa domain revealed that it is composed of anti-parallel α-helices that pack together to form a compact three helical bundle (Figure 1.7, panel C; Gouda *et al.*, 1992). Both the Spa-Fc and Spa-Fab complexes have been co-crystallized. The crystal structure of domain B of Spa in complex with an Fc fragment revealed that the Fc binding site spans helices I and II on a single face of the molecule and comprises 11 residues (Deisenhofer, 1981). The binding sites of vWF and TNFR-1 have been localised by site-directed mutagenesis. Both recognise a region in helices I and II that

overlaps the Fc binding site (O'Seaghdha et al., 2006, Gomez *et al.*, 2006). The crystal structure of domain D of Spa in complex with V_H3-Fab revealed that the V_H3 binding site spans helices II and III and the residues from helix II that bind Fc differ from those that bind V_H3 with the exception of glutamine 32 in Spa domain D (Graille *et al.*, 2000). Indeed it is possible for a single Spa domain to bind Fc and V_H3-Fab simultaneously (Figure 1.7, panel D; Graille et al., 2000, Starovasnik *et al.*, 1999).

Spa has multiple functions in thwarting the host immune response (see section 1.4) and is a virulence factor in murine infection models of subcutaneous infection, sepsis and septic arthritis and staphylococcal pneumonia (Patel et al., 1987, Palmqvist *et al.*, 2005b, Gomez et al., 2004). Spa is a potent immunomodulator due to its ability to bind to the V_H3 region adjacent to the antigen-binding domain of IgM molecules exposed on the surface of B lymphocytes (Sasso *et al.*, 1989, Sasano *et al.*, 1993). The cells bearing V_H3 are stimulated to proliferate and undergo apoptosis leading to a significant reduction in antibody-secreting B cells in the spleen and bone marrow. Spa can also act as an immune effector by binding to and activating TNRF-1 the receptor for tumour necrosis factor α (TNFα). Spa mimics TNFα by activating the TNFR-1 proinflammatory signalling cascade and stimulating shedding of TNFR-1 by TNF-converting enzyme (TACE) (Gomez *et al.*, 2004, Gomez *et al.*, 2006). This is important in the pathogenesis of pneumonia as TNFR-1 is widely distributed on the airway epithelium. Consequently mice infected with wild-type *S. aureus* have a significantly higher incidence of pneumonia than mice infected with a Spa-deficient strain. Furthermore the Spa-deficient strain is attenuated similarly in TNFR-1 null and wild-type mice (Gomez *et al.*, 2004). The Xr domain of Spa has been shown to induce type I INF signalling in airway epithelial cells, in particular INF-β transcription (Martin *et al.*, 2009). INF-α/β receptor 1 null mice cannot respond to type I INF signalling. INF-α/β receptor 1 null mice exhibit increased survival in a lethal *S. aureus* pneumonia model compared to wild-type mice indicating that Spa-induced type I INF signalling is important in the pathogenesis of *S. aureus* pneumonia (Martin *et al.*, 2009).

More recently the role of Spa in invasion of *S. aureus* across airway epithelial cells has been elucidated. Spa activates the RhoA/ROCK/MLC cascade that is involved in cytoskeleton rearrangement. ROCK is the effector molecule of RhoA and stimulates contraction of actin filaments by phosphorylation of MLC which increases myosin II

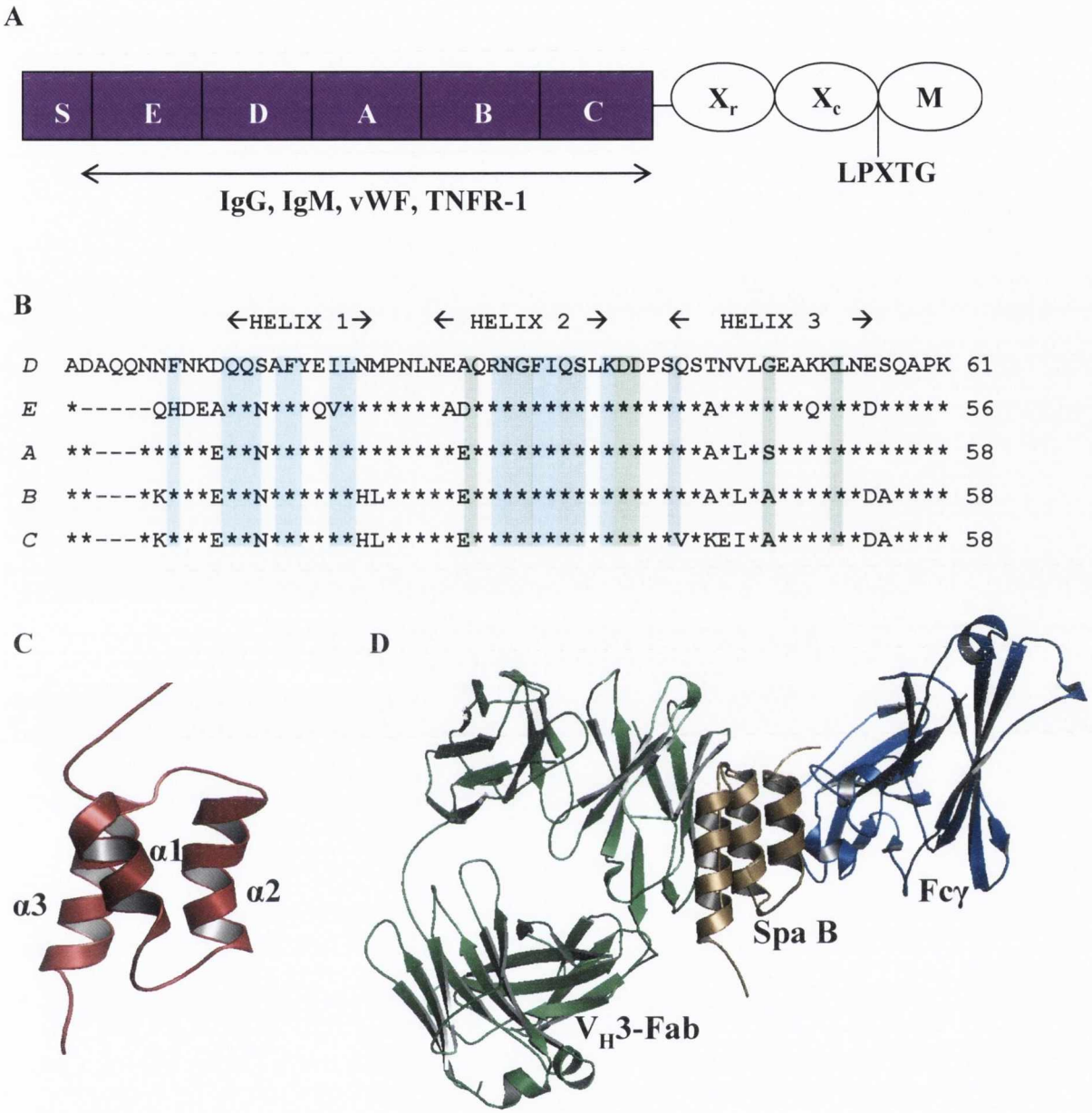


Figure 1.7 Schematic diagrams of Spa.

(**A**) Schematic diagram of the structure of Spa. S, signal sequence; IgG binding domains of Spa (E,D, A, B, C); Xr, proline-rich C terminal domains; LPXTG wall anchoring motif; M, transmembrane domain. (**B**) Sequence alignment of the five IgG-binding domains of Spa, with positions of the α-helices and residues involved in Fc (blue) or Fab (green) binding indicated. (**C**) Ribbon diagram showing the overall chain folds for the B domain of Spa. Helices are numbered. (**D**) Composite figure of a single Spa domain interacting simultaneously with Fc and a V_H3-Fab. Spa is coloured gold, Fc in blue and V_H3-Fab is coloured green.

ATPase activity. Inhibition of RhoA-MLC signalling with the ROCK inhibitor Y-27632 prevented transmigration of *S. aureus* across airway epithelial cells (Soong *et al.*, 2011).

Spa was also shown to activate epidermal growth factor receptor (EGFR) signalling which in turn activates RhoA and stimulates protease expression. Inhibition of EGFR phosphorylation significantly reduced the transmigration of *S. aureus* across airway epithelial cells (Soong *et al.*, 2011). A protein matrix spans the paracellular junction between adjacent epithelial cells, *S. aureus* must traverse this during invasion. EGFR signalling activates TACE and the calcium-dependent cysteine proteases the m-calpains. Two of the paracellular junction proteins E-cadherin and occludin are substrates for the calpains. Cleavage products of both of these proteins were detected in epithelial cells incubated with wild-type *S. aureus* but not in cells exposed to the Spa-deficient strain. In addition, mice pretreated with a calpain inhibitor had significantly increased numbers of *S. aureus* in the bronchoalveolar lavage than PBS treated control mice (Soong *et al.*, 2011). Taken together these results indicate that Spa mediates invasion across airway epithelial cells by activating RhoA and subsequent proteolytic cleavage of paracellular junction proteins.

S. aureus is responsible for greater than 80% of osteomyelitis cases. Recent studies have indicated a role for Spa in adherence to osteoblasts through interaction with TNFR-1 expressed on the surface of osteoblasts. It has been shown that *S. aureus* adherence to osteoblasts via Spa leads to inhibition of proliferation and subsequent apoptosis of these cells whilst also increasing the production of the bone remodelling molecule RANKL (Claro *et al.*, 2011). Thus Spa is an important factor in causing destruction of bone in the pathogenesis of osteomyelitis.

1.5.2 Clumping factor A

Clumping factor A (ClfA) of *S. aureus* is a fibrinogen-binding protein that is located on the bacterial cell surface. If promotes the formation of cell aggregates in soluble fibrinogen and the adherence of bacterial cells to fibrinogen-coated surfaces and fibrin clots (McDevitt *et al.*, 1994). The *clfA* gene is expressed predominately in the stationary phase of growth from a sigma factor B-dependent promoter (Nicholas *et al.*, 1999, Bischoff *et al.*, 2004). Weaker expression of ClfA occurs in the exponential growth phase and is dependent on transcription from a sigma factor A-dependent

promoter. The structural features of ClfA are similar to other Gram-positive bacterial surface-anchored proteins. A 40 residue N-terminal signal sequence is followed by the 520 residue fibrinogen-binding A domain (McDevitt *et al.*, 1995). C-terminal to the A domain is a serine-aspartate (SD) dipeptide repeat region which varies in length between strains (McDevitt & Foster, 1995). The function of the SD repeats is to act as a stalk allowing the ligand-binding A domain to be displayed away from the cell surface, permitting its interaction with fibrinogen (Hartford *et al.*, 1997). The C-terminal wall-spanning region, membrane-spanning region and LPDTG motif are involved in anchoring ClfA to the cell wall (Figure 1.8, panel A; McDevitt et al., 1994).

The A domain of ClfA is composed of three subunits N1, N2 and N3. The minimum fibrinogen-binding domain of ClfA comprises N2 and N3 (McDevitt *et al.*, 1997). Crystallisation of the N2N3 subdomain of ClfA in the apo-form identified a hydrophobic trench between N2 and N3 that forms the binding cleft for the extreme C-terminal of the γ-chain of fibrinogen (Deivanayagam *et al.*, 2002). Structural studies of the N2N3 subdomains of ClfA in complex with the fibrinogen γ-chain peptide indicated that binding occurs by a variation of the "dock-lock-latch" mechanism (Figure 1.8, panel B; Ganesh *et al.*, 2008).

The ClfA binding site in the γ-chain of fibrinogen is the same region of fibrinogen recognised by the integrin GPIIb/IIIa on platelets which is necessary for platelet aggregation (Farrell *et al.*, 1992, Hettasch *et al.*, 1992). Bacteria expressing ClfA stimulate platelet activation by forming a fibrinogen bridge between the bacterium and GPIIb/IIIa. ClfA-specific immunoglobulin is also required to interact with the platelet immunoglobulin Fc receptor (FcγRIIa) for platelet activation to occur (Loughman *et al.*, 2005).

ClfA is anti-phagocytic and protects *S. aureus* from phagocytosis by murine macrophages and human neutrophils *in vitro* (Palmqvist *et al.*, 2004a, Higgins *et al.*, 2006). The anti-phagocytic properties of ClfA are partially dependent on fibrinogen-binding. Coating of bacteria with fibrinogen may impair deposition and/or access of opsonins to the bacterial cell surface. However, bacteria expressing a non-fibrinogen binding derivative of ClfA (ClfAPY) were still partially protected from opsonophagocytosis (Higgins *et al.*, 2006). This is due to the ability of ClfA to capture and activate the complement regulatory protein factor I from human serum resulting in

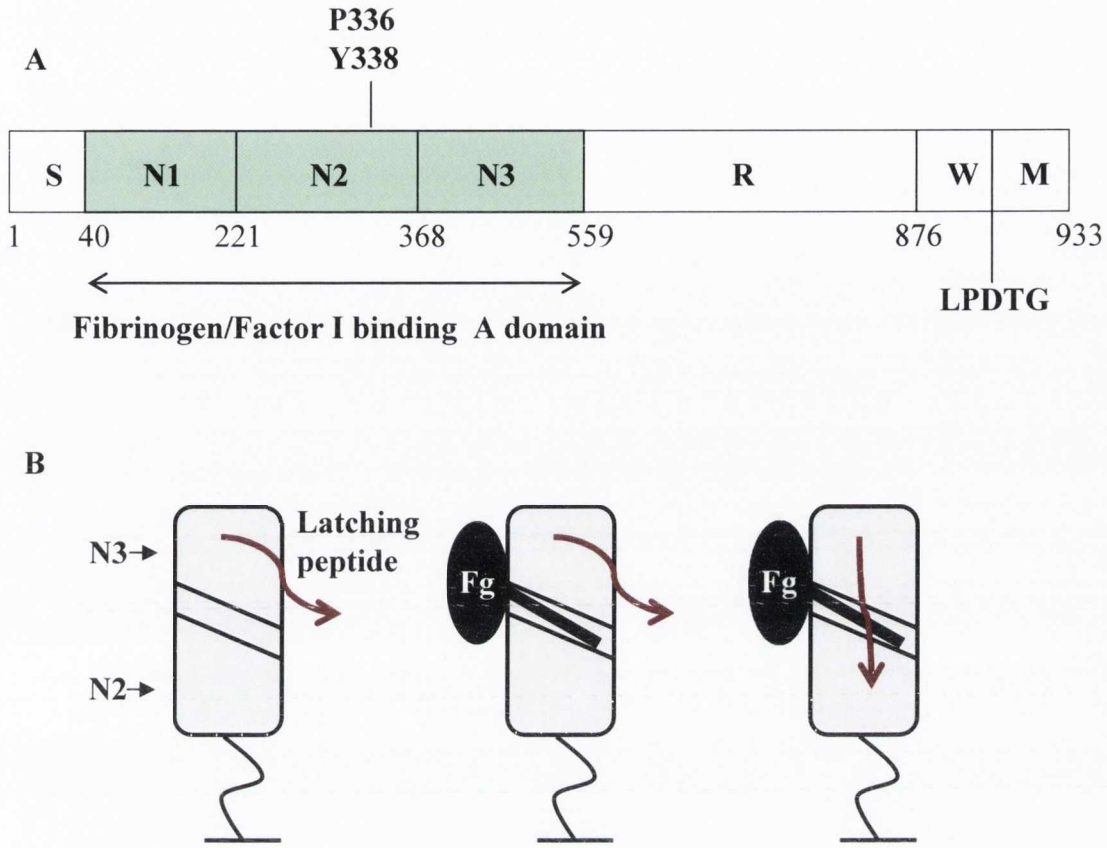


Figure 1.8 Schematic representation of ClfA (A) and the dock, lock and latch-binding mechanism (B)

(**A**) Schematic diagram of the structure of ClfA. S is the 40 residue signal sequence. The fibrinogen/factor I-binding domain (A domain) comprises subdomains N1, N2 and N3. The minimum ligand binding truncate of ClfA is N2N3 (residues 221-559). Amino acids P_{336} and Y_{338} within N2 have been identified as being essential for fibrinogen binding by ClfA. C-terminal to the A domain is a repeat region (R) consisting of Ser-Asp dipeptide repeats followed by the wall (W) and membrane (M) spanning domains and the LPDTG motif required for cell wall anchoring of ClfA. (**B**) The N2 and N3 subdomains are indicated. The flexible N3 latching peptide is represented by a red arrow. Fibrinogen 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

enhanced degradation of opsonin C3b to iC3b (Hair et al., 2008, Hair *et al.*, 2010). This leads to loss of opsonins whilst also inhibiting C3 convertase formation and the alternative pathway amplification loop, as surface bound iC3b can no longer be recognised by factor B. However, the ClfAPY mutant binds factor I differently to wild-type ClfA. ClfAPY sequesters inactive factor I on its surface preventing the release of factor I from the bacteria in its active form (Hair *et al.*, 2010). ClfAPYII-expressing *S. aureus* are more susceptible to complement-mediated phagocytosis than the wild-type strain suggesting that the substitutions at positions 336 and 338 of ClfA are important in modulating *S. aureus* susceptibility to phagocytosis (Hair *et al.*, 2010).

ClfA has been shown to be a virulence factor in several infection models. ClfA-deficient mutants were less infective in endocarditis models then parental strains (Moreillon *et al.*, 1995). ClfA is also a virulence factor in murine models of septic arthritis and sepsis (Palmqvist *et al.*, 2005a, Josefsson *et al.*, 2001). In experimental endocarditis function blocking antibodies to ClfA sterilize vegetations on heart valves when administered with vancomycin (Vernachio *et al.*, 2003). Active immunisation with recombinant ClfA or passive immunisation with polyclonal anti-ClfA antibodies protected mice from arthritis and sepsis-induced death (Josefsson *et al.*, 2001). The protein is expressed in 98% of *S. aureus* strains isolated from healthy blood donors and in 100% of those with invasive disease (Peacock *et al.*, 2002). This makes ClfA an ideal target for the development of novel immuno prophylactic or therapeutic agents to combat *S. aureus* infections.

1.5.3 Iron regulated surface determinants

The expression of a subset of staphylococcal genes is induced under iron-limiting conditions, which are likely to resemble growth conditions in serum and within the host during infection. The products of the iron-regulated surface determinant (*isd*) genes are involved in iron acquisition. The *isd* genes are located on five transcriptional units (*isdA, isdB, isdCDEFsrtBisdG and isdH*) (Mazmanian *et al.*, 2003, Dryla *et al.*, 2003). Expression of Isd proteins is controlled by the ferric-uptake-response (Fur) transcriptional regulator which inhibits transcriptions of *isd* genes in the presence of Fe^{3+}. IsdA, IsdB and IsdH each contain the LPXTG sortase A sorting signal, while IsdC appears to be buried in the cell wall and contains an NPQTN motif for sortase B-catalysed cell wall sorting (Mazmanian *et al.*, 2002).

The surface-exposed Isd proteins IsdA, IsdB, IsdC and IsdH contain variable numbers of conserved NEAT (near iron transporter) domains which are involved in binding to haem and haem-containing proteins. Collectively these proteins bind human heamoproteins, remove the haem molecule and transport haem through the cell wall and plasma membrane for accumulation in the bacterial cytoplasm (Skaar *et al.*, 2004). Haem is passed in a co-ordinated manner from the NEAT domains of IsdB and IsdH to IsdA (Muryoi *et al.*, 2008). IsdA then passes haem to IsdC, the central conduit for haem transport into the cytoplasm. IsdC passes haem to IsdE, a component of the ABC transporter composed of IsdD, IsdE and IsdF (Muryoi *et al.*, 2008). Once inside the cytoplasm haemoxygenases IsdG and IsdI degrade haem releasing free iron (Figure 1.9).

In addition to iron-containing compounds, Isd proteins have been shown to interact with other ligands. IsdA binds to fibrinogen and fibronectin and acts as an adhesin when *S. aureus* is growing in iron-limiting conditions (Clarke *et al.*, 2004). It also promotes bacterial adhesion to desquamated nasal epithelial cells and nasal colonisation of the cotton rat nares (see section 1.1.2). This may be due to the ability of IsdA to bind loricrin, involucrin and cytokeratin 10, all of which are associated with the outer membrane of desquamated nasal epithelial cells (Clarke *et al.*, 2009). Vaccination of cotton rats with IsdA and IsdH protected against nasal colonisation (Clarke *et al.*, 2006). IsdA also binds to the bactericidal protein lactoferrin and neutralises its antibacterial serine protease activity. IsdA mutants are more sensitive to killing by lactoferrin (Clarke & Foster, 2008). In addition, expression of IsdA by *S. aureus* confers resistance to the innate defenses of the human skin (see section 1.4.1) (Clarke *et al.*, 2007).

IsdH plays a role in the evasion of phagocytosis. An *S. aureus* IsdH-deficient strain was engulfed more readily by human neutrophils, survived poorly in fresh whole human blood and was less virulent in a mouse model of sepsis. This protective mechanism seems to be due to the ability of IsdH to bind to and activate complement factor I, which accelerates degradation of the serum opsonin C3b (see section 1.5.3) (Visai *et al.*, 2009). IsdB binds to the platelet integrin receptor GpIIb/IIIa and promotes *S. aureus* adhesion to and aggregation of platelets (Miajlovic *et al.*, 2010).

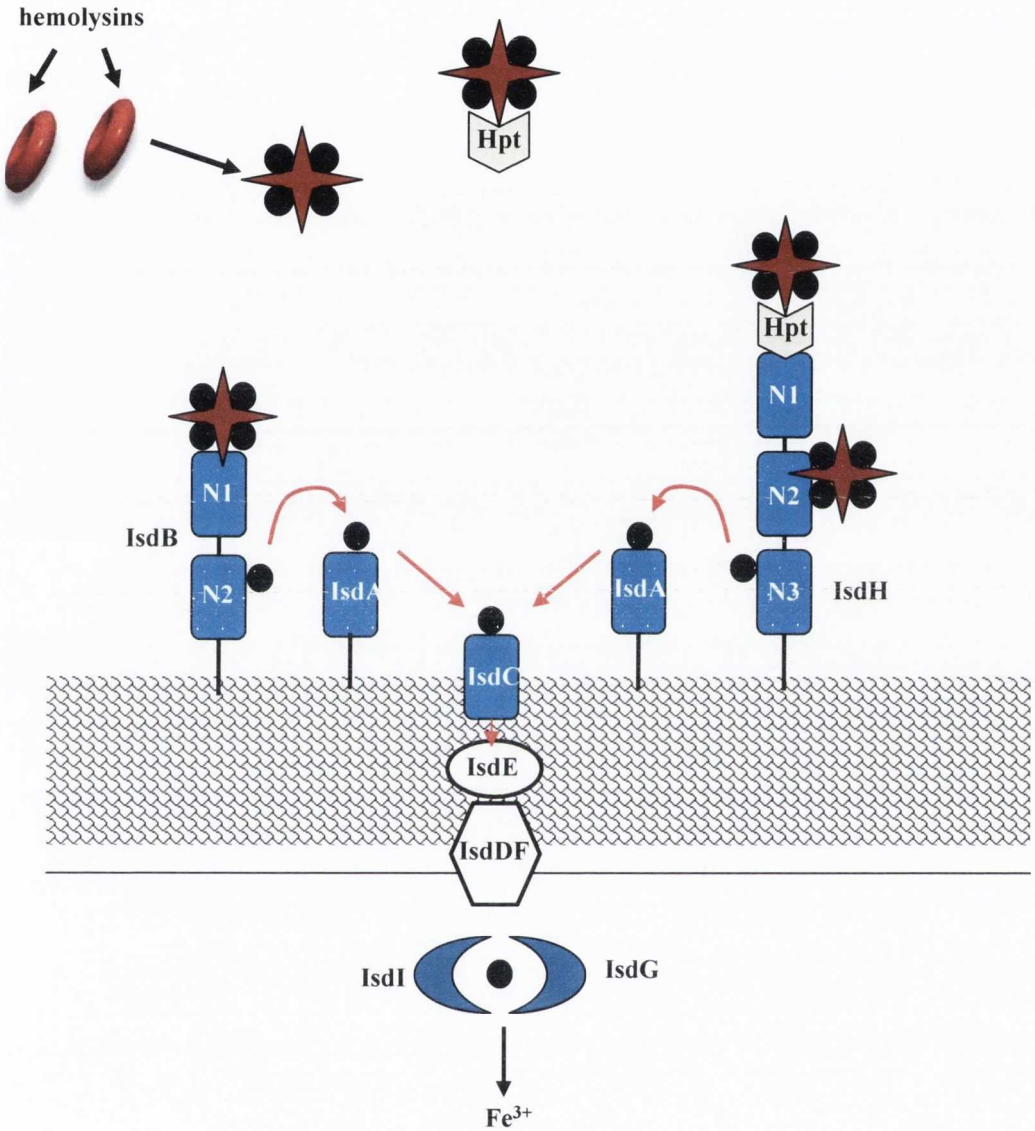


Figure 1.9 Putative pathway of haem transfer between Isd proteins of *S. aureus*

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

1.5.4 Sas proteins

Analysis of *S. aureus* genome sequences identified ten putative LPXTG-proteins (Mazmanian et al., 2001, Roche *et al.*, 2003a). These were named *S. aureus* surface (Sas) proteins. SasE, SasI and SasJ have since been renamed IsdA, IsdH and IsdB, respectively (see section 1.5.3). Antibodies to Sas proteins have been detected in sera from patients recovering from *S. aureus* infections indicating that these proteins are expressed *in vivo* (Roche *et al.*, 2003a).

SasG promotes bacterial adhesion to desquamated nasal epithelial cells and is also involved in the accumulation phase of biofilm formation (Roche *et al.*, 2003b, Corrigan et al., 2007, Geoghegan *et al.*, 2010a). In addition, SasG can mask the ability of *S. aureus* surface proteins such as ClfA, ClfB and FnBPs to bind their ligands (Corrigan *et al.*, 2007). The protein contains an N-terminal A domain followed by several homologous B-repeats. Full length SasG protein exposed on the cell surface is processed within the B domains resulting in cleaved proteins of various lengths being released into the extracellular environment. The length of SasG is crucial for its function. The binding of ligands was not masked in *S. aureus* strains expressing SasG proteins with fewer than 5 B-repeats. These strains showed reduced adherence to squamous cells and reduced biofilm formation (Corrigan *et al.*, 2007). Recent studies have shown that it is the B repeats and not the A domain of SasG that are required for biofilm formation (Geoghegan et al., 2010a). Recombinant B-repeat protein was found to bind to cells that have B domains anchored to their surface and to inhibit biofilm formation. In addition, the recombinant B-domains formed homodimers *in vitro* in a Zn^{2+}-dependent manner and it is proposed that homophilic association is necessary for biofilm formation (Geoghegan *et al.*, 2010b).

SasA (also known as SraP) can promote *S. aureus* binding to platelets and is a virulence factor in the rabbit endocarditis model (Siboo *et al.*, 2005). SasC was recently found to promote cell aggregation and biofilm formation (Schroeder *et al.*, 2009). SasH (also known as AdsA) converts AMP to adenosine which is an important regulator of neutrophil activity (Thammavongsa et al., 2009). SasH is anti-phagocytic supporting the survival of *S. aureus* in whole human blood. The generation of adenosine by SasH promotes the survival of *S. aureus* within the neutrophil (Thammavongsa *et al.*, 2009).

In the same study SasH was also required for kidney abscess formation in naive mice. No functions have been attributed to other Sas proteins (SasD, SasF and SasK).

1.5.5 Capsular polysaccharide

Capsular polysaccharide (CP) is produced by a majority of *S. aureus* clinical isolates (O'Riordan & Lee, 2004; Roghmann *et al.*, 2005). *In vitro* CP is expressed in the stationary phase of growth and has been detected *in vivo* in endocardial vegetations (Lee *et al.*, 1993). Eleven distinct CP serotypes have been identified. Heavily encapsulated strains express serotype 1 or 2 macrocapsules and form mucoid colonies on solid media. They are seldom encountered among clinical isolates. Expression of serotype 5 and 8 capsule (CP) is associated with increased virulence in animal infection models (Baddour *et al.*, 1992; Lee *et al.*, 1997; Luong & Lee, 2002; Nilsson *et al.*, 1997; Thakker *et al.*, 1998). Expression of capsule inhibits binding of antibodies to *S. aureus* cell surface components and impedes opsonisation and subsequent phagocytosis (Thakker *et al.*, 1998). High titres of anti-capsular polysaccharide antibodies overcome this effect and promote opsonophagocytosis (Lee *et al.*, 1997; O'Riordan & Lee, 2004). In a recent study expression of capsule inhibited *S. aureus* clumping factor A-mediated binding to fibrinogen and platelets (Risley *et al.*, 2007).

1.6 Secreted proteins involved in innate immune evasion

1.6.1 SCIN

The staphylococcal complement inhibitor (SCIN) protein of *S. aureus* is a 9.8kDa secreted protein expressed during the early exponential growth phase of the bacteria (Rooijakkers *et al.*, 2005a, Rooijakkers *et al.*, 2006). SCIN blocks human complement by specific interactions with the C3 convertases C3bBb and C4bC2a. SCIN is encoded on *S. aureus* pathogenicity island 5 (SaPI5) (van Wamel *et al.*, 2006). In addition to SCIN, SaPI5 also encodes the genes for the secreted immune evasion factors CHIPS and staphylokinase, both of which have been shown to be human-specific (de Haas *et al.*, 2004, Gladysheva *et al.*, 2003). In the presence of human serum SCIN inhibits phagocytosis of *S. aureus* by isolated human neutrophils as well as deposition of the serum opsonin C3b on the bacterial cell surface (Rooijakkers *et al.*, 2005a). Furthermore SCIN was shown to inhibit all three complement pathways and to be human specific. SCIN stabilises the C3 convertases preventing the dissociation of C2a

and Bb (Rooijakkers *et al.*, 2005a). Genome analysis of *S. aureus* revealed the presence of two 9.8kDA secreted SCIN homologs SCIN-B and SCIN-C, both of which inhibit opsonisation, complement-mediated phagocytosis and all three complement pathways (Jongerius *et al.*, 2007). As with SCIN, opsonisation of *S. aureus* in the presence of SCIN-B and SCIN-C results in increased levels of bacterial cell surface-bound C2a and Bb, indicating that all three SCIN proteins stabilise the C3 convertases (Jongerius *et al.*, 2007).

Structural analysis of SCIN has shown that it is composed of a triple α-helical bundle similar to the IgG binding domains of Spa (Figure 1.10, panel A; Rooijakkers *et al.*, 2007). The active site of SCIN was localised to residues 31-48, the majority of which are located on its α2-helix. Residues 31-48 have been shown to be essential for C3 convertase stabilisation and SCIN-mediated complement inhibition (Rooijakkers *et al.*, 2007). Further structural studies showed that SCIN induces the formation of stable C3bBb:SCIN dimers. Co-crystallisation of C3bBb:SCIN dimers revealed that SCIN stabilises the convertase dimers by interacting with C3b and Bb on one convertase and C3b on the opposing convertase (Figure 1.10, panel B; Rooijakkers *et al.*, 2009). However, convertase dimerisation is not essential for complement inhibition by SCIN. SCIN derivatives with altered C3b dimerisation contact sites generate only monomeric C3bBb:SCIN complexes. These SCIN derivatives retain the ability to stabilise C3bBb on the bacterial cell surface and inhibit complement activation (Rooijakkers *et al.*, 2009). SCIN-mediated dimerisation of C3bBb is important for inhibition of phagocytosis. Dimerisation of C3bBb:SCIN complexes on the bacterial cell surface inhibits phagocytosis by impairing the recognition of convertases by complement receptor 1 on the surface of neutrophils (Jongerius *et al.*, 2010).

To elucidate the mechanism by which SCIN prevents C3b formation surface plasmon resonance was used to measure the deposition of C3b on the surface of a sensor chip as C3 is passed over immobilised preformed C3bBb convertases. When SCIN was passed over the surface of the sensor chip prior to C3 a small binding response due to C3 binding to C3bBb is seen but no deposition of C3b (Ricklin *et al.*, 2009). In the absence of SCIN a significantly increased binding response is observed when C3 is passed over the surface as C3bBb cleaves C3 with subsequent deposition of C3b. This study indicates that SCIN does not inhibit the binding of C3 to C3bBb but

instead inhibits the formation of an active enzyme-substrate complex preventing proteolytic cleavage of C3 by C3bBb (Ricklin *et al.*, 2009). Recent studies have shown that SCIN can also bind directly to activated C3b and in doing so blocks the C3b binding site for factor B leading to inhibition of convertase formation (Garcia *et al.*, 2010).

1.6.2 CHIPS

The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14.1kDa secreted protein that is expressed during the early exponential growth phase of the bacteria (de Haas *et al.*, 2004, Rooijakkers *et al.*, 2006). CHIPS is encoded on SaPI5 and is found in over 60% of clinical isolates (van Wamel *et al.*, 2006). CHIPS binds directly to the C5a receptor (C5aR) and the formylated peptide receptor (FPR) blocking C5a and formylated peptide induced phagocyte activation and chemotaxis (Postma *et al.*, 2004). CHIPS reduced neutrophil recruitment towards C5a in a mouse peritonitis model. However, CHIPS activity is much more potent on human cells then mouse cells (de Haas *et al.*, 2004).

A two site binding model is purposed for C5a binding to C5aR. The N-terminus of C5a initially binds the N-terminus of C5aR followed by interaction of the C-terminus tail of C5a with residues in the transmembrane bundle of C5aR which is essential for activation of the receptor. CHIPS binds to residues 10-18 of the N-terminus of C5aR. (Postma *et al.*, 2005). The sulphated tyrosine residues 11 and 14 within the N-terminal of C5aR are essential for tight binding of CHIPS to C5aR (Ippel *et al.*, 2009). Substitution of these residues for phenylalanine completely abolishes CHIPS binding to C5aR (Liu *et al.*, 2011). The active site on CHIPS for C5aR has been localised to residues 31-121 (Haas *et al.*, 2005). The active binding on CHIPS for the FPR involves the first and third phenylalanines of its N-terminus (Haas *et al.*, 2004). Substitution of either of these residues abolished inhibition of FPR-mediated neutrophil chemotaxis by CHIPS.

1.6.3 Efb

The extracellular fibrinogen-binding protein (Efb) is a 15.6kDa protein secreted by *S. aureus* mainly during the post-exponential phase of growth (Wade *et al.*, 1998, Palma *et al.*, 1998). The protein binds to the α-chain of fibrinogen but does not support

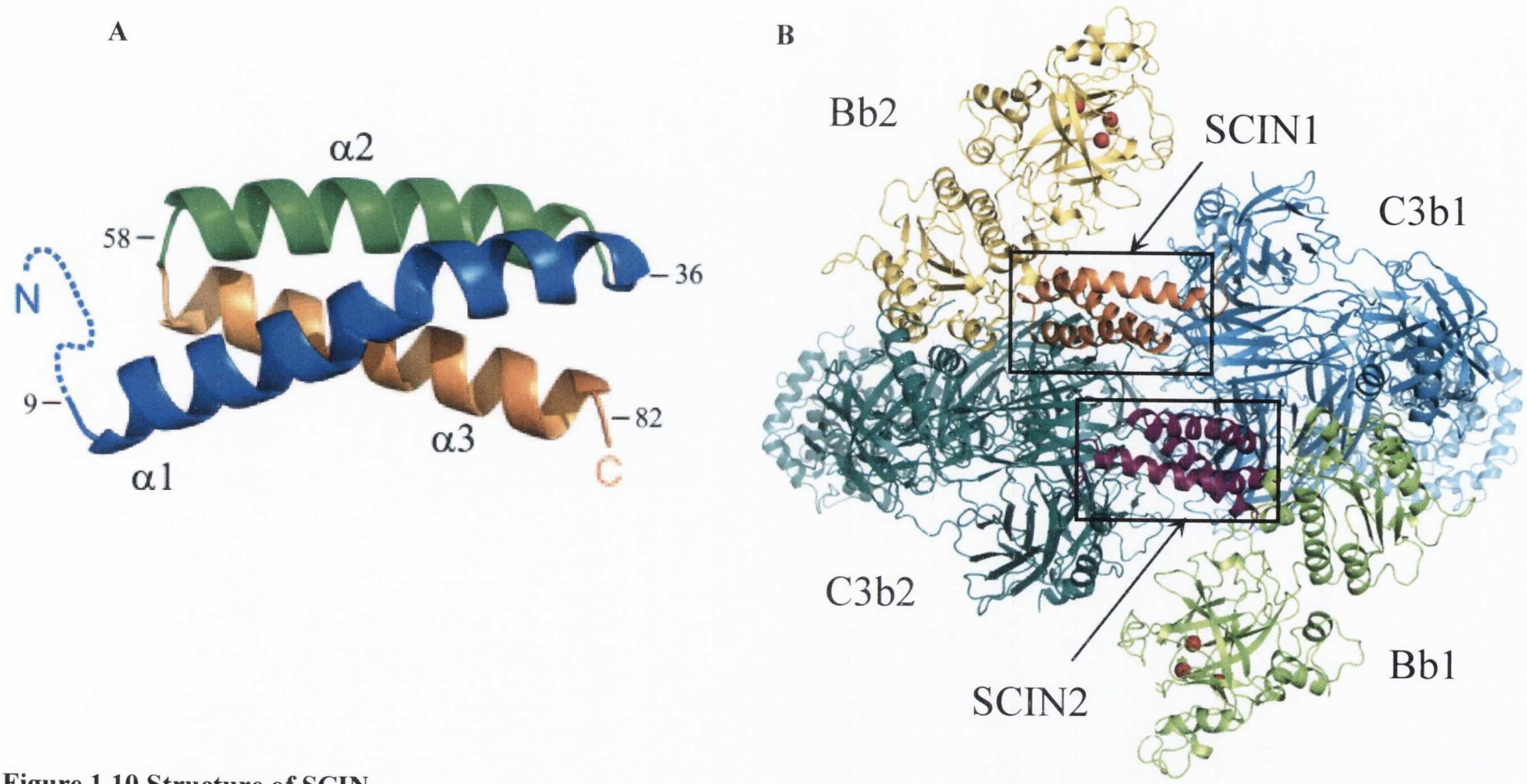


Figure 1.10 Structure of SCIN

(**A**) Ribbon diagram showing the overall structure of the three alpha-helical bundle of SCIN. Helices are numbered. α1 is coloured blue, α2 is coloured green and α3 is coloured orange. Numbers represent the residues that compose each helix. Diagram adapted from Rooijakkers *et al.*, 2007. (**B**) Ribbon diagram representing of the C3bBb:SCIN dimeric complex. C3b is coloured blue and turquoise, Bb is coloured green and golden and SCIN is coloured purple and orange. Diagram adapted from Rooijakkers *et al.*, 2009.

bacterial adhesion to immobilized fibrinogen *in vitro* (Palma *et al.*, 2001). The binding of Efb to fibrinogen is divalent with one binding site at the N-terminus of Efb and the other at the C-terminus (Wade *et al.*, 1998, Palma *et al.*, 1998). The N-terminus of Efb contains two homologous repeated regions that bear structural similarity to the fibrinogen-binding C-terminal domain of coagulase (Palma *et al.*, 1998). These regions of Efb and coagulase compete for the same binding site on fibrinogen, which is close to an RGD sequence recognized by platelet receptor GPIIb/IIIa (Palma *et al.*, 2001). Platelet aggregation is initially mediated by fibrinogen binding to activated GbIIb/IIIa via two RGD motifs on the α-chain and one on the γ-chain. The binding of Efb to this region of fibrinogen interferes with platelet activation and inhibits platelet aggregation. The binding of Efb to platelets may occur in a fibrinogen-dependent manner (via GPIIb/IIIa) or in a fibrinogen-independent manner, directly to the platelet surface (Shannon & Flock, 2004). Inhibition of platelet activation and aggregation by Efb is believed to contribute to delayed wound healing. The N-terminal domain of Efb inhibits neutrophil adhesion to immobilized fibrinogen. Efb binds fibrinogen blocking its interaction with the leukocyte integrin receptor α(M)β(2) inhibiting fibrinogen dependant leukocyte adhesion (Ko *et al.*, 2011).

Efb also binds to the complement protein C3, resulting in the inhibition of both the classical and alternative pathways of complement activation (Lee *et al.*, 2004a). Efb inhibited complement-mediated opsonophagocytosis of fluorescent beads conjugated to ClfA in the presence of serum opsonins and antigen specific antibodies. The C-terminus of Efb binds the C3d thioester-containing domain of C3. Efb-C has been crystallized in complex with C3d (Lee *et al.*, 2004b, Hammel *et al.*, 2007b). Recombinant Efb can bind to both C3 and fibrinogen simultaneously, forming a trimolecular complex (Lee *et al.*, 2004b). Efb-C has a domain organization similar to Spa. It is composed of three α-helices arranged in a three helical bundle. A single Efb-C molecule binds the residues comprising the acidic depression on the concave face of the dome-shaped C3d molecule via its α2-helix (Hammel *et al.*, 2007b). Substitution of amino acids Arg^{131} and Asn^{138} for alanine in the α2 helix of Efb-C abolishes C3d binding (Hammel *et al.*, 2007b).

To define the mechanism of Efb-C mediated inhibition of the alternative pathway, recombinant Efb-C was incubated with human plasma and the reactivity of C3 with a panel of monoclonal antibodies was measured. Following incubation with Efb-C

C3 was recognized by monoclonal antibodies to C3a and C3b indicating that C3 bound to Efb-C was not being activated to C3b (Hammel *et al.*, 2007b). However, the monoclonal antibody C3-9 which recognizes a neoantigen exposed during activation of C3 to C3b showed an increase in reactivity. This indicates that Efb-C binding to C3d induces a conformational change in C3 that prevents its processing to C3b (Hammel *et al.*, 2007b). Recently it has been shown that Efb-C binding to C3b induces conformational changes that spread across the molecule altering functional domains removed from the Efb-C binding site (Chen *et al.*, 2010). Efb-C binding to C3b has been shown to impair the binding of factor B inhibiting the formation of the C3 convertase C3bBb.

Efb has been shown to be an important virulence factor in infection models. Pre-treatment with Efb resulted in a significant prolongation of bleeding time in a mouse model (Shannon *et al.*, 2005). Antibodies against Efb blocked the binding of Efb to fibrinogen and prevented Efb-mediated inhibition of platelet aggregation (Shannon *et al.*, 2006). Immunization of mice with Efb resulted in the generation of high titer specific antibodies and vaccinated animals developed significantly less severe wound infection than the unvaccinated controls (Shannon *et al.*, 2006).

1.6.4 Ehp

The Efb homologous protein (Ehp) is a small 9.5kDa protein secreted by *S. aureus* primarily during the exponential growth phase of the bacteria (Hammel *et al.*, 2007a, Jongerius *et al.*, 2007). Ehp is also refered to as the extracellular complement binding protein (Ecp). Ehp shares 44% sequence identity with the C-terminal C3 binding domain of Efb. Ehp also binds the C3d thioester-containing domain of C3 (Hammel *et al.*, 2007a). Sequence alignments revealed that Arg^{75} and Asn^{82} in Ehp correspond to Arg^{131} and Asn^{138} in Efb-C. Substitution of Arg^{75} and Asn^{82} in Ehp resulted in reduced not complete inhibition of C3d binding as seen with Efb-C. This lead to the identification of a second C3d binding motif in Ehp at Asn^{63}. The combined substitution of Asn^{63}, Arg^{75} and Asn^{82} to glutamate abolished C3d binding by Ehp (Hammel *et al.*, 2007a).

Ehp was shown to inhibit the alternative pathway at a 2-3 fold greater level then Efb (Hammel *et al.*, 2007a, Jongerius *et al.*, 2007). Ehp-mediated complement

inhibition occurs via the same mechanism as Efb-mediated inhibition. Ehp binding to C3d results in a conformational change in the C3 molecule that renders it unable to be activated by proteolytic cleavage of C3a (Hammel *et al.*, 2007a). Incubation of Ehp in human serum prevented the formation of C5 convertases where as the Ehp $Asn^{63}/Arg^{75}/Asn^{8}$ substitution mutant had no inhibitory effect on C5 convertase formation. Ehp also blocked C5a-dependent neutrophil recruitment into the peritoneal cavity in a mouse model of immune complex peritonitis (Jongerius *et al.*, 2007).

1.7 Rational for this study

1.7.1 Sbi

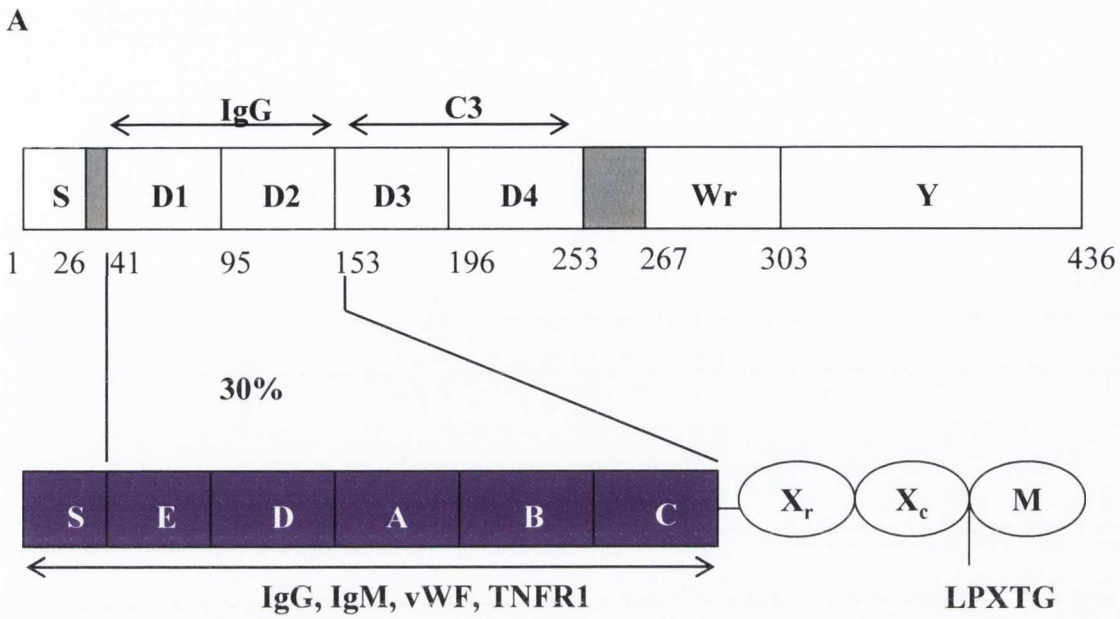
Phage display studies with *S. aureus* strain 8325-4 genomic DNA revealed a novel IgG binding peptide that was later characterised as part of the second binding protein of immunoglobulin (Sbi) (Zhang *et al.*, 1998). Sbi comprises 436 residues, including a 29 residue N-terminal signal sequence, four putative extracellular ligand binding domains and a proline rich repeat region beginning at position 267. Such sequences are usually found within cell wall-spanning domains. However, unlike the surface-anchored MSCRAMM proteins, the putative wall-spanning region is not followed by an LPXTG motif and hydrophobic domain in Sbi (Zhang *et al.*, 1998). Instead the proline rich region of Sbi is followed by a 130 residue tyrosine rich region (Y). A schematic diagram of the Sbi protein is shown in Figure 1.11, panel A. Characterisation of Sbi has been hindered by both its similar reactivity with mammalian IgGs and similar apparent molecular mass (~48kDa) to Spa (~50kDa).

Residues 41-152 of Sbi form two independent IgG binding domains, D1 and D2 (Zhang *et al.*, 1998). D1 and D2 are predicted to be composed of anti-parallel α-helices that pack together to form a three helical bundle similar to the homologous repeat domains of Spa (Atkins *et al.*, 2008). Sequence alignments of the IgG binding domains of Spa (EDABC) and Sbi (D1D2) revealed that they share 30% sequence homology (Figure 1.11, panel B). Almost all Spa residues involved in Fcγ-binding on helices I and II are identical in Sbi D1 and D2. There is no homology in the residues on helices II and III of Spa involved in V_H3 Fab binding with Sbi domains D1 and D2. Surface plasmon resonance studies (SPR) demonstrated that Sbi D1 and D2 bound the Fcγ region of IgG subclasses 1, 2 and 4 and as with Spa neither D1 nor D2 of Sbi bound IgG subclass 3

(Atkins *et al.*, 2008). Sbi domains D1 and D2 did not bind to V_H3 Fab in SPR experiments, thus ruling out Sbi as a B-cell superantigen. Use of Spa in immunoadsorption therapy is linked with severe side-effects believed to be caused by FcγR recognition and subsequent complement fixation. Sbi may offer a viable alternative to Spa as the precipitation of insoluble immune complexes by Sbi occurs solely via Fcγ binding. Thus free Fc regions are unlikely to be available for FcγR recognition and complement fixation (Atkins *et al.*, 2008).

Domains D3 and D4 of Sbi also interact with complement component C3 (Burman *et al.*, 2008). SPR analysis revealed that SbiD3D4 and SbiD4 but not SbiD3 bound the central complement component C3 through its thioester-containing C3d and anaphylatoxin C3a subunits (Burman et al., 2008). SbiD3D4 inhibited all three pathways of the complement system. However, in the absence of D3, Sbi D4 showed a 300-fold weaker inhibition of the classical and mannose-binding pathways but specific inhibition of the alternative pathway (Burman *et al.*, 2008). C3b is the first major cleavage product of native C3. In serum it is rapidly cleaved by factor I in the presence of factor H. The characteristic chains of iC3b are β, α'67 and α40. SDS-PAGE autoradiographic analysis revealed that the near total inhibition of all three complement pathways by SbiD3D4 is due to consumption of C3, as a prominent α'67 band was detected but no native C3 (Burman *et al.*, 2008). When SbiD3D4, but not SbiD4, is incubated with serum a large proportion of activated C3 becomes covalently bound to SbiD3D4 via its C3d thioester, forming a SbiD3D4:C3b adduct seen as a prominent band migrating below the α-band of native C3 in autoradiographic studies (Burman *et al.*, 2008). It has been proposed that SbiD3D4:C3b adduct formation provides a platform for the assembly of the alternative pathway C3 convertase, C3bBb, that is transiently resistant to inactivation by factor I and factor H leading to the consumptive cleavage of C3 (Isenman *et al.*, 2010).

Crystallisation of SbiD4 revealed that it shares structural similarities with Efb-C, Ehp and SCIN. All four molecules have a common three-helical bundle domain organisation (Figure 1.12). SbiD4 like Efb-C and Ehp binds the C3d thioester containing domain of C3 (Hammel *et al.*, 2007b, Hammel *et al.*, 2007a). The crystal structures for Efb-C, Ehp and SbiD4 in complex with C3d have been solved (Hammel *et al.*, 2007b, Hammel *et al.*, 2007a, Clark *et al.*, 2011). As with Efb-C and Ehp, SbiD4



B

```
          ←HELIX 1→     ← HELIX 2 →    ← HELIX 3 →
SpA-D:    DQQSAFYEILNMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPK
SpA-E:    A..N...QV.......AD.................A......Q...D.....
SpA-A:    E..N.............E.................A.L.S.............
SpA-C:    E..N......HL...T.E................V.KEI.A......DA....
SpA-B:    E..N......HL.....E................A.L.A......DA....
          50        60        70        80        90
Sbi-I:    D..K...QV.HLKGIT.E...QY.KT.REH.ERAQEVFS.S--.KD.KN.D
Sbi-II:   A..N...NV.KND..T.QEK.NY.AQI.EN.DR.QQVWV.S--VQS.K.KE
```

Figure 1. 11 Schematic diagrams of Spa and Sbi.

(**A**) The upper figure is Sbi and the lower is Spa. S, signal sequence; D1-D4, Sbi ligand binding domains that have sequence similarity to IgG binding domains of Spa (E,D, A, B, C); Wr and Xr, proline-rich C-terminal domains; Y, C-terminal domain likely to be involved anchoring Sbi to the cell envelope; LPXTG wall anchoring motif; M, transmembrane domain. (**B**) Sequence alignment of the five Ig-binding domains of Spa, with positions of the α-helices and residues involved in Fc (blue) or Fab (green) binding indicated.

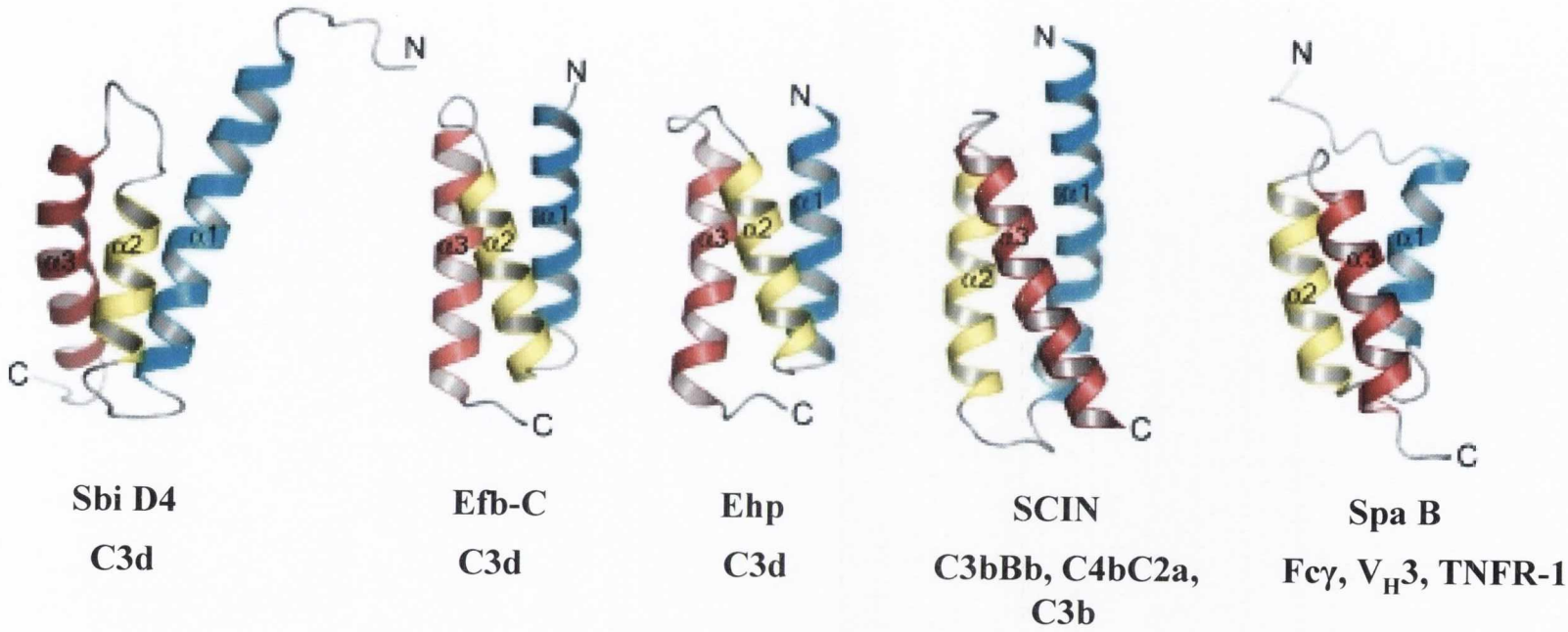


Figure 1.12. Structural comparison of *S. aureus* innate immune evasion proteins.

Ribbon diagrams depicting SbiD4, Efb-C, Ehp, SCIN and Spa domain B. Helices are labelled α1 (blue), α2 (yellow) and α3 (red). The ligands for each domain are indicated below each structure. This figure was adapted from Upadhyay *et al.*, 2008.

binds via its α2 helix to residues comprising the acidic depression on the concave face of the dome-shaped C3d molecule (Figure 1.13; Clark *et al.*, 2011). The SbiD4:C3d crystal structure validated previous mutational analysis that identified residues Arg^{231} and Asn^{238} of SbiD4 as being essential for binding to C3d (Upadhyay *et al.*, 2008). These residues were initial identified through sequence alignments with Efb-C and Ehp. As discussed in section 1.6.3 the combined alteration of Arg^{131} and Asn^{138} in Efb-C also resulted in the total loss of C3d binding activity by Efb-C (Hammel *et al.*, 2007b).

The crystal structure of SbiD4 in complex with C3d revealed a second low affinity binding interaction between helices α1 and α3 of SbiD4 and C3d (Figure 1.13; Clark *et al.*, 2011). It has been proposed that this interaction may be involved in the formation of the covalent adduct between Sbi and C3. Despite the fact SbiD3 is needed for activation of C3, it has been suggested that SbiD4 forms an important part of the C3b transacylation target (Clark *et al.*, 2011).

The C3d fragment on its own or as part of iC3b is the ligand for complement receptor 2 (CR2) present on B cells and follicular dendritic cells (Kalli *et al.*, 1991). SbiD3D4 competitively inhibited the binding of CR2 to C3d (Burman *et al.*, 2008). Substitution of residues located on the concave face of C3d affected its binding to both SbiD4 and CR2 revealing that the two molecules share overlapping contact residues on C3d (Isenman *et al.*, 2010). By inhibiting CR2 binding to C3d, SbiD4 interrupts the link between the innate and adaptive immune systems, as the interaction of CR2 with C3d is required for optimal antibody response and the induction of immunological memory (Nielsen *et al.*, 2002, Rickert, 2005).

The precise cellular location of Sbi has been the subject of debate with conflicting reports in the literature. If Sbi is indeed associated with the *S. aureus* cell the ability of SbiD4 to activate the alternative complement pathway on the bacterial cell surface would be detrimental (Zhang *et al.*, 2000). Sbi has been shown to be a secreted protein that is also abundant in the protoplast fraction (Burman *et al.*, 2008). Thus it was proposed that Sbi targets the alternative pathway by the distinct mechanism of fluid phase activation (Burman *et al.*, 2008) Changing the phospholipid composition of *S. aureus* by deletion of the *mprF* gene caused distinct changes in the membrane proteome including a reduction in the amounts of Sbi detected in the membrane fraction (Sievers *et al.*, 2010). The precise cellular location and surface exposure of the ligand-binding

domains of Sbi along with its mechanism of attachment to the cytoplasmic membrane are discussed in detail in Chapter 4.

Expression of Sbi appeared to be induced by growth of bacteria in human IgG (Zhang *et al.*, 2000). Microarray analysis of the CA-MRSA strain USA300 grown in whole human blood and serum revealed that *sbi* is up-regulated under these conditions (Malachowa *et al.*, 2011). A conserved palindromic consensus sequence specific to promoters of genes regulated by the two component system SaeR/S was identified upstream of *sbi*. Deletion of SaeR/S led to down-regulation of *sbi* and the other immunomodulatory genes *efb* and *chs* (Nygaard *et al.*, 2010). SprD is a small RNA that plays a major role in *S. aureus* virulence, contributing significantly to the disease process in murine models of infection (Chabelskaya *et al.*, 2010). SprD negatively regulates the expression of Sbi. The mechanism of repression is antisense pairings between SprD and 5'-end of *sbi* mRNA encompassing the Shine Delgarno sequence and the AUG start codon thus blocking translation initiation (Chabelskaya *et al.*, 2010).

1.7.2 Aims and objectives

The initial aim of this study was to isolate an Sbi-defective mutant of *S. aureus* and to combine this mutation with a mutant defective in Spa. This facilitated the precise location of Sbi within the bacterial cell to be measured by carefully controlled fractionation experiments. Expression of Sbi N-terminal truncates from the expression vector pRMC2 facilitated the surface exposure and ligand binding abilities of Sbi D1, D2, D3 and D4 on the bacterial cell surface to be investigated. The mechanism of attachment of Sbi to the cell envelope was investigated to determine if the C-terminal "Y" domain of Sbi is responsible.

In order for the D1D2 domains of Sbi to function like Spa and interfere with IgG deposition, phagocytosis and activation of the classical pathway of complement fixation they must be exposed on the cell surface. Conversely for futile consumption of C3 to be effective a significant proportion of Sbi must be secreted into the medium. Activation of C3b by Sbi anchored to the cell surface would be deleterious so presumably this is prevented. Therefore this study aimed to investigate the relative contribution of each Sbi ligand binding domain to survival of the bacteria whilst also establishing whether the activity occurs at the cell surface or extracellularly.

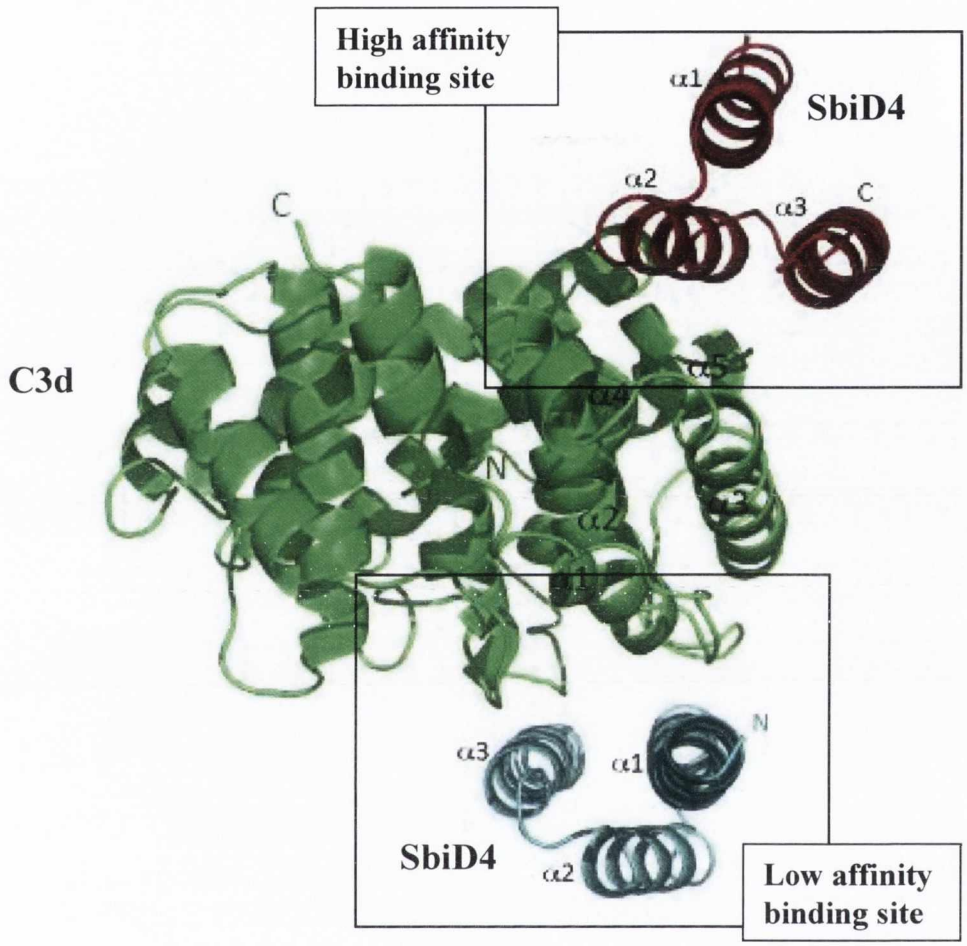


Figure 1.13 Structure of SbiD4 in complex with C3d

Composite figure of a SbiD4 interacting with C3d. Both the high (red) and low (blue) affinity interactions of SbiD4 with C3d (green) are shown. Helices α1, α2 and α3 of SbiD4 are labelled. SbiD4 α1 and α3 helices interact with C3d in the low affinity interaction. It is this interaction that is believed to support Sbi:C3 adduct formation Crystal structures adapted from Clark *et al.*, 2011.

Chapter 2
Methods and Materials

2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2.1. *S. aureus* was grown on trypticase soy agar (TSA, Oxoid) or broth (TSB) at 37° C with shaking (200 r.p.m.) for liquid cultures. *E. coli* strains were grown on Luria (L) agar or in L-broth (Difco) at 37° C with shaking (200 r.p.m.). Strains in L broth or TSB supplemented with 20% (v/v) glycerol were snap frozen and maintained at -70° C. The following antibiotics (Sigma) were added to the media as required: ampicillin (Ap) at 100 μg/ml chloramphenicol (Cm) at 10 μg/ml, erythromycin (Em) at 10 μg/ml, kanamycin (Ka) 50 μg/ml or 90 μg/ml and tetracycline (Tc) at 2 μg/ml.

2.1.1 Growth of bacteria in limiting conditions

To reflect growth conditions *in vivo*, bacterial strains were grown in RPMI 1640 (Sigma). RPMI medium is an iron deficient medium which was originally designed for the cell culture of human leukocytes (Moore *et al.*, 1967). Starter cultures were diluted in 20 ml of RPMI to an OD_{600} 0.05 and grown at 37°C with shaking (200 r.p.m).

2.1.2 Growth of bacteria in human plasma

Bacterial strains were also grown in pooled human plasma, an iron restricted environment. For expression studies RPMI starter cultures were diluted in 20 ml human plasma to an OD_{600} 0.05 and grown at 37°C with shaking (200 r.p.m). For input CFU controls in whole blood survival assays *S. aureus* Newman and its mutants were grown in RPMI to stationary phase, diluted in PBS and 100μl ($5x10^2$ CFU) was added to 500 μl of human plasma. Cultures were incubated at 37°C with gentle rocking and after 3 h serial dilutions were plated to determine the number of surviving CFU(s).

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

Plasmid pRMC2 is a anhydrotetracycline-inducible expression vector for *S. aureus*. *S. aureus* strains carrying pRMC2 derivatives were grown and induced with anhydrotetracycline as follows. Starter cultures of *S. aureus* Newman carrying pRMC2 derivatives were diluted to an OD_{600} 0.05 in 20 ml of RPMI, containing 1.28 μg/ml anhydrotetracycline, and grown at 37°C with shaking (200 r.p.m) for 16 h.

2.2 Plasmids

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

2.3 DNA manipulations

Standard methods were used in DNA manipulations (Sambrook & Russell 2001). Restriction endonucleases were purchased from Roche and Fermentas and were used according to the manufacturers' instructions. DNA ligase and shrimp alkaline phosphatase were purchased from Roche. Confirmatory DNA sequencing was carried out by GATC Biotech.

2.3.1 Preparation of plasmid and genomic DNA

Plasmid DNA was extracted from bacteria using WizardPlus™ Miniprep kit (Promega), according to the manufacture's instructions. Genomic DNA was prepared using Bacterial Genomic DNA purification kit (Edge BioSystems) according to the supplier's protocol. Plasmid and genomic extracts from *S. aureus* required the addition of 200 µg/ml of lysostaphin (AMBI, New York) for 20 min at 37°C to digest cell wall peptidoglycan.

2.3.2 Polymerase chain reaction

Polymerase chain reaction (PCR) amplification was carried out in a DNA thermal cycler (Techne). Plasmid DNA (10 ng) or *S. aureus* genomic DNA (100 ng) was used as a template. Primers were purchased from Sigma-Aldrich and Integrated DNA Technologies and are listed in Table 2.3. PCR reactions were typically carried out in 50 µl volumes using 1 U Phusion™ Hot Start DNA polymerase in Phusion HF buffer (Finnzymes). Primers and dNTPs (Bioline) were used at final concentrations of 0.2 mM and 200 µM, respectively. Initial denaturation was carried out at 98 °C for 30 sec followed by 30 cycles of denaturation for 10 sec at 98 °C, 30 sec annealing (temperature dependent on primer used) and extension at 72 °C, allowing 30 sec/kb for genomic DNA and 15 sec/kb for plasmid DNA. A final extension step was carried out at 72 °C for 5min. PCR products were purified using Wizard SV gel and PCR clean-up system (Promega).

Table 2.1 Bacterial Strains

Strains	Relevant Characteristics	Source/References
E. coli		
XL-1 Blue	Propagation of plasmids	Stratagene
TOPP3	Protease deficient strain used for recombinant protein expression	Stratagene
TB1	Used for expression of recombinant MBP-tagged proteins	Stratagene
S. aureus		
RN4220	Restriction-deficient derivative of 8325-4	(Kreiswirth *et al.*, 1983)
Newman	NCTC 8178.	(Duthie & Lorenz, 1952)
Newman *spa*	Newman *spa::*Ka^R	(Higgins *et al.*, 2006)
Newman *clfA*	Newman *clfA5* frameshift mutation	(Higgins et al., 2006)
Newman *cap*	Newman *cap::*Tc^R	(Wann *et al.*, 1999)
Newman *isdH*	Newman *isdH::*Em^R	(Visai *et al.*, 2009)
Newman *spa clfA*	Newman *spa::*Ka^R *clfA5*	(Higgins et al., 2006)
Newman *sbi*	Newman *sbi::*Em^R	This study
USA300 *sbi*	USA300 *sbi::*Em^R	This study
Newman *spa sbi*	Transduction of *sbi::*Em^R into Newman *spa::*Ka^R	This study
Newman *spa clfA cap*	Transduction of *cap::*Tc^R into Newman *spa::*Ka^R *clfA5*	This study
Newman *spa clfA cap isdH*	Allelic replacement of *isdH* in Newman *spa::*Ka^R *clfA5 cap::*Tc^R using pJH4*isdH2*	This study
Newman *spa clfA cap isdH sbi*	Transduction of *sbi::*Em^R into Newman *spa::*Ka^R *clfA5 cap::*Tc^R *isdH2*	This study
Newman *clfB*	Newman *clfB::lacZ* Em^R	(Fitzgerald *et al.*, 2006)
4S5	RN4220 *spa ltaS*, with a suppressor mutation in *gdpP*	(Corrigan *et al.*, 2011)

Table 2.1 Bacterial strains, continued

Strains	Relevant Characteristics	Source/References
MSSA 476	Community-acquired MSSA strain ST1	(Holden *et al.*, 2004)
MRSA 252	Hospital-acquired MRSA strain ST36	(Holden et al., 2004)
N315	Hospital-acquired MRSA strain ST5	(Kuroda *et al.*, 2001)
USA300	Community-acquired MRSA strain ST8	(Diep *et al.*, 2006)
P1	Rabbit virulent strain. Strong adherence to extracellular matrix proteins ST973	(Sherertz *et al.*, 1993)
COL	MRSA isolate, isolated from the operating theatre of a hospital in Colindale England ST250	(Gill *et al.*, 2005)
Cowan	Clinical isolate, isolated from a patient with septic arthritis	ATCC12598 (Grundmeier *et al.*, 2010)
LS-1	Mouse virulent strain. Caused septic arthritis	(Bremell *et al.*, 1991)
38	Human clinical isolate ST1	(Day *et al.*, 2001)
2	Human clinical isolate ST 7	(Day et al., 2001)
19	Human clinical isolate ST 10	(Day et al., 2001)
42	Human clinical isolate ST 20	(Day et al., 2001)
13	Human clinical isolate ST 22	(Day et al., 2001)
21	Human clinical isolate ST 36	(Day et al., 2001)
52	Human clinical isolate ST 188	(Day et al., 2001)

Table 2.2 Plasmids

Strains	Relevant Characteristics	Marker(s)	Source/References
pBluescript	*E. coli* cloning vector	Ap^R	Stratagene
pTS*erm*C	*S. aureus* plasmid containing temperature sensitive-replicon. Em^R	Em^R	(Josefsson *et al.*, 2008)
pTS*tet*K	*S. aureus* plasmid containing temperature sensitive-replicon. Tc^R	Tc^R	(Smith *et al.*, 2011)
pES2	*S. aureus* shuttle plasmid. Temperature sensitive-replicon. Sequences for creating *sbi*::Em^r mutation	Ap^R in *E.coli* Tc^R in *S.aureus*	This study
pJH4*isdH*	Temperature sensitive shuttle vector with *isdH2* mutations Y126A/H127E/F128A. *Eco*RV site codon 105	Ap^R in *E.coli* Em^R in *S.aureus*	(Visai et al., 2009)
pMAL-c2G	*E. coli* vector for the expression of maltose binding protein-tagged recombinant proteins	Ap^R	New England Biolabs
pMAL-c2G-$sbi_{41\text{-}436}$	pMAL-c2G derivative encoding $Sbi_{41\text{-}436}$	Ap^R	This study
pMAL-c2G-$sbi_{41\text{-}253}$	pMAL-c2G derivative encoding $Sbi_{41\text{-}253}$	Ap^R	This study
pMAL-c2G -$sbi_{254\text{-}436}$	pMAL-c2G derivative encoding $Sbi_{254\text{-}436}$	Ap^R	This study
pGEX-4T2	*E. coli* vector for the expression of glutathione S-transferase-tagged recombinant proteins	Ap^R	(Smith & Johnson, 1988)
pGEX-4T2-$sbi\Delta_{D1D2}$	pGEX-4T2 derivative encoding *sbi* with an in-frame deletion of D1D2 (residues 41-153)	Ap^R	This study
pRMC2	Derivative of the tetracycline-inducible expression vector pALC2073. Anhydrotetracycline-inducible expression vector for *S. aureus*.	Ap^R in *E. coli.* Cm^R in *S. aureus*	(Corrigan & Foster, 2009)

Table 2.2 Plasmids, continued

Strains	Relevant Characteristics	Marker(s)	Source/References
pRMC2-*sbi*	pRMC2 derivative encoding full length *sbi*.	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pRMC2-$sbi_{1\text{-}335}$	pRMC2 derivative encoding $sbi_{1\text{-}335}$.	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pRMC2-$sbi_{1\text{-}368}$	pRMC2 derivative encoding $sbi_{1\text{-}368}$.	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pRMC2-$sbi_{1\text{-}403}$	pRMC2 derivative encoding $sbi_{1\text{-}403}$.	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pRMC2-$sbi\Delta_{335\text{-}368}$	pRMC2 derivative encoding *sbi* containing an in-frame deletion of residues 335-368	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pRMC2-$sbi\Delta_{D1\text{-}D2}$	pRMC2 derivative encoding *sbi* containing an in-frame deletion of D1D2 (residues 41-153)	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pRMC2-*sbi* $\Delta_{D3\text{-}D4}$	pRMC2 derivative encoding *sbi* containing an in-frame deletion of D3D4 (residues 153-254)	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pRMC2-$sbi_{1\text{-}335}\Delta_{D1D2}$	pRMC2 derivative encoding $sbi_{1\text{-}335}$ containing an in-frame deletion of D1D2 (residues 41-153)	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pRMC2-$sbi_{1\text{-}335}\Delta_{D3D4}$	pRMC2 derivative encoding $sbi_{1\text{-}335}$ containing an in-frame deletion of D3D4 (residues 153-254)	Ap^R in *E. coli.* Cm^R in *S. aureus*	This study
pCN34	*E. coli-S. aureus* shuttle plasmid	Ap^R in *E. Coli* Ka^R in *S. aureus*	(Charpentier *et al.*, 2004)
pCN34-*ltaS*	pCN34 derivative encoding *ltaS* expressed from its natural promoter	Ap^R in *E. Coli* Ka^R in *S. aureus*	(Corrigan *et al.*, 2011)

^{R} Resistance

Table 2.3 Primers

Primer	Sequence (5'-3')[a, b]	5' Modifications
Sbi 5' F	CTGAAGCTTCATCGGCTCTTACAGCTG CAG	*Hind*III
Sbi 5' R	CTGAGAATCCTGCCCCAACTAGCAACT TCG	*Bgl*II
Sbi 3' F	CTGAGAATCCTCAAGAAACTGCATCAT CAATC	*Bgl*II
Sbi 3' R	CTGGGATCCGCTAATCGTTTGCTAGTA ATGTC	*Bam*H1
ErmC 5'	CTGAGATCTGTGCTATAATTAT	*Bgl*II
ErmC 3'	CTGAGATCTGGTGTCACAAGA	*Bgl*II
Em^r M' R	GAGCTATTCACTTTAGGTTTAGG	
ClfA 5' F	CAAATACATCAACATGACTTTGC	
ClfA 3' R	CTCTGGAATTGGTTCAATTTC	
CapA 5' F	CAAGCTAATACTCAAATTTTAGTG	
CapC 3' R	GCTGTTAGCAAATCTAAATTTTC	
IsdH 5' F	ACAAACATCACCCAAAATTAAGG	
IsdH 3' R	TTATTTAGATTCTTTTCTGAATTTAG	
Sbi_{1-436} 5' F	CTGGGTACCAAAAGAAAGGGAATACA CATGAAA	*Kpn*1
Sbi_{1-436} 3' R	CTGAGATCTTAATTTCCAGAATGATAA TAAATTAC	*Bgl*II
Sbi_{1-335} 3' R	CTGAGATCTTTACATATTTTTCTTTATA GCTTTTATA	*Bgl*II
Sbi_{1-368} 3' R	CTGAGATCTTTAGGATGTAAGATTTAG AACCAC	*Bgl*II
Sbi_{1-403} 3' R	CTGAGATCTTTAGTATATAATACTTTA CCAGTATTG	*Bgl*II
$SbiΔ_{335-368}$ 5' R	TTTTTCTTTATAGCTTTTATAAGTATC	5' phosphorylated
$SbiΔ_{335-368}$ 3' F	CAACCATTGAAAGTTGATGATAAA	5' phosphorylated

Table 2.3 Primers, continued

Primer	Sequence (5'-3')[a, b]	5' Modifications
$Sbi\Delta_{D1D2}$ 5' R	ATTGAAAATGCGGATAAAGCAATTA	5' phosphorylated
$Sbi\Delta_{D1D2}$ 3' F	TTGGTGCTTAGTTGAAGTTTGTTG	5' phosphorylated
$Sbi\Delta_{D3D4}$ 5' R	GCATTAGTTGCTCAAAAAGATGC	5' phosphorylated
$Sbi\Delta_{D3D4}$ 3' F	ATTTTGACGTTCTTTAGCTTTAG	5' phosphorylated
$Sbi\Delta_{D1D2}$ RP 5' F	CTGGGATCCAAAGCAATTAAAGATTTC CAAG	*Bam*H1
$Sbi\Delta_{D1D2}$ RP 3' R	CTGGAATTCTTTCCAGAATGATAATAA ATTACT	*Eco*R1
Sbi_{41-436} RP 5' F	CTGGGATCCACAACTCAAAACAACTAC GTAAC	*Bam*H1
Sbi_{41-436} RP 3' R	CTGCCCGGGTTATTTCCAGAATGTTAA TAAATTAC	*Pst*I
Sbi_{41-253} RP 3' R	CTGCCCGGGTTAGTCTAATTGTTTCTGT AAATGCT	*Pst*I
$Sbi_{254-436}$ RP 5' F	CTGGGATCCGCATTAGTTGCTCAAAAA GATGCT	*Bam*H1

[a] Restriction sites used for cloning are underlined.
[b] RP, Recombinant protein

Table 2.4 Antibodies

Antibody	Relevant features	Working dilution/ concentration	Source/ Reference
Polyclonal rabbit anti-SbiD3D4WrY	Raised against recombinant $Sbi_{154-436}$	1:5000	P. Speziale
Polyclonal rabbit anti-SbiE	Raised against recombinant $Sbi_{154-436}$, HRP-conjugated	1:100	M. Halsborough
Polyclonal rabbit anti-ClfA	Raised against recombinant $ClfA_{221-559}$	1:20,000	P. Speziale
Polyclonal rabbit anti-IsdH	Raised against recombinant $IsdH_{41-321}$	1:5000	P. Speziale
Polyclonal rabbit anti-SdrE	Raised against recombinant $SdrE_{41-544}$	1:2000	(Josefsson *et al.*, 1998)
Polyclonal rabbit anti-EbpS	Raised against recombinant $EbpS_{1-267}$	1:500	(Downer *et al.*, 2002)
Monoclonal mouse anti-LTA	Raised against the polyglycerolphosphate of LTA, Clone 55	1:4000	Hycult Biotechnologies
Polyclonal rabbit anti-human C3	Raised against native C3 purified from pooled human serum	1:2000	Abcam
Polyclonal goat anti-human C3	Raised against native C3 purified from pooled human serum	1:2000	Abcam
Chicken anti-GST	Chicken IgY, HRP-conjugated	1:500	Gallus Immunotech
Monoclonal mouse anti-MBP	Raised against the maltose binding protein of *E. Coli*, HRP-conjugated	1:2000	New England BioLabs
Monoclonal mouse anti-MBP	Raised against the maltose binding protein of *E. Coli*	1.5 μg/ml	Genscript
Rabbit IgG	HRP-conjugated	1:5000	Dako
Rabbit anti-human IgG	Secondary antibody, HRP-conjugated	1:2000	Dako

Goat anti-rabbit IgG	Secondary antibody with low Spa/Sbi-reactivity, HRP-conjugated	1:2000	Dako
Rabbit anti-mouse IgG	Secondary antibody, FITC-conjugated	1:20	Dako

2.4 Transformation

Competent E. coli cells were prepared by the $CaCl_2$ method (Sambrook and Russell, 2001). Competent cells were incubated on ice for 30 min with 2 ng of plasmid or 20 ng of DNA ligation reactions. The mixture was then incubated at 42°C for 1 min followed by a further 2 min on ice. Next, 500 µl L-broth was added and the mixture was incubated for 1 h at 37°C. The mixture was plated on L-agar plates containing ampicillin 100 µg/ml and incubated for 16-24 h. *E. coli* transformants were screened for the presence of recombinant plasmids using the rapid colony screening procedure developed by Le Gouill & Dery (1991).

2.4.1 Preparation and electroporation of *S. aureus*

Plasmids were introduced into *S. aureus* by electroporation into strain RN4220, Newman *spa* and Newman *spa sbi* (Augustin & Gotz, 1990). Electrocompetent cells were prepared by growing each *S. aureus* strain to mid-exponential phase in TSB. Cells were washed in 0.5 M sucrose and harvested by centrifugation at 4 °C. Electrocompetent cells (200 µl) were mixed with (0.1 µg) plasmid DNA in a 0.2 mm electrode gap cuvette. Electroporation was carried out at 200 Ω resistance, 25 µF capacitance and 25 V. Cells were incubated for 1 hour at 37 °C with shaking (200 r.p.m) in TSB containing 500 mM glucose prior to plating on antibiotic containing media. Transformants were screened by PCR, restriction mapping and Western immunoblotting.

2.5 Phage transduction

Phage 85 was used to transfer chromosomal mutations and plasmids marked with antibiotic resistance cassettes between *S. aureus* strains. Plasmids were introduced into strain RN4220 by electroporation prior to preparation of phage stocks. Bacterial cultures of donor strains were diluted in phage broth (20 g/l nutrient broth No. 2, Oxoid supplemented with 10 mM $CaCl_2$) and grown at 37°C for 4h with shaking. The donor strains were incubated with a phage 85 stock for 30 min at room temperature. Molten top agar (20 g/l Nutrient broth No. 2, 3.5 g/l Agar No. 1, Oxoid supplemented with 10 mM $CaCl_2$) was added to the bacteria and phage and poured over 2 phage base plates (20 g/l Nutrient broth No. 2, 7 g/l Agar No. 1, Oxoid supplemented with 10 mM $CaCl_2$). Following overnight incubation at 37°C the top agar was removed to an Oakridge tube

and centrifuged for 10 min at 20,000 x g. The supernatant containing the phage and the desired plasmid or mutation was filtered and stored at 4°C. Plasmids and chromosomal mutations were introduced into recipient strains by infection with phage 85 carrying the desired plasmid or mutation. A 10 ml overnight culture of the recipient strain was harvested and incubated for 40 min at 37 °C with phage stock (containing desired antibiotic marker) in L-broth supplemented with $CaCl_2$. Sodium citrate (0.02 M) was added at this point to eliminate contaminating phage particles and cells were harvested by centrifugation. Following resuspension in sodium citrate (0.02 M), bacterial cells were incubated on ice for 2 h. The recipient strains were plated on TSA containing 0.05% (w/v) sodium citrate and the appropriate antibiotic. Transductants were screened, as appropriate, by PCR, plasmid restriction, or Western immunoblotting.

2.6 Strain Construction

2.6.1 Allelic replacement mutagenesis of *sbi*

In order to inactivate the *sbi* gene, DNA fragments comprising 900 bp upstream and 740 bp downstream of *sbi* were amplified by PCR from Newman genomic DNA using primer pairs Sbi 5'F/Sbi 5'R and Sbi 3'F/Sbi 3'R, respectively (see Table 2.3). These fragments were designed to create a 1190 bp deletion in *sbi* when cloned together between the *Hind*III-*Bam*H1 sites of plasmid pBluescript. Both fragments were cloned into pBluescript and joined together using a *Bgl*II restriction site introduced by PCR. A PCR fragment expressing erythromycin resistance was amplified from pTS*ermC* using primers ErmC 5'F/ErmC 3'R and cloned into the *Bgl*II site between the upstream and downstream *sbi* sequences in pBluescript, generating an insertion mutation construct for *sbi*. Plasmid DNA was analysed by digestion with restriction enzymes and the *sbi* mutation was verified by DNA sequencing. The plasmid was designated pBlueΔ*sbi*. The construct was then ligated to pTS*tetK*, a plasmid that is temperature-sensitive for replication in *S. aureus*. pBlueΔ*sbi* and pTS*tetK* plasmid DNA was prepared using the WizardPlus™ Minipreps kit (Promega). Each plasmid was cut with *Hind*III. To minimise background due to religation of the vector, *Hind*III-cut pBlueΔ*sbi* was treated with shrimp alkaline phosphatase (Roche) according to manufacturer's instructions. After ligation for 2 hours, reactions were pooled, transformed into *E. coli* XL1-Blue and plated on L agar plates containing 2 μg/ml tetracycline. Due to the fact ligation of pBlueΔ*sbi* and pTS*tetK* was at a single common restriction site, two orientations of the

chimeric plasmid were possible. Because this was not important for the purpose of allelic replacement, chimeric plasmids were chosen arbitrarily. Plasmid constructs were verified by restriction analysis and the resulting chimeric plasmid was designated pES2.

pES2 was prepared from *E. coli* XL1-Blue using the WizardPlus™ Minipreps kit and 1 μg was electroporated into *S. aureus* RN4220 and then transduced into *S. aureus* strain Newman at the permissive temperature of 28 °C . *S. aureus* strain Newman containing pES2 was grown at 28 °C in TSB broth containing tetracycline. Serial dilutions were plated in duplicate on TSA agar containing erythromycin. One set of plates was incubated at 44 °C and the other at 28 °C. Plates growing at 44 °C had 10^3–fold less colonies growing on them than those incubated at the lower temperature. Putative integrants were single colony purified prior to inoculation into drug free TSB broth. Cultures were incubated at 28 °C overnight to encourage plasmid replication and excision. Cultures were then diluted 1/100 in fresh broth and grown for 8 hours at 44 °C to promote loss of the plasmid. Three rounds of temperature shifting were carried out. Cultures were finally diluted and plated on TSA agar and single colonies screened for sensitivity to tetracycline and resistance to erythromycin. The *sbi* mutation was validated by PCR and Western immunoblotting.

2.6.2 Allelic replacement mutagenesis of *isdH*

Newman *spa clfA cap isdH* was constructed by allelic replacement with the tempreture-sensitive plasmid pJH4*isdH2*. The *isdH2* mutation introduces a triple amino acid substitution (Y126A/H127E/F128A), in the NEAT-1 domain of the IsdH protein. pJH4-*isdH2* was prepared from RN4220 and was electroporated into *S. aureus* strain Newman *spa clfA cap* at the permissive temperature of 28 °C. *S. aureus* strain Newman *spa clfA cap* containing pJH4-*isdH2* was grown at 28°C in TSB broth containing erythromycin. Serial dilutions were plated in duplicate on TSA agar containing erythromycin. One set of plates was incubated at 44°C and the other at 28°C. Plates growing at 44°C had 10^3–fold less colonies growing on them than those incubated at the lower temperature. Putative integrants were single colony purified prior to inoculation into drug free TSB broth. Cultures were incubated at 28 °C overnight to encourage plasmid replication and excision. Cultures were then diluted 1/100 in fresh broth and grown for 8 hours at 44 °C to promote loss of the plasmid. Three rounds of temperature shifting were carried out. Cultures were finally diluted and plated on TSA agar and

single colonies screened for sensitivity to erythromycin. The *isdH2* mutation was validated by diagnostic PCR with primers IsdH 5'F/IsdH 3'R followed by restriction digest of the PCR product with *Eco*RV.

2.6.3 Construction of strains by transduction

The *cap*::Tc^R element was transduced from a Newman *cap* strain using selection for tetracycline resistance into *S. aureus* strain Newman *spa clfA* and validated by PCR with primers Cap 5'F/Cap 3'R to ensure that the *capB* gene was inactivated. Newman *spa sbi* and Newman *spa clfA cap isdH sbi* were constructed by transduction of the *sbi*::Em^R mutation from Newman *sbi* and validated by PCR with primers Sbi 5'F/Em^R M'R. Each construct was compared to its parental strain to ensure haemolytic activity had not been altered during strain construction. In addition, resistance to erythromycin, tetracycline and kanamycin was monitored.

2.6.4 Complementation of the *sbi*::Em^R mutation

The entire *sbi* gene including the ribosome binding site, but lacking the promoter was amplified from genomic DNA of strain Newman using primers Sbi_{1-436} 5' F/ Sbi_{1-436} 3' R. A *Kpn*1 site was incorporated into the 5' end of the forward primer, while a *Bgl*II site was incorporated into the 3' end of the reverse primer. Following cutting with the two restriction enzymes the *sbi* fragment was cloned between the *Kpn*1 and *Bgl*II sites in pRMC2 to create pRMC2-*sbi*. pRMC2-*sbi* was electroporated into *S. aureus* Newman *sbi* and Newman *spa sbi* and validated by measuring the expression of Sbi in the cytoplasmic membrane fraction.

2.6.5 Construction of *sbi* C-terminal truncates

C-terminal truncates of *sbi* were amplified by PCR from genomic DNA of strain Newman using the primer pairs Sbi_{1-335} 5' F/ Sbi_{1-335} 3' R, Sbi_{1-368} 5' F/ Sbi_{1-368} 3' R and Sbi_{1-403} 5' F/ Sbi_{1-403} 3' R. A *Kpn*1 site was incorporated into the 5' end of each forward primer, while a *Bgl*II site was incorporated into the 3' end of each reverse primer. Following restriction digest the amplimers were cloned between the *Kpn*1 and *Bgl*II sites in pRMC2 to create pRMC2-sbi_{1-335}, pRMC2-sbi_{1-368} and pRMC2-sbi_{1-403}. Plasmid constructs were verified by restriction analysis and DNA sequencing. All three constructs were electroporated into *S. aureus* Newman *spa sbi* and expression of Sbi in

the cytoplasmic membrane and supernantant fractions was measured by Western immunoblotting.

2.6.6 Construction of *sbi* N-terminal deletions

N-terminal deletions of *sbi* were created by inverse PCR from plasmids pRMC2-*sbi* and pRMC2-$sbi_{1\text{-}335}$ using the primer pairs $Sbi\Delta_{D1D2}$ 5'R/ $Sbi\Delta_{D1D2}$ 3'F and $Sbi\Delta_{D3D4}$ 5'R/ $Sbi\Delta_{D3D4}$ 3'F. All primers were 5' phosphorylated and following amplification PCR products were ligated and transformed into *E. coli* XL1-Blue to generate plasmids pRMC2-$sbi\Delta_{D1D2}$, pRMC2-$sbi\Delta_{D3D4}$, pRMC2-$sbi_{1\text{-}335}\Delta_{D1D2}$ and pRMC2-$sbi_{1\text{-}335}\Delta_{D3D4}$. Plasmid constructs were verified by restriction analysis and DNA sequencing. Constructs were electroporated into *sbi* mutants of *S. aureus* Newman. Expression of truncates in the cytoplasmic membrane and supernantant fractions were measured by Western immunoblotting.

2.7 Isolation of *S. aureus* cell wall and cell envelope components

2.7.1 Preparation of staphylococcal whole cell lysates

S. aureus cultures were grown in TSB. Cells were harvested by centrifugation at 2,000 x g, washed in PBS and adjusted to an OD_{600nm} of 10 in PBS containing complete EDTA free protease inhibitors (Roche) and DNase (80 µg/ml). Cell walls were digested by incubation at 37 °C for 30 min with lysostaphin (200 µg/ml). Cells were then mixed with an equal volume of final sample buffer (10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 3 % (w/v) SDS, 0.01 % bromophenol blue in 62.5 mM Tris-HCl, pH 6.8), boiled for 20 min and analysed by SDS-PAGE and Western immunoblotting.

2.7.2 Preparation of solubilised proteins associated with the staphylococcal cell wall

S. aureus cultures were harvested by centrifugation at 2,000 x g for 10 min and cells were washed twice in PBS. An OD_{600nm} of 10 was resuspended in 250 µl of digestion buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 30 % (w/v) raffinose, pH7.5). Complete EDTA-free protease inhibitor cocktail (70 µl of a 10x stock) and lysostaphin (200 µg/ml) were added to the cells and incubated at 37 °C for 10 min. Protoplasts were harvested by centrifugation at 3,500 x g for 10 min and the supernatant containing the

cell wall associated proteins was removed and analysed by SDS-PAGE or stored at -20°C.

2.7.3 Preparation of staphylococcal protoplasts and cytoplasmic membrane fractions

Protoplast pellets were isolated as described is section 2.7.2 and further fractionated into membrane and cytoplasmic fractions. Protoplast pellets were washed once in digestion buffer and resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5) containing complete EDTA-free protease inhibitor cocktail (70 µl of a 10x stock) and DNase (80 µg/ml). Protoplasts were lysed on ice by vortexing. The membrane fraction was obtained by centrifugation in a SM-24 rotor (Sorvall) at 40,000 x g for 1 h at 4° C. The supernatant was retained as the cytoplasmic fraction. The pellet was washed once with ice-cold lysis buffer and the pellet containing the cytoplasmic membrane fraction was finally resuspended in 250µl of ice-cold lysis buffer.

2.7.3.1 Preparation of *L. monocytogenes* and *E. faecalis* protoplasts and cytoplasmic membrane fractions

Bacterial cultures were harvested by centrifugation at 2,000 x g for 10 min and cells were washed twice in PBS. An OD_{600nm} of 10 was resuspended in 250 µl of digestion buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 500 mM sucrose, pH7.5). Complete EDTA-free protease inhibitor cocktail (70 µl of a 10x stock), mutanolysin (1000 U/ml) and lysozyme (1 mg/ml) were added to the cells and incubated at 37 °C for 20 min. Protoplasts were harvested by centrifugation at 3,500 x g for 15 min. The protoplast's cytoplasmic membrane fraction was then isolated as described in section 2.7.3.

2.7.3.1.2 Preparation of *E. coli* membrane fractions

Cultures were harvested by centrifugation at 2,000 x g for 10 min and cells were washed twice in PBS. An OD_{600nm} of 10 was resuspended in 10 ml phosphate-buffered saline (PBS) containing protease inhibitors (Roche), lysozyme (200 µg/ml) and DNase (3 µg/ml) and allowed to stand on ice for 1 h. Cells were lysed by repeated passage through a French Pressure Cell. The lysate was centrifuged at 20,000 x g for 15 min at 4 °C in a Sorvall SS-34 rotor and the pellet was retained as the cytoplasmic membrane fraction. The pellet was washed once with ice-cold lysis buffer and the pellet containing

the cytoplasmic membrane fraction was finally resuspended in 1 ml of ice-cold lysis buffer.

2.7.4 Preparation of extracellular proteins

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

2.7.5 Cell wall preparation

Cells from a stationary phase culture of strain Newman *spa sbi* were adjusted to an OD_{600nm}100 in 1.5 ml lysis buffer containing complete EDTA free protease inhibitors, DNase and RNase (80μg/ml). The cell suspension was transferred to a blue cap FastRNA tube and shaken in a Fastprep™ cell disrupter at speed 6 for 40 sec. This was repeated 12 times with cooling on ice for 1 min between cycles. Cell lysis was monitored by phase contrast microscopy. Lysates were then centrifuged for 2 min at 3000 x g to pellet any remaining whole cells. The supernatant containing the cell wall fragments was boiled in 4% SDS for 2 hrs to remove cytoplasmic and membrane material and then washed in deionized water to remove SDS. The resulting lysates were centrifuged for 15 mins at 17,000 x g to sediment the cell wall fragments.

2.8 Electrophoresis

2.8.1 Agarose gel electrophoresis

Gels containing 0.5-2 % agarose which was dissolved by boiling in TAE buffer (Invitrogen), and cooled to 65 °C were cast in mini trays (Life Technologies). DNA samples in loading buffer containing an elecrophoretic dye were pipetted into wells along with DNA size markers (Bioline). Electrophoresis of samples was routinely performed at 90 V. Gels were bathed in ethidium bromide for 10 min, washed and viewed under UV light. Gel images were analysed using Alpha Imager™ software.

2.8.2 SDS-PAGE

Samples for SDS-PAGE were diluted 2-fold in final sample buffer and boiled for 5 min. 10-20 µl volumes were separated by SDS-PAGE (Laemmli, 1970) using 3 % stacking and either 10 % or 15 % separating acrylamide gels. Electrophoresis was carried out at 120 V after which proteins were either visualised by Coomassie blue protein staining or electroblotted onto methanol-activated PVDF membranes (Roche) at 100 V for 1h using a wet transfer cell (BioRad) for immunodetection or ligand blotting.

2.9 Immunoblotting

2.9.1 Western immunoblotting

Non-specific binding to PVDF membranes was blocked by incubation for 2-16 h in 10% (w/v) skimmed milk powder (Marvel) in TS buffer (10mM Tris-HCl, pH 7.4, 150mM NaCl). Primary antibodies diluted in 10 % (w/v) Marvel/TS buffer were incubated with the membranes for 1 h at room temperature with shaking. Antibodies and their working dilutions are listed in Table 2.4. Unbound antibody was removed by three 10 min washes with TS buffer containing 0.05% Tween. Secondary antibodies (HRP-conjugated) diluted in 10 % (w/v) Marvel/TS buffer were incubated with the membranes for 1 h at room temperature with shaking. Unbound secondary antibody was removed by washing three times with TS buffer containing 0.05% Tween. The chemiluminesent substrate LumiGlo (New England Biolabs) was used according to the manufacturer's instructions. Blots were exposed to X-Omat autoradiographic film (Kodak) and visualised using manual development with developer and fixer solutions (Kodak). The intensity of protein bands resulting from equal loadings of bacterial cellular extracts or purified proteins was assessed visually or by ImageJ software (NIH).

2.9.2 Far Western blotting

PVDF membranes containing electroblotted cytoplasmic membrane samples of *S. aureus* Newman *spa sbi* (pRMC2-*sbi*Δ_{D1D2}) were blocked and washed as in Western immunoblotting (section 2.9.1). Membranes were then incubated with purified LTA (Sigma) 2.5µg/ml in TS/Marvel for 1 h at room temperature. Between each subsequent step membranes were washed 3 times for 10 min in TS/Tween 0.05%. Bound LTA was detected with anti-LTA (polyglycerolphosphate) monoclonal antibodies followed by

HRP-conjugated rabbit anti-mouse IgG. Membranes were washed and developed as in Western immunoblotting (section 2.9.1).

2.9.3 Whole cell dot immunoblotting

Cells were washed twice in PBS and adjusted to an OD_{600} nm of 50 in PBS. Doubling dilutions of cells (5 µl) were dotted onto a nitrocellulose membrane (Protran). The membrane was blocked for 1 h with 10% (w/v) skimmed milk powder (Marvel) in TS buffer. Primary antibodies were diluted in 10 % (w/v) Marvel/TS buffer and incubated with the membrane for 1 h at room temperature with shaking. Three 10 min washes with TS buffer were performed to remove unbound antibody. Secondary (HRP-conjugated) antibody was diluted in 10 % (w/v) Marvel/TS buffer and incubated with the membrane for 1 h at room temperature with shaking. Unbound secondary antibody was removed by washing three times with TS buffer. Antibodies and their working dilutions are listed in Table 2.4. The membrane was developed in the dark using the chemiluminescent substrate LumiGlo as described in section 2.9.1.

2.10 Recombinant protein purification

2.10.1 Purification of GST-tagged recombinant proteins

Recombinant domains of Sbi were expressed from pGEX-4T2 with an N-terminal glutathione-*S*-transferase (GST) fusion of 26 kDa protein. The pGEX-4T2 constructs were transformed into the protease-defective *E. coli* TOPP3 strain for large-scale purification. Cultures were grown to $OD_{600\ nm}$ of 0.5-0.6 and then induced with 1 mM IPTG for 3 h at 37°C. Cells were harvested by centrifugation at 7,000 x g for 10 min at 4°C in a Sorvall GS-3 rotor. The pellets were resuspended in 20 ml phosphate-buffered saline (PBS) containing protease inhibitors (Roche), lysozyme (200 µg/ml) and DNase (3 µg/ml) and allowed to stand on ice for 1 h. Cells were lysed by repeated passage through a French Pressure Cell. The lysate was centrifuged at 20,000 x g for 15 min at 4 °C in a Sorvall SS-34 rotor and the supernatant was filtered through a 0.45 µm filter. The GST-fusion proteins were purified using a GSTrap™ column (Amersham) according to the manufacturer's instructions with the flow rate controlled using a peristaltic pump (Amersham). Bound proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0 in 2ml fractions and samples were analysed by SDS-PAGE for presence of the recombinant protein. Positive fractions were pooled and dialysed against

PBS for 16 h at 4°C. Protein concentrations were determined using the BCA assay kit (Pierce) and by measuring absorbance at 280 nm using a Nanodrop™ 1000 spectrophotometer.

2.10.2 Purification of MBP-tagged recombinant proteins

Recombinant domains of Sbi were expressed from pMAL-c2G with an N-terminal maltose-binding protein (MBP) fusion of 42.5 kDa. The pMAL-c2G constructs were transformed into *E. coli* TB1 strain for large-scale purification. Cultures were grown to $OD_{600\ nm}$ of 0.5-0.6 and then induced with 1 mM IPTG for 3 h at 37°C. Cells were harvested by centrifugation at 7,000 x g for 10 min at 4°C in a Sorvall GS-3 rotor. The pellet was resuspended in 20 ml PBS containing protease inhibitors (Roche), lysozyme (200 µg/ml) and DNase (3 µg/ml) and allowed to stand on ice for 1 h. Cells were lysed by repeated passage through a French Pressure Cell. The lysate was centrifuged at 20,000 x g for 15 min at 4 °C in a Sorvall SS-34 rotor and the supernatant was filtered through a 0.45 µm filter. The MBP-fusion proteins were purified using an amylose column (Bio-Rad) according to the manufacturer's instructions. Bound proteins were eluted using 10 mM maltose in amylose column buffer (20mM Tris-HCL, 200mM NaCl, 1mM EDTA, 1mM DTT, p.H 7.4) in 2ml fractions and samples were analysed by SDS-PAGE for presence of the recombinant proteins. Positive fractions were pooled and dialysed against PBS for 16 h at 4°C. Protein concentrations were determined using the BCA assay kit (Pierce) and by measuring absorbance at 280 nm using a Nanodrop™ 1000 spectrophotometer.

2.11 Enzyme linked immunosorbent assays (ELISA)

2.11.1 Solid phase binding assay

Human complement factor C3 (CompTech) and purified *S. aureus* LTA (Sigma), diluted to various concentrations in PBS, were coated onto microtitre plates (Nunc) in sodium carbonate buffer (pH 9.6) for 16 h at 4 °C. Wells were washed three times with PBS and blocked at 37 °C for 2 h with 200 µl 5% (w/v) bovine serum albumin (BSA) in PBS. Wells were again washed and varying concentrations of recombinant domains of Sbi in 5% (w/v) BSA were added. Plates were incubated for 1 h at 37 °C. Unbound protein was removed by washing. Wells were incubated with the appropriate antibody (100 µl at recommended dilution) in 5% BSA and incubated for 1

h at 37 °C. After washing, 100 µl of a chromogenic substrate solution (1 mg/ml tetramethylbenzidine and 0.006% H_2O_2 in 0.05 M phosphate citrate buffer pH 5.0) was added and plates were developed for 5-10 min. The reaction was stopped by the addition of 2 M H_2SO_4 (50 µl/well) and plates were read at 450 nm. Data was graphed and analysed using GraphPad Prism version 4.00 for Windows.

2.11.1.2 Inhibition studies

Microtitre plates (Nunc) were coated with either purified LTA or the cytoplasmic membrane material of *S. aureus* strain Newman *spa sbi* in sodium carbonate buffer (pH 9.6) for 16 h at 4 °C. Wells were washed three times with PBS and blocked at 37 °C for 2 h with 200 µl 5% (w/v) bovine serum albumin (BSA) in PBS. Wells were incubated with mixtures containing increasing concentrations of inhibitor and a single concentration of ligand corresponding to that which gave half-maximal binding to the coated protein or cytoplasmic membrane material. Wells were washed and residual ligand binding was detected as described in section 2.11.1. Percentage inhibition was calculated from the amount of bound protein detected in the absence of inhibitor.

2.11.2 Bacterial cellular fraction ELISA

Microtitre plates (Nunc) were coated with cytoplasmic membrane and cell wall material, derived from OD_{600nm} 10 of *S. aureus* Newman *spa sbi* cells, overnight at 4 °C for 16 h in 50 mM sodium carbonate buffer (pH9.6.) The coating of membrane and cell wall material was demonstrated by ELISA with anti-EbpS and anti-ClfA antibodies, respectively. Non-adherent material was removed by washing plates with PBS. Plates were blocked at 37 °C with 5 % (w/v) BSA in PBS (200 µl). Plates were washed and serial dilutions of recombinant Sbi or antibody diluted in PBS (100 µl) were added followed by incubation at 37 °C for 1 h. Unbound material was removed by washing. Wells were incubated with the appropriate primary and secondary antibodies (100 µl at recommended dilution) in 5% BSA for 1 h at 37 °C, respectively. Plates were washed with PBS and developed as described previously (2.11.1).

2.12 Fractionation of cells following incubation with MBP-Sbi

Newman *spa sbi* cells (OD_{600nm} 5) were washed twice in PBS and incubated in

PBS with 5 μg/ml of MBP-Sbi_{41-436}, MBP-Sbi_{41-253} and MBP-$Sbi_{254-436}$ for 1 hr at 37°C. Bacteria were pelleted by centrifugation for 5 min at 17,000 x g. Cell fractionation was repeated as described previously in section 2.7. Equivalent amounts of material were separated by 10% SDS-PAGE and probed with HRP-conjugated anti-MBP antibodies and detected as described previously in section 2.9.1.

2.13 Displacement of Sbi from the cytoplasmic membrane by soluble LTA

Cytoplasmic membrane fractions of Newman *spa* were prepared as described previously in section 2.7.3. Cytoplasmic membrane material derived from OD_{600nm} 1 of cells was incubated in PBS with concentrations of *S. aureus* LTA ranging from 1 μg/ml-400 μg/ml for 1 hr at 37°C and then pelleted by centrifugation 40,000 x g for 1 hr at 4 °C. The pellets were resuspended in 50 mM Tris-HCl, pH 7.5. The supernatants were filtered through a 0.45μm filter and proteins were concentrated by addition of a 1:20 volume of ice-cold 100 (w/v) trichloroacetic acid as described previously in section 2.7.4. Equivalent amounts of material were separated by 10% SDS-PAGE and probed with HRP-conjugated anti-MBP antibodies and detected as described in section 2.9.1.

2.14 Polymorphonuclear leukocyte isolation

Fresh whole blood was obtained from healthy volunteers, heparinised and mixed with an equal volume of PBS. All reagents used in polymorphonuclear leukocyte (PMNL) isolation were certified endotoxin-free. 20-25 ml portions were centrifuged through step gradients of 12.5 ml Histopaque (ρ=1.077, Sigma) and 10 ml Ficoll-paque (ρ=1.119, Amersham). The PMNLs were aspirated from the buffy coat between the Ficoll and Histopaque layers into a fresh tube. Cells were washed in 50 ml RPMI 1640 medium (containing 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 25 mM glutamine and 0.05% (v/v) human serum albumin) and resuspended in 9 ml water. After a 30 second osmotic shock to lyse contaminating red blood cells, 1 ml of 10x PBS was added and the cells were again washed in RPMI. PMNL were counted in a haemocytometer (bright line, Neubauer) and adjusted to 5 x 10^6 cells/ml in RPMI. This procedure typically yielded >97% PMNL with >95% viability, as determined by trypan blue exclusion.

2.15 Phagocytosis assay

Bacterial cells grown to stationary phase were washed twice in PBS and labelled with 3μg/ml fluorescein isothiocyanate (FITC; Sigma) in PBS for 1 hour at 37 °C with shaking. Cells were washed three times in PBS and counted in a haemocytometer (Neubauer), adjusted to $1x10^9$ cfu/ml in RPMI and frozen at -20 °C. Bacteria were thawed on ice and diluted to $5x10^7$ cfu/ml in RPMI. Pooled human serum (CompTech) was diluted in RPMI to 10%. Bacteria (50 μl) were opsonised in 10 μl diluted serum for 10 min at 37 °C, followed by addition of 50 μl pre-warmed PMNL (isolated as in Section 2.14) and incubated at 37 °C with vigorous shaking. The final bacteria:PMNL ratio was 10:1. Reactions were stopped after 5-15 min by addition of 100 μl ice-cold 1% (w/v) paraformaldehyde in PBS. The percentage of PMNL bearing FITC-labelled bacteria was determined by flow cytometric analysis of 5,000 cells using a FACScan flow cytometer (Becton Dickinson). Quenching of extracellular fluorescence was carried out by addition of 20 μg/ml (final concentration) of trypan blue (Merck). Statistical analyses were performed using the student *t* test GraphPad Prism version 4.00 for Windows (GraphPad Software) *P* values < 0.05 were considered significant.

2.16 Whole blood survival assay

The ability of *S. aureus* to survive in human blood was measured as previously described (Visia *et al.*, 2009). Briefly, *S. aureus* Newman and its mutants were grown in RPMI to stationary phase and diluted in PBS. A 100 μl ($5x10^2$ cfu/ml) was added to 0.5 ml of fresh blood obtained from human healthy volunteers that had been treated with 50 μg/ml of the anti-coagulant hirudin (Refludan, Pharmion, Rome, Italy). A portion of each culture was plated on agar to determine the input CFU. Tubes were incubated at 37°C with gentle rocking and after 3 h samples were diluted in sterile water and plated to determine the number of surviving CFUs. To exclude the possibility that the differences in viable counts were due to differences in the ability to grow in plasma, viable counts of each strain were performed after growth for 3 h in plasma. Statistical analyses were performed using the student *t* test GraphPad Prism version 4.00 for Windows, *P* values < 0.05 were considered significant.

2.17 Experimental arthritis and sepsis

Seven week old female NMRI mice were obtained from B&K Universal AB

(Stockholm, Sweden) and maintained in the animal facility of the Department of Rheumatology and Inflammation Research, University of Göteborg, Sweden. All mice were maintained according to the local ethic board animal husbandry standards. The mice were housed 10 to a cage under standard conditions of temperature and light and were fed standard laboratory chow and water *ad libitum*. Bacteria were grown on RPMI 1640 agar plates with L-glutamine, glucose and sodium bicarbonate for 48 h, were harvested and kept frozen at -20°C in PBS containing 5% bovine serum albumin and 10% dimethyl sulfoxide. Before injection into animals, the bacterial suspensions were thawed, washed in PBS, and adjusted to appropriate cell concentrations. Mice were inoculated in the tail vein with 3.06 x 10^6 CFU of Newman wild-type or 3.102 x 10^6 CFU of Newman Sbi$^-$ in 0.2 mL of bacterial suspension. The number of viable bacteria was measured in conjunction with each challenge by counting colonies following culture at 37 °C for 24 hours on blood agar plates. The clinical evaluation was performed in a blinded manner. The overall condition of each mouse was examined by assessing signs of systemic inflammation, i.e., weight decrease, reduced alertness, and ruffled coat. In cases of severe systemic infection, when a mouse was judged too ill to survive another 24 h, it was killed by cervical dislocation and considered dead due to sepsis. Statistical evaluation was done by the Logrank test at survival analysis. $P<0.05$ was considered to be significant.

2.18 Ethics Statement

Approval for experiments using human blood was granted by the Royal College of Surgeons in Ireland Ethics Committee (REC License #0269). Donors gave informed consent in writing prior to the commencement of any procedures. All animal studies were carried out in strict accordance with the recommendations in the Regulations and General Advices on Animal Experiments of the Swedish Animal Welfare Agency (DFS 2004:4). The protocol was approved by the Ethical Committee for Animal Experimentation, Göteborg (Permit Number: 282-2006). All efforts were made to minimize suffering.

2.19 Determination of haemolytic activities

Qualitative evaluation of δ-hemolysin production was evaluated on sheep blood

agar as a surrogate for *agr* expression. Bacteria to be tested were streaked at a right angle to RN4220 and the plate incubated overnight. β-Hemolysin forms a turbid zone of hemolysis surrounding RN4220. δ-Hemolysin and β-hemolysin are synergistic resulting in a zone of clear hemolysis where they interact.

2.20 Binding of recombinant MBP-Sbi to *S. aureus* Newman *spa sbi* cells

Stationary phase cultures of Newman *spa sbi* were harvested by centrifugation at 2,000 x g for 10 min and washed twice in PBS. Cells were adjusted to an OD_{600nm} 10 in PBS and incubated with 6nM of MBP-Sbi_{41-436}, MBP-Sbi_{41-254}, MBP-$Sbi_{254-436}$ and MBP for 1 h at 37 °C. Cells were washed three times in PBS and incubated with 1.5 μg/ml anti-MBP IgG for 1 h at 37 °C. Cells were again washed three times in PBS and incubated with FITC-labelled rabbit anti-mouse IgG for 1 h at 37 °C. Finally cells were again washed three times in PBS and the mean flouresence of each cell set was determined by flow cytometric analysis of 5,000 cells using a FACScan flow cytometer (Becton Dickinson).

2.21 Densitometric analysis

Densitometric analysis was carried out using ImageJ software from the National Institute of Health (NIH). Integrated band densities were measured with correction of background noise.

2.22 Antibodies

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

2.23 Proteomics tools for prediction of protein topology

Membrane-spanning regions and topology of Sbi and EbpS based on their primary protein sequence was performed using the following online computer programs; Kyte-Doolittle Hydropathy plotting http://gcat.davidson.edu/rakarnik/kyte-doolittle.htm. Prediction of transmembrane α-helices TMHMM (www.cbs.dtu.dk/services/TMHMM) and DAS (www.sbc.su.se/~miklos/DAS/).

Chapter 3

Genetic manipulation of *sbi*

3.1 Introduction

The isolation of engineered mutations in staphylococcal genes has been necessary in order to study the phenotypic effects of individual surface expressed and secreted bacterial proteins and to investigate their roles, if any, in the virulence mechanisms of the bacteria. Comparing the virulence of specific isogenic mutants with their parental wild-type strains in animal models of infection has allowed the identification of numerous important virulence factors. A specific example is the recent study showing that the secreted plasma clotting factors coagulase and von Willebrand factor binding protein are both virulence factors in murine staphylococcal bacteraemia models (Cheng *et al.*, 2010). In contrast, the TrpP protein was shown not to be a virulence factor in a murine subcutaneous abscess model (Adhikari *et al.*, 2007).

Several methods for the construction of isogenic mutants in *S. aureus* are available, namely transposon mutagenesis, directed plasmid integration and allelic replacement. The main advantage of transposon mutagenesis is that it requires no prior knowledge of the target gene. Only a phenotypic screen to identify mutants is needed. Successful transposon mutagenesis relies upon a high transposition frequency and the lack of site-specificity with which the transposon inserts into the target genome. Not all transposons insert entirely at random, creating hot-spots for insertion which leaves some regions of the target genome devoid of insertions. The *bursa aurealis* element, a mariner-based transposon, can overcome the problem of hot-spots as it exhibits no sequence preference in the genome of *S. aureus* strain Newman (Bae *et al.*, 2004). The recent increase in the number of complete genome sequences for *S. aureus* strains has resulted in transposon mutagenesis becoming less important as site-specific mutations in any sequenced gene can now be designed.

Allelic replacement allows site-specific mutations to be introduced into the chromosomal genes by homologous recombination. Typically, regions 5' and 3' to the target gene are cloned in a multicopy plasmid in *E. coli* and a drug resistant determinant is inserted into a specific restriction site located between the fragments, forming a deletion-substitution mutation. The mutation is subsequently delivered into *S. aureus* and integrated into the chromosome by homologous recombination. A number of different drug resistant determinants have been used successfully to generate mutations in *S. aureus* including erythromycin resistance (ermC; O'Reilly *et al.*, 1986,

Phonimdaeng *et al.*, 1990, O'Brien *et al.*, 2002a, Visai et al., 2009), tetracycline resistance (*tetK*; O'Connell *et al.*, 1993, Greene *et al.*, 1995, Ni Eidhin *et al.*, 1998), kanamycin-neomycin resistance (Patel *et al.*, 1989) and ethidium bromide resistance (Patel et al., 1987).

Several different techniques have been utilised to stimulate allelic replacement. Plasmid incompatibility was originally used to generate mutations in the α-toxin (O'Reilly *et al.*, 1986), coagulase (Phonimdaeng *et al.*, 1990) and protein A genes (Patel *et al.*, 1987). The mutational cassette was introduced into *S. aureus* on a shuttle plasmid and a second incompatible plasmid was then introduced. Selection for the incompatible plasmid results in elimination of the shuttle plasmid. This allows for subsequent identification of allelic replacement mutations by screening for loss of resistance associated with the shuttle plasmid and a change in the phenotype of the mutant.

Suicide plasmids are plasmids that cannot replicate in a particular host. The only way the plasmid can be maintained is by integrating directly into the chromosome by recombination at a site of shared homology. Suicide plasmids can be used for directed integration and allelic replacement to disrupt genes. Directed integration involves insertion of a region of the target gene into the suicide plasmid, electroporation of the plasmid construct into the restriction-deficient strain RN4220 (Kreiswirth *et al.*, 1983) and integration into the chromosome by homologous recombination. A single cross-over leads to integration of the plasmid, duplication of the inserted region and disruption of the gene (Figure 3.1). The plasmid-associated resistance determinant can then be used for generalised transduction of the mutation to other *S. aureus* strains. Suicide plasmids have been used to generate mutations in the coagulase gene (McDevitt *et al.*, 1993) and the type 5 capsular polysaccharide operon (Sau *et al.*, 1997). Disadvantages of suicide plasmid integration are the low frequency at which integration occurs and being restricted to RN4220 (at least until improvements in transformation are available). Duplication of regions of the target gene, which is necessary for recombination, can result in residual gene function. The 5' region of the target gene remains attached to the promoter and expression of this fragment can occur. Additionally, duplications can in the absence of selection revert to wild-type by plasmid excision.

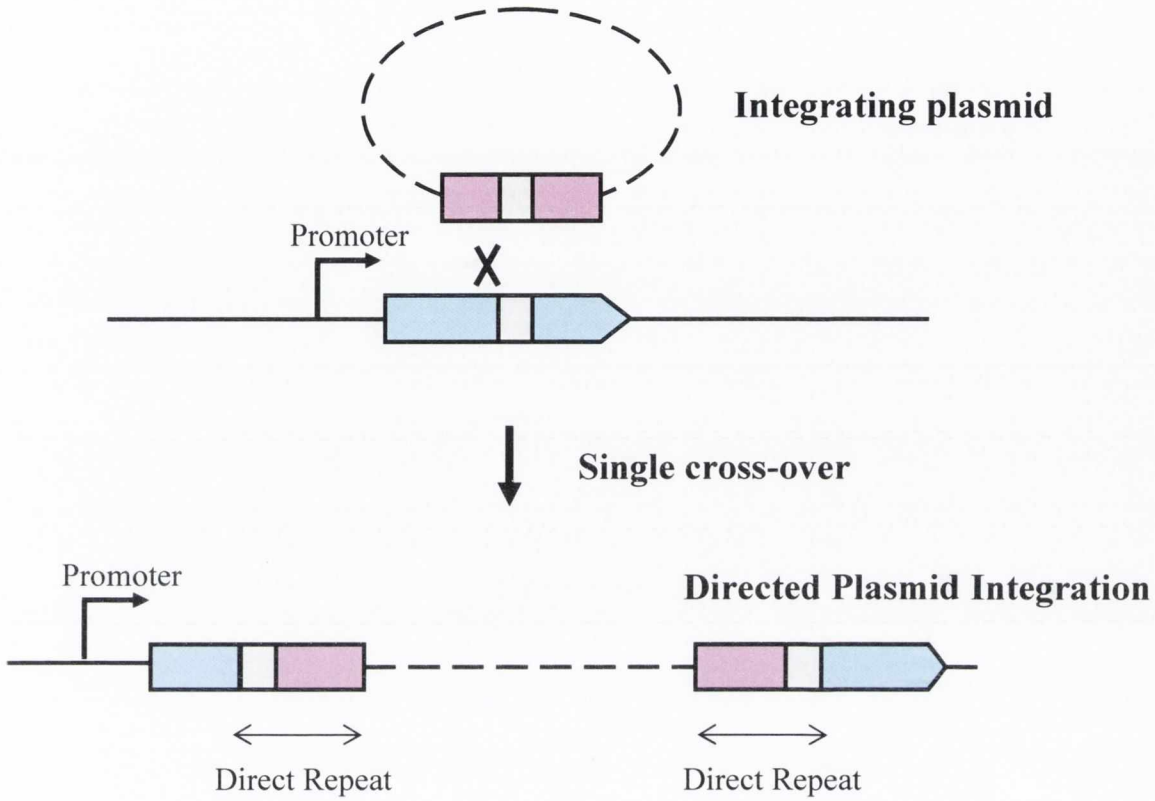


Figure 3.1 Suicide plasmid integration

A single cross-over event results in the integration of the plasmid and disruption of the target gene. Plasmid integration at the chromosomal locus by homologous recombination causes duplication of the inserted sequence, creating direct repeats. The suicide vector has no replication functions for *S. aureus* and is stably integrated. The sequence inserted in the plasmid determines the site of integration.

Another example of directed plasmid integration is the generation of transcriptional fusions using pAZ106 (Kemp *et al.*, 1991). Plasmid pAZ106 has been used to generate transcriptional reporter fusions to *clfB*, *spa* and α-haemolysin (*hla*) (McAleese *et al.*, 2001, Chan & Foster, 1998). The plasmid bears no *S. aureus* replication functions and contains a promoterless β-galactosidase gene (*lacZ*). A 5' gene DNA fragment is the only region of homology between the plasmid and the *S. aureus* chromosome and integration occurs in that region, creating a transcriptional fusion to the β-galactosidase gene (Figure 3.2). Transcription occurs from the chromosomal promoter but drives β-galactosidase production in the place of the wild-type gene product. The amount of β-galactosidase produced is measured quantitatively, and is a reporter of promoter activity.

Allelic replacement can also be achieved by directed plasmid integration. Suicide plasmid integration occurs at the site of introduced homology, single cross-over events result in the duplication of the inserted sequence. Double cross-over events can occur upon selection for mutation-associated, rather than plasmid-associated resistance, resulting in direct replacement of the wild-type allele. The mutation-associated resistance marker can be subsequently used for phage-mediated transduction of the mutation to other *S. aureus* strains.

Temperature-sensitive plasmids are now commonly used for allelic replacement in *S. aureus*. These plasmids contain thermosensitive replicons derived from pE194 (Horinouchi & Weisblum, 1982) and pT181 (Khan & Novick, 1983). Temperature-sensitive plasmids can replicate in *S. aureus* at the permissive temperature usually 28 °C, but not at the restrictive temperature, usually 44 °C. The plasmid construct containing the mutational cassette is propagated at the permissive temperature, followed by growth at the restrictive temperature revealing variants in which the plasmid has integrated into the chromosome (Figure 3.3, panel A). Selection for plasmid-associated resistance at the restrictive temperature selects for single cross-over integration. Growth of single cross-over integrants at the permissive temperature for replication results in plasmid excision by homologous recombination (Figure 3.3, panel B; Biswas *et al.*, 1993). Following a further temperature shift to the restrictive temperature, in the absence of selection for plasmid-associated resistance, the plasmid is eliminated from the bacterial population allowing the identification of recombinants. Plasmid excision

can result in the removal or retention of the mutation in the chromosome depending on the site of recombination (Figure 3.3, panel C). One advantage of temperature-sensitive plasmids is that plasmid constructs can be transferred between strains at the permissive temperature allowing allelic exchange by temperature shift in different backgrounds.

Several temperature-sensitive plasmids have been used to generate allelic replacement mutants in *S. aureus*. Plasmid pTS2 a derivative of pE194*ts* (Gryczan *et al.*, 1982) has been used to generate mutations in the *fnb* genes (Greene *et al.*, 1995), the *clfB* gene (Ni Eidhin *et al.*, 1998) and the *sdrCDE* locus of *S. aureus*. The broad range pG^+ host vectors from lactococci (Maguin *et al.*, 1996) have been used to generate mutations in the *hlg* gene of *S. aureus* (Supersac *et al.*, 1998) and the *sdrG* gene of *S. epidermidis* (Hartford *et al.*, 2001). Plasmids pTS*ermC* and pTS*tetK* are derivatives of pTS2 in which the chloramphenicol resistance determinant has been replaced with the erythromycin resistance determinant *ermC* from pE194 (in pTS*ermC*) or the tetracycline resistance determinant *tetK* from pT181 (in pTS*tetK*). pTS*ermC* has been used previously to isolate a mutation in the *clfA* gene (Fitzgerald *et al.*, 2006) whilst pTS*tetK* was used to generate a deletion-substitution mutation in the *sbi* gene of *S. aureus* in this study.

To facilitate the isolation of and identification of plasmid excisants following directed integration into the chromosomal locus, temperature-sensitive plasmids pMAD and pKOR1 were developed. pMAD carries the *bgaB* gene which encodes β-galactosidase enabling staphylococci to cleave the chromogenic substrate X-gal resulting in blue colonies (Arnaud *et al.*, 2004). Expression of *bgaB* provides a screening tool for the excision and loss of the plasmid, as colonies lacking the plasmid will have a normal colour on X-gal. pMAD has successfully been used to generate mutations in the ABC transporter genes *vraF* and *vraG* of *S. aureus* strain Mu50 (Arnaud *et al.*, 2004). Plasmid pKOR1 utilizes antisense *secY* RNA expression for counter-selection (Bae & Schneewind, 2006). SecY is a component of the SecYEG translocase that transports signal peptide bearing proteins across the cytoplasmic membrane (Manting & Driessen, 2000). Protein secretion and SecY expression are essential for bacterial growth. Expression of *secY* antisense RNA inhibits colony formation on agar plates (Ji *et al.*, 2001). Inducible expression of antisense *secY* RNA

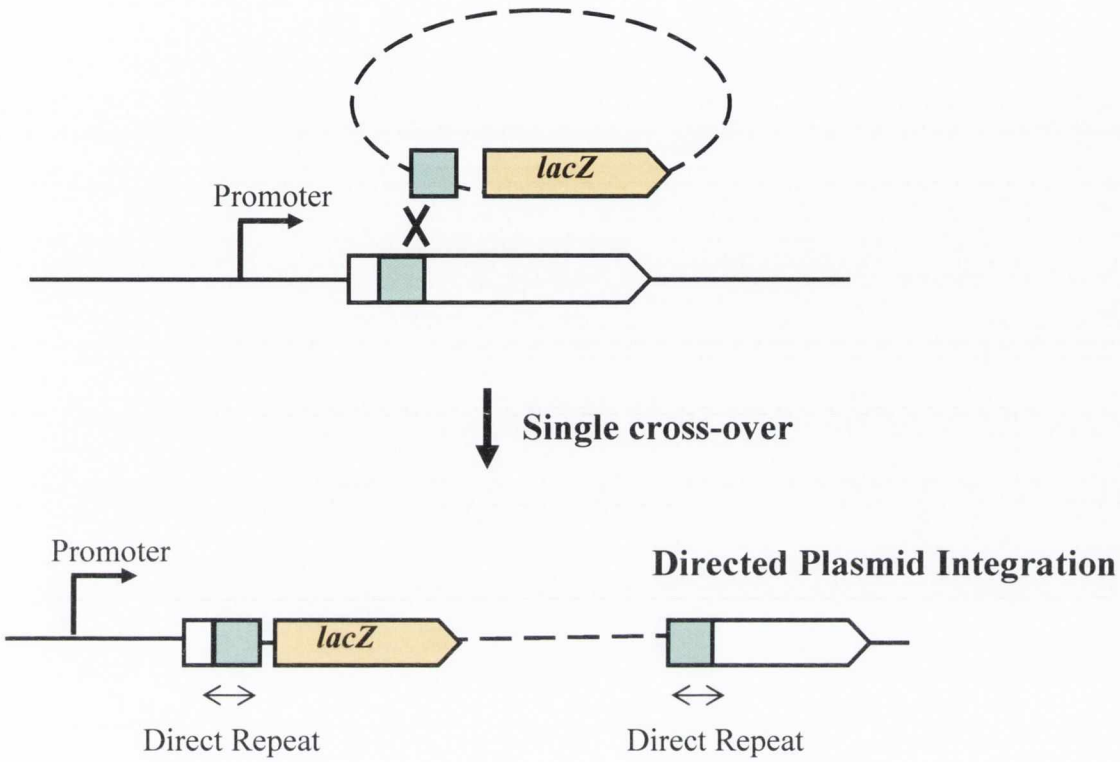


Figure 3.2 Transcriptional fusion to *lacZ*

The 5' end of the gene is inserted upstream of the *lacZ* gene in pAZ106. A single cross-over event results in the integration of the plasmid. This causes fusion of the 5' end of the chromosomal copy of the gene, and upstream sequences, to *lacZ.* The *lacZ* element is promoterless but carries a ribosome-binding site. The promoter of the gene drives transcription of the 5' end of the gene and *lacZ* as a single transcript.

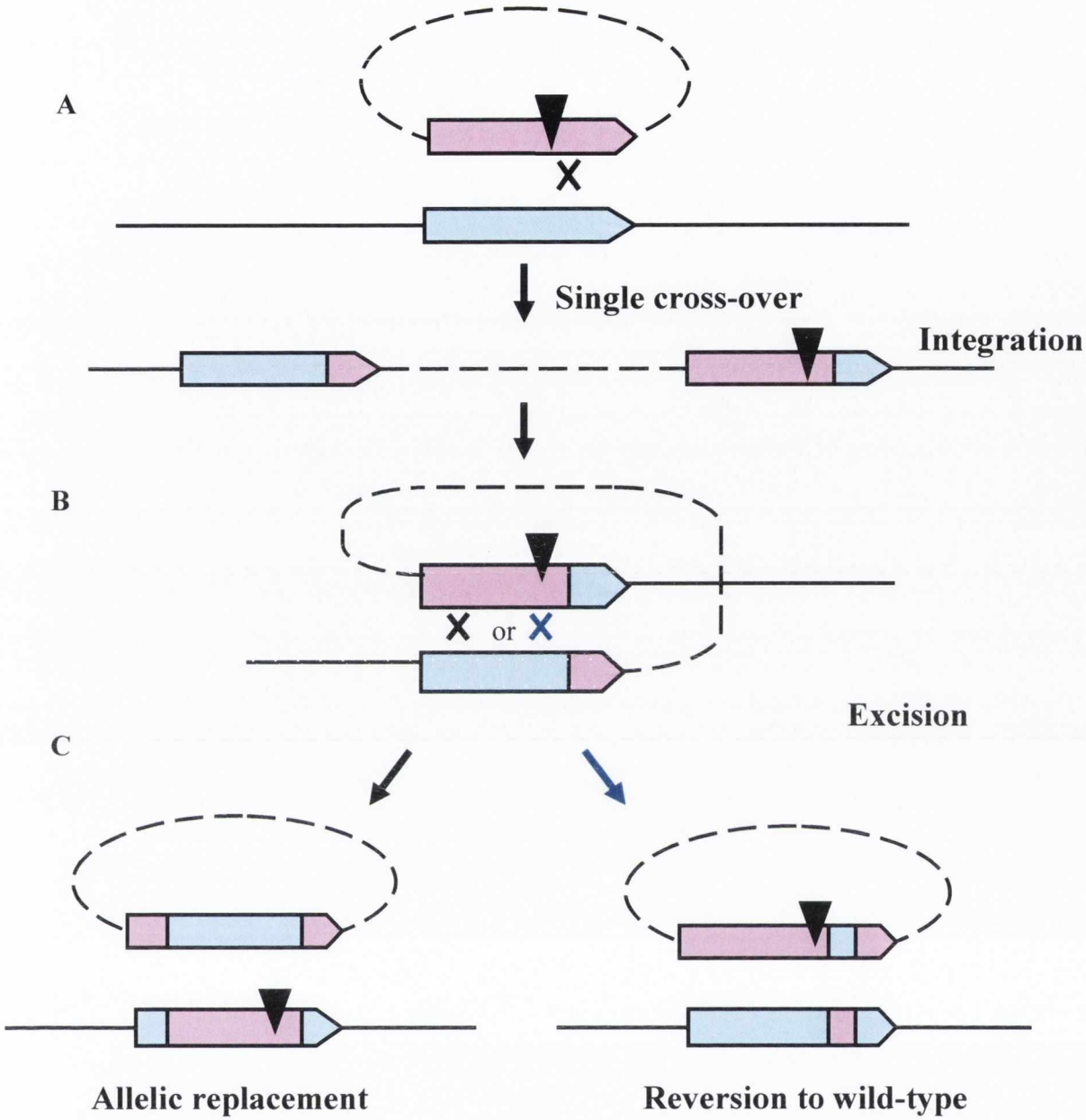


Figure 3.3 Schematic representation of plasmid integration and excision

(A) Plasmid integration at the chromosomal locus by single cross-over event on one side of the mutation causes duplication of the inserted sequence. **(B)** A second recombination event allows plasmid excision via an intermediate. **(C)** A second cross-over on the same side of the mutation (in blue) as the first causes removal of the mutation to the plasmid. A second cross-over on the opposite side (in black) to the first causes removal of the wild-type gene, resulting in successful allelic replacement.

suppresses growth of bacterial cells containing pKOR1 resulting in a mixture of large and small colonies on agar plates, with large colonies having lost the plasmid.

Allelic replacement mutagenesis using temperature-sensitive plasmids is more flexible than directed plasmid integration, as it does not require a mutation-associated resistance marker. This allows the construction of non-polar mutations by the generation of in-frame deletions (Sau *et al.*, 1997) or insertions (Rice *et al.*, 2001). Allelic replacement allows single-base pair substitutions in chromosomal loci, facilitating study of gene regulation (Kaatz *et al.*, 2005). In the absence of a mutation-associated marker a simple screen for successful replacement is desirable. For point mutations it is possible to generate mutation specific primers but this requires precision with PCR to differentiate between the wild-type and mutant. Another option is to create a novel restriction site nearby if a search reveals that a simple same-sense base change can be introduced, but it must be sufficiently close to be incorporated into one of the primers used to create the mutation by overlap exchange PCR.

This chapter describes the construction and validation of mutant strains and plasmids used in this study as follows (i) The generation of an isogenic mutation in the *sbi* gene of *S. aureus* strain Newman by allelic replacement with the temperature-sensitive plasmid pES2. (ii) Strains constructed by generalised transduction of null mutations in surface protein genes. (iii) The complementation of the *sbi* mutation using the anhydrotetracycline inducible expression vector pRMC2. (iv) The generation of deletions in the *sbi* coding sequence by inverse PCR.

3.2 Results

3.2.1 Allelic replacement mutagenesis of *sbi*

A deletion-substitution mutation (*sbi*::Em^r) was isolated in the *sbi* gene of strain Newman by allelic replacement. The mutation deleted all but 40 bp at the 5' end of the gene and 70 bp at the 3' end, creating a 1190 bp deletion into which a 1050 bp *ermC* fragment encoding erythromycin resistance was inserted (Figure 3.4, panel A). A 900 bp fragment located 5' to the *sbi* gene and 740 bp covering the 3' end of the gene were amplified by PCR, using the primer pairs Sbi 5'F/Sbi 5'R and Sbi 3'F/Sbi 3'R. The fragments were sequentially cloned into pBluescript and the *ermC* erythromycin resistance determinant was cloned into the unique *Bgl*II site that separated the 5' and 3' *sbi* sequences. The construct was converted to a temperature-sensitive shuttle plasmid by ligation with pTS*tetK*, which encodes tetracycline (Tc) resistance, generating plasmid pES2.

To allow for allelic replacement mutagenesis of the *sbi* gene, pES2 was transferred into *S. aureus* strain Newman. Following a temperature shift to the restrictive temperature (44 °C), cells were plated on 10μg/ml Em in order to select for single cross-over integration. In order to encourage plasmid excision, single cross-over integrants were grown in broth at the restrictive temperature with antibiotic selection, then diluted and grown without selection at the permissive temperature (28 °C) for replication. It is thought that rolling circle replication of a chromosomally integrated plasmid stimulates excision (Biswas *et al.*, 1993). Loss of excised plasmids was encouraged by dilution in drug-free broth and growth at the restrictive temperature. This process was repeated twice more to enrich for plasmid-free excisants. Double cross-over integrants (Tc sensitive, Em resistant) occurred in 1 in 50 colonies.

3.2.2 Validation of the *sbi*::Em^r mutation

Genomic DNA was prepared from parental strains and erythromycin resistant derivatives. Primers Sbi 5'F/Em^R M'R were designed to amplify a PCR product that spanned the junction of the mutation. If the mutated allele was retained in the chromosome a 1000 bp fragment would be amplified from Newman *sbi* but not from wild-type. As predicted a 1000 bp fragment was amplified from Newman *sbi*::Em^r and pES2 DNA. Plasmid pES2 was included as a control for the PCR reaction. In contrast

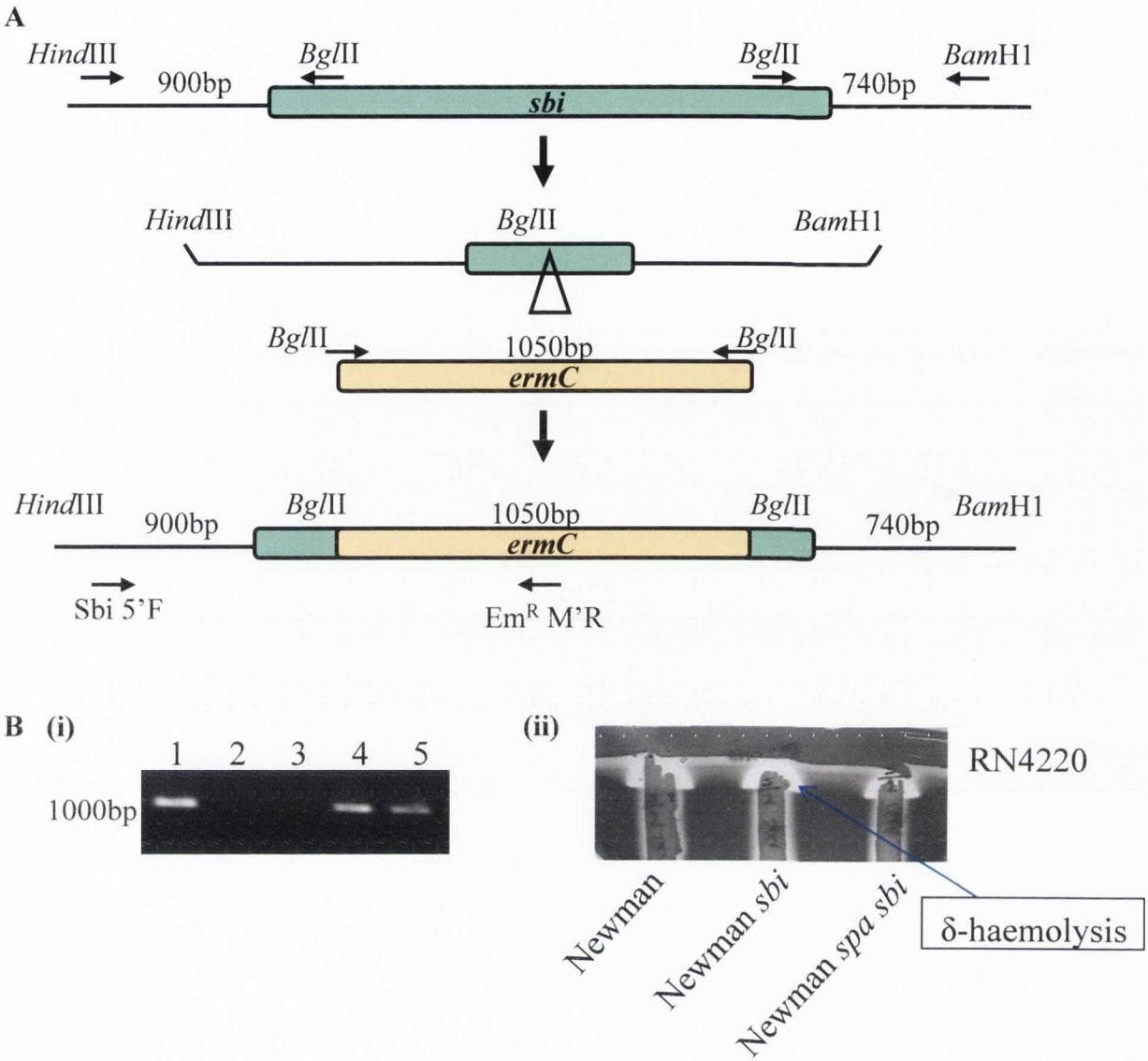


Figure 3.4. (A) Schematic representation of the *sbi*::Em^R mutation.

An 900bp fragment 5' to the *sbi* gene and 740bp fragment covering the 3' end of the gene were cloned into pBluescript and joined together using a *Bgl*II restriction site introduced by PCR. A PCR fragment expressing erythromycin resistance was inserted between the 5' and 3' *sbi* sequences. **(B) Validation of Newman *sbi* and Newman *spa sbi*.** (i) Diagnostic PCR with primers Sbi 5'F and Em^R M'R designed to amplify a 1000bp fragment from strains in which the *sbi*::Em^R mutation was retained in the chromosome. Primer positions are indicated in Figure A. Lane 1, pES2; lane 2, Newman; lane 3, Newman *spa*; lane 4, Newman *sbi*; lane 5, Newman *spa sbi*. (ii) Haemolytic patterns of Newman *sbi* and Newman *spa sbi* on sheep blood agar cross-streaked with RN4220. The clear arc is due to δ-toxin causing complete lysis of erythrocytes damaged by β-toxin.

wild-type Newman genomic DNA failed to generate a PCR product (Figure 3.4, panel B(i)).

Newman *sbi* was compared to its parental wild-type strain for expression of δ-toxin as a surrogate for *agr* function. Spontaneous *agr* mutations can occur at high frequency under stress and these could affect expression of other virulence factors and alter fitness which could affect the rate of growth. δ-haemolysin has very weak activity on sheep blood agar but is strongly synergistic with β-haemolysin, producing a zone of clear haemolysis where they interact. Newman and Newman *sbi* were cross-streaked against RN4220, which produces only β-haemolysin (Tegmark *et al.*, 2000). A zone of clear haemolysis was observed for both Newman and Newman *sbi* indicating normal *agr* function in these strains (Figure 3.4, panel B(ii)).

3.2.3 Construction and validation of combined mutations in *S. aureus* strain Newman

To assess unambiguously the cellular localisation of Sbi it was necessary to construct a strain lacking both Sbi and Spa. The *sbi*::Em^r mutation was introduced into Newman *spa* by generalised transduction to create Newman *spa sbi*. PCR was used to determine that the constructed strain had the appropriate genotype (Figure 3.4, panel B(i)). Newman *spa sbi* was compared to its parental wild-type strain for expression of δ-toxin to ensure normal Agr function. As with Newman *sbi* a zone of clear haemolysis was observed for Newman *spa sbi* indicating that Agr is intact (Figure 3.4, panel B(ii)).

To avoid the possibility that any anti-phagocytic effect of Sbi could be masked by the other known anti-phagocytic factors of *S. aureus* (Spa, ClfA, Capsular polysaccharide (Cap) and IsdH), the immune evasion properties of Sbi were also analysed in a strain lacking all of these factors. This necessitated construction of a mutant of Newman that is defective in *spa*, *clfA*, *cap*, and *isdH* as well as a second mutant defective in *spa*, *clfA*, *cap*, *isdH* and *sbi*. This was achieved in a step-wise manner by combining previously isolated mutations by generalised transduction or allelic replacement. PCR and restriction digests were used to determine that the constructed strains had the appropriate genotype.

The *cap* mutation (Table 2.1) was generated in strain Newman by disruption of the capsular polysaccharide operon by insertion of the *tetK* resistance determinant from

pT181 into the *capB* gene (Figure 3.5, panel A(i)). The *cap*::Tc^R mutation was transduced from Newman *cap* to Newman *spa clfA* (Higgins et al., 2006) selecting for tetracycline resistance and screened by diagnostic PCR with primers Cap 5'F/Cap 3'R which were designed to amplify a 3500 bp fragment from strains carrying the *cap*::Tc^R insertional mutation and a 1800 bp fragment from wild-type Newman (Figure 3.5, panel A(ii)).

Newman *spa clfA cap isdH* was constructed by allelic replacement with the temperature-sensitive plasmid pJH4-*isdH2* (Visai *et al.*, 2009). The *isdH2* mutation introduces a triple amino acid substitution (Y126A/H127E/F128A), in the NEAT-1 domain of the IsdH protein. The amino acid substitution mutant has previously been shown to have the same defect as the *isdH* null mutant in survival in whole human blood and phagocytosis *in vitro* by human neutrophils (Visai *et al.*, 2009). pJH4-*isdH2* was transformed into Newman *spa clfA cap*. Following growth at the restrictive temperature cells were plated on 10μg/ml Em in order to select for single cross-over integration. Integrants were grown through thirty generations by diluting 1/100 in broth at the permissive temperature followed by growth at the restrictive temperature, in order to promote plasmid replication and excision from the chromosome, coupled with allelic exchange of wild-type and mutant alleles. Colonies grown on drug-free agar were screened for loss of resistance to erythromycin. The *isdH2* mutation contains a same-sense nucleotide mutation at residues 315 and 316 to create a novel *Eco*RV restriction site that aids screening of allelic replacement mutants (Figure 3.5, panel B(i)). Primers IsdH 5'F/IsdH 3'R were designed to amplify the 2685 bp *isdH* fragment. If the mutated allele was retained in the chromosome, a novel *Eco*RV site would have been introduced. The *isdH* gene of Newman also contains a naturally occurring *Eco*RV site at residue 795. The fragment amplified from the parental strain was cut once with *Eco*RV. Fragments containing the mutation were cut twice by *Eco*RV into three fragments of 315 bp, 795 bp and 1575 bp. Plasmid pJH4-*isdH2* was used as a template for a control PCR reaction and *Eco*RV digestion (Figure 3.5, panel B(ii)).

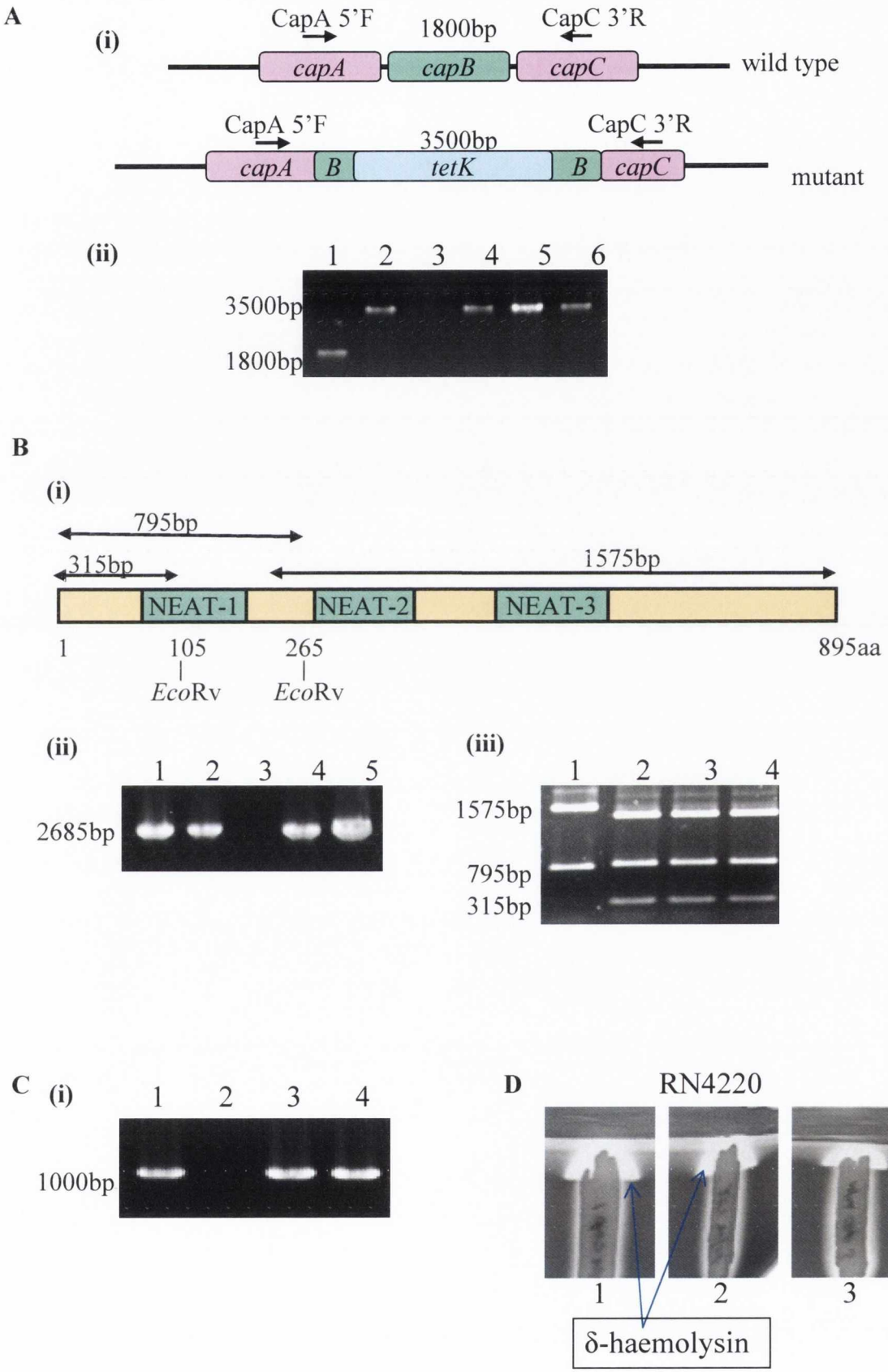
Newman *spa clfA cap isdH sbi* was constructed by transduction of the *sbi*::Em^R mutation from Newman *sbi* to Newman *spa clfA cap isdH*. Transductants were screened by PCR with primer pair Sbi 5'F/Em^R M'R as described in section 3.2.2 (Figure 3.5, panel C). Each mutant was tested for expression of δ-toxin. As with wild-type Newman

Figure 3.5. (A) Schematic representation and validation of the *cap*::Tc^R mutation. (i) The *cap* operon was disrupted by insertion of the *tetK* resistance determinant from pT181 into *capB*. (ii) Diagnostic PCR using primers CapA 5'F and CapC 3'R designed to amplify a 3500bp fragment from strains in which carried *cap*::Tc^R. Lane 1, Newman; lane 2, Newman *cap*; lane 3, template-free PCR control; lane 4, Newman *spa clfA cap*; lane 5, Newman *spa clfA cap isdH*; lane 6, Newman *spa clfA cap isdH sbi.*

(B) Schematic representation and validation of the *isdH2* mutation. (i) The *isdH2* mutation contains same-sense nucleotide mutations at resides 315 and 316 to create a novel *Eco*RV restriction site for screening of allelic replacement. (ii) PCR from strains carrying the *isdH2* mutation with primers IsdH 5'F and IsdH 3'R. Lane 1, Newman; lane 2, pJH4-*isdH2*; lane 3, template-free PCR control; lane 4, Newman *spa clfA cap isdH*; lane 5, Newman *spa clfA cap isdH sbi.* (iii) Following PCR products were digested with *Eco*RV. Lane 1, Newman; lane 2, pJH4-*isdH2*; lane 3, Newman *spa clfA cap isdH*; lane 4, Newman *spa clfA cap isdH sbi.* Arrows indicated the sizes of restriction digest fragments.

(C) Validation of the *sbi*::Em^R mutation in Newman *spa clfA cap isdH sbi.* Diagnostic PCR with primers Sbi 5'F and Em^R M'R designed to amplify a 1000bp fragment from strains harbouring the *sbi*::Em^R mutation. Lane 1, pES2 control; lane 2, Newman; lane 3, Newman *sbi*; lane 4, Newman *spa clfA cap isdH sbi.*

(D) Haemolytic patterns. Lane 1, Newman; Lane 2, Newman *spa clfA cap isdH* and lane 3, Newman *spa clfA cap isdH sbi* cross-streaked close to RN4220. δ-haemolysin is indicated by the blue arrows.


A
(i)
CapA 5'F
1800bp
CapC 3'R
capA
capB
capC
wild type
CapA 5'F
3500bp
CapC 3'R
capA
B
tetK
B
capC
mutant
(ii)
1 2 3 4 5 6
3500bp
1800bp
B
(i)
795bp
315bp
1575bp
NEAT-1
NEAT-2
NEAT-3
1
105
EcoRv
265
EcoRv
895aa
(ii)
1 2 3 4 5
2685bp
(iii)
1 2 3 4
1575bp
795bp
315bp
C
(i)
1 2 3 4
1000bp
D
RN4220
1
2
3
δ-haemolysin

a zone of clear haemolysis was observed for all mutants tested indicating Agr is functional (Figure 3.5, panel D)

3.2.4 Complementation of the *sbi*::Em^R mutation

In order to complement the *sbi*::Em^R mutation, the *sbi* gene including the ribosomal binding site, but lacking the promoter, was amplified from genomic DNA of strain Newman and cloned between the *Kpn*1 and *Bgl*II sites in pRMC2, an anhydrotetracycline-inducible vector, to create pRMC2-*sbi* (Figure 3.6). pRMC2-*sbi* was transferred into *S. aureus* Newman *spa* and Newman *spa sbi*.

3.2.5 Construction and validation of *S. aureus* strain USA300 *sbi*

To evaluate the relative contribution of Sbi to immune evasion in a clinically relevant strain of *S. aureus* the *sbi*::Em^r mutation was transduced into the community-acquired MRSA strain USA300 (Diep *et al.*, 2006) to create USA300 *sbi*. Transductants were screened by PCR with primer pair Sbi 5'F/Em^R M'R as described in section 3.2.2 (Figure 3.7, panel A). Western immunoblotting of whole cell lysates was used to determine that the constructed strain had the appropriate phenotype (Figure 3.7,panel B) USA300 *sbi* was compared to wild-type for expression of δ-toxin and was shown to have normal Agr activity (Figure 3.7, panel C).

3.2.6 Construction of *sbi* C-terminal 'Y' domain truncates

In order to investigate the mechanism of attachment of Sbi to the cell envelope a series of C-terminal truncates of *sbi* were generated by PCR and cloned between the *Kpn*1 and *Bgl*II sites in pRMC2, to create pRMC2-$sbi_{1\text{-}335}$, pRMC2-$sbi_{1\text{-}368}$ and pRMC2-$sbi_{1\text{-}403}$ (Figure 3.8, A). Plasmid constructs were verified by restriction analysis (Figure 3.8, B) and DNA sequencing. Plasmids were transferred into Newman *spa sbi*.

3.2.7 Construction of $sbi\Delta_{335\text{-}368}$

To further characterise the Sbi C-terminal binding domain for the *S. aureus* cell envelope, an in-frame deletion encoding a truncate lacking residues 335-368 was generated by inverse PCR (Figure 3.8, A). Plasmid pRMC2-*sbi* was used as the template for the inverse PCR reaction with the 5' phosphorylated primer pair Sbi$\Delta_{335\text{-}368}$ 5'R/Sbi$\Delta_{335\text{-}368}$ 3'F. The amplified 5'-phosphorylated product was circularized to

generate plasmid pRMC2-*sbi*$\Delta_{335\text{-}368}$. Plasmid constructs were verified by restriction analysis (Figure 3.8, D) and DNA sequencing. pRMC2-*sbi*$\Delta_{335\text{-}368}$ was transferred into Newman *spa sbi*.

3.2.8 Construction of *sbi* lacking N-terminal domains

To investigate the cell surface expression of the ligand binding domains D1-D4, in-frame deletion mutants encoding truncates lacking residues 41-153 (D1D2) and 154-253 (D3D4) were generated by inverse PCR. Plasmid pRMC2-*sbi* was used as the template for the inverse PCR reaction with the 5' phosphorylated primer pairs SbiΔ_{D1D2} 5'R/ SbiΔ_{D1D2} 3'F and SbiΔ_{D3D4} 5'R/ SbiΔ_{D3D4} 3'F (Figure 3.9, panel A). The amplified 5'-phosphorylated products were circularized by ligation to generate plasmids pRMC2-*sbi*Δ_{D1D2} and pRMC2-*sbi*Δ_{D3D4}. Plasmid constructs were verified by restriction analysis (Figure 3.9, panel B) and DNA sequencing. pRMC2-*sbi*Δ_{D1D2} and pRMC2-*sbi*Δ_{D3D4} were transferred into Newman *spa sbi*. Plasmids pRMC2-*sbi*Δ_{D1D2} and pRMC2-*sbi*Δ_{D3D4} were also transferred into Newman *sbi* to facilitate the study of domains D1D2 and D3D4 separately in immune evasion.

To determine the roles of the ligand binding domains of secreted Sbi in immune evasion, deletion mutants lacking residues 41-153 (D1D2) and 154-253 (D3D4) were combined with the C-terminal deletion in residues 335-436. Plasmid pRMC2-*sbi*$_{1\text{-}335}$ was used as the template for the inverse PCR reaction again with primer pairs SbiΔ_{D1D2} 5'R/ SbiΔ_{D1D2} 3'F and SbiΔ_{D3D4} 5'R/ SbiΔ_{D3D4} 3'F (Figure 3.9, panel A). The amplified 5'-phosphorylated products were circularized by ligation to generate plasmids pRMC2-*sbi*$_{1\text{-}335}\Delta_{D1D2}$ and pRMC2-*sbi*$_{1\text{-}335}\Delta_{D3D4}$. Plasmid constructs were verified by restriction analysis (Figure 3.9, panel B) and DNA sequencing. pRMC2-*sbi*$_{1\text{-}335}\Delta_{D1D2}$ and pRMC2-*sbi*$_{1\text{-}335}\Delta_{D3D4}$ were transferred into Newman *sbi*.

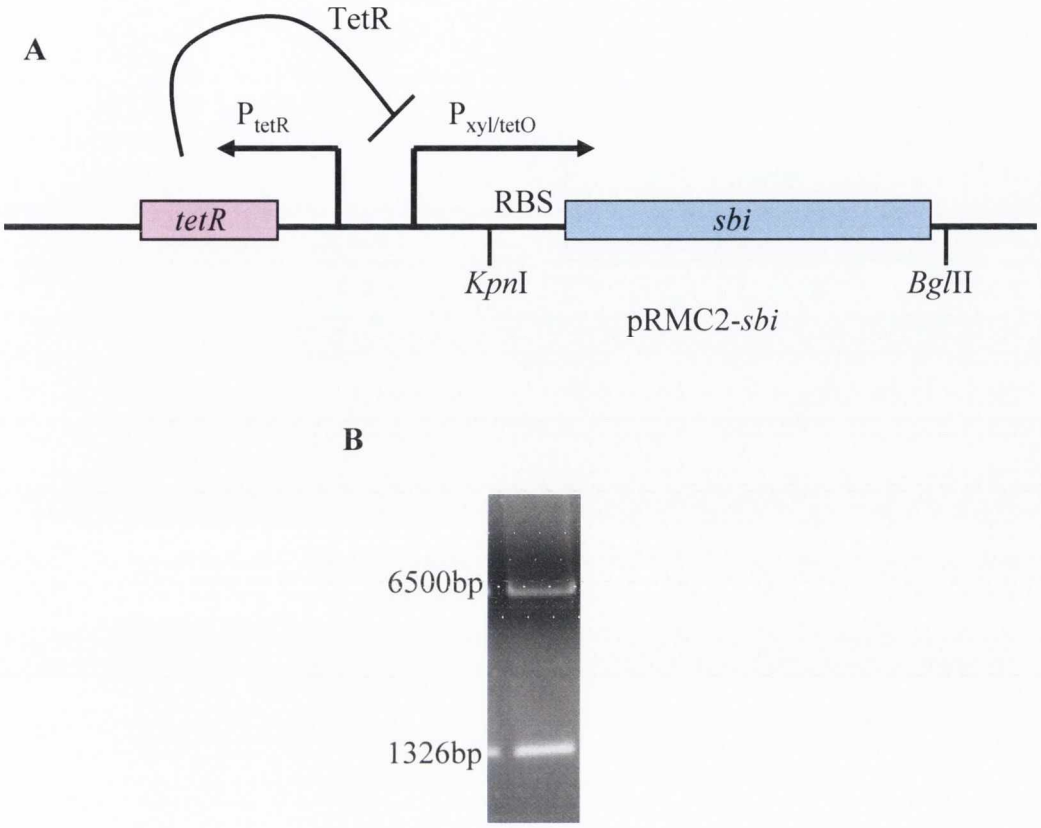


Figure 3.6. (A) Structure of pRMC2-*sbi*. The *sbi* gene including the ribosomal binding site (RBS) but lacking the promoter was amplified and cloned between the *Kpn*1 and *Bgl*II sites in pRMC2**. (B) Restriction analysis of plasmid pRMC2-*sbi*.** pRMC2 cut with *Kpn*I and *Bgl*II. 6500bp fragment; pRMC2 backbone, 1326bp fragment; *sbi*

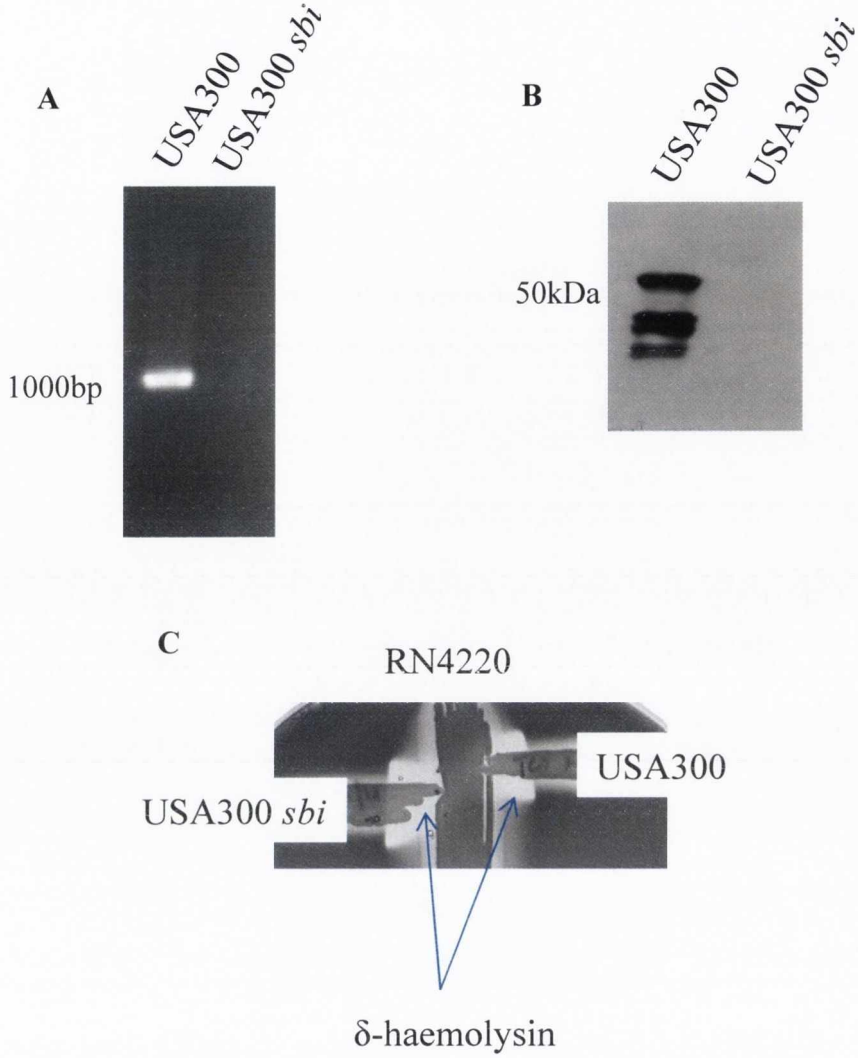


Figure 3.7. Validation of USA300 *sbi*

(**A**) Diagnostic PCR with primers Sbi 5'F and Em^R M'R designed to amplify a 1000bp fragment from strains in which the *sbi*::Em^R mutation was retained in the chromosome. (**B**) Western immunoblot of whole cell lysates. Blots were probed with rabbit anti-SbiD3D4WrY antibodies followed by HRP-conjugated goat anti-rabbit IgG. The 50kDa band is Sbi. The lower bands are likely to be breakdown products. (**C**) Haemolytic patterns of USA300 and USA300 sbi on sheep blood agar cross-streaked with RN4220. The clear arc is due to δ-toxin causing complete lysis of erythrocytes damaged by β-toxin.

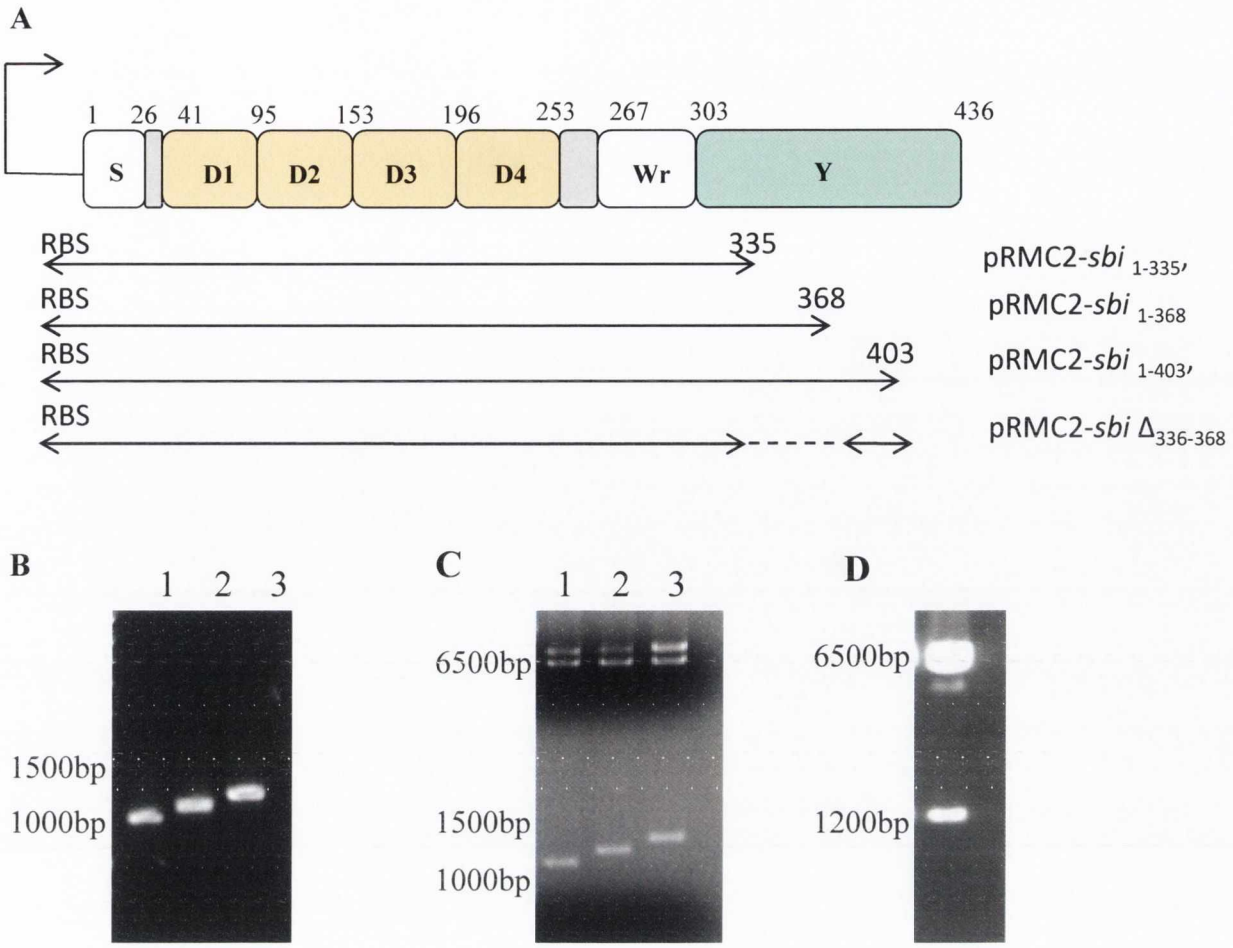
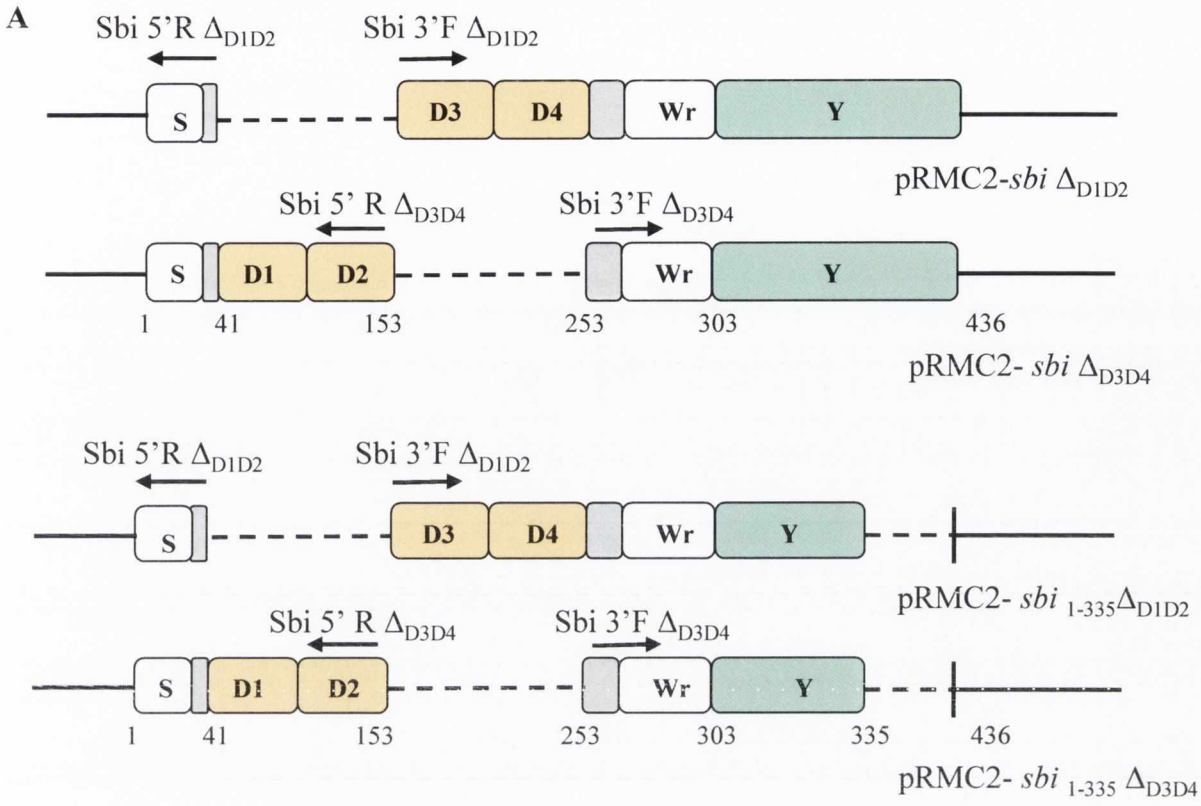


Figure 3.8. (A) Schematic representation of *sbi* deletions. The *sbi* C-terminal truncates including the ribosomal binding site (RBS), were amplified and cloned between the *Kpn*1 and *Bgl*II sites in pRMC2. **(B) PCR of amplified *sbi* C-terminal truncates.** Lane 1, 1005bp fragment $sbi_{1\text{-}335}$; lane 2, 1104bp fragment $sbi_{1\text{-}368}$ and lane 3, 1209bp fragment $sbi_{1\text{-}403}$. **(C) Restriction analysis of plasmids pRMC2-$sbi_{1\text{-}335}$, pRMC2-$sbi_{1\text{-}368}$ and pRMC2-$sbi_{1\text{-}403}$.** Plasmids, were cut with *Kpn*I and *Bgl*II. 6500bp fragment pRMC2 backbone in all three lanes. Lane 1, 1005bp fragment $sbi_{1\text{-}335}$; lane 2, 1104bp fragment $sbi_{1\text{-}368}$ and lane 3, 1209bp fragment $sbi_{1\text{-}403}$. Upper bands are undigested plasmid. **(D) Restriction analysis of plasmid pRMC2-*sbi* $\Delta_{335\text{-}368}$.** The *sbi* C-terminal in-frame deletion was amplified by inverse PCR from plasmid DNA of pRMC2-*sbi* to generate pRMC2-*sbi* $\Delta_{335\text{-}368}$. pRMC2-*sbi* $\Delta_{335\text{-}368}$ was cut with *Kpn*I and *Bgl*II. 6500bp fragment corresponds to the pRMC2 backbone in all lanes.



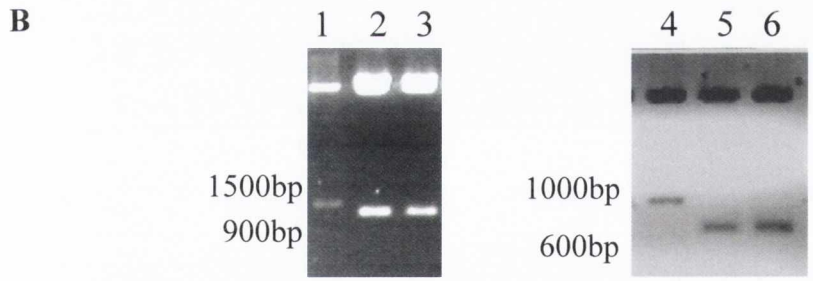


Figure 3.9. (A) Schematic diagram of *sbi* N-terminal in-frame deletions. The *sbi* N-terminal deletions were amplified by inverse PCR from plasmid DNA of pRMC2-*sbi* and pRMC2-*sbi*$_{1-335}$. Primer positions are indicated by black arrows. Plasmids generated were pRMC2-*sbi* Δ_{D1D2}, pRMC2-*sbi* Δ_{D3D4}, pRMC2- *sbi* $_{1-335}\Delta_{D1D2}$ and pRMC2- *sbi* $_{1-335}\Delta_{D3D4}$. The dashed lines indicate the regions deleted. **(B) Restriction analysis of plasmids.** Plasmids, constructs cut with *Kpn*I and *Bgl*II. The 6500bp fragment corresponds to the pRMC2 backbone in all lanes. Lane 1, pRMC2-*sbi*; lane 2, pRMC2-*sbi* Δ_{D1D2}; lane 3, pRMC2-*sbi* Δ_{D3D4},; lane 4, pRMC2-*sbi* $_{1-335}$; lane 5, pRMC2-*sbi* $_{1-335}$ Δ_{D1D2}; lane 6, pRMC2- *sbi* $_{1-335}\Delta_{D3D4}$.

3.3 Discussion

A deletion-substitution mutant was isolated by allelic replacement in the *sbi* gene of *S. aureus* strain Newman using the temperature-sensitive shuttle plasmid pES2. An advantage of generating mutations directly in the strain of interest is that no linked mutations or polymorphisms are likely to be introduced. This is a risk when using directed plasmid integration with suicide vectors which must be integrated in strain RN4220 and then transduced into the target strains. As growth at the restrictive temperature required for allelic replacement can result in secondary mutations, the activity of δ-haemolysin produced by Newman *sbi* was screened on sheep blood agar as a surrogate for Agr function.

The Agr system is a two-component quorum-sensing system which is activated in the late-exponential growth phase. The *agr* locus consists of two divergent transcriptional units, driven by promoters P2 and P3. The P2 operon encodes AgrACDB, components of the sensing system, while the P3 transcript is RNAIII, the effector molecule. AgrC is a transmembrane protein and sensor of the Agr system (Lina *et al.*, 1998). It is activated upon extra cellular accumulation of the secreted auto-inducing peptide (AIP). AIP is the product of AgrD and is post-translationally modified by AgrB (Zhang *et al.*, 2002). AIP binds to the extracellular N-terminal domain of AgrC. At a specific concentration binding induces autophosphorylation of AgrC (Ji *et al.*, 1995). The phosphate moiety is subsequently transferred to the response regulator AgrA which then binds to both the P2 and P3 promoters, inducing production of AIP and transcription of RNAIII (Novick *et al.*, 1995). RNAIII is a 514 nucleotide RNA that as well as acting as a global regulator, encodes the toxin δ-haemolysin. RNAIII positively regulates α-haemolysin by binding to *hla* mRNA. Complementary sequences at the 5′ end of RNAIII prevent *hla* mRNA folding into an untranslatable configuration, thus promoting translation (Novick, 2003). RNAIII negatively regulates the repressor of toxins (Rot). Translation of *rot* is inhibited by the base pairing of RNAIII with the ribosome binding region of *rot* mRNA. Subsequent cleavage of *rot* mRNA is mediated by RNase III (Boisset *et al.*, 2007, Geisinger *et al.*, 2006). A similar mechanism inhibits translation of *spa* mRNA (Huntzinger *et al.*, 2005).

S. aureus produces four haemolytic toxins (α, β, δ and γ) of which the first three can be detected on SBA. β-haemolysin forms a wide turbid zone of haemolysis and is

weakly regulated by Agr. In contrast α- and δ-haemolysins are strongly up-regulated by Agr. δ-haemolysin has very weak activity on SBA but it is strongly synergistic with β-haemolysin. Newman *sbi* was crossed-streaked against *S. aureus* strain RN4220, a strain that produces only β-haemolysin. β-haemolysin forms a turbid zone of haemolysis surrounding the growth of RN4220. A zone of clear haemolysis was produced where β- and δ-haemolysin interact. All mutants of Newman and USA300 described in this chapter were shown to produce the same level of δ-haemolysin as their corresponding wild-type strain indicating that Agr is still functional.

The *sbi*::Em^R mutation was introduced into strain Newman *spa* to create a strain that lacks all IgG binding directly on the bacterial cell surface. Detection of Spa and Sbi is complicated by the fact their molecular weights are very similar and that they both bind the Fc region of IgG. However, the availability of *spa*, *sbi* and *spa sbi* mutants allowed the unambiguous identification of Spa and Sbi by the comparison of mutants by Western blotting using HRP-conjugated rabbit IgG. This approach allowed the precise cellular location of Sbi to be identified and is discussed in detail in Chapter 4.

Analysis of the amino acid sequence of the C-terminal of Sbi revealed that it lacks the typical LPXTG motif that promotes covalent attachment of proteins to the cell wall peptidoglycan of Gram positive bacteria (Zhang et al., 1998). Motifs that occur in proteins such as autolysins that attach non-covalently with the cell wall peptidoglycan and sequences that promote binding of proteins in teichoic acids are not evident. In order to investigate sequences that promote anchorage of Sbi to the cell envelope C-terminal 'Y' domain truncates of *sbi* were cloned in the anhydrotetracycline inducible expression vector pRMC2. Anchoring of Sbi to the cell envelope is discussed in detail in Chapter 4.

Sbi domains D1D2 bind IgG in a non-immune manner (Zhang *et al.*, 1998). Sbi domain D4 binds complement factor C3, whilst domains D3D4 promote futile consumption of C3 (Burman et al., 2008). Therefore it seemed likely that Sbi aids *S. aureus* in avoiding the innate immune defences of the host. The ability of Sbi to contribute to immune evasion will be compared to the well established immune evasion factors Spa, ClfA, IsdH, and Cap using isogenic mutants that were constructed by transduction and allelic replacement. Transduction of the *sbi*::Em^R mutation into the community-acquired MRSA strain USA300 (Diep *et al.*, 2006) allowed the relative

contribution of Sbi to immune evasion to be evaluated in a clinically relevant genetic background. Furthermore the availability of an isogenic *sbi* mutation enabled the comparison of the wild-type and mutant strains in murine models of septic arthritis and sepsis.

The construction of in-frame deletions lacking *sbi* domains D1D2 and D3D4 enabled the relative contribution of both the IgG and C3 binding domains to immune evasion by *S. aureus* to be evaluated separately. The N-terminal truncations of *sbi* were combined with a C-terminal truncation resulting in the induced proteins being secreted. This allowed for the relative contribution, to immune evasion, of both the secreted and cell surface-associated forms of Sbi to be investigated as well as defining the sites of action of the ligand binding domains of Sbi. The immune evasion properties of Sbi are discussed in detail in Chapter 5.

If domains D3D4 are to promote conversion of C3 to C3b they must do so at a distance from the cell, otherwise they would actually promote opsonin formation. The availability of the *spa sbi* double mutant expressing the SbiΔ_{D1D2} protein from pRMC2, allowed the cell surface expression of Sbi domains D3D4 to be measured with specific antibodies without interference by Spa and Sbi D1D2 binding the Fc region of the antibodies. Furthermore the comparison of *spa* and *spa sbi* mutants allowed the ability of Sbi to bind to C3 on the bacterial cell surface to be investigated. Cell surface expression of Sbi and C3 binding on the bacterial cell surface are discussed in detail in Chapter 4.

Chapter 4

Analysis of the cellular localisation of Sbi

4.1 Introduction

Proteins can be anchored to the cell envelope of Gram positive bacteria by several mechanisms as discussed in section 1.3 (Cabanes *et al.*, 2002). Briefly, covalent linkage to cell wall peptidoglycan occurs by the action of sortases (Srt) on the LPXTG motif that is part of a carboxy terminal wall-anchoring domain (Mazmanian et al., 2001). The sorting signal also comprises a hydrophobic membrane-spanning domain followed at the extreme C-terminus by positively charged residues. SrtA covalently attaches the cleaved protein via the C-terminus threonine to the N-terminus of the fifth glycine in the pentaglycine bridge of the nascent peptidoglycan subunits (Ton-That et al., 2000).

Lipoproteins are anchored to the outer face of the cytoplasmic membrane by a hydrophobic interaction between the protein's attached acyl groups and the long chain fatty acids of the membrane (Inouye *et al.*, 1977, Bubeck Wardenburg *et al.*, 2006). Lipoproteins have a conserved motif or lipobox, (L(A/S)(G/A)C) at the C-terminus of the signal peptide (Kovacs-Simon et al., 2011, Halegoua et al., 1977). Lipoprotein diacylglycerol transferase catalyzes transfer of diacylglycerol from phosphatidyl glycerol in the outer face of the membrane to the sulphydryl moiety of the cysteine residue of the lipobox (Sankaran & Wu, 1994). The mature lipoprotein is then anchored to the membrane via its lipid moiety.

Proteins may be anchored non-covalently to the cell wall components peptidoglycan and teichoic acids. Internalin B (InlB) of *Listeria monocytogenes* has C-terminal "GW" repeat domains of ~80 residues that bind to lipoteichoic acid (Jonquieres et al., 1999). Thus InlB is associated with the cytoplasmic membrane in cell fractionation experiments but can also occur extracellularly (Braun et al., 1997). Furthermore, cell-bound InlB can be displaced by soluble LTA and by the highly negatively charged heparin sulphate proteoglycan (Jonquieres *et al.*, 1999). Autolysins such as Alt from *S.aureus* and AtlE from *S.epidermidis* are also attached to the cell envelope via GW repeats (Oshida et al., 1995, Heilmann et al., 1997). The extracellular adherence protein Eap (also known as Map) has repeated domains that can bind to several different ligands (Chavakis et al., 2002). The protein can also bind to the bacterial cell surface by an unknown mechanism and promote attachment to and

invasion of mammalian cells, most likely by binding to fibronectin and forming a bridge to the α5β1 integrin (Hussain *et al.*, 2008, Harraghy et al., 2003)

The teichoic acids (TA) comprising wall teichoic acid (WTA) and lipoteichoic acid are major components of the *S. aureus* cell wall. TAs are constitutively produced and are either covalently linked to peptidoglycan in the case of WTA, or associated with the cytoplasmic membrane in the case of LTA. LTAs are macroamphiphiles composed of glycerol phosphate polymers attached to the cytoplasmic membrane by a glycolipid anchor (Wicken & Knox, 1975, Fischer, 1988). LTA biosynthesis occurs by the transfer of glycerol phosphate units form phosphatidylglycerol, a major constituent of bacterial membranes. LTA is polymerised directly on the glycolipid membrane anchor (Fischer, 1988). Three genes have been identified that are necessary for the biosynthesis of the LTA backbone (Figure 4.1). The *ypfP* gene encodes the YpfP enzyme that is responsible for synthesis of the anchor. YpfP transfers two glucose residues from UDP-glucose to diacylglycerol (Kiriukhin *et al.*, 2001). Inactivation of *ypfP* does not inhibit biosynthesis of LTA but results in LTA with diacylglycerol as the membrane anchor (Fedtke *et al.*, 2007). The *ltaA* gene encodes a membrane protein that is believed to translocate the glycolipid from the inner to the outer leaflet of the cytoplasmic membrane (Grundling & Schneewind, 2007a). The *ltaS* gene encodes the LTA polymerase LtaS which utilises the glycerol phosphate units from phosphatidylglycerol to synthesise the LTA polymer backbone in the outer leaflet of the cytoplasmic membrane (Grundling & Schneewind, 2007b). LTA is modified with D-alanine at the 2-hydroxyl group of glycerol. D-alanine modification of LTA is controlled by the *dltABCD* genes. The *dltC* encodes a D-alanyl carrier protein (Dcp). The *dltA* gene encodes a ligase that catalyses the formation of a D-alanyl-Dcp complex in the cytoplasm. The *dltB* gene encodes an integral membrane protein whilst the *dltD* gene encodes a hydrophilic membrane anchored protein (Figure 4.1). Both are required for translocation and incorporation of D-alanine into LTA (Debabov *et al.*, 2000, Neuhaus & Baddiley, 2003).

Sbi comprises four N-terminal ligand-binding domains. The first two N-terminal domains D1 and D2 have 30% sequence similarity to the IgG binding domains of protein A (Zhang et al., 1998). Sbi domains D3 and D4 are also separately folded and contribute to the elongated structure of the protein (Upadhyay et al., 2008). Sbi domain

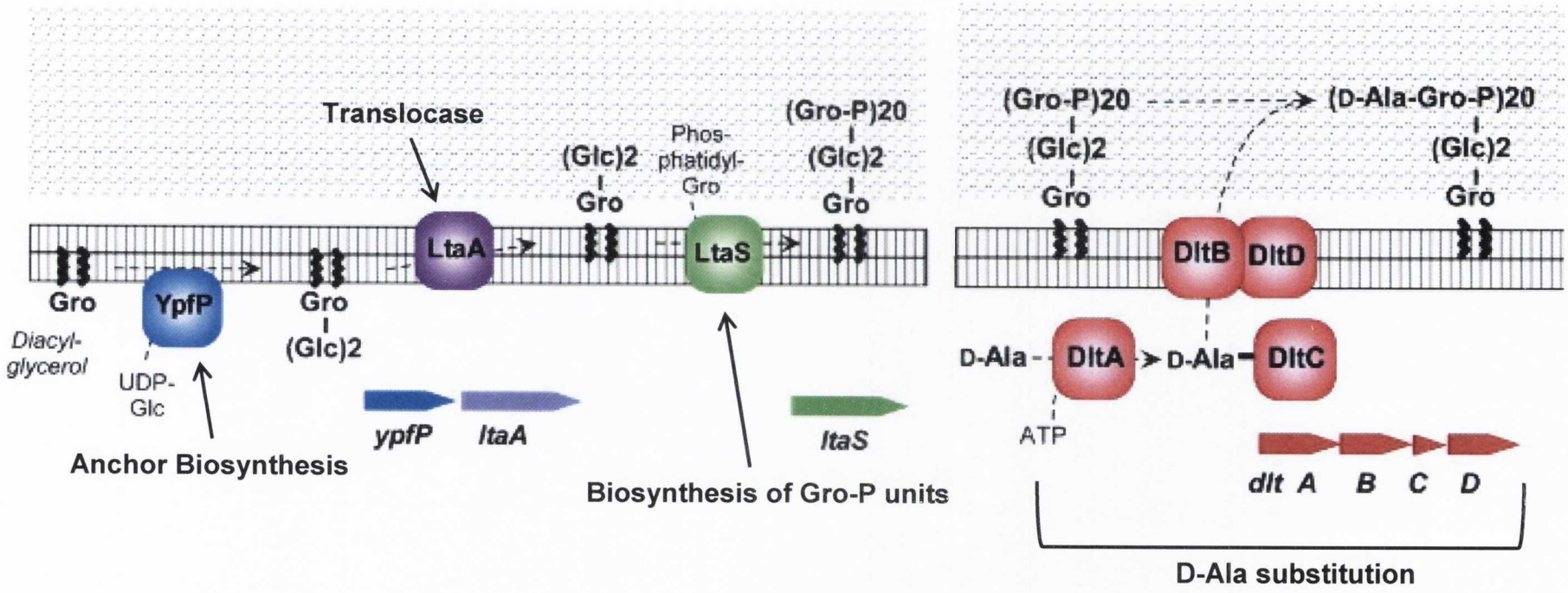


Figure 4.1 Biosynthesis of Lipoteichoic Acid

Biosynthesis pathways of lipoteichoic acid (LTA) and D-Alanine activation and incorporation. The glycolipid anchor is generated by the YpfP enzyme. LtaA translocates the glycolipid anchor from the inner to the outer membrane. LtaS synthesis the Gro-P backbone of LTA on the outer face of the membrane by utilizing Gro-P subunits from phosphatidylglycerol. The DltABCD proteins facilitate the activation and incorporation of D-Alanine residues into the Gro-P backbone. Gro, glycerol; UDP-Glu, uridine-5-diphosphate-glucose; Glc, glucose. Diagram reproduced from Xia *et al.*, 2010

D4 binds to complement factor C3 whilst domains D3D4 in complex have been shown to result in the consumption of C3 (Burman et al., 2008). The C-terminus of Sbi consists of a proline rich repeat region "Wr" followed by a tyrosine and threonine rich region "Y". The Sbi C-terminus lacks motifs associated with anchorage to the cell wall or cytoplasmic membrane.

The exact cellular location of Sbi has not yet been elucidated although it appears to be present on the *S. aureus* cell surface and also in the extracellular media (Burman et al., 2008). The pKa of Sbi is 9.8 indicating the protein is basic, and it has been suggested that electrostatic interactions play a role in its anchoring to the cell surface via acidic residues in teichoic acids, like InlB of *L. monocytogenes* (Jonquieres et al., 1999). In this chapter *in silico* analyses of the primary sequence of Sbi was carried out to predict the likely structure and localisation of Sbi. In parallel with *in silico* analyses, fractionation studies of the *S. aureus* cell envelope were undertaken to localise Sbi. By analyzing mutants lacking Sbi and Spa, the precise cellular localisation of Sbi was elucidated. The ability of the four Sbi ligand binding domains to bind their respective ligands on the bacterial cell surface was investigated. By expressing truncated Sbi in *S. aureus* and using purified recombinant Sbi, the role of the C-terminal Y domain in anchoring Sbi to the cell envelope was investigated. Finally the ability of lipoteichoic acid to mediate anchoring of Sbi to the cell envelope was examined.

4.2 Results

4.2.1 Cellular location of Sbi

The Sbi protein does not contain domains that are found in proteins that are covalently or non-covalently associated with cell wall peptidoglycan or the cytoplasmic membrane of Gram-positive bacteria. It is not clear from previous studies if Sbi is cell surface-associated. This study set out to determine unambiguously which cellular compartment(s) carry Sbi and whether Sbi is expressed on the cell surface. To achieve this, a mutant defective in Sbi was constructed by allelic exchange to delete base pairs 20-1270 and replace them with an *erm*C cassette as discussed in section 3.2.1. The *sbi* mutation was combined with a mutation in the *spa* gene. Cells were grown to the mid-exponential and stationary phases. After centrifugation to sediment cells, a sample of supernatant was concentrated ten-fold by TCA precipitation. The cells were adjusted to the same density in a high concentration of raffinose to protect protoplasts when the cell wall peptidoglycan was dissolved with lysostaphin. This solubilised any cell wall-associated proteins. The stabilised protoplasts were lysed and the membrane fragments were sedimented and the supernatant was retained as the cytoplasmic fraction. Each fraction was analysed by SDS-PAGE and immunoblotting. Detection of Spa and Sbi is complicated by the fact that their molecular weights are very similar and that they both bind the Fc region of IgG. Identification of Spa and Sbi relied upon the comparison of mutants using HRP-conjugated rabbit IgG. Samples were also probed with antibodies specific for the cell wall-anchored protein SdrE (Josefsson *et al.*, 1998) and the integral membrane protein EbpS (Downer *et al.*, 2002) which served as controls for both sample loading and the purity of fractions. These proteins are sufficiently different in size from Spa and Sbi to be well separated from them by SDS-PAGE.

The solubilised cell wall material probed with HRP-IgG had a ~50kDa band present in the wild-type and *sbi* mutant samples which was missing in the *spa* mutant fraction (Figure 4.2, panel A(i)). This is clearly protein A. The absence of any reactive protein in the *spa* mutant sample showed that Sbi is not a wall-anchored protein. Analysis of the membrane fraction revealed a ~50kDa band in the wild-type and *spa* mutant samples that was absent in the *sbi* mutant (Figure 4.2, panel B(i)). This is Sbi and strongly suggests that the protein is associated with the cytoplasmic membrane. The culture supernatant samples were also probed to reveal both Sbi and Spa in the samples

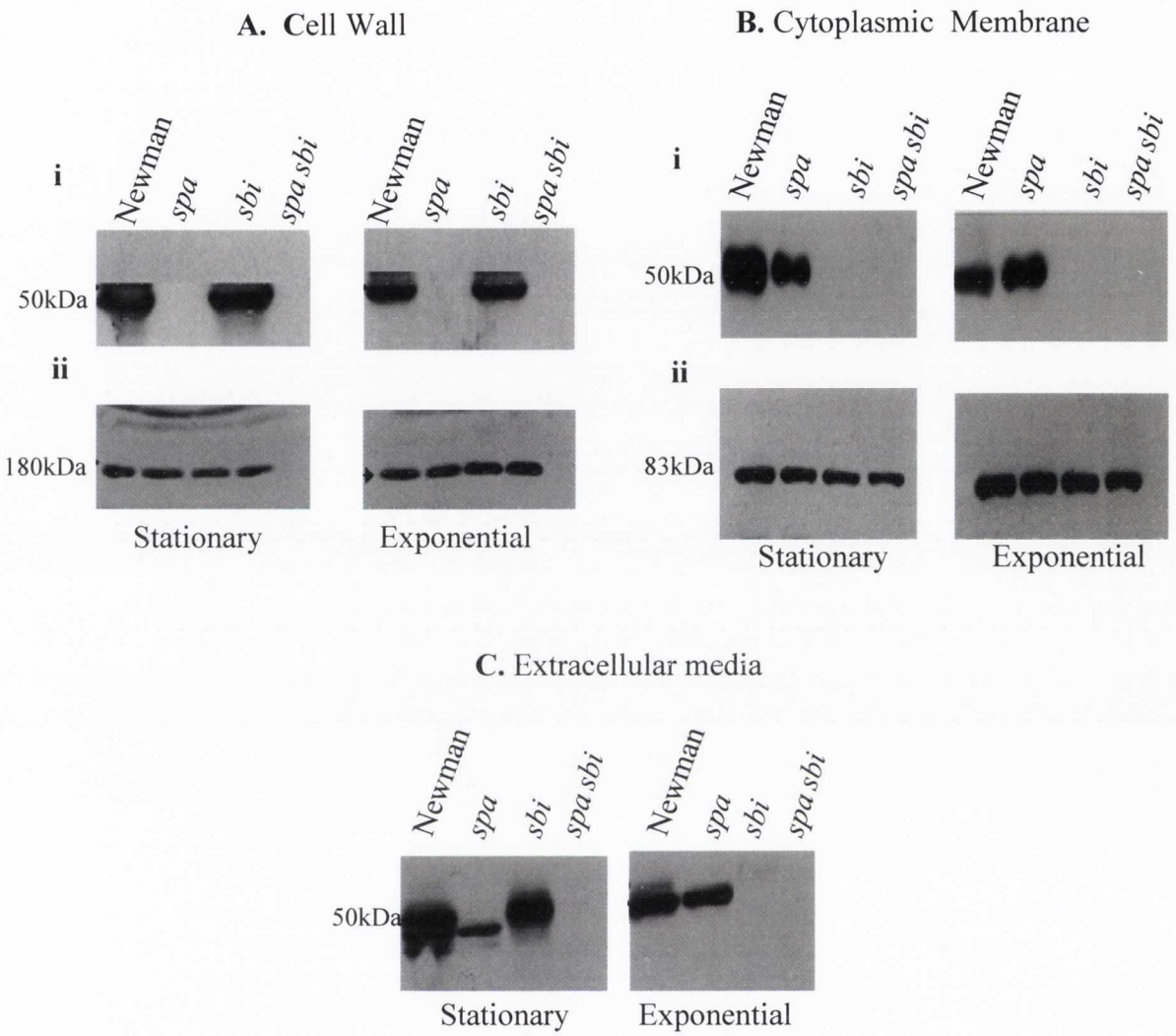


Figure 4.2 Cellular localisation of Sbi

Sbi and Spa have similar molecular weights and co-migrate in SDS-PAGE minigels. Both proteins were recognized by HRP-IgG that bound to the Fc binding domains. Cells from stationary and exponential phase were tested. (**A**) Cell wall fractions. Gels were analysed by Western blotting with HRP-labelled rabbit IgG or rabbit anti-SdrE antiserum followed by HRP-labelled goat anti-rabbit IgG. (**B**) Cytoplasmic membrane fraction. Gels were analysed by Western blotting with HRP-labelled rabbit IgG or rabbit anti-EbpS antiserum followed by HRP-labelled goat anti-rabbit IgG. (**C**) Supernatant fraction probed with HRP-labelled rabbit IgG. These are representatives of 3 independent experiments.

from the stationary phase but only Sbi was detected in the exponential phase samples (Figure 4.2, panel C).

4.2.2 *In silico* analysis of Sbi

As determined by fractionation studies Sbi is present in the extracellular media and the cytoplasmic membrane of *S. aureus*. Therefore, Sbi could be an integral membrane protein. However the sequence does not possess the stretch of 15-30 hydrophobic residues necessary to traverse the membrane. The Sbi sequence was analysed by Kyte-Doolittle hydropathy plotting for putative transmembrane regions. Kyte-Doolittle plots provide information about the putative structure of a protein based on hydrophobic residues in the amino acid sequence. Residues are given a hydrophobicity score between -4.5 (very hydrophilic) and 4.5 (very hydrophobic). After input of a protein sequence, a window size is set to for displaying the data. A window size of 19 residues gives the best results when searching for a potential transmembrane sequence. The hydrophobicity score for this sequence is averaged and the mean score assigned to the middle residue in the window. Then the computer program calculates the average of all the hydrophobicity scores in the next window, which is one amino acid down from the previous window. The pattern continues to the end of the sequence, computing the average score for each window and assigning it to the middle amino acid in the sequence. The averages are then plotted on a graph. Analysis of the Sbi sequence from *S. aureus* Newman did not reveal any significant regions of hydrophobicity in the entire protein other than the N-terminal signal sequence (Figure 4.3, panel A(i)). The elastin-binding protein (EbpS) of *S. aureus*, which is an integral membrane protein, was also analysed by this method. EbpS produced three peaks that correspond to the reported hydrophobic domains H1 (205-224), H2 (265-280) and H3 (315-342) (Figure 4.3, panel A(ii); Downer et al., 2002).

Hidden Markov Models (TMHMM) predict transmembrane α-helices by combining the hydrophobic signal and the charge bias signal in which an abundance of positively charged residues are usually located in the protein sequence at the cytoplasmic side of a cell membrane (the 'positive-inside' rule). The Hidden Markov Model (a pattern recognition algorithm) is well suited for the prediction of transmembrane helices because it can incorporate hydrophobicity, charge bias, helix lengths, and biological 'rules' (e.g. that loops of transmembrane proteins must alternate

between the cytoplasmic and non-cytoplasmic sides) into one model for which algorithms for parameter estimation and prediction already exist. This program was successfully used to predict two membrane spanning domains (H1 and H3) in EbpS (Figure 4.3, panel B(i)). However it failed to identify any putative membrane-spanning regions in Sbi (Figure 4.3, panel B(ii)).

Another web-based program for the prediction of possible transmembrane regions is the dense alignment surface (DAS) method. A scoring matrix previously derived from a collection of non-homologous membrane proteins is used to generate low-stringency dot-plots of the query sequence. In this way, the DAS method improves the prediction abilities of protein sequences that have no known homologues. When analysed in this way, the Sbi sequence yields a potential 9-residue membrane-associated region, at position 354 (Figure 4.3, panel C(i)). The DAS method predicts three transmembrane regions in the EbpS sequence, corresponding to H1, H2 and H3 of the protein (Figure 4.2, panel C(ii)).

4.2.3 Surface expression of Sbi ligand binding domains

In order to determine if Sbi is exposed on the cell surface a Newman *spa* mutant and a Newman *spa sbi* mutant expressing Sbi from the anhydrotetracycline-inducible expression vector pRMC2 were compared by whole cell immunoblotting (Figure 4.4, panel A). Doubling dilutions of cell suspensions were probed with HRP-conjugated rabbit IgG that bound to the N-terminal domains D1D2 of Sbi (Figure 4.4, panel B). The specificity of the detection method is indicated by the absence of any reaction in the *spa sbi* (pRMC2) sample. This showed that the IgG binding domains of the cell-associated form of Sbi are exposed on the cell surface, despite fractionating with the cytoplasmic membrane.

A mutant of pRMC2-*sbi* was constructed which expressed a truncate that lacked the IgG binding D1D2 domains. Western immunoblotting demonstrated that the protein was expressed at the same level as wild type Sbi and that it could be detected in the membrane fraction (Figure 4.4, panel A). Cells were resuspended in buffer at an OD_{600nm} of 50 and serial dilutions were pipetted onto a nitrocellulose membrane and probed with anti-Sbi D3D4WrY antibodies. Cells expressing wild-type Sbi could bind antibodies via domains D1 and D2 by a non-immune reaction as well as by a specific

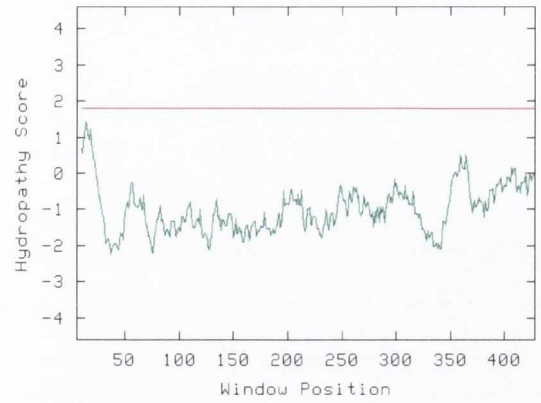
Figure. 4.3 Bioinformatic analysis of Sbi

(**A**) Kyte-Doolittle hydropathy plotting for putative transmembrane regions of Sbi. (i) The primary sequence of Sbi from *S. aureus* Newman was analysed by Kyte-Doolittle hydropathy plotting. Putative transmembrane regions in the protein sequence exceed a score of 1.8 (red line). (ii) The *S. aureus* cytoplasmic membrane-associated protein EbpS was also analysed.

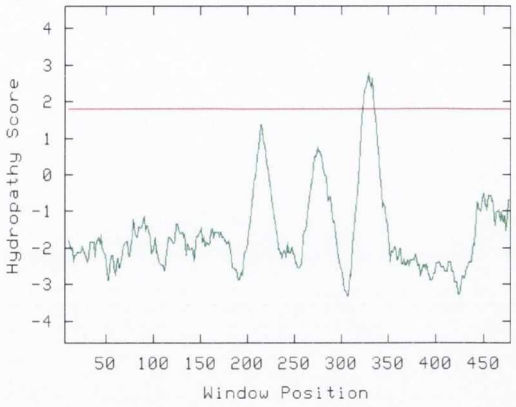
(**B**) Prediction of transmembrane α-helices on Sbi using the TMHMM program. (i) The primary sequence of Sbi from *S. aureus* Newman was analysed for potential transmembrane α-helices using the TMHMM program which utilises both the hydropathy index of the protein sequence and current knowledge of membrane insertion mechanisms. (ii) The program correctly predicted two membrane spanning domains (H1 and H3) in EbpS.

(**C**) Prediction of possible transmembrane regions of a given peptide sequence using the dense alignment surface (DAS) programme. (i) Again the primary sequence of Sbi from *S. aureus* Newman was analysed for potential transmembrane regions by DAS. A scoring matrix previously derived from a collection of non-homologous membrane proteins is used to generate low-stringency dot-plots of the query sequence. (ii) EbpS was also analysed by DAS. The program correctly predicted two membrane associated domains (H1 and H3).

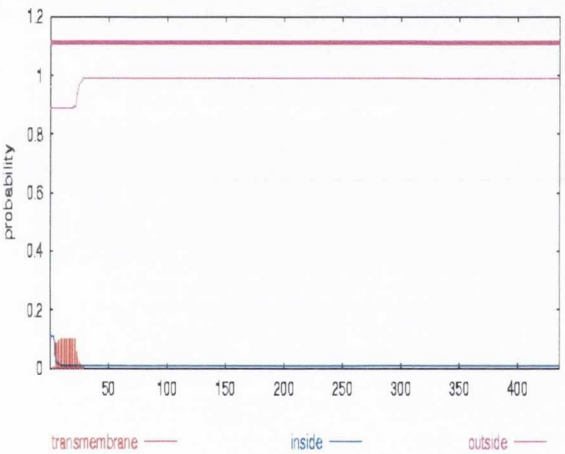
A (i)



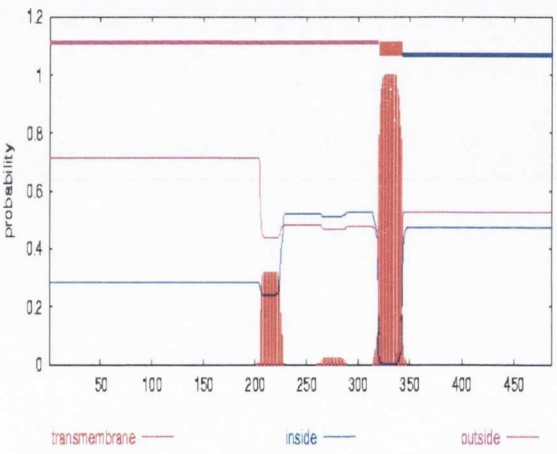
(ii)



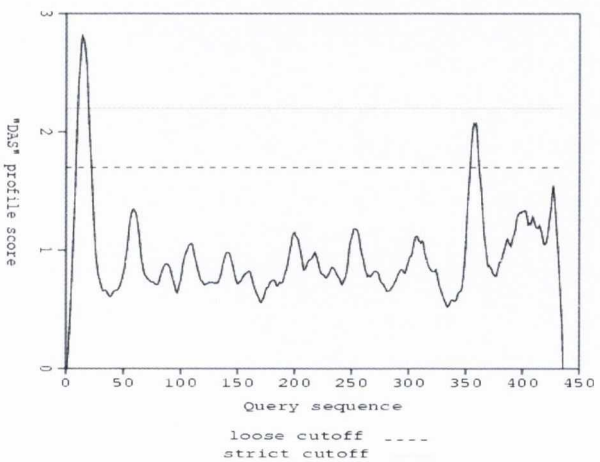
B (i)



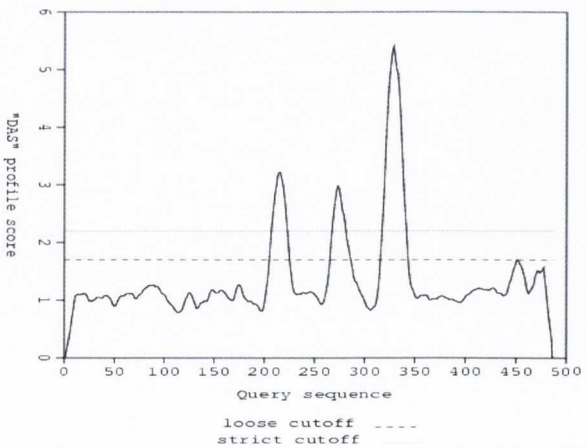
(ii)



C (i)



(ii)



immune reaction with domains D3 and D4. The same reactivity was seen with cells expressing wild-type Sbi from the chromosomal gene and when induced from pRMC2-*sbi* (Figure 4.4, panel C). In contrast cells expressing the truncated form of Sbi reacted 16-32-fold less. Given that D1 and D2 can bind to a single Fc region each whereas D3D4 most likely has several epitopes for polyclonal IgG Fab, it can be concluded that the majority of D3D4 epitopes are buried within the cell wall and are not exposed on the cell surface.

4.2.4 Can Sbi domains D3D4 bind complement factor C3 on the cell surface?

Analysis of intact whole cells showed that the IgG-binding domains D1 and D2 are surface-exposed and by binding IgG could contribute to immune evasion. Conversely domains D3 and D4 are only partly exposed on the cell surface. Recombinantly expressed domains D3D4 of Sbi have been reported to bind complement component C3 with high affinity (Burman *et al.*, 2008). This was confirmed here in an ELISA with recombinant GST-tagged Sbi lacking D1D2 binding to purified human C3 in a dose-dependent and saturable fashion with a half maximum concentration of 11 nM (Figure 4.5, panel A).

In order to determine if the D3D4 domains of Sbi can bind purified human C3 to the bacterial cell surface, *S. aureus* Newman *spa* and Newman *spa sbi* cells were coated onto microtitre plates and incubated with purified human C3 followed by polyclonal goat anti-C3 antiserum and HRP-conjugated protein A. The two strains bound equally well to the plastic surface when controls were probed with antibodies specific for the cell wall-anchored protein ClfA (data not shown). C3 bound weakly to whole cells of both strains with no difference between the single and the double mutant indicating that Sbi D3D4 cannot bind C3 detectably on the bacterial cell surface (Figure 4.5, panel B). Therefore, in serum, cell-bound Sbi D3D4 would be unlikely to be able to promote complement fixation.

4.2.5 Sbi binding to the cytoplasmic membrane

To address the importance of the C-terminal domain of Sbi in membrane anchoring, three maltose binding protein (MBP) fusion proteins were constructed (Figure 4.6, panel A). These comprised the entire Sbi protein (residues 41-436), the N-terminal ligand binding domains (residues 41-253) and the C-terminal domains Wr and

Y (residues 253-436). The proteins were expressed in *E.coli* and purified by affinity chromatography. Their purity and integrity were verified by SDS-PAGE and Western blotting with anti-MBP antiserum (Figure 4.6, panel B and C).

Cytoplasmic membrane material purified from *S. aureus* Newman *spa sbi* was incubated in microtitre plates. Coating of the surface was verified with antibodies recognizing the integral membrane protein EbpS (Figure 4.6, panel D). The membranes were incubated with MBP-Sbi_{41-436} and MBP-$Sbi_{254-436}$ which were able to bind in a dose-dependent and saturable manner with half maxima of 0.32 nM and 0.16 nM, respectively, whilst MBP-Sbi_{41-253} and the MBP control were unable to bind (Figure 4.6, panel E). These results indicate that the C-terminal WrY domain of Sbi binds strongly to purified cytoplasmic membrane material from a *spa sbi* mutant.

4.2.6 Recombinant Sbi binds to whole cells and fractionates with the cytoplasmic membrane

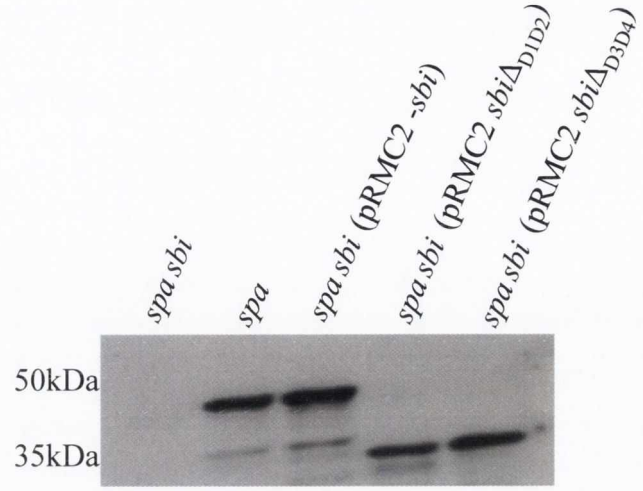
Recombinant MBP-Sbi binds to purified cytoplasmic membrane material with high affinity. To address whether this mode of association is similar to that of Sbi expressed by *S. aureus*, recombinant proteins MBP-Sbi_{41-436}, MBP-Sbi_{41-253} and MBP-$Sbi_{254-436}$ were separately incubated with whole cells of Newman *spa sbi*. MBP-Sbi_{41-436} and MBP-$Sbi_{254-436}$ both bound to whole cells dose-dependently and saturably with similar affinities (0.87 nM and 0.47 nM, respectively) (Figure 4.7, panel A). Binding of recombinant MBP-Sbi_{41-436}, MBP-Sbi_{41-253}, MBP-$Sbi_{254-436}$ and MBP to whole cells of strain Newman *spa sbi* was also investigated by flow cytometry. Recombinant Sbi derivatives were separately incubated with whole cells of Newman *spa sbi* and binding was detected with monoclonal mouse anti-MBP antiserum followed by FITC-labelled rabbit anti-mouse IgG. MBP-Sbi_{41-436} and MBP-$Sbi_{254-436}$ both bound to whole cells confirming the ELISA result. (Figure 4.7, panel B and C).

In order to determine if recombinant Sbi that bound to the bacterial cells fractionated with the cell wall or membranes during protoplast formation, and to determine if a receptor for Sbi is exposed on the cell surface, Newman *spa sbi* was incubated with MBP-Sbi_{41-436} and MBP-$Sbi_{254-436}$, washed and then fractionated to isolate the cytoplasmic membrane. Figure 4.7, panel D shows the purified cytoplasmic membrane fraction probed with HRP-conjugated anti-MBP antiserum. The ~86kDa

Figure 4.4 Surface expression of Sbi.

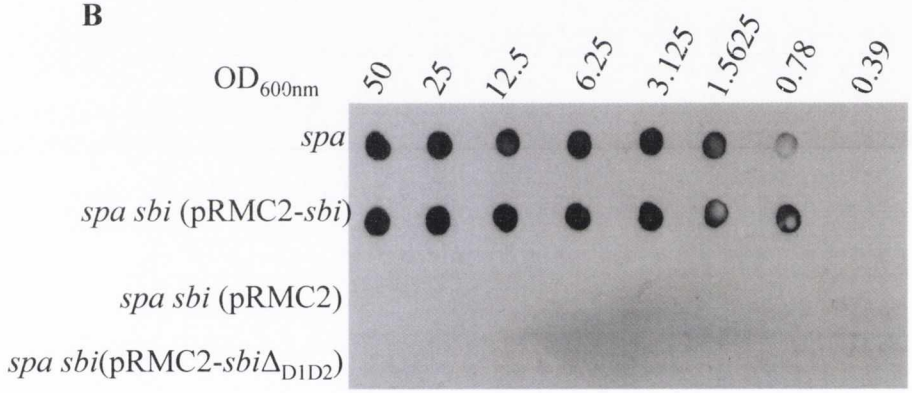
(**A**) Cytoplasmic membrane fractions were analysed by Western blotting with rabbit anti-SbiD3D4WrY antiserum followed by HRP-labelled goat anti-rabbit IgG. (**B**) Serial dilutions of cells were applied to a nitrocellulose membrane and probed with HRP-labelled rabbit IgG. (**C**) Serial dilutions of cells were applied to a nitrocellulose membrane and probed with rabbit anti-Sbi D3D4WrY antiserum followed by HRP-conjugated goat anti-rabbit IgG.

A



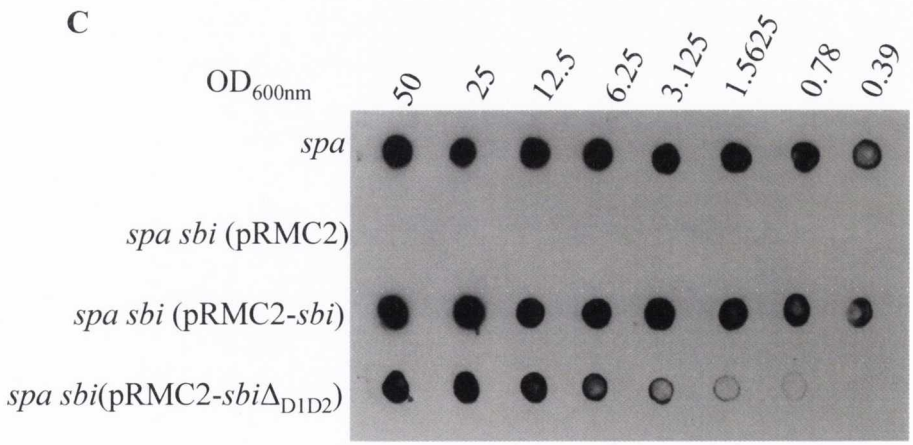
Cytoplasmic membrane

B



Probe: Rabbit IgG

C



Probe: Rabbit anti-Sbi D3D4WrY

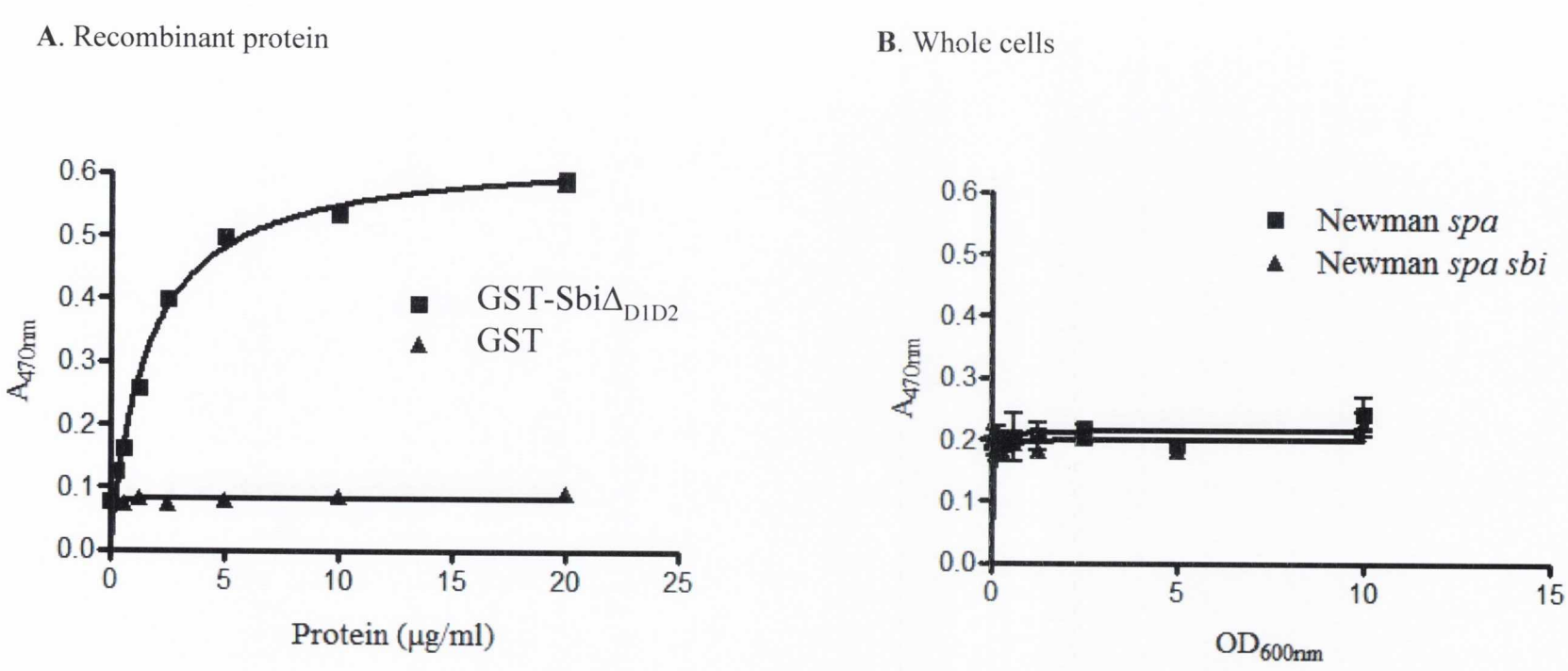
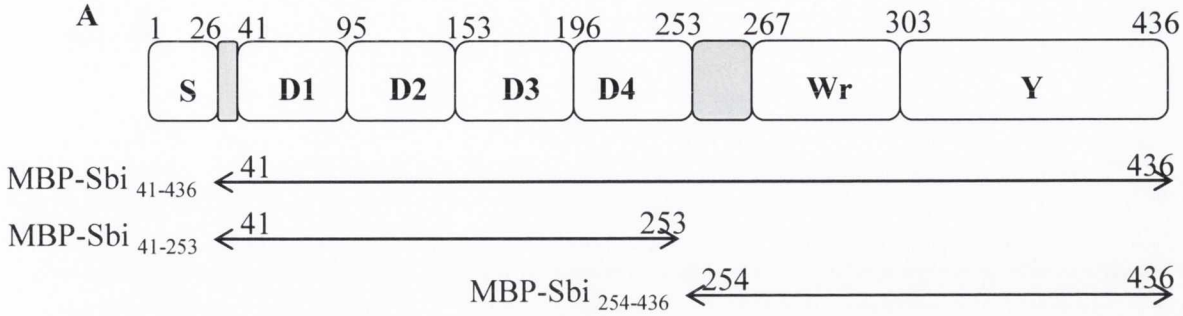


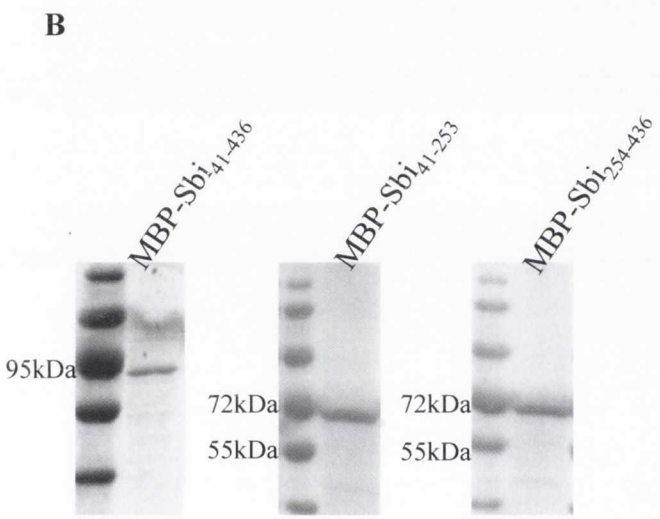
Figure 4.5 Complement factor C3 binding

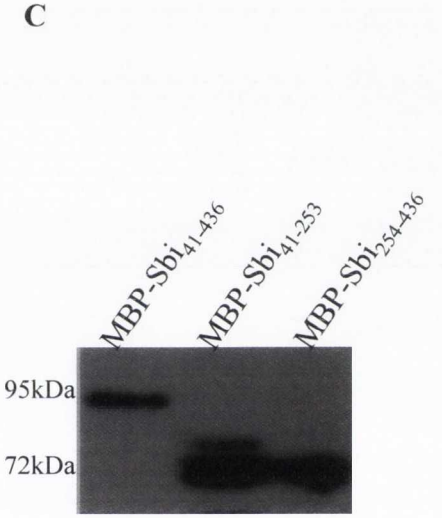
Complement factor C3 binding to (**A**) recombinant GST-tagged SbiΔ_{D1D2} and (**B**) whole cells of strains Newman *spa* and Newman *spa sbi*. C3 was incubated with wells coated with concentrations of GST-SbiΔ_{D1D2} ranging from 0-20 nM or whole cells OD_{600nm} ranging from 0-10. Binding was detected with rabbit anti-C3 antiserum followed by goat anti-rabbit IgG. Graphs shown are representative of three independent experiments.

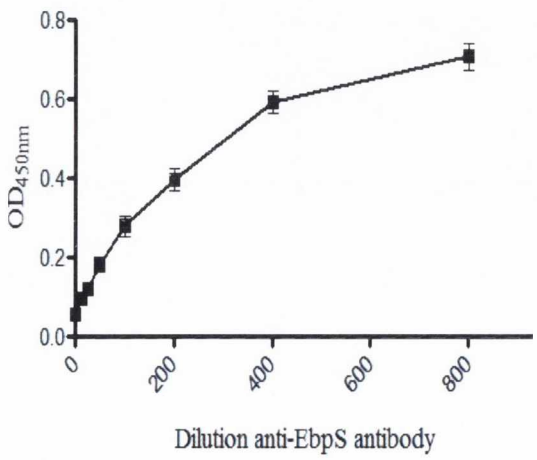
Figure 4.6 Binding of MBP-Sbi$_{41-436}$, MBP-Sbi$_{41-253}$ and MBP-Sbi$_{254-436}$ to purified cytoplasmic membrane

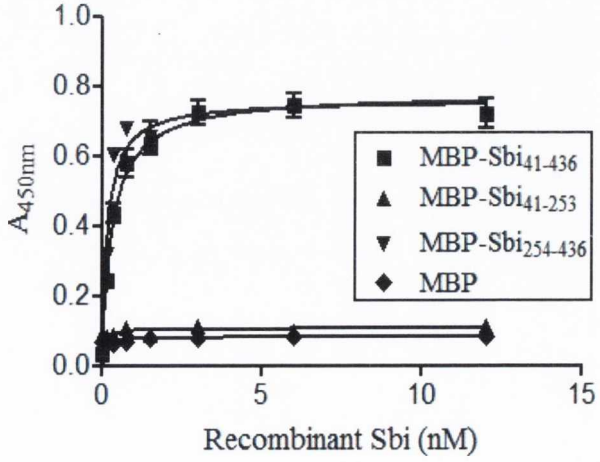
(**A**) Schematic diagram of Sbi showing the residues present in each recombinant MBP-tagged protein. (**B**) Coomassie stain of an SDS-PAGE gel of MBP-Sbi recombinant proteins. (**C**) Western immunoblot of MBP-Sbi recombinant proteins probed with HRP-conjugated anti-MBP antiserum. (**D**) Cytoplasmic membrane fragments purified from *S. aureus* Newman *spa sbi* were incubated in microtitre plates. Coating of the surface was verified with rabbit antibodies recognizing the integral membrane protein EbpS followed by HRP-conjugated goat anti-rabbit IgG. (**E**) Binding of MBP-Sbi$_{41-436}$, MBP-Sbi$_{254-436}$ MBP-Sbi$_{41-253}$ and MBP to cytoplasmic membrane-coated wells. Recombinant protein binding was detected with HRP-conjugated anti-MBP antiserum. The graphs shown are representatives of three separate experiments. Error bars show the standard deviation.


A
1 26 41 95 153 196 253 267 303 436
S
D1
D2
D3
D4
Wr
Y
MBP-Sbi 41-436
41
436
MBP-Sbi 41-253
41
253
MBP-Sbi 254-436
254
436


B
MBP-Sbi41-436
MBP-Sbi41-253
MBP-Sbi254-436
95kDa
72kDa
55kDa
72kDa
55kDa


C
MBP-Sbi41-436
MBP-Sbi41-253
MBP-Sbi254-436
95kDa
72kDa


D
OD450nm
0.0
0.2
0.4
0.6
0.8
0
200
400
600
800
Dilution anti-EbpS antibody


E
A450nm
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
Recombinant Sbi (nM)
MBP-Sbi41-436
MBP-Sbi41-253
MBP-Sbi254-436
MBP

band present in lane 3 corresponds to recombinant MBP-Sbi_{41-436} whilst the ~63kDa band in lane 4 corresponds to recombinant MBP-$Sbi_{254-436}$ (Figure 4.7, panel D). These data show that a receptor for the C-terminal domain of Sbi is exposed on the cell surface and yet the added recombinant protein is associated with the membrane following fractionation of protoplasts.

4.2.7 Sbi binds to lipoteichoic acid

The C-terminal region of Sbi has neither a sequence of hydrophobic residues sufficient to span the cytoplasmic membrane typical of integral or membrane-spanning proteins nor a lipoprotein consensus sequence that could be involved in anchoring the protein to the cytoplasmic membrane, raising the possibility that the association of Sbi with the cytoplasmic membrane could be due to binding to a membrane-associated component. Lipoteichoic acid (LTA) is an anionic polymer linked to a glycolipid anchor in the outer face of the cytoplasmic membrane and a poly(glycerophosphate) chain that extends across the cell wall (Neuhaus & Baddiley, 2003). LTA remains associated with the protoplast after removal of the cell wall peptidoglycan by lysostaphin (Neuhaus & Baddiley, 2003). To determine if LTA could be the target for the C-terminal Y domain of Sbi, wells of microtitre plates were coated with a constant amount of purified *S. aureus* LTA and incubated with increasing concentrations of recombinant MBP-Sbi_{41-436}, MBP-Sbi_{41-253} and MBP-$Sbi_{254-436}$. Proteins containing the C-terminal domain Wr-Y (Sbi_{41-436} and $Sbi_{254-436}$) were able to bind LTA in a dose-dependent and saturable manner with half maximal concentrations of 2.9 nM and 1.5 nM, respectively (Figure 4.8, panel A). Furthermore preincubation of Sbi with different concentrations of *S. aureus* LTA inhibited binding to immobilized LTA and to purified cytoplasmic membranes in a dose-dependent manner (Figure 4.8, panel B).

To investigate the specificity of the interaction between Sbi and LTA, MBP-Sbi was preincubated with different concentrations of heparin sulfate, an anionic glycosaminoglycan which consists of a repeating disaccharide unit of glucosamine and uronic acid residues and which occurs on the surface of mammalian cells (Li & Vlodavsky, 2009). Heparin sulphate (HS) was able to displace InlB that was bound to LTA from the surface of *L.monocytogenes* but was not able to inhibit the interaction of MBP-Sbi with immobilized LTA or purified cytoplasmic membrane material (Figure 4.8, panel C), suggesting that the interaction between LTA and Sbi is specific and not

simply due to the positively charged protein binding to negatively charged residues in surface polymers.

4.2.8 Can Sbi bind to purified cell wall?

The cell wall contains wall teichoic acid, a polymer of ribitol phosphate that is covalently anchored to the peptidoglycan (Neuhaus & Baddiley, 2003, Xia et al., 2010) as well as covalently anchored wall-associated proteins such as ClfA and Spa. The cell wall fraction of *S. aureus* Newman *spa sbi* was purified following disruption of the cells by mechanical shearing and by boiling with detergent to solubilise the cytoplasmic membrane.

The coating affinity and purity of the cell wall fraction was assessed by ELISA using (i) polyclonal anti-ClfA antiserum, (ii) polyclonal anti-EbpS antiserum and (iii) monoclonal anti-LTA antiserum (Figure 4.9, panel A, B and C). This showed that the wall fraction bound to the ELISA wells efficiently and that the level of contamination with membrane was low. MBP-Sbi_{41-436} and MBP-$Sbi_{254-436}$ was added to the cell wall-coated ELISA wells but did not bind detectably. This shows that Sbi cannot bind to the purified cell wall fraction (Figure 4.9, panel D).

4.2.9 Displacement of Sbi from purified cytoplasmic membrane by soluble LTA

In order to determine if soluble LTA could displace Sbi from the membrane of *S. aureus* cells, purified cytoplasmic membranes of the Sbi^+ strain Newman *spa* were incubated with different concentrations of LTA. Some Sbi was displaced into the supernatant in a concentration-dependant manner whilst the majority remained associated with the membrane (Figure 4.10, panel A). This indicates that Sbi is attached to the membrane with high affinity at least in part by binding to LTA and suggests the possibility of a second ligand.

4.2.10 Detection of the Sbi-LTA interaction by Far Western blotting

The experiment described above in section 4.2.8 shows that recombinant Sbi can bind to LTA. In order to determine if Sbi expressed by *S. aureus* binds LTA a Far Western blotting approach was taken. The membrane fraction was purified from *S. aureus* Newman *spa sbi* lacking the ability to express the IgG binding proteins Spa and Sbi from chromosomal genes but expressing $Sbi\Delta_{D1D2}$ from pRMC2. The membrane

Figure 4.7 Binding of MBP-Sbi$_{41-436}$, MBP-Sbi$_{41-253}$ and MBP-Sbi$_{254-436}$ to whole cells of Newman *spa sbi*.

(**A**) Binding of MBP-Sbi$_{41-436}$, MBP-Sbi$_{254-436}$ MBP-Sbi$_{41-253}$ and MBP to wells coated with whole Newman *spa sbi* cells. Recombinant protein binding was detected with HRP-conjugated anti-MBP IgG. (**B**) Washed whole cells of Newman *spa sbi* were incubated with 0.5μM of MBP-Sbi$_{41-436}$, MBP-Sbi$_{254-436}$, MBP-Sbi$_{41-253}$ and MBP followed by mouse anti-MBP antiserum and FITC-labelled rabbit anti-mouse IgG. Fluorescence intensity was measured by flow cytometry. (**C**) A representative flow cytometry trace of recombinant Sbi derivatives binding to Newman *spa sbi*. (**D**) Recombinant MBP-Sbi$_{41-436}$, MBP-Sbi$_{254-436}$ MBP-Sbi$_{41-253}$ and MBP were incubated with whole cells of Newman *spa sbi* and fractionated. Cytoplasmic membrane fractions were analysed by Western blotting with HRP-conjugated anti-MBP IgG or rabbit anti-EbpS IgG followed by HRP-conjugated protein A. These are representatives of three separate experiments. Error bars show the standard deviation.

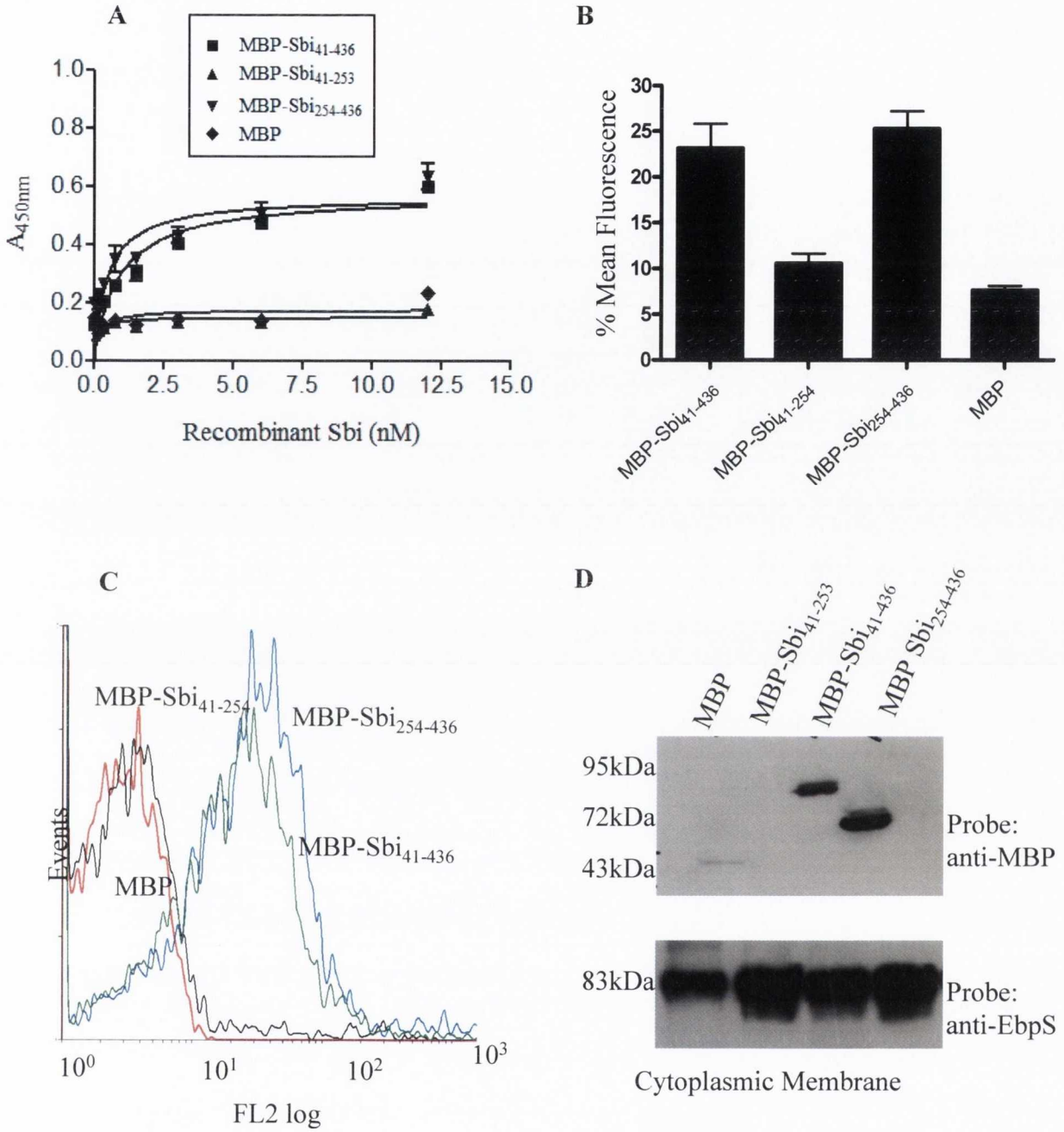
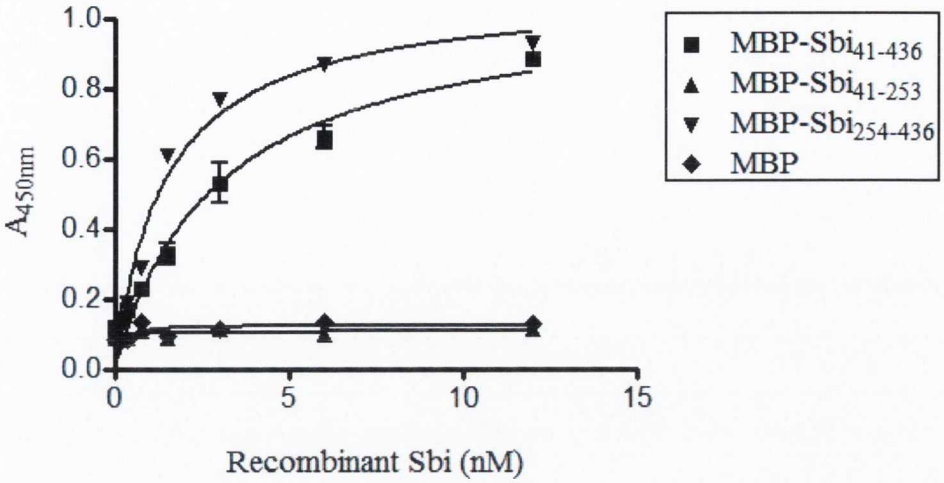

A
MBP-Sbi41-436
MBP-Sbi41-253
MBP-Sbi254-436
MBP
A450nm
Recombinant Sbi (nM)
B
% Mean Fluorescence
MBP-Sbi41-436
MBP-Sbi41-254
MBP-Sbi254-436
MBP
C
MBP-Sbi41-254
MBP-Sbi254-436
MBP-Sbi41-436
MBP
Events
FL2 log
D
MBP
MBP-Sbi41-253
MBP-Sbi41-436
MBP Sbi254-436
95kDa
72kDa
43kDa
Probe:
anti-MBP
83kDa
Probe:
anti-EbpS
Cytoplasmic Membrane

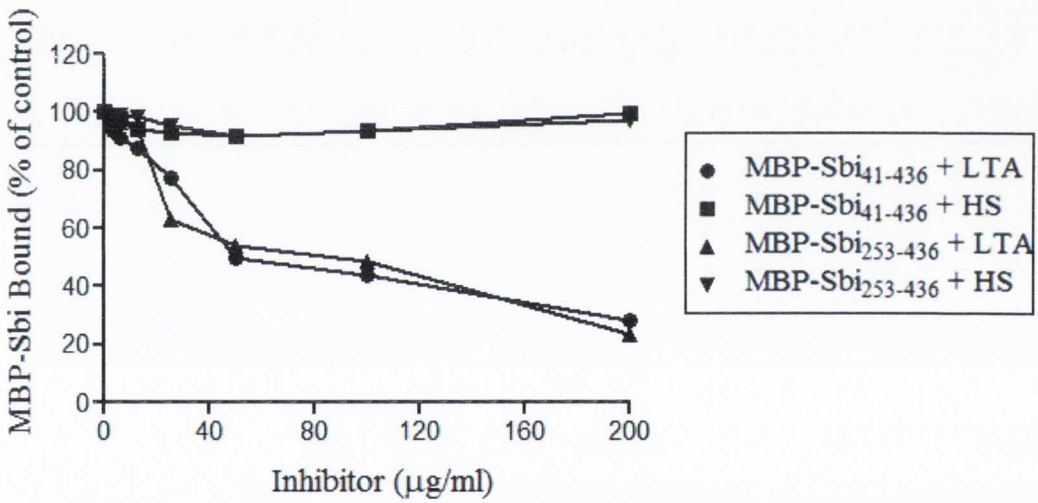
Figure 4.8 Interaction of MBP-Sbi with LTA.

(**A**) Binding of MBP-Sbi_{41-436}, MBP-$Sbi_{254-436}$ MBP-Sbi_{41-253} and MBP to LTA-coated wells. Recombinant protein binding was detected with HRP-conjugated anti-MBP IgG. (**B**) Inhibition of binding of MBP-Sbi_{41-436} and MBP-$Sbi_{254-436}$ to LTA and (**C**) to the purified cytoplasmic membrane fraction from *S. aureus* Newman *spa sbi*. Recombinant proteins were preincubated with increasing concentrations of either *S. aureus* LTA or heparin sulfate (HS) (0-200μg/ml) before being added to coated microtitre plates Each assay was preformed three times with similar results. Error bars show the standard deviation.

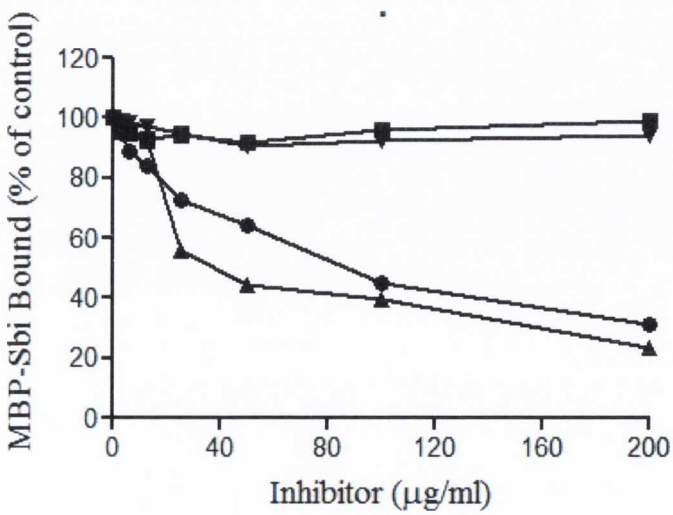
A. LTA



B. Inhibition of binding to LTA



C. Inhibition of binding to cytoplasmic membrane



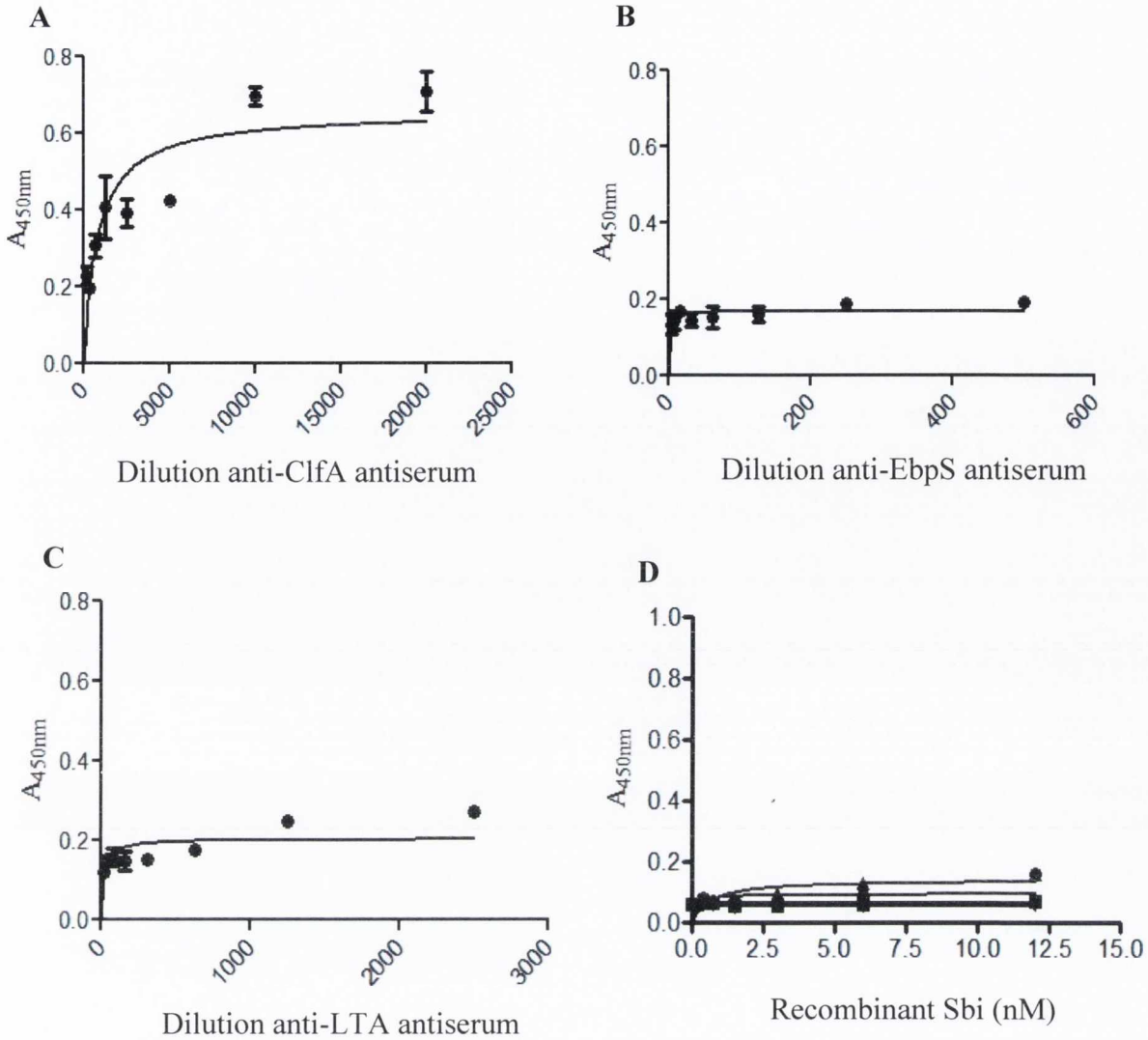


Figure 4.9 Interaction of MBP-Sbi$_{41-436}$, MBP-Sbi$_{41-253}$ and MBP-Sbi$_{254-436}$ with purified cell wall material

(**A**) Cell wall fragments purified from *S. aureus* Newman *spa sbi* was incubated in microtitre plates and coating of the surface verified with rabbit antibodies recognizing the cell wall-anchored protein ClfA followed by HRP-conjugated goat anti-rabbit IgG. Cytoplasmic membrane contamination of the cell wall fraction was evaluated with (**B**) rabbit antibodies recognizing the integral membreane protein EbpS followed by HRP-conjugated goat anti-rabbit IgG and (**C**) mouse antibodies recognizing LTA followed by HRP-conjugated rabbit anti-mouse IgG. (**D**) Binding of MBP-Sbi$_{41-436}$, MBP-Sbi$_{254-436}$ MBP-Sbi$_{41-253}$ and MBP to cell wall-coated wells. Recombinant protein binding was detected with HRP-conjugated anti-MBP antiserum The graphs shown here are representatives of three separate experiments. Error bars show the standard deviation.

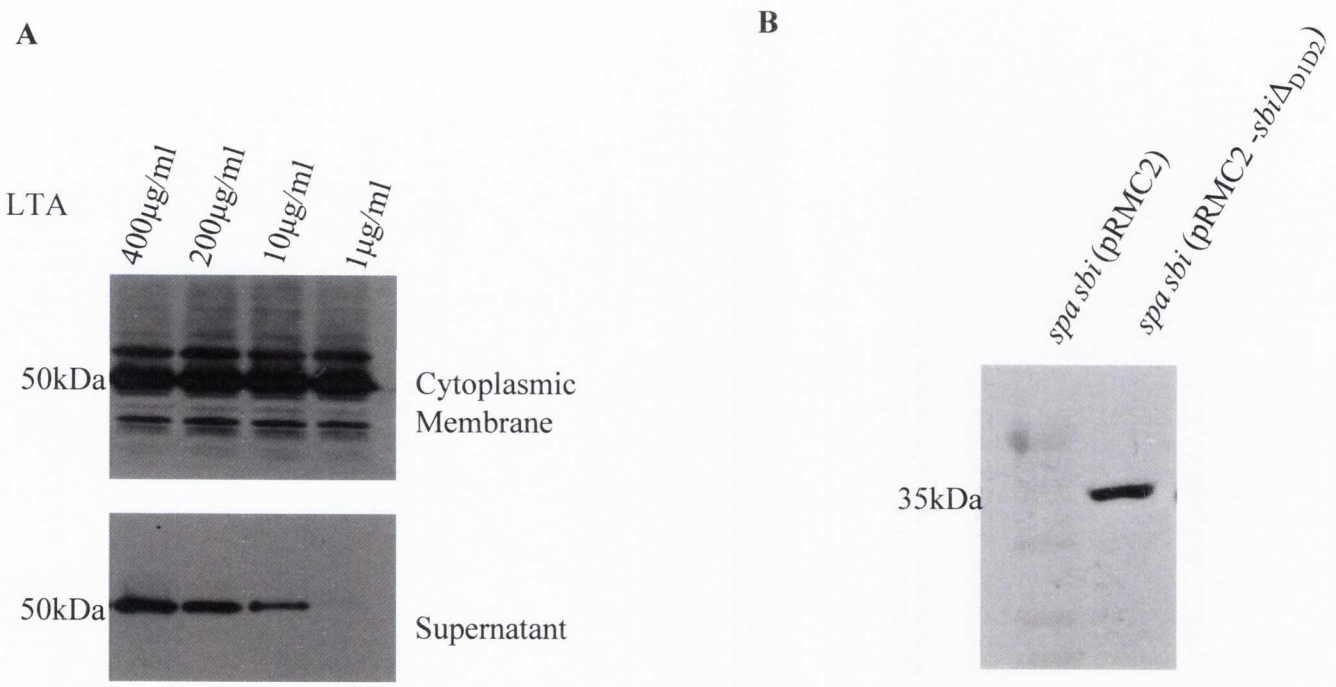


Figure 4.10 Analysis of the Sbi-LTA interaction.

(**A**) Displacement of Sbi from the cytoplasmic membrane by soluble LTA. Cytoplasmic membrane from Newman *spa* was incubated with increasing amounts of *S. aureus* LTA (0-400µg/ml). Sbi bound to the cytoplasmic membrane or released into the supernatant was detected using rabbit anti-Sbi D3D4WrY antiserum followed by HRP-conjugated goat anti-rabbit IgG. (**B**) Far Western blotting of *S. aureus* cytoplasmic membrane fractions with LTA. Newman *spa sbi* and Newman *spa sbi* (pRMC2-*sbi*Δ_{D1D2}) cytoplasmic membrane material was fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, incubated with purified LTA and bound LTA was detected with mouse anti-LTA monoclonal antiserum followed by HRP-conjugated rabbit anti-mouse IgG.

fraction was separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with LTA followed by a mouse monoclonal antibody to LTA and HRP-conjugated rabbit anti-mouse IgG. The reactive band at 37kDa corresponds to SbiΔ_{D1D2} demonstrating that Sbi expressed from *S. aureus* binds LTA (Figure 4.10, panel B).

4.2.11 An LTA-defective mutant has decreased levels of Sbi in the cytoplasmic membrane

S. aureus mutants that lack LTA can only grow under osmotically stabilizing conditions or by the acquisition of compensatory mutations (Oku et al., 2009, Corrigan et al., 2011). The LTA-deficiant strain of RN4220 *spa* (4S5) contains a complete deletion of the LTA synthase gene *ltaS* and has acquired two additional mutations that permit it to grow and divide in the absence of LTA in a similar fashion to the wildtype (Corrigan *et al.* 2011). To assess the role of LTA in the localisation of Sbi to the cell membrane, cytoplasmic membrane and culture supernatant fractions of wildtype RN4220 *spa* and 4S5 cells grown to mid-exponential phase were isolated and analysed by SDS-PAGE and Western immunoblotting using rabbit anti-Sbi D3D4WrY IgG. An immunoreactive band of ~50 kDa was detected in the membrane and supernatant fractions of both the wildtype and LTA-negative suppressor strain (Figure 4.11, panel A). However, strain 4S5 exhibited 3.4±0.4-fold lower levels of Sbi in its cytoplasmic membrane but contained a 1.8±0.2-fold higher level of Sbi in the supernatant fraction as compared to the wild-type strain (Figure 4.11, panel B).

To confirm the role of LTA in Sbi localisation and to rule out any involvement of the suppressor mutations present in 4S5, pCN34-*ltaS* (Corrigan *et al.* 2011), which expresses *ltaS* from its native promoter, was transferred into 4S5 in order to complement the *ltaS* mutation. This resulted in the restoration of LTA synthesis as judged by Western immunoblotting (Figure 4.11, panel A). Complementation with pCN34-*ltaS* also restored Sbi expression in the cytoplasmic membrane to wild-type levels (Figure 4.11, B). Furthermore, the level of the integral membrane protein EbpS in the cytoplasmic membrane of all strains was the same. This indicates that the inhibition of LTA expression does not affect another membrane associated proteins (Figure 4.11, panel B). This shows that the suppressor mutations are not responsible for the reduction in Sbi in the membrane fraction and directly implicates LTA in attaching Sbi to the cell envelope.

4.2.12 Anchorage of Sbi to the membrane by the C-terminal Y domain

It seemed likely that the C-terminal domain Y is involved in attaching Sbi to the cytoplasmic membrane. To test this a fourth MBP fusion protein was constructed that comprised the C-terminal Y domain (residues 303-436), (Figure 4.12, panel A). The protein was expressed in *E.coli* and purified by affinity chromatography. The protein's purity and integrity were verified by SDS-PAGE and Western blotting with anti-MBP antiserum (Figure 4.12, panel B and C).

Cytoplasmic membrane material purified from *S. aureus* Newman *spa sbi* was incubated in microtitre plates and coating of the surface verified with antibodies recognizing the integral membrane protein EbpS (data not shown). The membranes were incubated with MBP-$Sbi_{254-436}$ and MBP-$Sbi_{303-436}$ which were able to bind in a dose-dependent and saturable manner with half maxima of 1.15 nM and 1.23 nM, respectively, whilst the MBP control was unable to bind (Figure 4.12, panel B). These results indicate that the C-terminal Y domain of Sbi binds strongly to purified cytoplasmic membrane.

To localize the residues involved, the full length *sbi* gene and a series of deletions were cloned into the expression vector pRMC2 so that the *sbi* gene and truncates were expressed from the anhydrotetracycline-inducible promoter on the vector. The plasmids expressing full length Sbi and the C-terminal truncates Sbi_{1-335}, Sbi_{1-368} and Sbi_{1-403} were introduced into Newman *spa sbi*, bacteria were grown in the presence of the inducer and cytoplasmic membrane and culture supernatant fractions analysed by SDS-PAGE and Western blotting. An inducible protein of 50kDa was detected in the membrane and supernatant fractions of the strain expressing the wild type Sbi. The three truncates were detected in the culture supernatant, but in the case of the shortest, Sbi_{1-335}, the protein was not present in the membrane fraction and was only found in the supernatant (Figure 4.12, panel C).

An in-frame deletion lacking amino acids 335-368 was constructed in pRMC2-*sbi*. The plasmid expressing $SbiΔ_{335-368}$ was introduced into Newman *spa sbi* and the cytoplasmic membrane and culture supernatant fractions were analysed as described above. $SbiΔ_{335-368}$ was detected in the cytoplasmic membrane but at a 2.4±0.5-fold lower level than wild-type Sbi as shown by densitometry. In contrast, $SbiΔ_{335-368}$ was

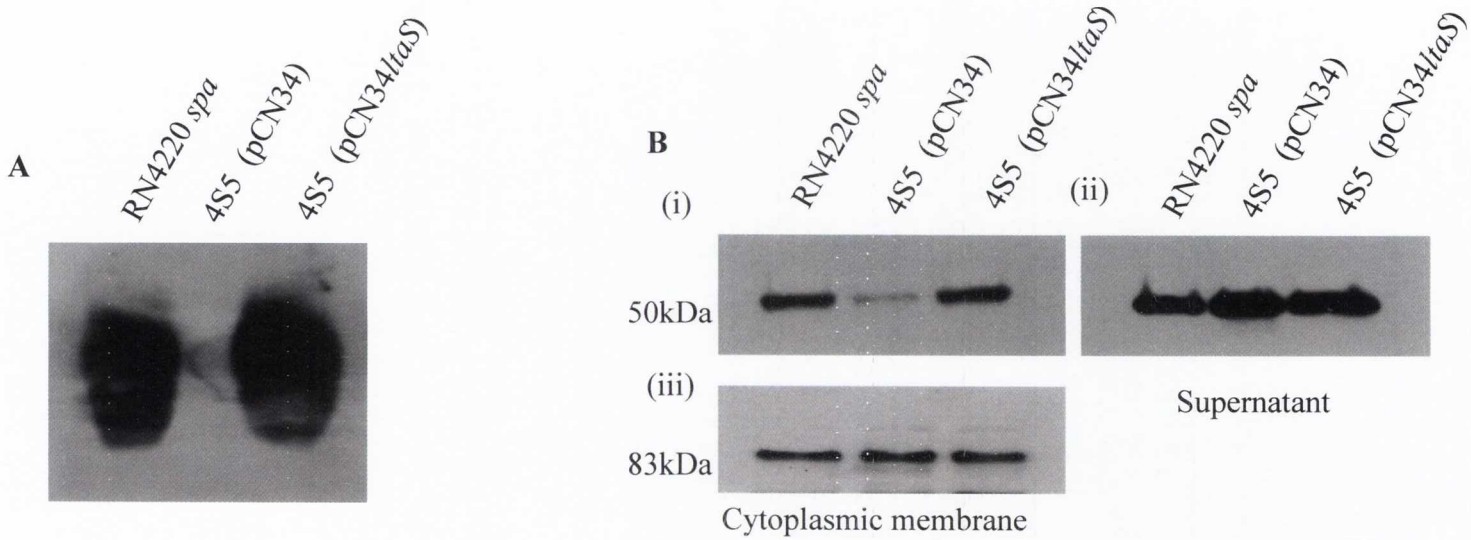
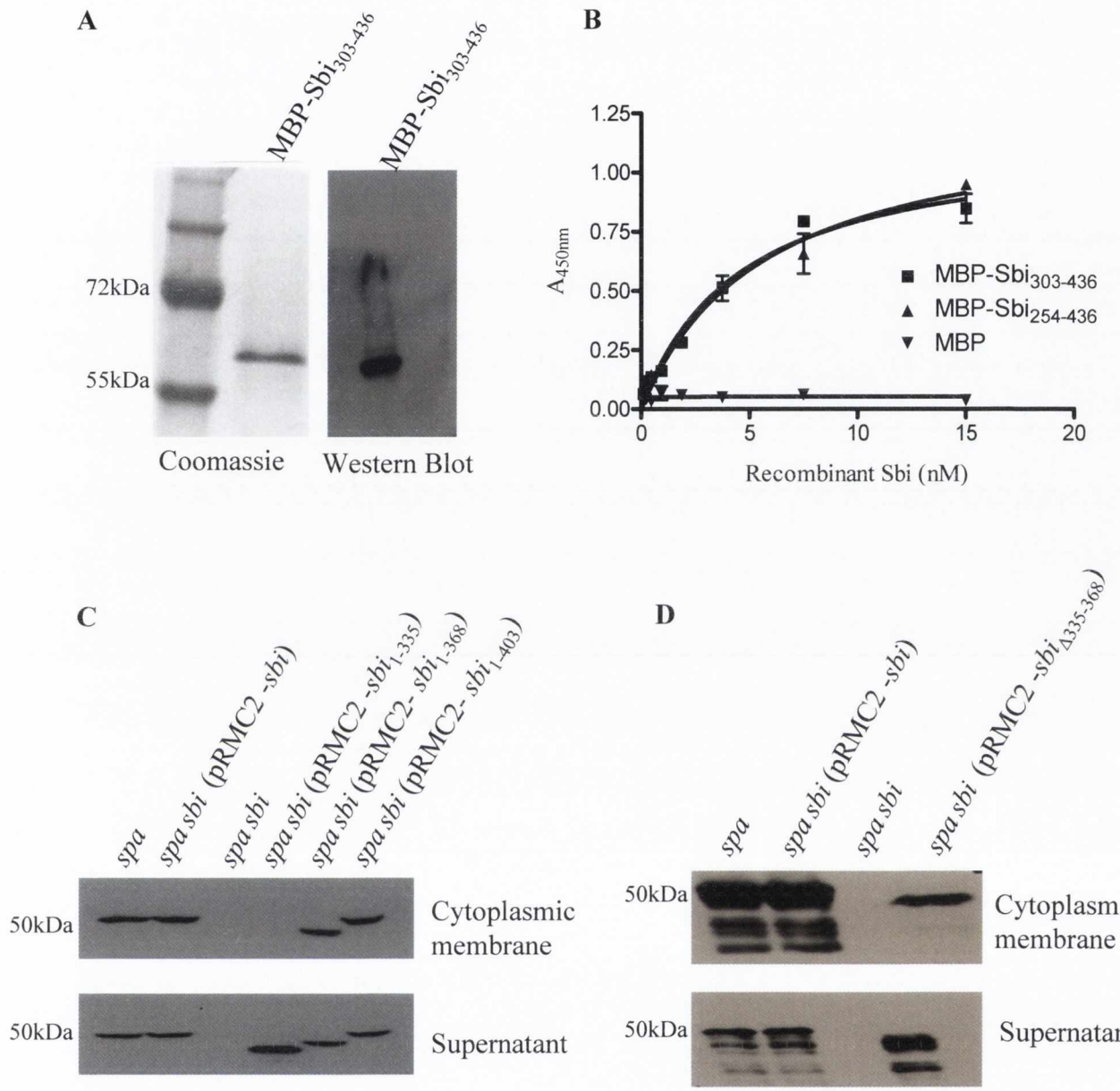


Figure 4.11 Sbi cellular location in LTA negative strains

(**A**) Whole cell lysate fractions of RN4220 *spa*, 4S5 and 4S5 (pCN34-*ltaS*) analyzed by Western immunoblotting with monoclonal mouse anti-LTA antiserum followed by HRP-conjugated rabbit anti-mouse IgG. (**B**) Cytoplasmic membrane and culture supernatant fractions of RN4220 *spa*, 4S5 and 4S5 (pCN34-*ltaS*) analyzed by Western immunoblotting with rabbit anti-Sbi D3D4WrY antiserum and HRP-conjugated goat anti-rabbit IgG (i and ii) and rabbit anti-EbpS antiserum followed by HRP-conjugated goat anti-rabbit IgG (iii). Blots shown are representative of three independent experiments.

Figure 4.12 Cellular localisation of Sbi C-terminal truncates.

(A) Coomassie stain of an SDS-PAGE gel of MBP-$Sbi_{303-436}$. Western immunoblot of MBP-$Sbi_{303-436}$ probed with HRP-conjugated anti-MBP antiserum. (B) Binding of MBP-$Sbi_{254-436}$, MBP-$Sbi_{303-436}$ and MBP to cytoplasmic membrane-coated wells. Recombinant protein binding was detected with HRP-conjugated anti-MBP antiserum. The graph shown here is a representative of three separate experiments. Error bars show the standard deviation. (C) Cytoplasmic membrane and culture supernatant fractions of Newman *spa sbi* (pRMC2-*sbi*) C-terminal truncates analysed by Western immunoblotting with HRP-labelled rabbit IgG. (D) Cytoplasmic membrane and culture supernatant fractions of Newman *spa sbi* (pRMC2-*sbi*$\Delta_{335-368}$) analysed by Western immunoblotting with HRP-labelled rabbit IgG. Blots shown are representative of three independent experiments. The blot is overexposed compared to that in panel B and shows additional bands that are presumably breakdown products. Densitometry was performed on the 50kDa bands


A
MBP-Sbi303-436
MBP-Sbi303-436
72kDa
55kDa
Coomassie
Western Blot
B
1.25
1.00
0.75
0.50
0.25
0.00
A450nm
0
5
10
15
20
Recombinant Sbi (nM)
MBP-Sbi303-436
MBP-Sbi254-436
MBP
C
spa
spa sbi (pRMC2 -sbi)
spa sbi
spa sbi (pRMC2 -sbi1-335)
spa sbi (pRMC2- sbi1-368)
spa sbi (pRMC2- sbi1-403)
50kDa
Cytoplasmic membrane
50kDa
Supernatant
D
spa
spa sbi (pRMC2 -sbi)
spa sbi
spa sbi (pRMC2 -sbiΔ335-368)
50kDa
Cytoplasmic membrane
50kDa
Supernatant

detected at a 2.6±0.7-fold higher level in the culture supernatant than wild-type Sbi (Figure 4.12, panel D). This suggests that the C-terminal domain Y is required to anchor the protein to the membrane and that residues 335-368 are required for efficient membrane anchoring but are not solely responsible.

4.2.13 Why does Sbi occur extracellularly?

The C-terminus of Sbi has a high affinity for LTA and the recombinant MBP-Sbi protein can bind to LTA exposed on the bacterial cell surface. If this is the case it is perhaps surprising that Sbi can occur extracellularly unless the cell-bound form saturates the surface-exposed LTA or the C-terminus of the secreted form is modified so that it cannot recognize LTA. To address this issue Newman *spa sbi* and Newman *spa* cells were coated onto the surface of ELISA wells and incubated with MBP-Sbi_{41-436} and MBP-$Sbi_{254-436}$. A reduction in absorbance of 2.3- and 3-fold occurred, respectively, when binding of MBP-Sbi to the Sbi^+ strain was compared to the Sbi^- mutant (Figure 4.13, panel A). This suggests that a significant fraction of the LTA on the surface of Sbi^+ cells is unavailable for binding by the secreted form of Sbi. Similar results were obtained when purified membranes from Newman spa and Newman *spa sbi* cells were immobilized and incubated with MBP-Sbi proteins, with a 2.4-fold and 2.7-fold reduction in binding of MBP-Sbi_{41-436} and MBP-$Sbi_{254-436}$, respectively (Figure 4.13, panel B). This suggests that a significant number of sites were unavailable in the membrane material purified from the Sbi^+ cells.

4.2.14 Can the Sbi Y domain support binding to the cytoplasmic membranes of other LTA type I expressing strains?

The ability of Sbi to bind to membranes (presumably via LTA) of other Gram positive bacteria was investigated. Cytoplasmic membrane material purified from *S. aureus*, *S. epidermidis*, *S. lugdunensis*, *L. monocytogenes*, *E. faecalis* and the Gram negative bacteria *E. coli* were incubated in microtitre plates. The membranes were incubated with either MBP-$Sbi_{303-436}$ or MBP. MBP-$Sbi_{303-436}$ bound the cytoplasmic membranes of all five Gram positive bacteria with a similar affinity whilst the MBP control was unable to bind (Figure 4.14, panel A). All five Gram positive bacterial strains tested express type I LTA which has a poly(glycerophosphate) backbone (Neuhaus & Baddiley, 2003). In contrast MBP-$Sbi_{303-436}$ did not bind to the cytoplasmic

membrane material of *E. coli* which does not express LTA. These results indicate that the C-terminal Y domain of Sbi binds strongly to purified cytoplasmic membranes of strains expressing LTA which has a poly(glycerophosphate) backbone. Furthermore MBP-$Sbi_{303-436}$ binding to the cytoplasmic membranes of all five Gram positive bacteria was inhibited by preincubating MBP-$Sbi_{303-436}$ with soluble purified LTA from *S. aureus* (Figure 4.14, panel B).

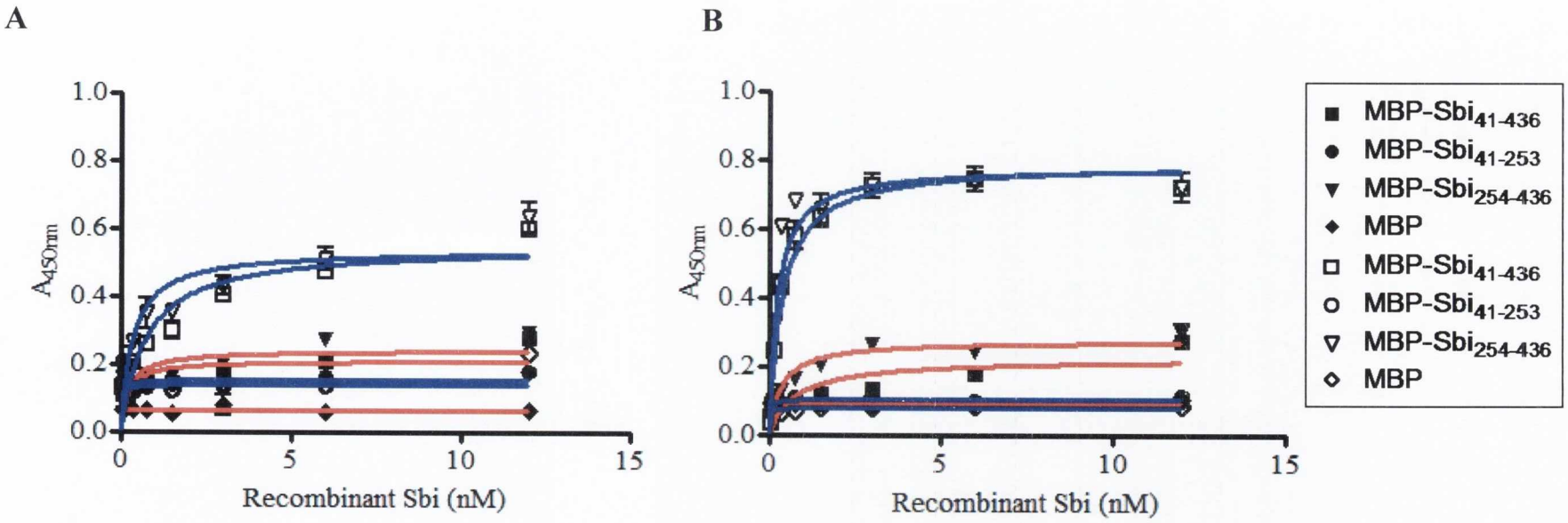


Figure 4.13 Binding of MBP-Sbi to whole cells and purified cytoplasmic membrane

Binding of MBP-$Sbi_{41-436,}$ MBP-$Sbi_{254-436}$ MBP-Sbi_{41-253} and MBP to wells coated with (**A**) *S.aureus* Newman Sbi^+ or Sbi^- cells and (**B**) wells coated with cytoplasmic membrane material from *S.aureus* Newman Sbi^+ or Sbi^-. Recombinant protein binding was detected with HRP-conjugated anti-MBP IgG. Closed symbols and red lines refer to Newman *spa*. Open symbols and blue lines refer to Newman *spa sbi*. Each assay was preformed three times with similar results. Error bars show the standard deviation

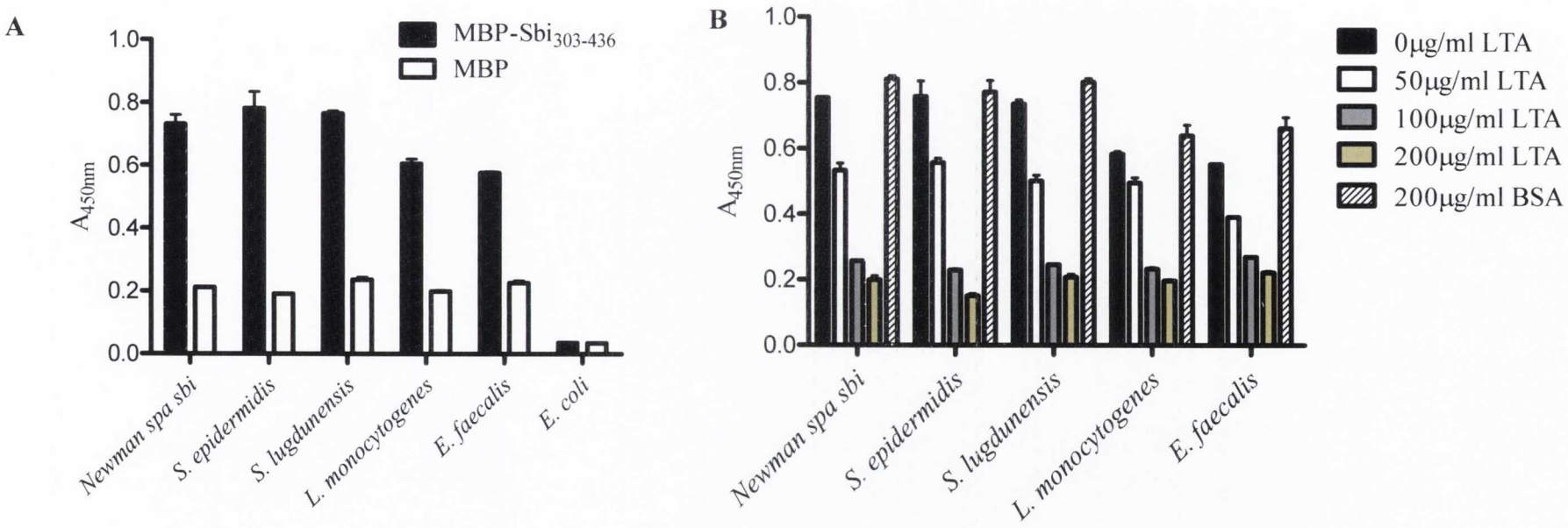


Figure 4.14 Binding of MBP-$Sbi_{303-436}$ to the cytoplasmic membranes of Gram-positive bacteria.

(**A**) Cytoplasmic membrane material purified from *S. aureus*, *S. epidermidis*, *S. lugdunensis*, *L. monocytogenes*, *E. faecalis* and *E. coli* were incubated with MBP-$Sbi_{303-436}$ and MBP in microtitre plates. Recombinant protein binding was detected with HRP-conjugated anti-MBP antiserum. (B) Recombinant MBP-$Sbi_{303-436}$ was preincubated with increasing concentrations of either *S. aureus* LTA or bovine serum, albumin (BSA) (0-200μg/ml) before being added to cytoplasmic membrane-coated microtitre plates. Recombinant protein binding was detected with HRP-conjugated anti-MBP antiserum. Each assay was preformed two times with similar results. Error bars show the standard deviation.

4.3 Discussion

The notion that the function of the Sbi protein is to help protect *S.aureus* from innate immune defences of the host was initially based on the *in vitro* activities of the recombinant D1 and D2 domains that bind to the Fc region of IgG and the D3 and D4 domains which can bind to complement factor C3 in serum and can promote its futile consumption (Burman et al., 2008, Zhang et al., 1998, Zhang *et al.*, 1999). If Sbi protects cells in the same manner as protein A by binding IgG at the Fc region so that the immunoglobulin cannot act as an opsonin or promote complement fixation, the protein must be bound to the cell envelope with domains D1 and D2 exposed on the cell surface. However, if domains D3 and D4 are to promote futile consumption of C3 in the fluid phase they must do so at a distance from the cell otherwise they would actually promote opsonin formation. This requires the protein to be secreted from the cell and for the D3D4 domains of the cell-associated Sbi to be inactive.

The results described in this chapter demonstrate that Sbi is both associated with the cell envelope and is also present extracellularly. Unexpectedly, cell-associated Sbi was found exclusively in the cytoplasmic membrane fraction when cells were treated with lysostaphin to create stable protoplasts. No Sbi was found among the solubilised proteins in the cell wall fraction. In contrast, protein A was only detected in the wall fraction, as expected for an LPXTG-anchored protein. Bioinformatic analysis of the amino acid sequence of Sbi did not reveal a putative membrane-spanning stretch of 15-30 hydrophobic residues. Comparative *in silico* analysis of Sbi with the cytoplasmic membrane-anchored protein EbpS by Kyte-Doolittle hydropathy plotting or the TMHMM program did not identify any possible membrane-spanning domains. A possible anchoring mechanism was identified when the Sbi sequence was analysed by dense alignment scattering (DAS), which highlighted a 9 residue stretch as potentially being membrane-associated.

Examination of the level of exposure of Sbi on the surface of whole cells indicated that the N-terminal D1 and D2 domains are available to bind IgG. In contrast only a small proportion of the D3 and D4 domains are recognized by the Fab regions of anti-SbiD3D4WrY antibodies and the Sbi domains do not appear to be able to promote binding of the bulky C3 molecule. This makes sense as any surface exposed SbiD3D4 could trigger complement activation that would promote rather than prevent

opsonisation. Even if C3 was attracted to D3D4 it might not be able to undergo the massive conformational change that occurs when it is converted to C3b (Gros *et al.*, 2008).

The Sbi protein is attached to the cell envelope by an unusual mechanism. When cells were converted to protoplasts the cell-associated protein was not solubilised like the LPXTG-anchored protein A or ClfA. Instead it was attached to the cytoplasmic membrane fragments following lysis of protoplasts and sedimentation. Purified recombinant MBP-Sbi bound with high affinity to immobilized membrane fragments prepared from a *sbi* mutant. Finding that recombinant Sbi bound to purified lipoteichoic acid both dose-dependently and saturably with a high affinity suggests that LTA is the ligand in the cell envelope bound by Sbi when it is secreted across the membrane of growing cells. Importantly the affinity of rSbi for purified LTA is very similar to that of purified membrane fragments. Also, reaching saturation in the binding assays is important because it is indicative of a specific interaction. Furthermore, MBP-Sbi could not bind to purified cell wall material effectively ruling out WTA as a ligand for Sbi.

In support of the contention that LTA is a ligand for attaching Sbi to the cell envelope is (i) the high affinity and specific binding of rSbi both to purified LTA and to the membrane fraction, (ii) inhibition of binding of rSbi to the membrane fraction by soluble LTA, (iii) partial displacement of Sbi from the membrane fraction by soluble LTA, (iv) binding of rSbi to the surface of whole cells where LTA is known to be exposed, (v) a reduction in the level of Sbi in the cytoplasmic membrane of an LTA negative mutant and (vi) the ability to bind to the membranes of other Gram positive bacteria with type I LTA in their cytoplasmic membranes. However, LTA might not be the only ligand for Sbi in the membrane. The membrane has a 3-fold higher affinity for rSbi compared to purified LTA and high concentrations of soluble LTA could only partially displace bound Sbi.

The behaviour of Sbi in cell fractionation experiments and its ability to bind to LTA resembles internalin B (InlB) of *Listeria monocytogenes* (Jonquieres et al., 1999). InlB binds to the human growth hormone receptor Met on mammalian cells and triggers bacterial internalization by receptor-mediated endocytosis (Mengaud *et al.*, 1996, Shen *et al.*, 2000, Braun *et al.*, 1998). In order to act as an invasin InlB must be able to promote clustering of the Met receptor in the host cell membrane and trigger the

signalling that leads to cytoskeletal rearrangements and endocytosis (Niemann, 2011). The ability of a secreted protein to perform this seems paradoxical until one considers the role of heparin sulphate proteoglycans that are bound on the surface of mammalian cells (Marino *et al.*, 2002). HSP is able to displace cell-associated InlB and to promote its release from the cell surface. It does so by binding to the GW repeats that contain the binding sites for LTA. It is likely that HSP-InlB complexes cooperate to trigger Met receptor clustering. A major difference between the association of InlB and Sbi with LTA is that the latter does not bind to HSPs *in vitro* and cannot be displaced easily from the membrane. This is likely to be due to the high affinity specific interaction that occurs between Sbi and LTA whereas the binding of InlB to LTA appears to be much weaker and is probably non-specific and based on binding of GW repeats to highly negatively charged polymers.

In conclusion Sbi is present both in the extracellular medium and in the cytoplasmic membrane where it is displayed on the surface of the bacterium where it is capable of binding the Fc region of IgG but not complement factor C3. Binding to the cytoplasmic membrane and cell surface expression involves a high affinity interaction with LTA and possibly to another component of the membrane.

Chapter 5

Analysis of the immune evasion properties of Sbi

5.1 Introduction

The innate immune system plays a vital role in the host's defence against invading pathogens. The core elements of the innate immune system are the complement cascade, phagocytes and antimicrobial peptides (Beutler, 2004). Activation of the complement cascade is initiated by the classical and lectin recognition pathways, which are amplified by the alternative pathway. All three pathways converge at the formation of the C3 convertases, C4bC2a and C3bBb. The C3 convertases cleave C3 into C3a and C3b (Gasque, 2004, Walport, 2001a, Walport, 2001b). Complement factor H binds immobilized C3b and activates complement factor I which cleaves C3b into iC3b (Walport, 2001a, Walport, 2001b). Deposition of C3b and iC3b on the bacterial cell surface promotes opsonophagocytosis by neutrophils and macrophages. Neutrophils recognise the Fc region of IgG via their FcγR1 receptor and complement factors bound to the bacterial cell surface by receptors CR1 and CR3. High concentrations of immobilized C3b trigger a shift in the substrate specificity of the C3 convertase as C3b binds C4bC2a to form the C5 convertase which cleaves C5 into C5b on the bacterial cell surface and releases the potent neutrophil chemoattractant C5a (Beutler, 2004, Gasque, 2004). The alternative pathway C3 convertase C3bBb in turn binds C3b to form the alternative pathway C5 convertase C3bBbC3b. C5b forms a complex with complement proteins C6, C7, C8 and C9 to form the membrane attack complex that directly lyses gram-negative bacteria (Beutler, 2004).

Neutrophils, or polymorphonuclear leucocytes (PMNL), have been shown to be crucial in the host defence against staphylococcal disease. In mouse models of *S. aureus* sepsis and septic arthritis, neutrophil-depleted mice died of sepsis within 3 days at an inoculum level that allowed normal mice to survive (Verdrengh & Tarkowski, 1997). In addition, it was reported that individuals that suffer from chronic granulomatous disease, an inherited disorder of neutrophil function, frequently present with recurrent *S. aureus* pneumonia and abscesses (Liese *et al.*, 1996). *S. aureus* expresses numerous factors that interfere with the complement cascade and opsonophagocytosis by neutrophils, as discussed in section 1.4.

Four surface components of *S. aureus* have been shown to promote evasion of neutrophil-mediated phagocytosis. Protein A inhibits opsonophagocytosis by binding IgG by the Fc region which prevents classical complement fixation and recognition by

the neutrophil Fc receptor (Foster, 2005). The classical pathway is triggered by the binding of the C1 complex to antibodies that are bound to antigens on the bacterial cell surface. The C1 complex is composed of three subunits C1q, C1r and C1s. C1q binds to the Fc region of immunoglobulin bound to the bacterial surface. This results in activation of C1r which cleaves and activates C1s. Activation of the C1 complex results in the cleavage of the complement proteins C4 and C2 allowing the formation of the C3 convertase C4bC2a.

Clumping factor A (ClfA) is a major fibrinogen-binding surface protein. It is found predominantly on cells from the stationary phase of growth. ClfA binds to the γ-chain of fibrinogen via its N-terminal A-domain (McDevitt et al., 1997). *S. aureus clfA* mutants are significantly attenuated in murine models for sepsis and arthritis (Josefsson et al., 1998), a rat endocarditis model (Que *et al.*, 2001) and a rabbit infective endocarditis model (Vernachio et al., 2003). Virulence is likely to be increased by bacterial cells becoming coated with fibrinogen, which inhibits deposition of, or access to, opsonins (Higgins et al., 2006). In addition, ClfA can capture and activate the serum complement regulator factor I and convert C3b to iC3b and C3d resulting in loss of opsonin (Cunnion et al., 2004, Hair et al., 2008). This will also prevent C3 convertase formation and terminal pathway activation (Cunnion *et al.*, 2004).

The majority of clinical isolates of *S. aureus* produce serotype 5 or serotype 8 capsular polysaccharide (Cap) (O'Riordan & Lee, 2004). Expression of capsular polysaccharide reduces phagocytosis *in vitro* and is associated with increased virulence in a murine bacteraemia model (Luong & Lee, 2002, Thakker et al., 1998). Capsule inhibits binding of antibodies to *S. aureus* cell surface components, which hinders opsonisation (Thakker et al., 1998). Capsule expression reduces the uptake of bacteria by neutrophils in the presence of normal serum opsonins (Nilsson *et al.*, 1997, Luong & Lee, 2002). Although complement factors can accumulate on the cell wall surface beneath the polysaccharide layer they are inaccessible to complement receptors on neutrophils (Cunnion et al., 2003). Reduced O-acetylation of capsular polysaccharide decreases antiphagocytic activity, due to increased antibody penetration and cell surface recognition (Bhasin et al., 1998).

The iron regulated surface determinant protein IsdH is part of a complex of proteins that are only expressed under iron-restricted conditions. They extract haem

from haemoglobin and transport it into the cytoplasm (Visai et al., 2009). *S. aureus isdH* mutants are engulfed more rapidly by human neutrophils in the presence of serum opsonins, they survive poorly in fresh whole human blood and are less virulent in a mouse model of sepsis (Visai *et al.*, 2009). IsdH promotes accelerated degradation of the serum opsonin C3b by enhancing conversion of C3b to C3d (Visai *et al.*, 2009). As with ClfA this will also prevent C3 convertase formation and terminal pathway activation.

Sbi consists of four ligand binding domains, of which domains D1 and D2 bind the Fc region of immunoglobulin, potentially blocking Fc-receptor-mediated phagocytosis (Zhang et al., 1998). It has been suggested that Sbi also blocks the binding of C1q preventing activation of the classical complement pathway. When Sbi domains D3 and D4 together, are incubated with serum, a significant proportion of activated C3 becomes covalently bound to SbiD3D4 via its C3d thioester forming a SbiD3D4:C3b adduct (Burman et al., 2008). It has been proposed that SbiD3D4:C3b adduct formation provides a platform for the assembly of the alternative pathway convertase, leading to futile consumption of C3 (Burman *et al.*, 2008). This could only be an effective defence if Sbi domains D3D4 are only active when secreted from the bacteria while the D3D4 domains of cell-bound Sbi are unable to bind C3. Any surface exposed SbiD3D4 could trigger complement activation that would promote rather than prevent opsonisation. Sbi domain D4 and complement receptor CR2 share overlapping contact residues on C3d (Isenman *et al.*, 2010, Clark *et al.*, 2011). By inhibiting CR2 binding to C3d, Sbi D4 interrupts the link between the innate and adaptive immune systems, as the interaction of CR2 with C3d is required for optimal antibody responses and the induction of immunological memory (Nielsen et al., 2002, Rickert, 2005). These observations led to the current investigation to determine if Sbi promotes evasion of phagocytosis and killing by neutrophils and it so to compare this with the protection offered by Spa, ClfA, Cap and IsdH. In addition, the role of secreted Sbi in immune evasion and the site of action of the four ligand binding domains of Sbi were investigated.

5.2 Results

5.2.1 Expression of Sbi by clinical isolates

Sixteen clinical isolates of *S. aureus* representing the genetic diversity of the species based on Multi Locus Sequence Typing (MLST) were screened for expression of Sbi. The cytoplasmic membrane and culture supernatant were prepared and probed with HRP-conjugated rabbit anti-SbiE antiserum (Table 2.4). The antibodies did not react with Spa in the fractions as indicated by the absence of any reactive protein band in the Spa$^+$ Newman *sbi* mutant sample (Figure 5.1). A ~50kDa immunoreactive band was detected in the membrane fraction of all strains (except Cowan) and in the supernatant of all but four (TL210, LS-1, 52 and 21). There was considerable variation in the level of Sbi expressed, particularly in the culture supernatant.

5.2.2 Expression of anti-phagocytic factors in iron limited conditions

The ability of *S. aureus* grown in the iron restricted media RPMI and in human plasma to express the known anti-phagocytic factors Spa, ClfA, Cap, IsdH and Sbi was tested by Western immunoblotting of cell wall or cell membrane fractions. In each case wild-type Newman was compared with the appropriate null mutant. The gels were probed with specific antibodies recognizing ClfA or IsdH and with HRP-conjugated IgG to detect Spa and Sbi. It is clear from data shown in Figure 5.2 that each of the proteins was expressed when bacteria were iron-restricted.

5.2.3 Expression of *S. aureus* strain Newman anti-phagocytic determinants in mutant strains.

S. aureus is prone to acquiring mutations in the Agr global regulator (Villaruz *et al.*, 2009, Adhikari et al., 2007). In order to ensure that mutants affecting immune evasion factors Spa, ClfA, IsdH and Sbi remained Agr$^+$, each was tested for the expression of δ-toxin, a marker that is often used to reflect the integrity of Agr. It was important to ensure that inactivating one anti-phagocyctic factor did not affect the expression of any of the other factors. Each mutant in turn was screened by Western immunoblotting to determine the levels of expression of the remaining anti-phagocytic proteins to ensure that attenuation in a mutant was due to loss of their specific protein.

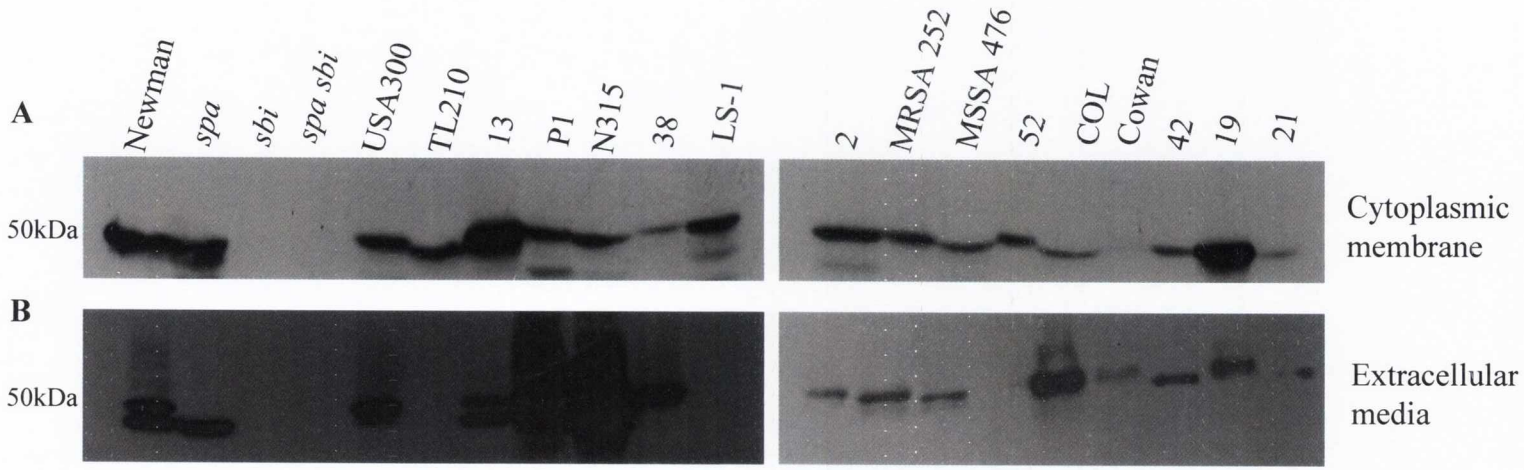


Figure 5.1 Expression of Sbi by clinical isolates of *S. aureus*.

Clinical isolates of *S. aureus* were analyzed by Western immunoblotting for the expression of Sbi in (**A**) the cytoplasmic membrane and (**B**) the culture supernatant. Samples were separated by SDS-PAGE and analyzed by Western blotting with HRP-conjugated rabbit anti-SbiE antiserum (Table 2.4).

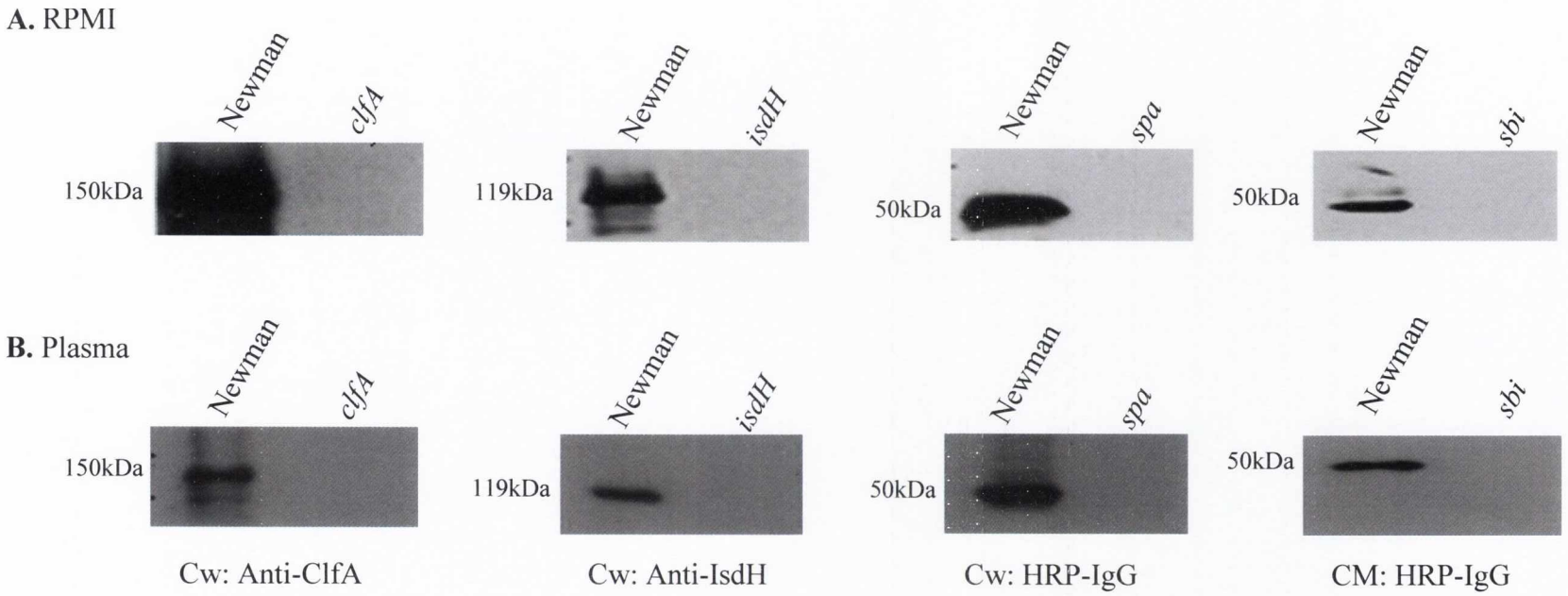


Figure 5.2. Expression of anti-phagocytic factors in iron limited conditions.

Bacteria were grown in (A) RPMI and (B) human plasma. In each case wild-type *S.aureus* Newman was compared with the appropriate null mutant. Solubilised cell wall (CW) associated proteins or in the case of Sbi the cytoplasmic membrane (CM) were analyzed by SDS-PAGE and Western blotting, probing with rabbit antibodies recognizing ClfA or IsdH followed by HRP-labelled goat anti-rabbit IgG or with HRP-labelled rabbit IgG to detect Spa and Sbi.

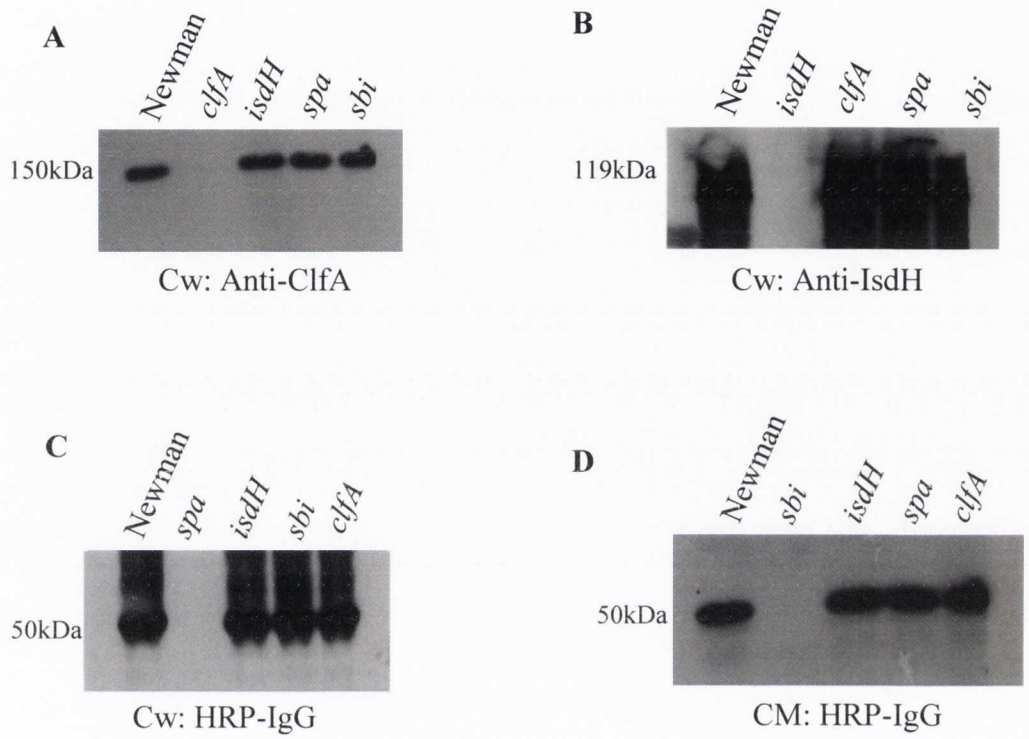


Figure 5.3 Expression of *S. aureus* strain Newman anti-phagocytic determinants in mutant strains.

Each mutant was screened by SDS-PAGE and Western immunoblotting probing with rabbit antibodies recognizing ClfA or IsdH followed by HRP-labelled goat anti-rabbit IgG (A-B) or with HRP-labelled rabbit IgG to detect Spa and Sbi (C-D). CW; cell wall CM; cytoplasmic membrane.

Each mutant expressed the same levels of the remaining anti-phagocyctic factors as the wild-type strain (Figure 5.3, panel A-D).

5.2.4 Expression of Sbi inhibits killing by neutrophils in whole human blood.

In order to determine if expression of Sbi contributes to survival of *S. aureus* in human whole blood, bacterial cells were incubated in blood obtained from several healthy donors. Hirudin was used as the anti-coagulant in order to preserve complement activity. Protein A, ClfA, IsdH and type 5 capsular polysaccharide are known to be important anti-phagocyctic factors so the ability of the *sbi* mutant to survive was compared to *spa, clfA, isdH* and *cap* mutants. This also allowed the relative contribution of each anti-phagocyctic factor in strain Newman to be compared under the same conditions, something that has never been done before. Furthermore, a mutant defective in *spa clfA cap isdH* was compared to a *spa clfA cap isdH sbi* mutant. None of the seven selected donors had detectable anti-Sbi, anti-Spa, anti-ClfA or anti-IsdH antibodies as determined by ELISA (Figure 5.4, panel A). This is important because if a donor has antibodies against the antigen being tested, enhanced opsonisation of the bacteria would occur leading to greater phagocytosis. With wild type *S. aureus* Newman, an average of 80% of the initial inoculum survived in blood for 3 hours. The survival of the *sbi* and *spa* mutants was significantly impaired with only 40% surviving (Figure5.4, panel B). In contrast, the *isdH and clfA* mutants were severely compromised with only 25% of the inoculum surviving. The survival of the *cap* mutant, although significantly impaired, was greater than the other mutants with 60% of the initial inoculum surviving. The survival of the *spa clfA cap isdH* and *spa clfA cap isdH sbi* mutants was 20% and 10% respectively (Figure 5.4, panel B). These experiments demonstrate that Sbi is important for the ability of *S. aureus* to survive in human blood. This is most likely because of its ability to resist opsonophagocytosis by neutrophils was lost and that the presence of Spa, ClfA and IsdH could not compensate.

It was important to show that the ability of strains to grow in plasma was not impaired. Bacteria were incubated in plasma for 3 hours and growth was measured by viable counting. All five single null mutants and both the multiple mutants grew to the same extent as the wild-type confirming previous results that growth in plasma resulted in cell numbers increasing to 130% of the initial inocula (Visai *et al.*, 2009).

5.2.5 Phagocytosis by human neutrophils

In order to show that expression of Sbi reduced the ability of human neutrophils to engulf *S. aureus* cells, Newman *sbi* was compared to the wild-type strain, and to Newman defective in Spa, ClfA, IsdH, Cap and a multiple mutant defective in all five factors in *in vitro* phagocytosis assays. IsdH and ClfA are established anti-phagocyctic factors in this assay and thus represent positive controls in the experiment (Higgins et al., 2006, Visai *et al.*, 2009). Bacteria were grown in RPMI and incubated with purified human neutrophils from three healthy donors in the presence of 10% normal human serum (NHS) to provide opsonins. Wild type *S. aureus* Newman resulted in 59% phagocytosis. The *spa*, *sbi*, *clfA*, *isdH* and *cap* mutants were engulfed at significantly greater rates than wild-type resulting in 75.3%, 72.1%, 82.4%, 78.5% and 71.9% phagocytosis, respectively. In contrast, the multiple *spa clfA cap isdH sbi* mutant was severely compromised resulting in 92.8% phagocytosis (Figure 5.5). This experiment is consistent with the results described for the whole blood survival assays and confirms that expression of Sbi is important for *S. aureus* to avoid opsonophagocytosis.

5.2.6 Complementation of the *sbi* mutation

In order to complement the *sbi*::Em^R mutation, pRMC2-*sbi* was transferred into Newman *sbi*. After growth in RPMI containing the inducer anhydrotetracycline the presence of Sbi in the membrane fraction was detected by Western blotting. To determine it complementation restored the ability of *S. aureus* to survive uptake and killing by neutrophils, bacteria were incubated in blood containing the inducer anhydrotetracycline. A mutant defective in ClfB, a surface protein with no known antiphagocytic properties, was included as a control. As before about 80% of Newman wild-type cells survived while only 40% of the *sbi* mutant remained. The *sbi* mutant carrying pRMC2-*sbi* was restored to 70% survival showing that Sbi expressed from an inducible promoter in a plasmid vector could complement the mutant phenotype (Figure 5.6, panel A). The *clfB* mutant suffered no defect in survival showing that immune evasion by surface proteins is specific.

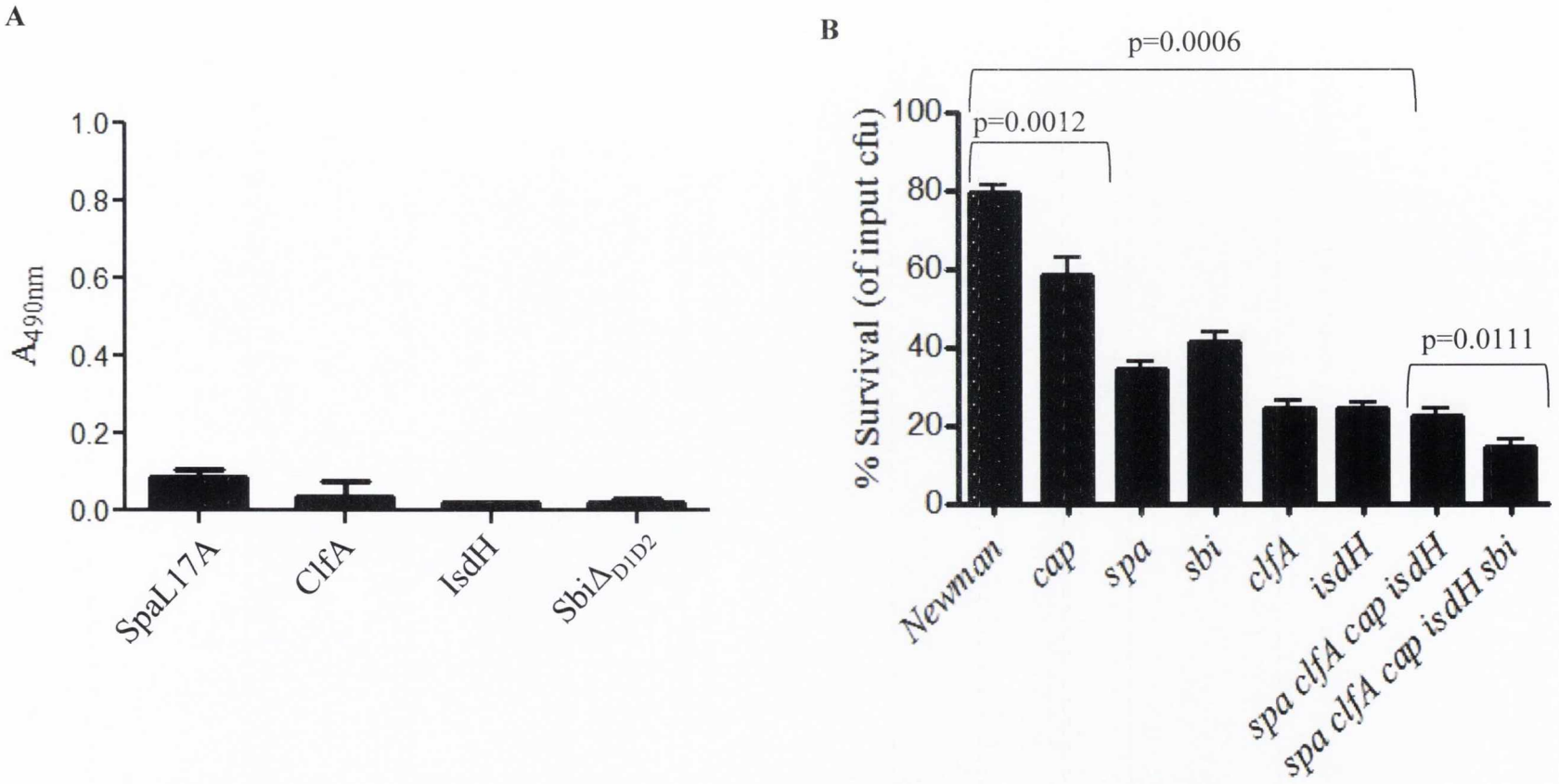
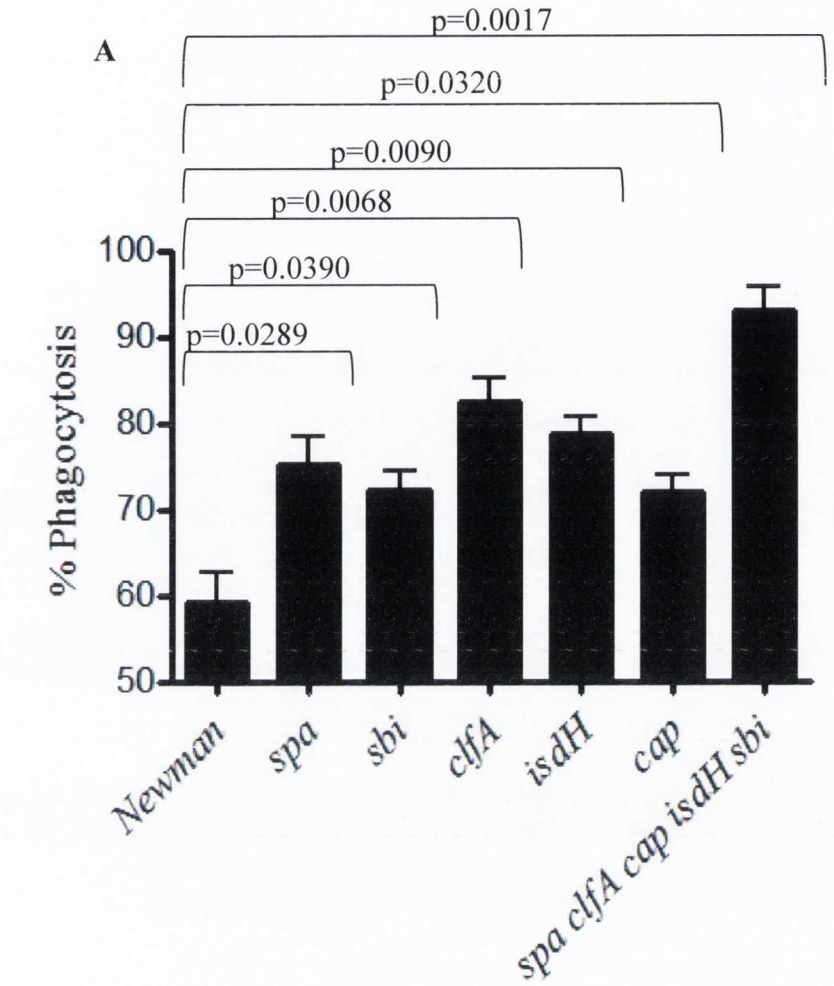


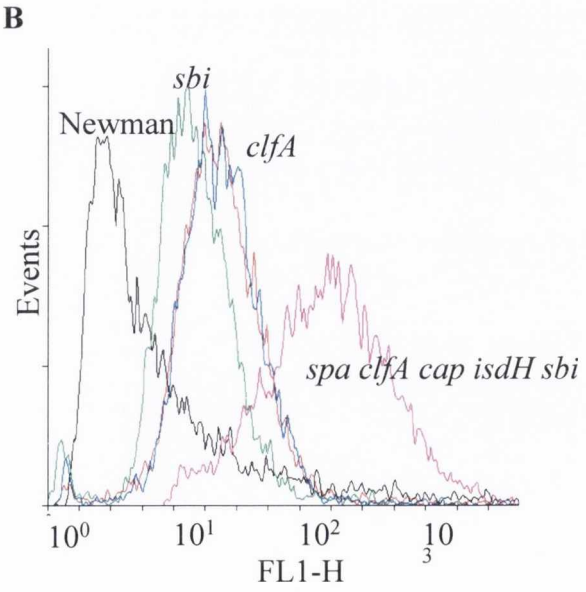
Figure 5.4 Survival of *S.aureus* in whole human blood: 1

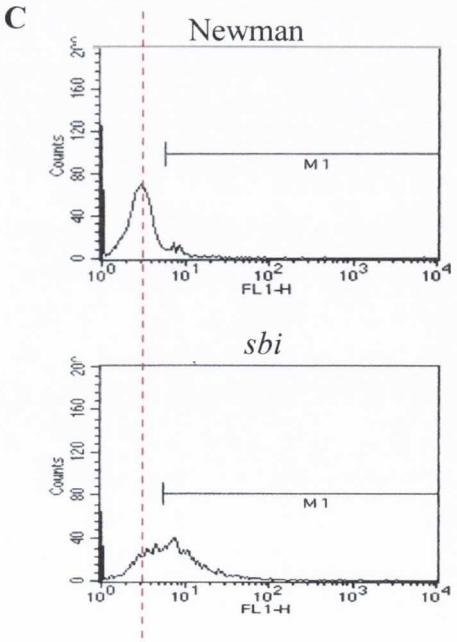
(**A**) Microtitre plates were coated with each antigen to be tested. Spa L17A and SbiΔ_{D1D2} are derivatives of Spa and Sbi, respectively that do not bind the Fc region of IgG. Wells were incubated with plasma from each blood donor followed by HRP-conjugated anti-human IgG. Data points represent the mean of duplicate wells. The graph is a representative of separate experiments preformed on blood from individual donors. (**B**) Bacteria grown in RPMI were tested for their ability to survive 3 h incubation in human blood. Surviving bacteria were measured by viable counting. Data is presented as the mean % surviving cfu ± SD (n=7).

Figure 5.5 Resistance of *S. aureus* to phagocytosis by human neutrophils

(**A**) Bacterial cells were labelled with FITC, adjusted to 5 x 10^7 cfu/ml, opsonized with 10% pooled human sera and incubated with polymorphonuclear leucocytes from three individual donors. After stopping the reaction, the percentage of PMNLs bearing fluorescent bacteria was measured by flow cytometry. Data is presented as mean % phagocytosis ± SD (n=3). (**B**) A representative flow cytometry trace of phagocytosis of fluorescently labelled bacteria. (**C**) Histograms of phagocytosis of selected representative datapoints of Newman strains. Histograms plot number of PMNL against fluorescence. A shift in fluorescence indicates phagocytic uptake of fluorescent bacteria. Gate (M1) was manually applied and identical for all datasets. Dashed red line indicates a shift in fluorescences.


A
p=0.0017
p=0.0320
p=0.0090
p=0.0068
p=0.0390
p=0.0289
% Phagocytosis
100
90
80
70
60
50
Newman
spa
sbi
clfA
isdH
cap
spa clfA cap isdH sbi


B
sbi
Newman
clfA
spa clfA cap isdH sbi
Events
10^0
10^1
10^2
10^3
FL1-H


C
Newman
M1
Counts
FL1-H
sbi
M1
Counts
FL1-H

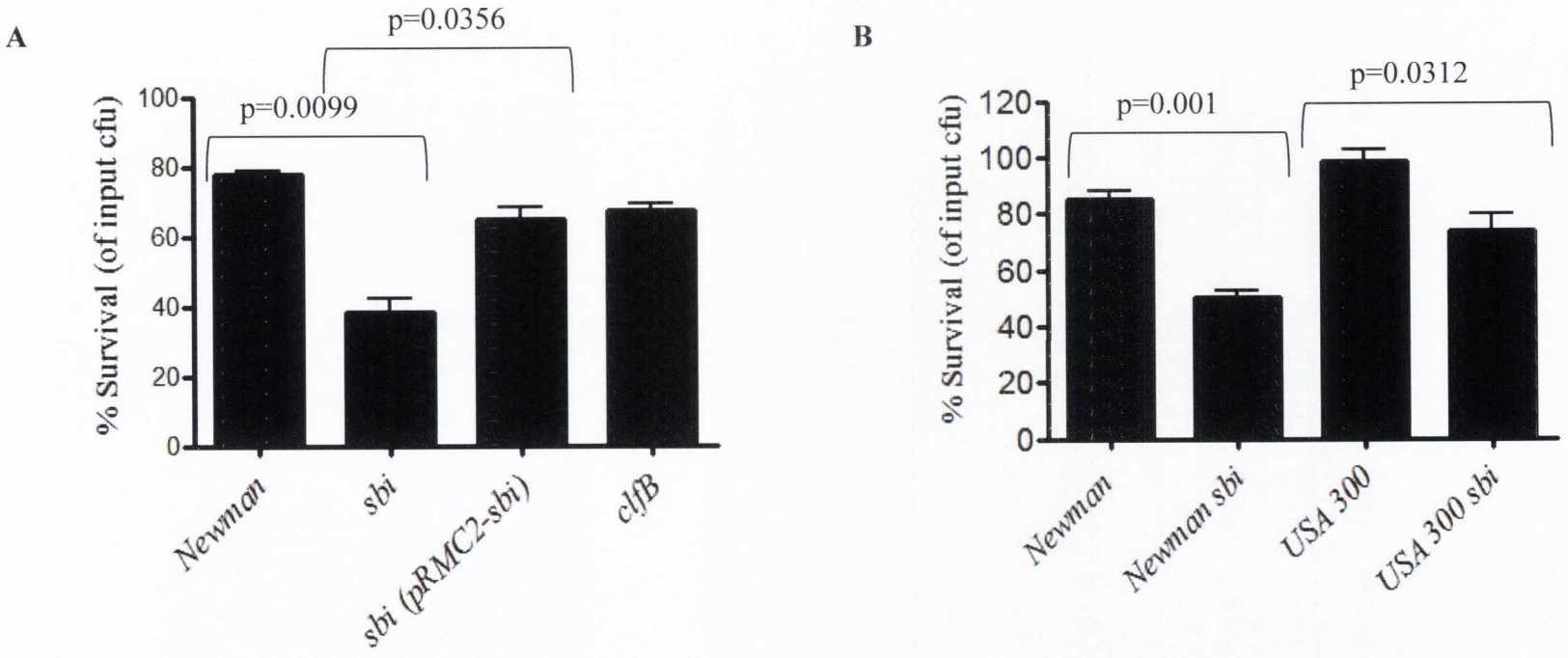


Figure 5.6 Survival of *S. aureus* in whole human blood: 2

(**A**) Complementation of *sbi*::Em^R in strain Newman by pRMC2-*sbi.* (**B**) Survival of clinical *S. aureus* strain USA300 harbouring the *sbi*::Em^R mutation. In both cases bacteria grown in RPMI were tested for their ability to survive 3 h incubation in human blood. Surviving bacteria were measured by viable counting. Data is presented as the mean % surviving cfu ± SD (n=3).

5.2.7 Expression of Sbi inhibits killing of USA300 by neutrophils in whole human blood

In order to show that expression of Sbi reduced the ability of human neutrophils to engulf the clinically relevant *S. aureus* strain USA300, USA300 *sbi* was compared to the wild-type strain. Bacterial cells were incubated in blood obtained from three healthy donors. Hirudin was used as the anti-coagulant in order to preserve complement activity. None of the three selected donors had detectable anti-Sbi antibodies as determined by ELISA. With wild type *S. aureus* USA300, an average of 80% of the initial inoculum survived in blood for 3 hours. The survival of the *sbi* mutant was significantly impaired with only 40% surviving (Figure 5.6, panel B). This experiment demonstrates the importance of Sbi for survival in human blood in clinically relevant strains of *S. aureus* and not just the commonly used lab strain Newman.

5.2.8 Inhibition of killing in human blood by truncates of Sbi

To investigate if both the IgG binding domains D1D2 and the C3 binding domains D3D4 of Sbi were required for protection against phagocytosis, bacteria expressing truncates of Sbi lacking D1D2 or D3D4 (Figure 5.7, panel A) were incubated in human blood. Partial complementation of resistance to phagocytosis was conferred by both SbiΔD1D2 and SbiΔD3D4 with 51.25% and 52.5% survival, respectively, compared to 38.75% for Newman *sbi* (Figure 5.7, panel B). This demonstrates that both the IgG (D1D2) and C3 (D3D4) binding domains contribute to survival.

Plasmid pRMC2-$sbi_{1\text{-}335}$ was constructed which expressed a variant of Sbi where the four binding domains were only found in the growth medium. It was interesting to determine if the exclusively extracellular form of Sbi was protective. Indeed about 56.2% of the cells survived in blood indicating that the extracellular form of the protein was partially protective (Figure 5.8, panel B). This implies that the wall-associated form is also active in immune evasion. It has been suggested that Sbi domains D3D4 can function in futile consumption of C3 when secreted from the cell. To study this further, pRMC2-$sbi_{1\text{-}335}$ was further manipulated to delete the regions encoding D1D2 or D3D4. These truncates were only found in the culture supernatant (Figure 5.8, panel A). Expression of the truncated protein comprising only secreted D1D2 failed to protect

bacteria from phagocytosis compared to the secreted D3D4 domains which were partially protective (51.6% survival) (Figure 5.8, panel B). This suggests that only the D3D4 domains are biologically active when secreted.

5.2.9 Sbi is not a virulence factor in the murine infection model for septic arthritis

The ability of Newman wild-type and a Newman *sbi* null mutant to provoke septic arthritis was investigated in eight week old female NMRI mice. Septic arthritis was induced by intravenous inoculation of 3 x 10^6 colony-forming units (cfu) of Newman wild-type and the *sbi* mutant. The development of arthritis was studied clinically for eight days. No significant difference in the severity of arthritis was observed between the two groups over the entire experimental period (Figure 5.9, panel A). Mice infected with both Newman wild-type and Newman *sbi* lost up to 30% of their body weight (Figure 5.9, panel B), with no significant difference between the two groups occurring at any time during the experimental period. Also, there was no significant difference in bacterial burden in the kidneys between the wild-type and *sbi* mutant (Figure 5.9 panel C). Thus expression of Sbi does not increase bacterial load *in vivo*. The percentage survival was 75% in Newman wild-type infected mice and 37% in the Newman *sbi* null mutant group although the difference was not significant (Figure 5.9, panel D). In conclusion no evidence was found that Sbi is a virulence factor in this model, supporting data previously reported (Chabelskaya et al., 2010).

To investigate this further, bacteria were incubated in mouse and human blood and their ability to survive phagocytosis was measured. This allowed the efficiency of opsonophagocytosis in mouse blood to be compared with human blood. Bacteria were phagocytosed much more efficiently in human blood and both *spa* and *sbi* mutants showed significant attenuation in survival compared to the wild-type agreeing with earlier results. In mouse blood the wild type strain proliferated and the *spa* and *sbi* mutants were only marginally attenuated (Figure 5.9, panel E).

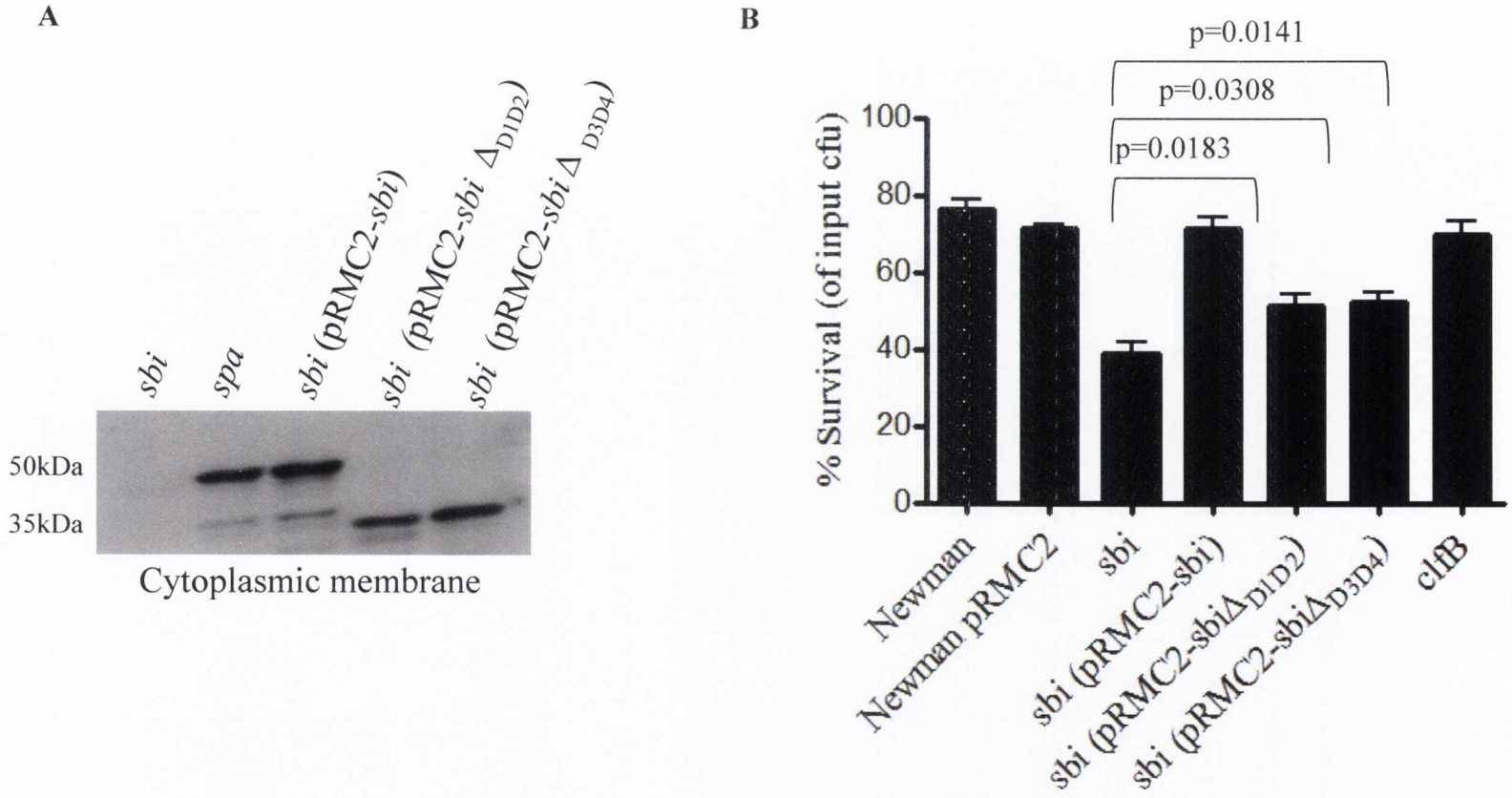


Figure 5.7 Expression of Sbi truncates and survival in blood.

(**A**) Cytoplasmic membrane fractions of Newman *sbi*, Newman *spa*, Newman *sbi* (pRMC2- *sbi*), Newman *sbi* (pRMC2-*sbi*Δ_{D1-D2}), Newman *sbi* (pRMC2-*sbi*Δ_{D3-D4}) were analyzed by SDS-PAGE and Western blotting by probing with HRP-conjugated anti-SbiE antiserum (Table 2.4) (**B**) The same bacterial strains were grown in RPMI and were tested for their ability to survive 3 h incubation in human blood. Surviving bacteria were measured by viable counting. Data is presented as mean % surviving cfu ± SD (n=3).

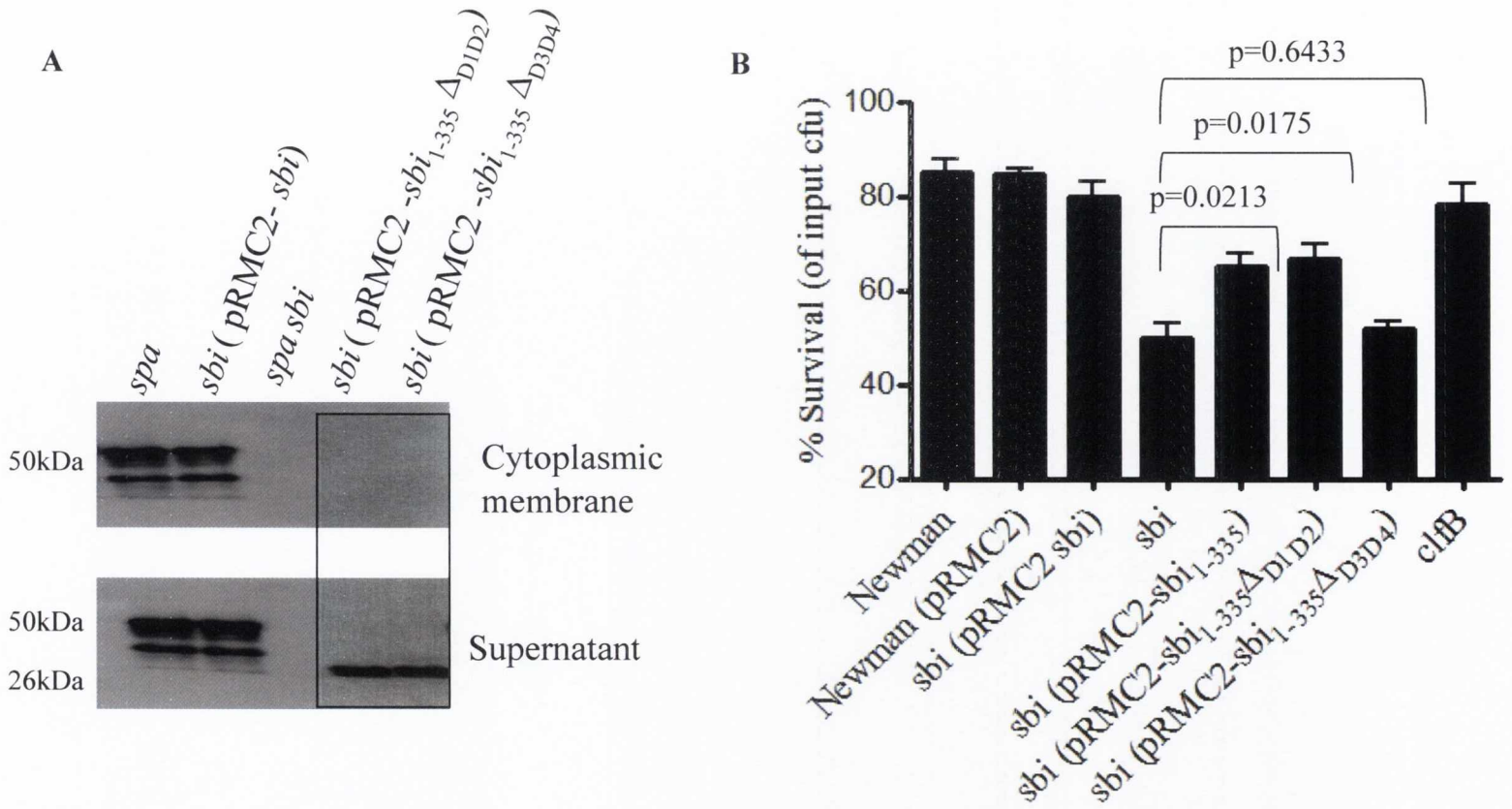
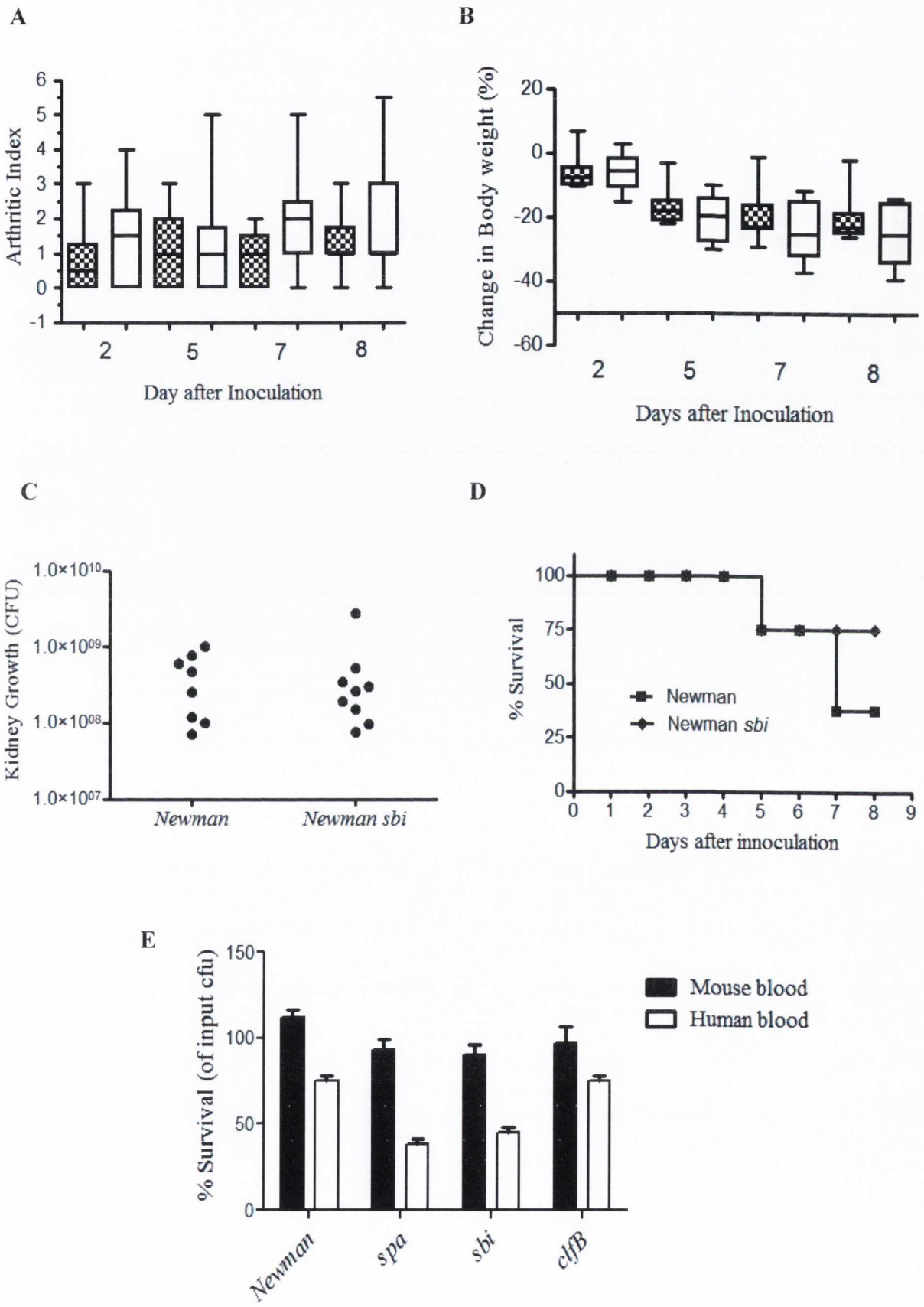


Figure 5.8. Expression of secreted Sbi truncates and survival in blood.

(**A**) Cytoplasmic membrane fractions of Newman *spa*, Newman *sbi* (pRMC2- *sbi*), Newman *spa sbi*, Newman *sbi* (pRMC2-$sbi_{1\text{-}335}\Delta_{D1\text{-}D2}$), Newman *sbi* (pRMC2-$sbi_{1\text{-}335}\Delta_{D3\text{-}D4}$) were analyzed by SDS-PAGE and Western blotting by probing with HRP-labelled anti-SbiE antiserum (Table 2.4). (**B**) Bacteria grown in RPMI were tested for their ability to survive 3 h incubation in human blood. Surviving bacteria were measured by viable counting. Data is presented as mean % surviving cfu ± SD (B, n=4).

Figure 5.9 Murine infection model. Severity of arthritis (**A**) measured as arthritic index, and weight loss (**B**) in mice inoculated with 3 x 10^6 cfu of *S. aureus* strains Newman and Newman *sbi*. Data are represented as medians (centre lines) and interquartile ranges (boxes). The Mann-Whitney *U*-test was performed on data from days 2, 5, 7 and 8 (ns). (**C**) Bacterial growth in kidneys (Data are represented as cfu per kidney pair. A circle represents the kidney counts from one mouse. (**D**) Survival of mice, *N*=10 per group. (**E**) Inhibition of killing in mouse blood compared to human blood. Bacteria grown in RPMI were tested for their ability to survive 3 h incubation in either mouse blood or human blood. Surviving bacteria were measured by viable counting. Data is presented as mean % surviving cfu ± SD. (N=3).


A
Arthritic Index
6
5
4
3
2
1
0
-1
2
5
7
8
Day after Inoculation
B
Change in Body weight (%)
20
0
-20
-40
-60
2
5
7
8
Days after Inoculation
C
Kidney Growth (CFU)
1.0×10^10
1.0×10^09
1.0×10^08
1.0×10^07
Newman
Newman sbi
D
% Survival
100
75
50
25
0
Newman
Newman sbi
0 1 2 3 4 5 6 7 8 9
Days after innoculation
E
% Survival (of input cfu)
150
100
50
0
Mouse blood
Human blood
Newman
spa
sbi
clfB

5.3 Discussion

Freshly isolated human blood treated with an anticoagulant that leaves the complement system intact is an excellent *in vitro* model for bacteraemia. Hirudin is a highly specific thrombin inhibitor derived from the leech *Hirudo medicinalis* that binds to and irreversibly inactivates thrombin whilst leaving the complement system unaffected (Bexborn *et al.*, 2009). In addition, neutrophils are intact and have not been damaged by the manipulations that occur when they are separated from plasma proteins for *in vitro* phagocytosis experiments. Small numbers of bacteria are incubated in whole blood for three hours where their survival depends on their ability to evade complement fixation, phagocytosis and killing by neutrophils.

It has been postulated from *in vitro* studies that Sbi is involved in innate immune evasion by *S. aureus*. Domains D1 and D2 at the N-terminus of the elongated protein bind to the Fc region of human IgG in the same manner as Spa (Zhang *et al.*, 1998). Detailed biochemical and biophysical analysis of the interaction of domains D3 and D4 with complement protein C3 led to the hypothesis that extracellular Sbi could trigger futile consumption of the central component of complement activation (Upadhyay et al., 2008, Burman et al., 2008). Sbi domains D3D4 together covalently bind to C3 via its C3d thioester forming a SbiD3D4:C3b adduct (Burman *et al.*, 2008). It has been proposed that SbiD3D4:C3b adduct formation provides a platform for the assembly of the alternative pathway convertase, that is transiently resistant to inactivation by factor H and factor I leading to futile consumption of C3 (Burman *et al.*, 2008). It has also been proposed that Sbi domains D3D4 bind to the complement regulatory protein factor H (FH). However, this interaction requires the presence of C3b with a tripartite SbiD3D4:C3b:FH complex being formed. In the proposed tripartite complex factor H is biologically active and displays complement regulatory activity by promoting factor I cleavage of C3b (Haupt *et al.*, 2008). This mechanism of complement utilization is different from the observation that SbiD3D4:C3b adduct formation acts as a platform for C3 convertase assembly resulting in the complete consumption of C3 in serum incubated with Sbi D3D4 (Burman *et al.*, 2008). If the tripartite structure is correct C3 consumption would not occur as any C3b available for formation of the C3 convertase C3bBb would be cleaved to inactive iC3b and C3d.

This study shows that Sbi contributes to the ability of *S. aureus* to evade opsonophagocytosis and to avoid killing by neutrophils. This was shown primarily by measuring survival of bacteria in fresh whole human blood and was supported by studies with purified human neutrophils. Firstly, Sbi was compared with known surface-located immune evasion factors. Isogenic mutants defective in Sbi, ClfA, Spa, IsdH and Cap each showed a reduced ability to survive in blood and were taken up more avidly by purified neutrophils *in vitro*. The protective effect of Sbi was also demonstrated by comparing a strain that was defective in Spa, ClfA, IsdH and Cap with a mutant lacking all five factors. In addition, the comparison of Spa, ClfA, IsdH, Cap and Sbi mutants in a uniform genetic background, under identical growth conditions and in the same in vitro assays allowed the relative contribution of each immune evasion factor to survival of *S. aureus* to be elucidated. Based on survival in whole human blood and *in vitro* phagocytosis assays ClfA and IsdH are the most important cell surface exposed immune evasion factors in *S. aureus* strain Newman. Furthermore the importance of Sbi in immune evasion was also demonstrated in the clinically relevant community-acquired MRSA strain USA300.

Another question to be addressed was whether both the secreted and the cell surface-associated forms of Sbi contributed to immune evasion. *S. aureus* strains were constructed where Sbi was expressed from an inducible promoter. The cloned *sbi* gene was mutated to express derivatives of Sbi that lacked residues in the C-terminus required for retaining the protein in the cell envelope as discussed in section 4.2.12. Mutants of Sbi were also constructed which lacked the IgG binding domains D1D2 or the C3 binding domains D3D4. Survival of bacteria expressing truncated Sbi proteins led to the conclusion that both the envelope-associated and the secreted forms of the protein contributed to survival. However, an exclusively secreted form of Sbi that lacked D3D4 and only contained D1D2 did not protect whereas a secreted protein carrying only D3D4 was protective. This implies that D1D2 provides protection only when it is anchored to the cell surface whereas only the secreted D3D4 domains are biologically active. These results concur with the fact that Sbi exposed on the bacterial cell surface binds IgG by its Fc domain as discussed in Chapter 4 and as a result inhibits opsonisation and phagocytosis as described here. The proposed futile consumption of C3 by SbiD3D4:C3b adduct formation and the subsequent generation of the C3 convertase could only be an effective defence if Sbi is extracellular and the D3D4

domains of any cell-bound Sbi are not capable of binding C3 on the bacterial cell surface. Any surface-exposed Sbi D3D4 could trigger complement activation that would promote rather than prevent opsonisation. As discussed in Chapter 4 Sbi is extracellular and cell surface-exposed Sbi does not bind C3 on the bacterial cell surface. These results agree with the finding that Sbi domains D3D4 are biologically active in whole blood survival assays only when secreted, supporting the hypothesis that secreted Sbi causes futile consumption of C3.

Bacterial survival in whole human blood was relied upon to fulfil Koch's Postulates at the molecular level for Sbi. The ability of Sbi to provoke septic arthritis and sepsis was investigated in 8 week old female NMRI mice. No statistically significant difference in the severity of arthritis, weight loss or bacterial burden in the kidneys of Newman or Newman *sbi* infected mice was observed at any time point during the experimental period. The percentage survival was 75% in Newman wild-type infected mice and 37% in the Newman *sbi* null mutant group. Thus no evidence was found that Sbi is a virulence factor in *S. aureus* induced arthritis and septic death, a result that concurs with a previous report (Chabelskaya et al., 2010). It is important to note that it was difficult to demonstrate that Spa is a virulence factor in the same mouse infection model. Statistically significant differences were only seen in the severity of arthritis on one day and only when groups of 30 mice were tested (Palmqvist et al., 2002). This study has shown that survival of *S. aureus* in mouse blood is significantly greater than in human blood and that *sbi* and *spa* mutants are much less attenuated in mouse blood than human blood. On the one hand natural opsonins for *S.aureus* in mouse blood might be less effective than those in human blood and on the other hand immune evasion proteins Spa and Sbi are less protective in the mouse. This illustrates the problems sometimes encountered when attempting to fulfil Molecular Koch's Postulates in murine models (Rooijakkers *et al.*, 2005b, van Wamel et al., 2006). This problem could be overcome by employing an animal where staphylococcal immune evasion factors are more proficient. Alternatively investigators have constructed transgenic mice that express the human version of a target protein or have murinized the pathogen by engineering a virulence factor that can recognize the murine ligand (Lecuit *et al.*, 2001, Bhaskaran & Stebbins, 2007, Wollert *et al.*, 2007).

In summary, this chapter suggests that Sbi has an important role in determining the ability of *S.aureus* to avoid phagocytosis in human blood. The relative contribution of each anti-phagocyctic factor of *S. aureus* to immune evasion under the same growth conditions and in the same genetic background has been established. Moreover we have shown that Sbi domains D1D2 provide protection only when anchored to the cell surface whereas only the secreted D3D4 domains are biologically active.

Chapter 6
Discussion

6.1 Discussion

6.1.1 Innate immune evasion mechanism of Gram-positive bacteria

S. aureus accounts for approximately 10 million skin and soft tissue infections annually in the United States (McCaig *et al.*, 2006) and is the leading cause of hospital acquired infections (Jones, 2003). The Gram-positive group A *Streptococcus* (GAS) accounts for approximately 700 million throat and skin infections and 650 000 invasive infections worldwide (Carapetis *et al.*, 2005). Both *S. aureus* and GAS can result in life threatening infections including endocarditis, sepsis, toxic shock syndrome and necrotizing fasciitis. The ability of both pathogens to cause severe invasive infections in otherwise healthy individuals is due to their capability to evade the host's innate immune responses. The innate immune system plays a vital role in the host's defence against invasive bacteria. The complement system is an essential part of the innate immune system that swiftly recognises and opsonises bacteria to trigger phagocytosis whilst producing chemoattractants to recruit phagocytes to the site of infection. To counteract this, *S. aureus* and GAS have evolved an extraordinary repertoire of complement evading mechanisms and it is informative to consider the similarities and differences in their approaches to innate immune evasion.

The classical and lectin pathways are responsible for the recognition of invading bacteria as nonself. The classical pathway is initiated by the binding of the C1q subunit of the C1 complex to the Fc regions of clustered immunoglobulins bound to antigens on the surface of the bacteria. The lectin pathway is triggered by the binding of the mannose-binding lectin or ficolin to sugar moieties on the surface of the bacterial cell (Walport, 2001a, Walport, 2001b). As discussed earlier the cell wall-anchored protein A (Spa) of *S. aureus* has four or five immunoglobulin binding domains that each bind the Fcγ region of IgG blocking the neutrophil Fc receptor and preventing classical complement fixation (Foster, 2005). GAS express several immunoglobulin binding proteins including Arp, Sir and Mrp, all belonging to the M protein family, which bind the Fc region of IgG inhibiting IgG dependant complement activation (Stenberg *et al.*, 1992, Berge *et al.*, 1997, Carlsson *et al.*, 2003).

An alternative mechanism for bacteria to avoid recognition by the host's innate immune system is to remove opsonins from their surface by proteolytic degradation. *S. aureus* secretes staphylokinase which binds to and activates the serine protease activity of host plasminogen molecules bound to the bacterial cell surface. The serine protease of plasminogen cleaves surface bound C3b and IgG resulting in reduced phagocytosis by neutrophils (Rooijakkers et al., 2005c). GAS expresses several proteases that directly cleave IgG. The cysteine protease SpeB cleaves IgG and is involved in the breakdown of C3 (Collin & Olsen, 2001a, Terao *et al.*, 2008). SpeB has been shown to block neutrophil recruitment thus inhibiting phagocytosis (Lukomski *et al.*, 1998). Mac-1, another cysteine protease of GAS cleaves IgG and binds FcγRIII3 on the surface of neutrophils inhibiting phagocytosis and the oxidative burst pathway (Agniswamy *et al.*, 2004, Lei *et al.*, 2001). The IgG endopeptidase Mac-2 binds the Fcγ receptor on the surface of neutrophils and inhibits neutrophil recognition of IgG on the GAS cell surface (Agniswamy *et al.*, 2004). GAS also secrete the endoglycosidase EndoS which hydrolyses the asparagine-linked glycan in the C_H2 domain of IgG preventing recognition of IgG by neutrophil Fc receptors and complement fixation by the classical pathway (Collin & Olsen, 2001a, Collin & Olsen, 2001b) .

The classical, lectin and alternative pathways of complement fixation converge at the formation of the C3 convertases. The surface-bound C3 convertases catalyse the fundamental reaction in complement activation cleavage of C3 into the chemoattractant C3a and the opsonin C3b (Walport, 2001a, Walport, 2001b, Gasque, 2004). Formation of C3 convertase is crucial as C3b and its inactive derivative iC3b aid phagocytosis. In addition, the surface-deposited C3b can bind factor B. The resulting complex is recognised by factor D which cleaves factor B to release Ba and generate the surface bound C3 convertase C3bBb, thereby amplifying the opsonisation process. High concentrations of locally deposited C3b trigger a shift in substrate specificity of the convertases from C3 to C5. The cleavage products of C5 are the potent chemoattractant C5a and C5b which activates the lytic pathway (Gasque, 2004, Beutler, 2004). *S. aureus* and GAS interfere with the actions of the C3 convertases by cleavage of C3, direct inactivation of the C3 convertases and by acquisition of convertase regulators.

S. aureus secretes a 10kDa protein called staphylococcal complement inhibitor (SCIN) which binds to and stabilizes the C3 convertases C4bC2a and C3bBb inhibiting

cleavage of C3 (Rooijakkers et al., 2005a). The C3 convertases are normally transiently active with dissociation leaving the bound C3b and C4b subunits free to act as catalysts for further cleavage of factor B and C2, respectively. Thus stabilisation of the C3 convertases blocks the amplification loop of the complement cascade, whilst inhibiting C3b formation ultimately blocks C5a formation (Rooijakkers et al., 2009). SCIN has been shown to block phagocytosis and killing of *S. aureus* cells by neutrophils (Rooijakkers *et al.*, 2005a). The *S. aureus* extracellular fibrinogen-binding protein Efb and the extracellular complement-inhibitory protein Ehp both inhibit convertases containing C3b. X-ray crystallography studies demonstrated that both Efb and Ehp bind to native C3 via its C3d thioester and alter the solution confirmation of C3 so that it can no longer be cleaved to generate C3b (Hammel et al., 2007b, Hammel et al., 2007a).

Complement regulatory proteins naturally down-regulate complement fixation, preventing uncontrolled activation and damage to host tissues. *S. aureus* cells can capture and activate factor I independently of factor H resulting in increased levels of iC3b on the bacterial cell surface, a decrease in total C3 fragments on the cells and a reduction in phagocytosis (Cunnion et al., 2004, Cunnion et al., 2005). The *S. aureus* fibrinogen-binding protein ClfA has been shown to recruit and activate factor I resulting in the conversion of C3b to iC3b and C3d (Hair *et al.*, 2008). This leads to loss of opsonins whilst also preventing C3 convertase formation and terminal pathway activation. The streptococcal M protein is a cell wall-anchored protein that binds to the human complement regulators factor H, factor H like-1 (FHL-1), membrane cofactor protein (CD46) and C4-binding protein (C4BP). The M protein consists of two polypeptide chains arranged in an α-helical coiled-coil complex. It comprises four regions the hypervariable (a), variable (b) and conserved regions (c and d) (Robinson & Kehoe, 1992, McNamara *et al.*, 2008). Multiple serotypes of GAS M proteins bind factor H and FHL-1 via their conserved c-repeat region and/or hypervariable N-terminal region (Johnsson *et al.*, 1998). The biological significance of M protein-mediated acquisition of factor H and FHL-1 is controversial as GAS strains of the M1 serotype do not require M protein to bind factor H or FHL-1. In M1 strains the cell wall-anchored protein Fba is responsible for binding factor H and FHL-1 (Pandiripally *et al.*, 2002). Fba promotes M1 GAS survival in whole human blood and prevents deposition of C3b on the bacterial cell surface (Pandiripally *et al.*, 2002). In addition, the streptococcal collagen-like proteins (Scl) of GAS bind factor H and complement factor H-related

protein 1 (Reuter *et al.*, 2010). The hypervariable regions of several M proteins can selectively capture C4BP from human serum. This results in the inhibition of the classical pathway as C4BP interferes with formation of the C4bC2a C3 convertase (Morfeldt *et al.*, 2001, Berggard *et al.*, 2001).

The ultimate reaction in the complement cascade is the cleavage of complement factor C5 into the potent chemoattractant C5a and C5b which is responsible for the initiation of the lytic pathway. *S. aureus* modulates the C5a response by blocking the C5a receptor (C5aR). The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a small secreted protein that binds C5aR inhibiting neutrophil recruitment towards C5a *in vitro* (de Haas et al., 2004, Postma et al., 2005). Staphylococcal superantigen-like 7 (SSL7) is a secreted protein that specifically binds C5a and IgA. SSL7 inhibits the generation of C5a by binding C5 and preventing the binding of C5 to the C5 convertase. The inhibitory effect of SSL7 on C5 convertase binding is increased in the presence of IgA (Bestebroer et al., 2010). ScpA is a cell-associated peptidase expressed by GAS that directly targets C5a. ScpA cleaves C5a within its neutrophil receptor recognition domain blocking chemotaxis of phagocytes towards the site of infection (Laarman *et al.*, 2010).

Among the numerous immune evasion factors of *S. aureus* is the second immunoglobulin binding protein Sbi. Sbi consists of four ligand binding domains D1-D4. D1 and D2 bind the Fcγ region of IgG on the bacterial cell surface effectively blocking the neutrophil Fc receptor and inhibiting opsonophagocytosis. Extracellular Sbi interacts with complement protein C3 via its C3d thioester domain (Upadhyay et al., 2008, Burman et al., 2008). Sbi domain D4 binds C3b and in the presence of Sbi domain D3 forms an Sbi:C3b adduct. It has been proposed that the Sbi:C3b adduct acts as a platform for the formation of the C3 convertase C3bBb resulting in futile consumption of C3 in the fluid phase (Burman *et al.*, 2008). This study demonstrated that an exclusively secreted form of Sbi that lacked domains D3D4 and only contained domains D1D2 did not protect *S. aureus* from killing in whole human blood whereas a wholly secreted protein carrying only D3D4 was partially protective. Furthermore this study also established that surface-exposed Sbi does not bind C3 on the bacterial cell surface. These results correlate with Sbi domains D3D4 being biologically active only when secreted, supporting the hypothesis that secreted Sbi could elaborate its protective

effect by the futile consumption of C3 in the fluid phase. Alternatively the Sbi:C3b adduct could also act as a platform for the formation of the alternative pathway C5 convertase C3bBbC3b. However this would ultimately be detrimental to the bacteria as it would facilitate the recruitment of neutrophils to the site of infection through the generation of C5a. C5 convertase formation would also fail to account for the previously reported complete degradation of C3 in human serum incubated with recombinant Sbi D3D4. Crystallization studies of Sbi D4 in complex with C3d revealed that Sbi D4, Efb and Ehp all share overlapping binding sites on C3d and that all three block the interaction between C3d and complement receptor 2, thus interfering with the link between the innate and adaptive immune systems (Clark et al., 2011, Isenman et al., 2010). This raises the possibility that Sbi could have a similar mechanism of complement evasion in the fluid phase to that of Efb and Ehp. Recently it was been reported that Sbi binds both factor H and factor H like-1 (FHL-1) in a stable tripartite complex with C3 and that this interaction inhibits the alternative pathway (Haupt et al., 2008). The precise mechanism of complement evasion by Sbi in the fluid phase remains to be elucidated and is the next important question to be addressed in the Sbi story.

Figure 6.1 summarises our current understanding of the association of Sbi with the cell envelope and its role in immune evasion. It shows cytoplasmic membrane associated Sbi partially exposed on the cell surface with the N-terminal D1D2 domains able to bind IgG, providing partial protection from opsonisation. In addition the secreted form of Sbi triggers conversion of C3 to C3b in the fluid phase ("futile consumption"). Extracellular Sbi cannot attach to the cell because most of the LTA is already occupied ensuring that at least some Sbi is available to trigger C3 metabolism.

It has been proposed that bacterial complement inhibitors represent an untapped reservoir for the development of novel therapeutics to combat inflammatory diseases that direct complement against our own cells. The high level of specificity of bacterial complement inhibitors for the effectors of the innate inflammatory response coupled with the fact their mechanisms of action rely on protein-protein interactions singles out these bacterial proteins as possible anti-inflammatory drugs. However, a potential drawback to their use is the presence of pre-existing antibodies in individuals who have been exposed to these common human commensals or pathogens. Furthermore some of the complement inhibitors such as SCIN and CHIPs are human specific complicating *in*

vivo studies in animal models (Rooijakkers et al., 2005a, de Haas et al., 2004). Alternatively since the 3D structures of a number of bacterial complement inhibitors in complex with their ligands have been resolved by crystallography perhaps it is more viable for these molecules to act as blueprints for the design of synthetic peptide inhibitors (Hammel et al., 2007a, Hammel et al., 2007b, Rooijakkers et al., 2009, Ippel et al., 2009, Clark et al., 2011). It would seem prudent that these molecules are considered in future strategies to fight infectious diseases. Successful inhibition of bacterial complement modulators during infection could tip the scales in favour of the host allowing a complete immune response and facilitating elimination of the pathogen. In essence a greater knowledge of bacterial immune evasion mechanisms will be an advantage in the design of future therapeutics for both inflammatory and infectious diseases.

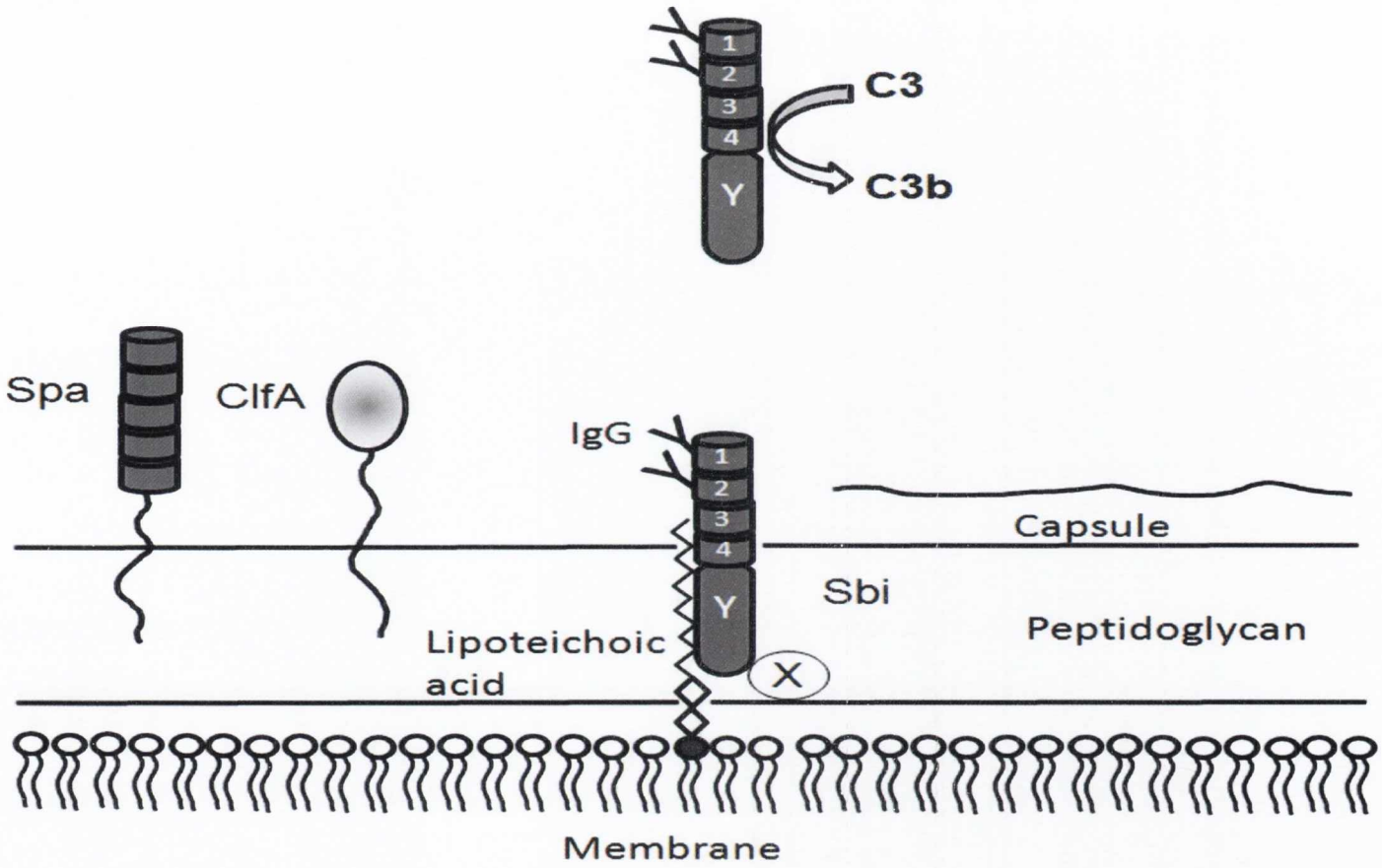


Figure 6.1. Proposed model for Sbi cellular localisation and surface expression

The diagram shows Sbi binding to LTA and to a putative second membrane component (X) as discussed in section 4.3. Only one face of the lipid bilayer is shown. For LTA the jagged line represent polyglycerol phosphate repeats and the rhombus the disacharride linker. The IgG binding domains are biologically active when Sbi is anchored to the cell membrane. Whereas the C3 binding are biologically active only when secreted. The wall-anchored proteins Spa and ClfA employed in this study are shown.

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