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Characterisation of SdrC, SdrD and SdrE from the Serine-Aspartate Repeat Family of *Staphylococcus aureus*.

A thesis submitted for the degree of Doctor of Philosophy

by

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April 2001

Declaration

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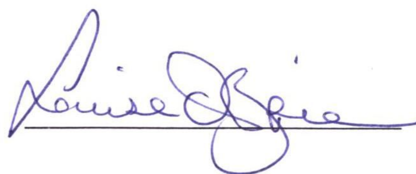

Louise Marie O' Brien.

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Summary

Staphylococcus aureus is an important human pathogen. The ability of *S. aureus* to initiate infection is attributed at least partly to the expression of cell wall-associated proteins known as MSCRAMMs (microbial surface components recognising adhesive matrix molecules). MSCRAMMs mediate the adherence of *S. aureus* to a variety of host ligands such as fibrinogen, fibronectin, collagen, elastin, thrombospondin, vitronectin, laminin, von Willebrand factor and mucin. This allows the bacterium to establish infections at various tissue sites and on biomaterial implants that have become coated with host proteins.

The fibrinogen-binding MSCRAMMs, clumping factors ClfA and ClfB, are members of a family of *S. aureus* proteins known as the serine-aspartate repeat protein family. There are at least three additional members of this family, which are known as SdrC, SdrD and SdrE. The Sdr proteins have a similar domain organisation to the Clf proteins, with the presence of between two and five additional B-repeats. The fibrinogen-binding regions of ClfA and ClfB are located in a unique region at the N-terminus of each protein, known as region A. It is thought that the A regions of SdrC, SdrD and SdrE also contain ligand-binding sites.

Characterising the Sdr proteins at the molecular level was the subject of this project. This required three separate approaches. (1) Isogenic mutants in each of the *sdr* genes were constructed in the two *S. aureus* laboratory strains Newman and 8325-4. Mutants were required in order to study protein expression patterns in *S. aureus* and to provide a tool to study novel ligand interactions by comparing the phenotype of wild type and mutant cells.

(2) Polyclonal mono-specific antibodies were raised against the A domain of each protein. These antibodies were used to identify each of the Sdr proteins in the *S. aureus* strains Newman and 8325-4. The temporal expression and proteolytic cleavage patterns of SdrC, SdrD and SdrE were characterised. SdrC was detected on the surface of strain Newman throughout the growth cycle and was not subject to proteolytic cleavage. However, in strain 8325-4, SdrC was only expressed during stationary phase and was subject to proteolytic cleavage. In strain Newman, SdrD was only detected on stationary

phase cells and was subject to extensive proteolytic cleavage. However, in strain 8325-4, SdrD was detected throughout the growth cycle. Progressive proteolytic cleavage of SdrD was detected as the cells entered stationary phase. Analysis of SdrE expression by strain Newman showed that it was expressed throughout the growth cycle with progressive proteolytic cleavage occurring as the cells entered stationary phase.

(3) Each of the Sdr-Clf proteins were expressed in the Gram-positive surrogate host *L. lactis*. Positive clones were identified by whole cell dot blot using mono-specific antibodies raised against the unique A region of each protein. The Sdr-Clf proteins were expressed constitutively by *L. lactis* and appeared to be present on the cell surface in their full-length form. These clones were used to identify novel phenotypes for the Sdr proteins.

L. lactis cells expressing the Sdr-Clf proteins were used to identify staphylococcal proteins that were involved in activating human platelet aggregation. Results showed that ClfA, ClfB and SdrE could independently activate platelet aggregation when expressed on the surface of *L. lactis*. Studies with *S.aureus* Newman mutants defective in different surface proteins supported these observations. It was shown that ClfA played a major role in the activation of platelet aggregation, as *L. lactis* and *S. aureus* cells expressing ClfA induced platelet aggregation with very short lag times. ClfB appeared to be of intermediate importance, as the lag time observed with *L. lactis* cells expressing ClfB was longer than that of the ClfA-expressing *L. lactis* cells. SdrE activated platelet aggregation with significantly longer lag times than either ClfA or ClfB. However, this reaction was specific as it could be inhibited by anti-SdrE antibodies and recombinant SdrE protein. In contrast, protein A appeared to play an auxiliary role in the process of activating platelet aggregation contrary to earlier reports claiming that it had a major role.

Several novel interactions between the Clf-Sdr proteins and immobilized host matrix/plasma proteins were identified using *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD and SdrE. While none of the proteins appeared to interact with collagen, vitronectin or mucin, ClfA was shown to interact with von Willebrand factor, ClfB was shown to interact with κ-elastin and SdrC was shown to interact with fibronectin.

These results show that the Sdr proteins are expressed on the surface of *S. aureus* and that they are responsible for mediating interactions between the bacterium and the

host. These interactions may be important to the pathogenicity of the organism and should be considered in future design of anti-staphylococcal therapies.

Publications

Kaatz, G. W., Seo, S. M., O'Brien, L., Wahiduzzaman, M. and Foster, T. J. (2000). Evidence for a multidrug efflux transported distinct from NorA in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **44**, 1404-1406.

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O' Brien*, L., Kerrigan*, S., Foster, T. J. and Cox, D. (2001). The ClfA, ClfB, SdrE and protein A proteins of *Staphylococcus aureus* are involved in the activation of human platelet aggregation. Manuscript in preparation. (*These authors contributed equally).

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Key to Abbreviations

Single Letter Amino Acid Code

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

Bases

A	Adenine
T	Thymine
C	Cytosine
G	Guanine

Key to Abbreviations

Antibiotics

Amp	Ampicillin
Cm	Chloramphenicol
Em	Erythromycin
Tet	Tetracycline
Kan	Kanamycin

aa	amino acid residues
bp	base pair(s)
BSA	bovine serum albumin
DNA	Deoxyribonucleic acid
DIG	Digoxygenin
dNTP	deoxy-nucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
h	hour(s)
Ig	Immunoglobulin
IPTG	isopropyl-β-D-thio-galactoside
kb	kilobase pair
kDa	kilodalton
LB	Luria broth
min	minute(s)
nt	nucleotide(s)
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline

Key to Abbreviations

PCR	polymerase chain reaction
PMN	polymorphonuclear leucocytes
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	tris-buffered saline
Tris	trishydroxymethylaminomethane
Ts	temperature sensitive
TSA	trypticase soya agar
TSB	trypticase soya broth
v/v	volume per volume
w/v	weight per volume
wt	wild type
X-gal	5-bromo-4-chloro-3-indoyl-b-D-galactoside.

FOR MY PARENTS

Chapter 1

Introduction

1.1 Biology of the staphylococci

1.1.1 Classification and Identification

Bacteria that are members of the *Staphylococcus* genus are gram-positive cocci about 1 μm in diameter, characteristically dividing 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"). Staphylococci are members of the family *Micrococcaceae* (Schleifer, 1986; Stackebrandt and Woese, 1979). However, this is a nonphylogenetically coherent grouping. Molecular taxonomic studies place the genus *Staphylococcus* in the broad *Bacillus-Lactobacillus-Streptococcus* cluster (Ludwig *et al.*, 1985; Stackebrandt and Teuber, 1988). Staphylococci are most closely related to *Enterococcus, Bacillus* and *Listeria*. The family *Micrococcaceae* contains genera with both high and low G+C content DNA. The G+C content of Staphylococcus DNA is in the range of 30-38%, while the G+C content of *S. aureus* DNA is 36 %.

Staphylococci have a unique cell wall peptidogylcan characterised by multiple glycine residues in the interpeptide bridge, making them susceptible to lysostaphin (Schliefer and Kandler, 1972). The cell wall also contains ribitol teichoic acid which is substituted with N-acetyl glucosamine (Ward, 1981). They are non-motile, non-sporing, catalase-positive and usually oxidase negative. Staphylococci are tolerant of high concentrations of NaCl, with most species able to grow in the presence of up to 3.5 M NaCl. The ability of halotolerant staphylococci to grow on mannitol salt agar containing 7.5% NaCl is often used to distinguish staphylococci from other organisms. Staphylococci can sometimes be distinguished on the basis of their colony morphology. *S. aureus* forms glistening, smooth, entire, raised, translucent colonies that often have a golden pigment.

Coagulase is widely used as a marker for virulent *S. aureus*, although there is little evidence that it is actually a virulence factor (Phonimdaeng *et al.*, 1990). The tube coagulase test involves testing the ability of a culture supernatant to clot rabbit plasma, while the slide test involves testing the ability of a bacterial suspension to agglutinate in the presence of plasma. However, the slide test is actually a test for clumping factor, as

cell-bound coagulase does not promote bacterial clumping in the presence of plasma (McDevitt *et al.*, 1994). Several commercial kits are available for the identification of staphylococcal species. These include tests for heamagglutination (Staphyloslide), latex-particle agglutination (Sero-STAT Staph, Staphaurex, Staph-a-Lex, Accu-Staph, Pastaurex Staph-Plus) and acid production from carbohydrate substrates (API STAPH-IDENT, STAPH Trac System, ID32 STAPH). Larger clinical laboratories may use automated systems (Vitek, Micro Scan Pos ID), antimicrobial susceptibility (PosCombo, Sceptor Staphylococcus MIC/ID, Sceptor Gram-Positive Breakpoint/ID and PGP Micro Plate) or fatty acid analysis by gas chromatography. The AccuProbe is a DNA probe-based system that can be used for the identification of *S. aureus* strains. This system is very accurate but expensive and may be used to identify strains that were negative in other tests.

1.1.2 Small Colony Variants

Staphylococcal small colony variants (SCVs) are slow growing forms of *S. aureus* that are often isolated from patients with persistent or recurring infections (Baddour and Christensen, 1988; Proctor *et al.*, 1994, 1995; Kahl *et al.*, 1998; Proctor and Peters, 1998) and can readily be selected *in vivo* and *in vitro* due to their intrinsic resistance to aminoglycoside antibiotics (Balwit *et al.*, 1994). The unusual colony morphology and altered biochemical profile of SCVs often leads to their misidentification in the clinical laboratory, causing major problems in the design of disease treatment (Kahl *et al.*, 1996, 1998; Proctor *et al.*, 1995). SCVs are defective in hemin and menaquinone biosynthesis, resulting in defective electron transport (Proctor *et al.*, 1994). In addition to the slow growth phenotype, SCVs have less pigmentation and have lowered coagulase. They also produce reduced amounts of lytic toxins (α-toxin) and are able to persist within host endothelial cells (Balwit *et al.*, 1994, Proctor, 1994, 1998; Proctor *et al.*, 1994, 1995, 1996). In fact, it has been shown that the host intracellular environment actually induces formation of SCVs (Vesga *et al.*, 1996), where after uptake by endothelial cells, SCVs occur at a 10,000 fold higher frequency than in the *in vitro*-grown culture. However, the SCV phenotype is often unstable. Slow-growing variants that are selected following

penicillin treatment are particularly unstable and often revert to the wild type phenotype following a single passage on solid media (Schnitzer at al., 1943). It is not known if these SCVs are defective in electron transport or if their phenotype is due to another mechanism (Proctor, 2000). SCVs that are selected on aminoglycosides such as streptomycin are more stable and have characteristics very similar to those of the now well-characterised SCVs that are defective in electron transport (Barbour, 1950).

1.1.3 Stress Resistance

S. aureus can colonise a wide variety of environmental niches. It is primarily a human pathogen that can colonise the skin and nasopharynx of healthy individuals. However, *S. aureus* can also persist outside the host in dust, soil, water and other environments (Clements and Foster, 1999). As *S. aureus* cycles from its host to the terrestrial milieus, it must survive a diverse range of stresses. It is the adaptability of this organism that allows it to be so successful as an opportunistic pathogen.

S. aureus usually enters the host at the site of a wound, caused either accidentally or during surgery. The bacterium has developed a number of mechanisms by which it avoids host defences, involving specific surface proteins and cell-surface charge (Foster and Hook, 1998; Peschel *et al.*, 1999). Professional phagocytes are responsible for most of the initial killing of *S. aureus*. Therefore, the ability to avoid or survive neutrophil or other phagocytic attack has important implications for the pathogenicity of the bacterium.

The production of superoxide (O_2^-) within host phagocytes is one mechanism by which bacteria are killed inside the phagolysosome. *S. aureus* produces at least two superoxide dismutases (SODs) for the destruction of exogenous O_2^- (Clements and Foster 1999). H_2O_2 is also produced by phagocytes. This molecule is reactive and can cause cell damage and cell death. *S. aureus* produces a single major catalase (KatA), which accumulates extracellularly and may be responsible for limiting the effects of H_2O_2 in the phagolysosome (Watson *et al.*, 1998a). *S. aureus* also contains an uncharacterised alkyl hydroperoxide reductase (AphC), which may protect the cell from the bactericidal $OONO^-$, also generated indirectly within neutrophils and macrophages (Armstrong-Buisseret *et al.*, 1995).

S. aureus can also survive and grow in a variety of environments not associated with pathogenesis. This is due to the ability of the bacterium to tolerate desiccation, osmotic stress and starvation. This in turn contributes to the organisms' dissemination and to the problem of eradication in the hospital environment (Clements and Foster, 1999). Although it is well known that staphylococci are extremely desiccation tolerent, the exact mechanism for this is unknown. However, a correlation between resistance to desiccation and pigmentation has been observed (Lacey and Lord, 1981).

S. aureus is also tolerant to high osmotic stresses and is able to grow at high concentrations (up to 3.5 M) of NaCl (Wilkinson., 1997). When exposed to high osmolarity, *S. aureus* accumulates the compatible solutes glycine, proline and betaine in the cytoplasm (Townsend and Wilkinson, 1992). Shortening of peptidoglycan interpeptide bridges also occurs at high osmolarity and this is thought to confer mechanical strength to the cell to resist changes in turgor pressure (Vijarankul *et al.*,1995).

Nutrient limitation in *S. aureus* induces a starvation survival state. After two days of growth in limiting nutrients at 37°C, 99% of the bacterial population will die. However, the remaining population can survive for several months due to induction of the starvation survival state. This is controlled by the interaction of several loci, including *sodA*, heam A synthase (*ctaA*) and a component of the SOS response (*umuC*) (Watson *et al.*, 1998a; Watson *et al.*, 1998b). This state is reversible and starved cells respond rapidly to the availability of nutrients, leading to a reversal of many of the starvation-associated cellular changes (Watson *et al.*, 1998b). However, mutants have been isolated that are defective in recovery (Clements and Foster, 1998; Clements *et al.*, 1999a, b). A sigma factor (σB) is required for stress resistance but not for starvation survival or recovery. σB mutants are defective in resistance to oxidative stress (Kullik *et al.*, 1998).

1.1.4 Identification of Genes Expressed or Required *In Vivo*

The identification and characterisation of genes that contribute to the survival and replication of a bacterial pathogen within its host are essential to understanding the

pathogenesis of the organism. Studying the effects of specific bacterial proteins has contributed greatly to the understanding of the pathogenicity of *S. aureus*, however *in vitro* analysis does not reflect the *in vivo* environment. In recent years, techniques such as signature-tagged mutagenesis (STM) and *in vivo* expression technology (IVET) have been successful in identifying genes that contribute to the virulence of *S. aureus* in murine models of infection.

1.1.4.1 Signature Tagged Mutagenesis (STM)

STM was initially developed to identify genes that were responsible for the virulence of *Salmonella typhimurium* in a mouse model of infection (Hensel *et al*., 1995). The technique was subsequently used to identify genes that contributed to the virulence of *S. aureus* in mouse models of bacteraemia, abscess and wound infection (Mei *et al.*, 1997; Schwan *et al.*, 1998). In *S. aureus* STM, a large number of insertion mutants are generated using the transposon Tn*917*. Each insertion is marked by a unique DNA tag, which allows mutants to be differentiated from each other. The tags comprise 40 bp variable central sequences flanked by invariant "arms" of 20 bp, which enable the central portions to be amplified by the polymerase chain reaction (PCR). Tagged mutant strains are assembled in microtitre dishes and are then pooled to form the "inoculum pool" for infection studies. At an appropriate time after inoculation, bacteria are isolated from the animal and are pooled to form the "recovered pool". The tags in both the inoculum pool and the recovered pool are separately amplified, labeled and used to probe filters arrayed with the different tags representing the mutants in the inoculum. Mutants with attenuated virulence are those with tags that give hybridisation signals when probed with tags from the inoculum pool, but not with tags from the recovered pool. STM has shown that several genes are important for the *in vivo* growth of *S. aureus*. These include *femA, femB* and *femX*, which are involved in the formation of the interpeptide pentaglygine cross-bridges of peptidoglycan (Mei *et al*., 1997), *recA* (Mei *et al*., 1997), *lsp* which encodes the *S. aureus* prolipoprotein signal peptidase (Mei *et al*., 1997), the *putP* proline permease (Schwan *et al*., 1998) as well as other genes of unknown function. One disadvantage associated with STM is that the technique cannot identify secreted proteins

(such as toxins) as virulence factors due to cross complementation by neighbouring bacterial cells in the infection pool.

1.1.4.2 *In Vivo* Expression Tecnnology (IVET)

IVET is a recently described strategy for identifying bacterial genes that are active during mamalian infection but relatively inactive at other times (Mahan *et al.*, 1993). IVET differs from STM in a number of ways. While STM identifies genes that are important for virulence *in vivo*, it does not select genes that are only expressed *in vivo* and therefore may identify genes that are constitutively expressed both during infection and outside the host. IVET allows identification of genes that are only expressed *in vivo* during bacterial infection. In addition, while the STM screen does not allow detection of possible secreted virulence factors due to cross-complementation by neighbouring cells in the infection pool, IVET allows the detection of any gene that is expressed strongly during mammalian infection. The principle of IVET is based on the use of genetic recombination as a reporter of gene activation *in vivo* (Camilli *et al.*, 1994). A gene library of bacterial genomic DNA fragments is constructed, where the fragment is fused to a promoterless copy of *tnpR*. *tnpR* encodes the site-specific recombinase ("resolvase") of transposon γδ (Newman and Grindley 1984). A second plasmid containing the kanamycin resistance casette flanked by direct *res* sites (substrate for resolvase) is integrated into the chromosome. Cells that contain this integrated plasmid are resistant to kanamycin. Transcriptionally active fragments that have been inserted into the *tnpR* plasmid will direct resolvase-mediated excision of the kanamycin casette from the chromosome. Kanamycin sensitive cells are selected following recovery from the infected animal, as these cells will contain *in vivo* active promoters fused to the *tnpR* gene. Several *S. aureus* genes that are expressed strongly *in vivo* have been identified (Lowe *et al.*, 1998). These include *cap8C* (type 8 capsular polysaccharide production), *spsAB* (type I signal peptidase), pbp2 (penicillin-binding protein 2), *lip* (glycerol ester hydrolase), *agrA* (accesory gene regulator). Other *S. aureus* genes of unknown function were also identified. IVET has previously been attempted in *S. typhimurium, Vibrio cholerae* and *Pseudomonas aeruginosa* (Camilli and Mekalanos, 1995; Heithoff *et al.*,

1997; Mahan *et al.*, 1993, 1995; Wang *et al.*, 1996). Both IVET and STM provide powerful tools for studying the genes that are important for bacterial pathogenesis *in vivo*.

1.2 Virulence Factors.

S. aureus is a major pathogen in human infectious diseases with many clinical manifestations. Infections associated with this organism can range from minor wound infections to severe sepsis. The ability of *S. aureus* to cause infection is due to the large number of extracellular virulence factors and other proteins which it produces in order to colonise and survive within the host.

1.2.1 Extracellular toxins

1.2.1.1 Alpha-toxin

Alpha-toxin (encoded by the *hla* gene) is a cytolytic pore-forming toxin that is secreted by *S. aureus* into the extracellular environment as a monomeric water-soluble protein of 293 aa residues (Bhakdi and Tranum-Jensen, 1991). Some cells such as rabbit erythrocytes, human platelets and human monocytes have high affinity binding sites for α-toxin, while other cells such as human erythrocytes have only low affinity sites (Hildebrand *et al.*, 1991). The identity of the high-affinity binding site is unknown, but it is probably a membrane protein. Upon binding to the membrane, the monomer oligomerises to form a ring-shaped heptameric pore. Only part of the toxin penetrates the bilayer while the bulk of it remains on the surface. The pore is a water-filled channel which is estimated to be 11.4 Å in diameter from conductance studies (Menestrina, 1986).

A model for α-toxin assembly on the mammalian cell surface has been proposed based on crystallographic data and structure-fuction experiments (Summarised by Bohach and Foster, 2000). The first step in assembly involves the binding of a single monomer (α1) to the target membrane. Once bound, α1 promotes the assembly of the heptamer (α7*). The final stage of assembly involves the insertion of β-sheets present in

each monomer into the target membrane (α7). This final step is crucial for channel formation (Valeva *et al.*, 1996). Although the α1 and α7* forms are susceptible to proteases, the fully assembled heptameric pore (α7) is resistant (Walker *et al.*, 1992, 1995).

The crystal structure of the α-toxin heptamer formed in detergent solution has been solved (Song *et al.*, 1996). These data show a mushroom-shaped heptamer forming a solvent-filled channel ranging from 14-46 Å. The lower half of a 14-stranded β-barrel forms the stem domain which penetrates and spans the lipid bilayer to form the pore.

Most *S. aureus* strains possess the *hla* gene, but are variable in the amount of toxin expressed. Animal model studies have shown that α-toxin is an important virulence factor in staphylococcal infection (Foster *et al.*, 1990).

1.2.1.2 Beta-toxin

Beta-toxin (encoded by the *hlb* gene) is a Mg^{2+}-dependent sphingomyelinase that is secreted into the extracellular environment by *S. aureus* (Mollby, 1983, Arbuthnott, 1982). Unlike lesions induced by pore-forming toxins, β-toxin causes invaginations of selected regions of the host cell membrane. It has been shown that β-toxin is at least 10- to 160-fold less toxic than α-toxin (Low and Freer, 1977). The sensitivity of erythrocytes to β-toxin is dependent on the sphingomyelin content of the cell membrane. Cow, sheep and goat erythrocytes are most sensitive to β-toxin, while human erythrocytes have intermediate sensitivity. Murine and canine erythrocytes are resistant to β-toxin. The toxin produces "hot-cold" heamolysis. Incubation of sensitive erythrocytes with β-toxin at 37°C results in little or no lysis. However, when the erythrocytes are chilled below 10°C, rapid lysis ensues. The toxin acts as a type C phosphatase, hydrolysing sphingomyelin to phosphorylcholine and ceramide (Low and Freer, 1977). This hydrolysis is dependent on divalent cations. Mg^{2+} is most effective, although either Co^{2+} or Mn^{2+} can enhance activity. Ca^{2+} and Zn^{2+} are inhibitory. Hot-cold lysis appears to have two stages. Following sphingomyelin hydrolysis at 37°C, the ceramide is held in the membrane with Mg^{2+} ions preventing the collapse of the weakened bilayer. However,

upon cooling, a phase separation occurs with condensation of the ceramide into pools and collapse of the bilayer (Low and Freer, 1977; Smyth *et al.*, 1975).

The *hlb* gene carries the attachment site for serological group F converting phages (Coleman *et al.*, 1986). Lysogens contain a disrupted *hlb* gene caused by integration of a prophage (Coleman *et al.*, 1986, 1989). Insertion of this prophage results in the non-expression of β-toxin, which occurs in the majority of human isolates. However, the prophage is strongly associated with the expression of enterotoxins and superantigens.

β-toxin is also leukotoxic. Like erythrocytes, leukocytes are susceptible to hot-cold lysis. β-toxin also inhibits monocyte migration and stimulates the release of IL-1β, IL-6 receptor and soluble CD14 from human monocytes (Walev, 1996). Impaired chemotaxis and Fc binding in neutrophils is also caused by β-toxin. Although β-toxin has been shown to contribute to pathogenesis in the murine mastitis model (Foster *et al.*, 1990) and the ocular keratitis model (O'Callaghan *et al.*, 1997), its effects are thought to be less significant that that of α-toxin.

1.2.1.3 Delta-toxin

The delta-toxin (encoded by the *hld* gene) is a small heat-stable 26 aa residue cytolytic peptide secreted into the extracellular environment by *S. aureus* (Arbuthnott, 1982; Mollby, 1983). The toxin, which has structural similarities to the bee venom melliton (Fitton *et al.*, 1980) has surfactive properties and is cytolytic towards a wide variety of membranes including erythrocytes, tissue culture cells and bacterial protoplasts (Kreger *et al.*, 1971). The *hld* gene is located near the 5' end of the Agr RNAIII transcript. It is produced by nearly all *S. aureus* isolates and by a high percentage of other staphylococcal species. The exact orientation and structure of the δ-toxin in the host cell membrane is not known. However, it is thought that the peptides insert into the lipid bilayer, disordering lipid chain dynamics. It is possible that the mode of lysis involves formation of channels in membranes composed of aggregates of six δ-toxin peptides (Mellor *et al.*, 1988). Because δ-toxin has such a broad specificity for a wide variety of mammalian cell types, it is likely that it has a role in pathogenicity. However, this has not been shown.

1.2.1.4 Gamma-toxin, Leukocidin and other biocompetent toxins

Gamma toxin, leukocydin and other biocompetent toxins are a family of proteins encoded by the *hlg* and *luk-PV* loci that are secreted into the extracellular environment by *S. aureus*. All of the toxins in this family contain two synergistically acting proteins: one S component (LukS-PV, HlgA and HlgC) and one F component (LukF-PV and HlgB), designated on their mobility (fast or slow) in ion-exchange chromatography (Summarised by Bohach and Foster, 2000).

The Panton and Valentine (PV) leukocidin is a two component cytotoxin released by some strains of *S. aureus*. It is composed of two polypeptide chains (LukS-PV and LukF-PV) that act together to damage the membrane of neutrophils and macrophages (Prevost *et al.*, 1995). Binding of the two subunits to the host cell membrane is sequential, with the LukF-PV component binding first, followed by the LukS-PV component. PV leukocydin is non-heamolytic and does not damage the membrane of other normal cells. However, PV leukocidin is strongly associated with severe skin infections such as furunculosis. It is a potent leukocidin, active against both human and rabbit leukocytes and has been shown to act as a virulence factor in several animal models.

The γ-toxin locus expresses three polypeptides. HlgA and HlgC are related to LukS-PV, while HlgB is related to LukF-PV (Cooney *et al.*, 1993; Supersac *et al.*, 1993). The γ-toxin, which consists of HlgA + HlgB, is active against erythroctyes. HlgB binds to the erythrocyte membrane, followed by the sequential binding of HlgA. It is thought that the two components form a ring-shaped pore in the membrane in a 1:1 ratio (Ozawa *et al.*, 1995).

More than 99% of clinical isolates carry the *hlg* locus, while only 2% of isolates carry the *luk* genes (Prevost *et al.*, 1995). Any S-component (LukS-PV, HlgA, HlgC) can interact with any F-component (LukF-PV, HlgB) to form an active toxin. Therefore, strains that carry the *luk* and *hlg* loci (rare), can manufacture up to six different PVL toxins. Strains that carry only the *hlg* locus produce only two different types of these toxins.

1.2.1.5 Epidermolytic (Exfoliative) toxin

S. aureus expresses two antigenically distinct forms of the epidermolytic or exfoliative toxin (ETA and ETB). Both of these toxins have been implicated in staphylococcal scalded skin syndrome (SSSS). SSSS includes a spectrum of staphylococcal illnesses in patients, predominantly children, characterised by the formation of blisters and widespread epidermal splitting (Bailey *et al.*, 1995; Gemmell, 1995). In patients that do not have antibodies to ET, both blistering and epidermal peeling occurs, however, patients that have ET antibodies in their sera only develop localised skin lesions, as in bullous impetigo (Melish and Glasgow, 1970).

ETA is encoded by the chromosomally-located *eta* gene, while the *etb* gene is carried on a plasmid. The proteins are approximately 40% identical, with conserved residues clustered in three discrete regions. In addition, both toxins are approximately 25% identical to the *S. aureus* serine protease (Prevost *et al.*, 1992). The catalytic triad H-72, D120 and S-195 which is present in each of these proteins is thought to be important for activity. This was demonstrated by site-directed mutagenesis of the S-195 residue, where mutants in this single residue showed no epidermolytic activity (Redpath *et al.*, 1991). Mutations in the H-72 and D-120 residues also showed similar results (Prevost *et al.*, 1992). Although the ETA and ETB toxins are similar to serine protease, they do not have detectable proteolytic activity. They do, however, have esterase activity (Bailey and Redpath, 1992).

The mode of action of ETA and ETB remains unclear and it is not certain which cells the toxins bind to. It has been shown that ETs act as super antigens, inducing T-cell proliferation via a mechanism that requires antigen presenting cells and occurs in a Vβ-dependent manner (Monday *et al.*, 1999). However, it is not known if this super antigenic property contributes to SSSS.

1.2.1.6 Enterotoxins and Toxic Shock Syndrome Toxin-1

The enterotoxins and toxic shock syndrome toxin-1 (TSST-1) are members of a family of pyrogenic toxins produced by *S. aureus* and *Streptococcus pyogenes* which behave as superantigens (Ulrich *et al.*, 1995; Bohach *et al.*, 1990; Marrack and Kappler, 1990). There are at least 9 antigenically distinct staphylococcal enterotoxins (SEs) produced by *S. aureus*, designated SEA-SEJ. The SEs are associated with staphylococcal food poisoning, which is one of the leading causes of food-borne illness usually associated with improper food storage (Holmberg and Blake, 1984). SEs are stable in the gastrointestinal tract and indirectly stimulate the emetic reflex center. When food that is contaminated with SEs is ingested, vomiting, nausea, diarrhea and abdominal pain usually follow. This illness is self-limiting and usually only lasts 24 hours. TSST-1 is not implicated in food poisoning.

Toxic shock syndrome (TSS) is an acute illness. Patients present with fever, rash, hypotension and desquamation during convalescence and have involvement of at least three additional organ systems (Rheingold *et al.*, 1982). TSS can manifest in two different forms: menstrual or non-menstrual. Menstrual TSS is associated with the usage of high-absorbency tampons and occurs in women whose vaginal or cervical mucosa is colonised with TSST-1-producing *S. aureus*. Non-menstrual TSS may result from *S. aureus* infection elsewhere in the body. The non-menstrual form of TSS may be mediated by either TSST-1 or SEs.

Superantigens are not processed by antigen-presenting cells (APCs), but bind as intact molecules to major histocompatibility complex II (MHC-II) molecules at a region adjacent to the peptide binding groove (Dellabonna *et al.*, 1990; Jardetsky *et al.*, 1994; Kim *et al.*, 1994). Once bound to the MHC-II molecule, the superantigen (SEs and TSST-1) stimulates T-cells bearing certain variable β (Vβ) domains on the α/β T-cell receptor (TCR) (Choi *et al.*, 1989). While conventional antigens activate 10^4-10^6 T-cells, superantigens can activate up to 30% of all α/β T-cells. This results in a massive elevation of cytokine levels leading to TSS and certain autoimmune diseases (Schlievert, 1993).

1.2.2. Extracellular Proteins and Enzymes

1.2.2.1. Coagulase

Coagulase production is the principle criterion used in the clinical laboratory for the identification of *S. aurues*. However, while most strains of *S. aureus* possess the *coa* gene, some do not produce detectable amounts of coagulase. Coagulase is predominantly an extracellular protein that binds prothrombin stoichiometrically in a 1:1 molar ratio to form a complex called staphylothrombin which activates the conversion of fibrinogen to fibrin clots (Hemker *et al.*, 1975; Kawabata *et al.*, 1985). Coagulase also binds to fibrinogen (Boden and Flock, 1989). The prothrombin-binding domain is located at the N-terminus of the protein, while the fibrinogen-binding domain is in the C-terminal repeated region (McDevitt *et al.*, 1992). A small proportion of coagulase becomes firmly attached to the staphylococcal cell wall by an unknown LPXTG-independent mechanism where it activates prothrombin (Boden and Flock, 1989). While bound coagulase is not responsible for fibrinogen-dependent cell clumping (McDevitt *et al.*, 1992), some reports suggest that cell-bound coagulase may be responsible for binding to soluble fibrinogen (Wolz *et al.*, 1996).

Production of coagulase is negatively regulated by the *agr* locus. In wild type cells coagulase is detected maximally during the exponential phase of growth, while in an *agr* mutant coagulase is detected throughout the growth cycle. However, the levels of the *coa*-specific transcript and coagulase activity are lower in an *agr* mutant, suggesting that *agr* also has a positive effect on *coa* (Lebeau *et al.*, 1994). Transcription of *coa* is also effected by the *sae* locus. In an *agr-sae-* double mutant, *coa*-specific mRNA levels were lower than in the *agr* mutant, suggesting that *sae* is a positive regulator (Guriado *et al.*, 1997).

The role of coagulase in pathogenesis is unclear. While coagulase does not appear to be an important virulence factor in the mouse mastitis model (Phonimdaeng *et al.*, 1990), it does appear to have a role in the mouse model of blood-borne pneumonia (Sawai *et al.*, 1997). These data suggest that coagulase may be more important in some types of infections than in others.

1.2.2.2 Staphylokinase

Staphylokinase (Sak) is a potent activator of plasminogen. Plasminogen is a 90 kDa plasma protein which is a key component of the mammalian fibrinolytic system (Stephens and Vaheri, 1993). It is the precursor of plasmin, which is a broad-spectrum serine protease. Plasmin is formed from plasminogen through activator-catalysed cleavage of a single peptide bond (R517-V562), resulting in two polypeptide chains that are held together by two disulphide bonds. Plasminogen is normally activated by the host activators urokinase and tissue plasminogen activator (tPA). Both of these host activators are tightly controlled by specific regulators. In the absence of fibrin, plasmin is rapidly inactivated by α_2-antiplasmin (Dano *et al.*, 1985).

Sak is expressed extracellularly by *S. aureus* and binds plasminogen/plasmin to form a 1:1 stoichiometric complex (Sako *et al.*, 1983; Behnke and Gerlach, 1987). The Sak-plasminogen complex is inactive and must be converted to Sak-plasmin to become active. Activation of the complex is thought to be dependent on residue Lys-11 of Sak, as deletion of this residue or substitution with Cys eliminates plasminogen activator potential (Collen, 1998). In the absence of fibrin, the Sak-plasmin is rapidly inactivated by α_2-antiplasmin. This results in the dissociation of Sak, which can then be recycled (Lijnen *et al.*, 1991). Inactivation of the Sak-plasmin complex by α_2-antiplasmin is decreased 100-fold in the presence of fibrin (Lijnen *et al.*, 1992). The active Sak-plasmin complex can convert both Sak-bound plasminogen and free plasminogen to plasmin, resulting in fibrin clot lysis (Silence *et al.*, 1995).

Sak can attach to the staphylococcal cell wall. This can occur after the secreted Sak has bound plasmin(ogen) or the normally secreted Sak can bind to the cell wall where it can directly capture plasminogen and bind it to the bacterial cell surface (Kuusela and Saksela, 1990; Christner and Boyle, 1995). Cell-associated plasmin is not regulated by α_2-antiplasmin and is very effective at solubilising fibrin clots. The presence of an active Sak-plasmin complex on the bacterial cell surface may be a mechanism for *S. aureus* to acquire invasive potential (Lottenburg *et al.*, 1994)

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1.2.2.3 Proteases

Most strains of *S. aureus* have extracellular protease activity. Analysis of genome sequences (http://www.genome.ou.edu/stap.html and http://www.tigr.org) has shown that there are at least six different classes of proteases present in *S. aureus* strains 8325 and COL (S. Foster, personal communication). Three of these have been characterised. The role of proteases in bacterial virulence is not clear. However, they may play a role in the modulation of bacterial surface-associated proteins (McAleese *et al.*, 2001)

1.2.2.3.1 Metalloprotease

The *S. aureus* metalloprotease (aureolysin, protease III) is a 33 kDa calcium-binding, zinc-dependent endopeptidase with optimal activity at neutral pH. This protease cleaves peptide bonds on the N-terminal side of bulky hydrophobic amino acid residues. The three-dimensional structure of the *S. aureus* metalloprotease has been solved and it appears to have structural similarities to the thermolysin of *Bacillus thermoproteolyticus*, the neutral protease of *Bacillus cereus* and the elastase of *Pseudomonas aeruginosa* (Banbula *et al.*, 1998). All the aa residues involved in substrate binding and catalytic activity among these proteases are conserved. However, some differences were seen in the active-site cleft, which may explain the lack of elastase activity observed with the *S. aureus* metalloprotease as compared with the other enzymes. The metalloprotease is involved in activating the V8 or serine protease proenzyme (Drepeau, 1978) and may be involved in the post-translational modification of other proteases. Very low levels of protease activity are detected in the metalloprotease mutant of *S. aureus* strain Newman (McAleese *et al.*, 2001).

1.2.2.3.2 Serine Protease

The *S. aureus* serine or V8 protease belongs to a small group of enzymes that preferentially cleave glutamoyl peptide bonds. A weak activity is also seen against peptide bonds following aspartic acid residues. Because of its narrow substrate

specificity, V8 protease has been used widely for site-specific fragmentation of proteins before amino acid sequencing. While the V8 protease has only low sequence identity to other bacterial serine proteases, the catalytic triad (His-51, Asp-93 and Ser-168) is well conserved. V8 protease is secreted as a preproenzyme that has no proteolytic activity, but is activated by proteolytic cleavage by the *S. aureus* metalloprotease (Drapeau, 1978). Sequence analysis of the *S. aureus* strain COL genome (http://www.tigr.org) has shown that the V8 structural gene (*sspA*) is followed closely by an open reading frame encoding a cysteine protease, designated *sspB* (Rice *et al.*, 2001). The *sspA* and *sspB* proteases are transcribed in an operon, which also includes a third open reading frame *sspC*, of unknown function. Non-polar inactivation of *sspA* resulted in a pleiotropic effect on the profile of secreted proteins, including autolysin activity and proteolytic maturation of the cysteine protease. However, no attenuation in a mouse tissue abscess model was observed (Rice *et al.*, 2001). This is curious, as *sspA* mutants have been identified by STM. However, the STM approach used a mouse bacteremia model, suggesting that SspA may be more important in certain types of infections.

1.2.2.3.3. Thiol Protease

There are three different extracellular thiol (cysteine) proteases in *S. aureus*. The first characterised enzyme (protease II) is an alkaline protein (pI 9.4) with an apparent molecular mass of 12-13 kDa (Arvidson, 1983). It is inactivated by thiol-acting agents and has a broad substrate-specificity, similar to papain. It also has strong activity against elastin (Potempa *et al.*, 1988). The gene and amino acid sequence of this enzyme are unknown.

A larger enzyme of 19 kDa with similarities to the papain family of thiol proteases was cloned from *S. aureus* strain V8. This protease, named staphopain, is thought to be distinct from protease II based on its larger molecular weight. Analysis of the *S. aureus* 8325 genome sequence (http://www.genome.ou.edu/stap.html) revealed that staphopain is probably secreted as a larger preproenzyme.

A third thiol protease (SspB) is co-transcribed with the V8 protease (SspA) (Rice *et al.*, 2001). SspB appears to be secreted as a proproenzyme that is activated by SspA.

It has approximately 47 % identity to mature staphopain, however little is known about its proteolytic activity.

1.2.2.4 Lipases

1.2.2.4.1 Glycerol Ester Hydrolase

True lipases are glycerol ester hydrolases that degrade water-soluble long-chain triacylglycerols at the lipid-water interface. Related enzymes hydrolysing water-soluble glycerol esters are also called lipases, but should properly be referred to as short-chain glycerol ester hydrolases, or esterases. Both types of enzymes are produced by *S. aureus*. Esterases are produced by nearly all strains, while true lipases are less common. True lipase, encoded by the *geh* gene is produced by strains TEN5 and PS54 (Kotting *et al.*, 1988; Rollof and Normark, 1992). It is secreted as a preproenzyme which is cleaved by an extracellular protease after secretion. The preproenzyme and the mature lipase have the same activity (Rollof and Normark, 1992). The production of lipase by strain PS54 is subject to negative lysogenic conversion by bacteriophage L54α, which inserts within the lipase coding sequence (Lee and Iandolo, 1986).

The gene encoding the staphylococcal esterase is also termed *geh*. However this is not entirely accurate and this gene should possibly be renamed (Arvidson, 2000). This enzyme is also secreted as a preproenzyme and the mature form has 54% identity to true lipase, with the highest homology around the active residues (Ser-412, Asp-603 and His-645).

Lipases and esterases may be required for bacterial nutrition. Their impairment of granulocyte function may also suggest a role in virulence (Rollof and Normark, 1992).

1.2.2.4.2. Phospholipase C

S. aureus produces two different types of phsopholipase C, β-toxin (described in section 1.2.1.2) and phosphatidylinositol specific phospholipase (PI-PLC). PI-PLC can degrade membrane-associated inositol phospholipids and release glycan-PI-anchored cell

surface proteins, thereby interfering with important eukaryotic cell functions (Marques *et al.*, 1989). The gene encoding PI-PLC (*plc*) has been cloned and sequenced and the gene product characterised (Daugherty and Low, 1993). Most *S. aureus* strains produce PI-PLC, however the amount of enzyme produced can vary substantially (Daugherty and Low, 1993). The exact role of PI-PLC in the virulence of *S. aureus* is not understood.

1.2.2.4.3 Fatty Acid-Modifying Enzyme (FAME)

FAME is produced by about 80% of *S. aureus* isolates and is capable of inactivating long-chain bacteriocidal lipids, often found in abcesses, by catalysing the esterification of these lipids to alcohols, preferably cholesterol (Kapral *et al.*, 1992; Long *et al.*, 1992). Production of FAME appears to be important for virulence as *S. aureus* FAME-producing isolates showed increased virulence in a mouse infection model (Mortensen *et al.*, 1992).

1.2.2.5 Hyaluronate Lyase

S. aureus produces an extracellular hyaluronate lyase, encoded by the *hysA* gene, which is capable of degrading hyaluronic acid by a β-eliminating mechanism yielding disaccharides that contain glucouronosyl residues with a double bond (Farrell *et al.*, 1995). The staphylococcal hyaluronate lyase shares homology with hyaluronate lyases from other bacterial species such as *Streptococcus pneumoniae, Streptococcus agalactiae* and *Propionobacterium acne* (Steiner *et al.*, 1997). A catalytic His-479 residue is present in the enzymes from the four different species (Lin *et al.*, 1997).

Unlike most other extracellular enzymes from *S. aureus*, hyaluronate lyase does not appear to be regulated by *agr* (Taylor and Holland, 1991).

1.2.2.6 Nuclease

Thermostable nuclease (TNase), encoded by the *nuc* gene, is produced by nearly all strains of *S. aureus*. It hydrolyses single or double-stranded DNA or RNA at the 5'

position of phosphodiester bonds by a calcium-dependent mechanism. The secreted form of the enzyme has a 19 kDa propeptide that is cleaved off following secretion by an extracellular protease. The propeptide appears to function as a specific secretion enhancer for the nuclease (Suciu and Inouye, 1996).

1.2.3 Surface Components

1.2.3.1 Capsular Polysaccharide

More than 90% of *S. aureus* clinical isolates produce capsular polysaccharide (CP), of which there are 11 distinct serotypes (Lee and Lee, 2000). These capsules can be divided into 2 distinct groups on the basis of colony morphology. Serotypes 1 and 2 are associated with heavily encapsulated strains (macrocapsules) which are mucoid on solid media (Wilkinson, 1983). Mucoid strains are rarely associated with strains causing disease in man or animal. Microcapsules include the remaining serotypes 3-11 CP, which have a thin capsular layer and form non-mucoid colonies on solid media. While macrocapsules can be detected by light microscopy and india ink staining (Karakawa *et al.*, 1985; Sompolinsky *et al.*, 1985), microcapsules can only be detected by agglutination, with specific sera or by immunoelectron-microscopy. The majority of human and ruminant isolates produce CP5 or CP8. Approximately 16-26% of human isolates produce CP5, while 55-65% produce CP8 (Arbeit *et al.*, 1984; Boutonnier *et al.*, 1989; Hochkeppel *et al.*, 1987; Sompolinsky *et al.*, 1985).

CP of serotypes 1, 2, 5 and 8 have been purified and their biochemical structures have been determined (Fournier *et al.*, 1984; Hanessian and Haskell, 1964; Moreau *et al.*, 1990; Murthy *et al.*, 1983). All of these CP serotypes are uronic acid polymers. The structures of CP5 and CP8 are very similar, with trisaccharide repeats of one molecule of N-acetylmannosaminuronic acid (ManAcA) and two of N-acetylfucosamine (FucNAc). The only difference is in the linkage between the amino sugars and the position of the O-acetylation. However, despite their similarities, CP5 and CP8 are immunologically different.

The genetic determinants of CP1, CP5 and CP8 have been cloned and sequenced (Lin *et al.*, 1994; Sau *et al.*, 1997). The *cap1* locus has 13 genes designated *cap1A-cap1M.* These genes are all transcribed in the same orientation into a 14.6 kb transcript (Lin *et al.*, 1994; Ouyang and Lee, 1997) (Figure 1.1). Transcription appears to be driven be a primary promoter located upstream of *cap1A*. However, several active weak promoters were also detected within the locus (Ouyang and Lee, 1997). The *cap5* and *cap8* loci each contain 16 closely-linked genes (*cap5*(*8*)*A*-*cap5*(*8*)*P*), transcribed in one orientation. Twelve of the 16 genes comprising the two gene clusters are almost identical, whereas the four remaining genes in each of the loci are type-specific (Sau *et al.*, 1997) (Figure 1.1).

CP1 appears to be constitutively expressed and is only modestly affected by environmental factors (Albus *et al.*, 1991). However, production of mucoid-type capsules has been shown to be unstable both *in vitro* and *in vivo* (Lin and Lee, 1996; Lee and Lee, 2000). In contrast, the production of CP5 and CP8 is heavily influenced by environmental factors. The production of CP8 is increased >300-fold when *S. aureus* is grown on solid media or in vivo compared with broth-grown *S. aureus* (Lee *et al.*, 1993). In addition, when *S. aureus* is grown in iron-limiting conditions, the production of CP8 is increased 4-8-fold (Lee *et al.*, 1993). CP5 production is enhanced under high oxygen tension, but limited under alkaline growth conditions, in the presence of CO_2 or in the presence of yeast extract (Stringfellow *et al.*, 1991; Herbert *et al.*, 1997). Both CP5 and CP8 are positively regulated by *agr* (Dassy *et al.*, 1993; Lee and Lee, 2000).

The role of staphylococcal capsules in virulence has been studied extensively. Mucoid capsules are important antiphagocytic virulence factors, which mask C3b deposited on the bacterial cell surface, preventing its recognition by phagocytic cells (Peterson *et al.*, 1978). The role of microcapsules as virulence factors is not as clear and results appear to depend on the animal model of infection tested. While two reports (Albus *et al.*, 1991; Xu *et al.*, 1992) showed evidence that CP5 and CP8 were not important virulence factors, Nilsson and co-workers (1997) showed that mice inoculated with CP5 positive strains had a higher incidence of arthritis and a more severe form of the disease than animals inoculated with the non-encapsulated mutant strain. In addition, a

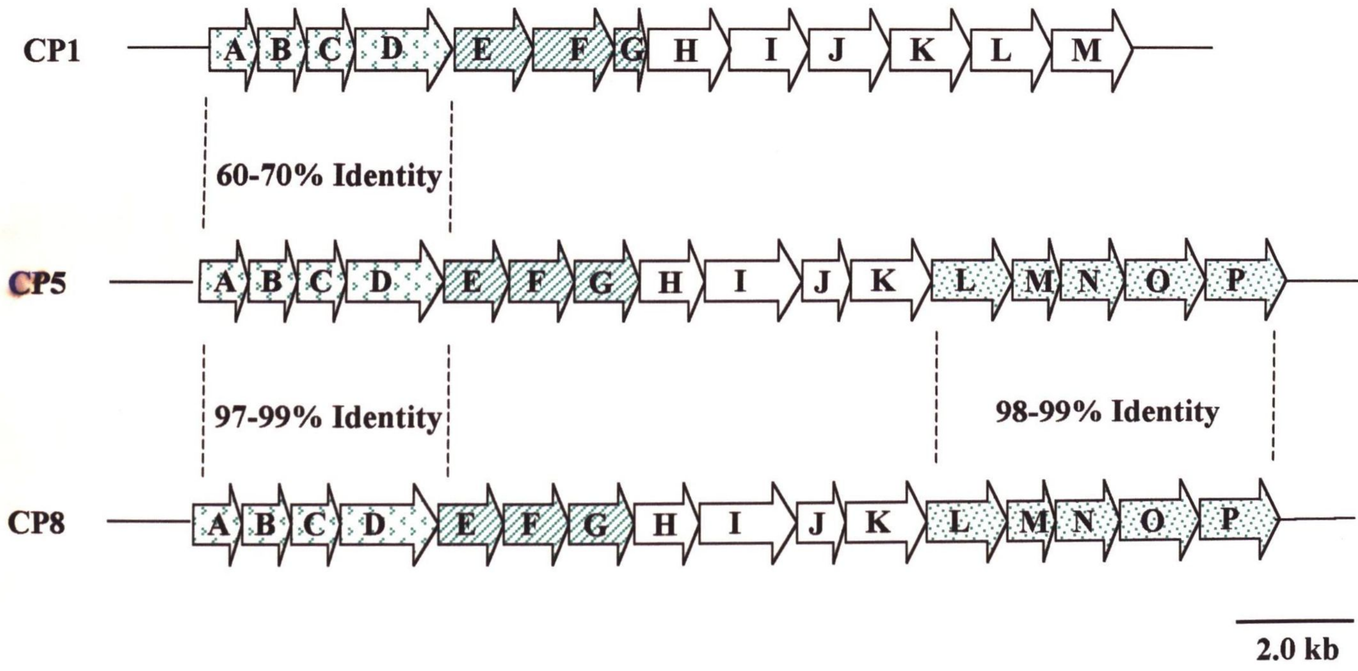

Figure 1.1 Structural organisation of the CP1, CP5 and CP8 loci of *S. aureus*

CP5-expressing strain was shown to be less readily cleared from the blood stream than the corresponding *cap5-* mutant (Thakker *et al.*, 1998).

The use of capsular polysaccharide in the development of a staphylococcal vaccine has been investigated. However, as most *S. aureus* clinical isolates express CP5 and CP8, further studies in defining their role as virulence factors is required.

1.2.3.2 Surface Proteins

Bacterial adhesion to host tissues is an important step in the initiation of bacterial infection. This process also plays an important role in bacterial colonisation of implanted medical devices such as intravenous lines and artificial heart valves that have become coated with extracellular host components (Vaudaux et al, 1984, 1989). *S. aureus* is primarily an extracellular pathogen that expresses a number of specific protein adhesins called MSCRAMMs (Microbial Surface Components Recognising Adhesive Matrix Molecules) which allow the organism to interact specifically with a variety of host extracellular matrix or plasma proteins present on normal tissues or adsorbed to biomedical devices (Patti *et al.*, 1994a; Foster and Höök, 1998; Höök and Foster, 2000). Most of these proteins share common features such as an N-terminal secretory signal sequence, repeated domains, a common cell wall anchoring motif (LPXTG) and a hydrophobic membrane spanning region followed by several charged residues (Figure 1.2).

1.2.3.2.1 Anchoring of Surface Proteins to the Cell Wall

Although the mechanism by which *S. aureus* surface proteins are secreted and anchored to the cell wall is not completely understood, the principle steps have been revealed. After synthesis in the cytoplasm, surface proteins of *S. aureus* and other Gram-positive bacteria are exported via a cleavable N-terminal signal peptide through the Sec pathway (Schneewind *et al.*, 1993). The presence of positively charged residues at the C-terminal end of the protein allow it to be retained in the cell wall, with the hydrophobic C-terminal region spanning the membrane. The LPXTG motif, also located at the C-

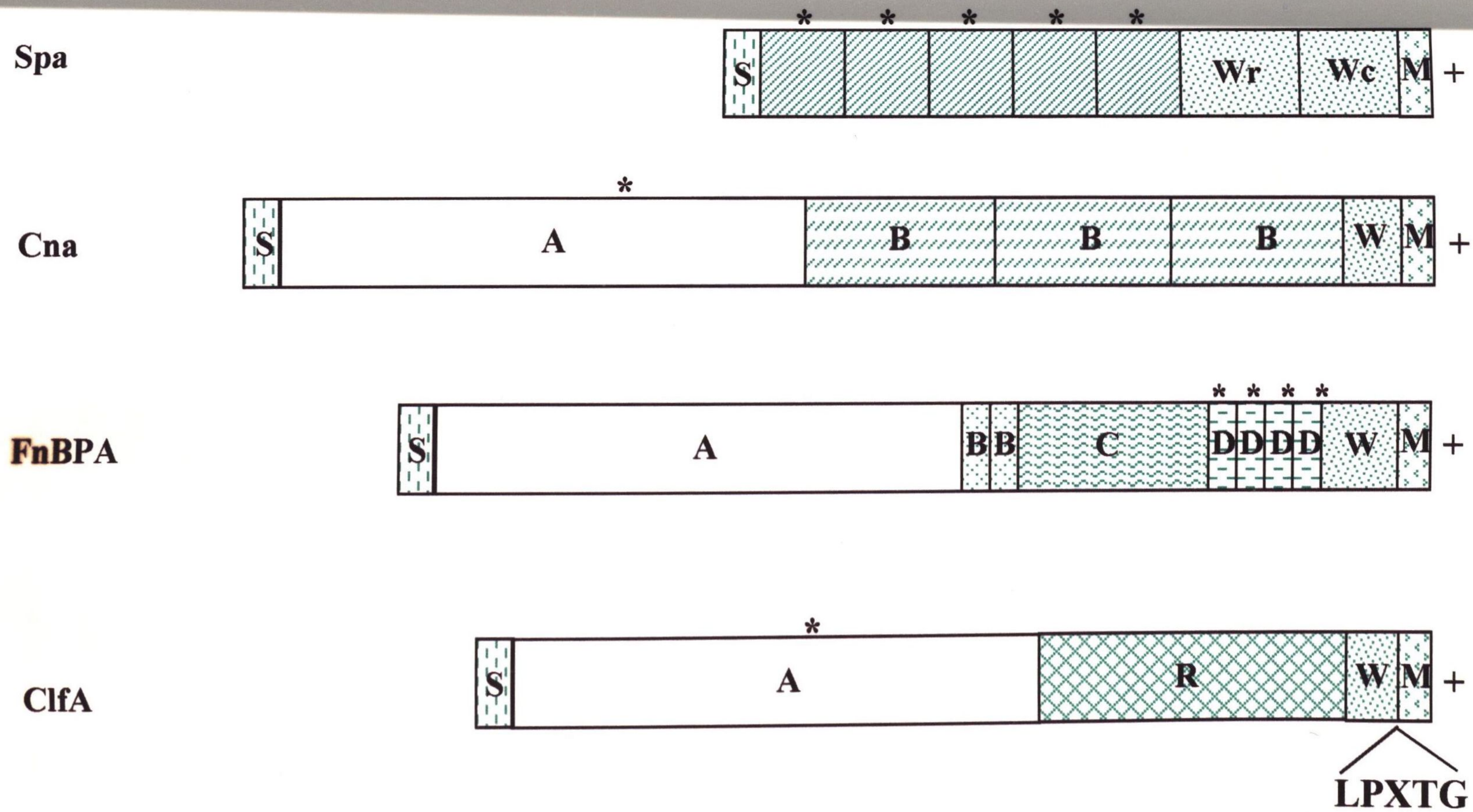

Figure 1.2 Domain organisation of surface-associated proteins in *S. aureus*. Spa, Protein A; Cna, Collagen-binding protein; FnBPA, fibronectin-binding protein; ClfA, fibrinogen-binding protein (Clumping factorA). The signal sequences (S) are removed during secretion. Each protein contains a common C-terminus: LPXTG motif, a membrane anchor (M) and positively charged residues. Region W is thought to span the cell-wall peptidogylcan. * indicates the position of the ligand-binding region.

terminal end of the protein, is recognised by an enzyme known as sortase (Mazmanian *et al.*, 1999). This enzyme cleaves the polypeptide between the threonine and glycine residues of the LPXTG motif (Navarre and Schneewind, 1994). The liberated carboxyl group of the threonine then forms an amide bond with the amino group of the pentaglycine cross-bridge of peptidoglycan (Schneewind *et al.*, 1995), thereby tethering the C-terminus of the protein to the bacterial peptidoglycan (Ton-That *et al.*, 1998; Navarre *et al.*, 1998). (See Figure 1.3). *S. aureus* mutants defective in the sortase gene (*srtA*), fail to anchor several surface proteins, including protein A, fibronectin-binding protein A and clumping factor A. These strains are less virulent in animal models of *S. aureus* infection (Mazmanian *et al.*, 2000).

1.2.3.2.2 Protein A

Protein A was the first *S. aureus* surface-associated proteins to be characterised and it is primarily known for its ability to bind to the Fc region of mammalian IgG (Söjdahl, 1977; Moks *et al.*, 1986). However, recent reports have shown that protein A is also responsible for binding to human von Willebrand factor (Hartlieb *et al.*, 2000). Protein A is thus a member of the MSCRAMM family of surface proteins and has all of the common features associated with this class of proteins (Figure 1.2). Indeed protein A was used as an archetypal model for studying the sorting of *S. aureus* surface proteins to the cell wall (Schneewind *et al.*, 1994).

Protein A contains five homologous tandem repeats of approximately 60 aa residues, each of which bind the Fc region of mammalian IgG molecules. Each of these binding domains forms an alpha-helix (Starovasnik *et al.*, 1996). X-ray analysis of a subdomain B and IgG co-crystal showed that this interaction involved 9 aa residues in the IgG fragment and 11 aa residues in the B domain (Gouda *et al.*, 1998).

Protein A appears to be expressed at high levels in most *S. aureus* strains. However, its role in virulence is somewhat unclear. It is thought that the binding of IgG molecules by protein A via the Fc region could interfere with phagocytosis of opsonised bacteria, as spa- mutants are phagocytosed more efficiently by PMNLs in the presence of serum opsonins (Höök and Foster, 2000). Protein A is also known to adhere to platelets

Figure 1.3 Representation of the cell wall anchoring mechanism of surface proteins in *S. aureus* and other Gram-positive bacteria. Proteins are secreted across the cell membrane by the Sec pathway and are cleaved at the N-terminus by a signal peptidase. An enzyme known as sortase cleaves the C-terminus of the protein between the threonine and glycine residues. The protein is then covalently linked to the pentaglycine cross-bridge of peptidoglycan through the formation of an amide bond between the carboxyl group of the threonine and the free amino group of the glycine residue. It is thought that linkage of surface proteins to the cross-bridge of peptidoglycan occurs with the lipid-linked peptidoglycan precursor molecule, prior to polymerisation.

Medium

NH2

NH2

NH2

NH2

Cell Wall

Membrane

Cytoplasm

1
Signal peptidase

NH2

LPXTG +

Sorting signal

L
P
X
T
G

+

2

Sortase

L
P
X
T

G

+

3

L
P
X
T–Gly5 – L-Lys

MurNac-PP
L-Ala
D-Glu
D-Ala
D-Ala

MurNac-PP
L-Ala
D-Glu
Gly5 – L-Lys
D-Ala
D-Ala

4

5

GlcNac
MurNac
L-Ala
D-Glu
L
P
X
T –Gly5– L-Lys
D-Ala–Gly5 – L-Lys

GlcNac
MurNac
L-Ala
D-Glu
D-Ala

-P

(Herrmann *et al.*, 1993; Nguyen *et al.*, 2000) and may have a role to play in their activation, a phenomenon that could have implications in the development of infective endocarditis. The effect of the protein A interaction with von Willebrand factor on bacterial virulence has not yet been characterised and may be important in the pathogenicity of *S. aureus*.

A second IgG-binding protein distinct from protein A (Sbi) has also been identified in *S. aureus*. Sbi, which has a molecular weight of approximately 45 kDa, has similar IgG-binding properties to protein A and has been shown to be expressed at high levels in *S. aureus* strains 8325-4 and Newman (Zhang *et al.*, 1998). However, this novel protein has not been characterised in detail at the molecular level and its role in virulence is undetermined.

1.2.3.2.3 Collagen binding protein

Collagen is the major structural protein of mammals and is present in many distinct types (Olsen and Ninomiya, 1993). The architecture of various tissues is dependent on collagen and is regulated by intricate interactions between different collagen molecules and non-collagenous components (Heinegard and Oldberg, 1989). Type II collagen is found preferentially in cartilage, while type IV collagen is distributed almost exclusively in the basement membrane (Miller and Gay, 1983; Olsen and Ninomiya, 1993).

The *S. aureus* collagen binding protein Cna is both necessary and sufficient for adherence of the bacterium to collagen substrates and collagenous tissues (Patti *et al.*, 1992). Cna recognises several collagen types, including types I, II, III and IV (Switalski *et al.*, 1993). However, the *cna* gene is only found in approximately 50% of *S. aureus* strains (Smeltzer *et al.*, 1997). The structural organisation of Cna is similar to that of most other *S. aureus* MSCRAMMs (Figure 1.2). Following the N-terminal signal peptide is the unique ligand-binding A domain that consists of approximately 500 aa. This domain is followed by region B which has a tandemly repeated structure. Region B is composed of approximately 180 aa. The number of B repeats varies from 1-4 copies,

depending on the *S. aureus* strain (Gillaspy *et al.*, 1997). The function of the B region is not known.

The Cna collagen-binding domain has been localised to a 19 kDa subdomain of region A between residues 151-318 (Patti *et al.*, 1995). Smaller truncates of this region lost the ability to bind to collagen. However, a synthetic peptide mimicking a segment in this subdomain has been shown to inhibit collagen binding to bacteria (Patti *et al.*, 1993; Patti *et al.*, 1995).

The crystal structure of the 19 kDa subdomain had been solved. This subdomain is presented in a "jelly-roll" fold composed of two anti-parallel β–sheets and two short α–helices (Symersky *et al.*, 1997). A trench traverses one of the β–sheets, which can accommodate the collagen triple helix. Site specific mutations designed to disrupt the wall of the binding trench reduced binding affinity.

The Cna adhesin was shown to be a virulence determinant in experimental septic arthritis (Patti *et al.*, 1994b). Furthermore, vaccination with the recombinant A domain of Cna provides protection against *S. aureus*-mediated septic death (Nilsson *et al.*, 1998b). However, the use of the recombinant Cna molecule alone as a potential vaccine candidate may not be successful due to the absence of Cna from 50% of *S. aureus* strains.

Expression of Cna is controlled primarily by the staphylococcal accessory regulator (*sar*) locus (Gillaspy *et al.*, 1998).

1.2.3.2.4 Fibronectin-Binding Proteins

Fibronectin is a high molecular-weight dimeric glycoprotein found in blood, body fluids (e.g. cerebrospinal fluid and urine), on many cell surfaces and in connective tissue matrices (Yamada and Olden, 1978). Fibronectin is thought to play a role in the regulation of cell adhesion, cell substratum adhesiveness and cell motility (Mosesson and Amrani, 1980; Yamada and Olden, 1978). Adhesion of fibronectin to eukaryotic cells is mediated by integrin receptors that bind to a specific site in the central part of fibrinogen.

Fibronectin-binding is a very common property of *S. aureus* isolates. This binding is mediated by two closely-related cell wall-associated fibronectin-binding proteins, FnBPA and FnBPB, which are encoded by two closely linked, but

independently transcribed genes, *fnbA* and *fnbB* (Signas *et al.*, 1989; Jonsson *et al.*, 1991). Both proteins are expressed by most strains of *S. aureus*. However some strains have a single *fnb* gene that is closely related to *fnbA* (Greene *et al.*, 1995; Peacock *et al.*, 1999). The primary binding site for *S. aureus* FnBPs on fibronectin is located in the 29 kDa N-terminal domain (N29) of the molecule (Sottile *et al.*, 1991) (Figure 1.4). This fibronectin N29 domain is a rigid structure composed of 5 type I modules each of about 45 aa residues (Potts and Campbell, 1994). A second weaker binding site for *S. aureus* FnBPs is located in a C-terminal type III repeat (Bozzini *et al.*, 1992).

FnBPA and FnBPB are both typical MSCRAMMs (Figure 1.5) that are anchored to the staphylococcal cell wall at the C-terminal LPXTG motif. The C-terminal regions of both proteins (C, D, W, M) are very similar with >95% identity. However, the N-terminal A regions are quite dissimilar with only 45 % identical residues (Figure 1.5). Until recently, the function of the FnBP A region was unknown. However, it has now been shown that a domain present in the FnBPA A-region can interact with fibrinogen (Wann *et al.*, 2000). This interaction is rather weak and can only be demonstrated in *S. aureus* when FnBPA is over-expressed from a multi-copy plasmid. Therefore, its biological significance is uncertain. The main fibronectin binding region has been localised to the D repeat region. This region comprises 3 complete repeats of 38 residues (D1, D2, D3) and a partial repeat, D4. Synthetic peptides corresponding to each repeat can inhibit fibronectin-FnBP interactions suggesting that each repeat can bind to fibronectin (Raja *et al.*, 1990). Repeat D3 has the strongest interaction. Furthermore, a recombinant protein comprising the complete D region (D_{1-4}) is a much more potent inhibitor than any single D peptide (Raja *et al.*, 1990), suggesting that multiple contacts occur between region D and fibronectin. Also, mutations in any single type I module in fibronectin N29 domain reduced interactions with FnBPA, indicating that each module is important in the reaction (Sottile *et al.*, 1991).

The ligand-binding D-repeat region of the FnBPs appear to lack a folded secondary structure (House-Pompeo *et al.*, 1996). However, when this repeat domain binds to fibronectin, it takes on ordered secondary structure comprising β–sheets. The presence of a ligand-induced conformation is supported by the properties of certain monoclonal antibodies that only recognise the bound form of the ligand-binding region of

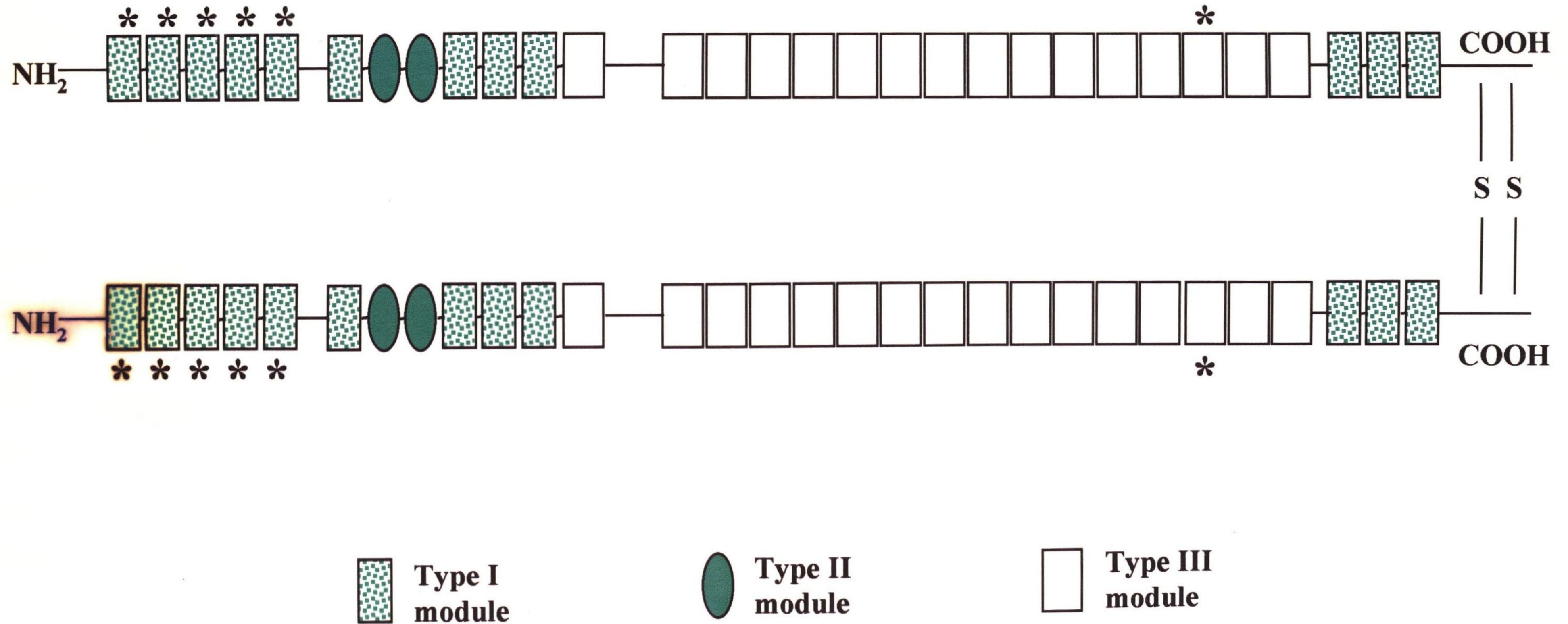

Figure 1.4 Structure of fibronectin. * indicates *S. aureus*-binding sites.

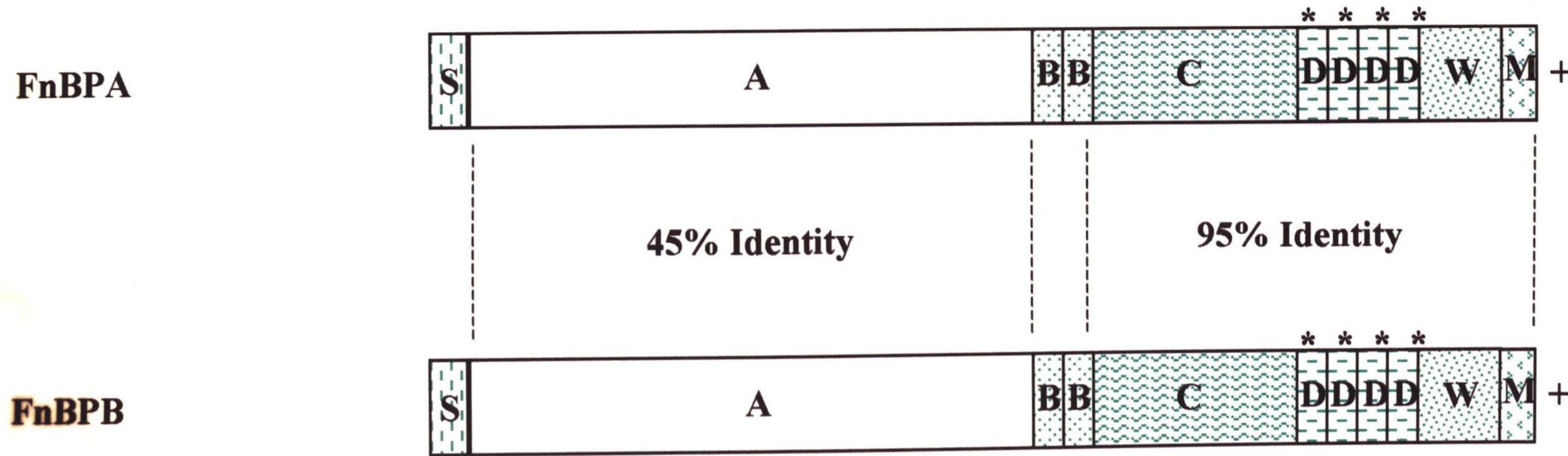

Figure 1.5 Schematic representation of the *S. aureus* fibronectin-binding proteins, FnBPA and FnBPB. The N-terminal regions of the FnBPA and FnBPB proteins share only 45% identical residues. Both proteins are ~30% identical to the A domain of ClfA and bind to fibrinogen. However, the C-terminal regions C, D, W and M are 95% similar in aa sequence.

fibronectin-binding proteins from *Streptococcus dysgalcties* (Speziale *et al.*, 1996), and the fact that immunisation with recombinant FnBPA does not generate efficient blocking antibodies (Ciborowski *et al.*, 1992; Rozalska *et al.*, 1994; Speziale *et al.*, 1996).

In addition, FnBPs also appear to play a major role in the adherence to and the invasion of host endothelial cells (Peacock *et al.*, 1999; Sinha *et al.*, 1999, 2000). FnBPs recruit and bind soluble fibronectin, which is subsequently recognised by integrins on the host cell, resulting in phagocytosis of the host protein-coated bacteria. As *S. aureus* can survive within endothelial cells, internalisation is thought to provide a mechanism for evading the host immune system. It is thought that region A of FnBPA is responsible for mediating internalisation by host endothelial cells.

The ability of *S. aureus* to bind to fibronectin is very important to the pathogenicity of the organism. Fibronectin is ubiquitous and is present at almost every mammalian body site. The fact that *S. aureus* can bind to both soluble and immobilised fibronectin means that the bacterium is extremely efficient at colonising the host and inducing infection. This is reflected in the wide variety of diseases caused by *S. aureus*.

Expression of the *fnbA* in *S. aureus* is regulated through a complex interaction between the *agr* and *sar* regulatory loci (Wolz *et al.*, 2000).

1.2.3.2.5 Fibrinogen-Binding Proteins

Fibrinogen is a large 340 kDa protein that is found predominantly in blood. It is composed of pairs of three polypeptide chains (α, β and γ) that are extensively linked by disulphide bonds to form an elongated dimeric structure (Doolittle, 1984; Ruggeri, 1993) (Figure 1.6). It is the most abundant ligand for the interaction with the platelet αIIb/β3 integrin. Binding of fibrinogen to the αIIb/β3 integrin on activated platelets results in platelet aggregation *in vitro* and in the formation of platelet-fibrin thrombi *in vivo* (Hawiger, 1995).

It has long been known that *S. aureus* cells clump in the presence of blood plasma (Much, 1908). This reaction is now known to be mediated by fibrinogen (Hawiger *et al.*, 1982). Two MSCRAMMs, ClfA and ClfB are primarily responsible for the clumping of *S. aureus* cells in the presence of soluble fibrinogen, (McDevitt *et al.*, 1994; Ní Eidhin *et*

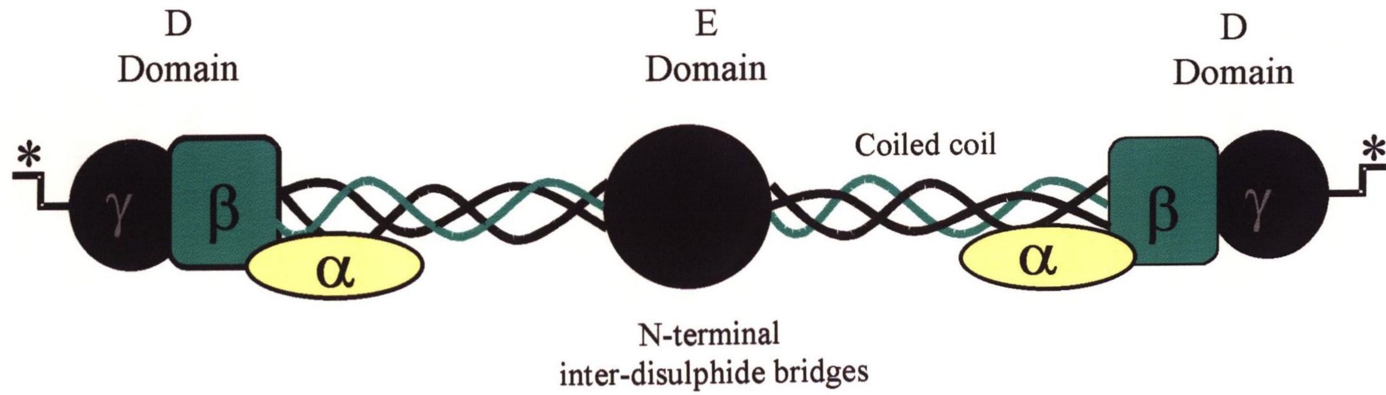

Figure 1.6 Structure of fibrinogen. * indicates binding site for *S. aureus* ClfA.

al., 1998), although FnBPA can also promote cell-clumping in a solution of fibrinogen if expressed at a sufficiently high level (Wann *et al.*, 2000). ClfA (clumping factor A) and ClfB (clumping factor B) are typical MSCRAMMs that are both structurally related (Figure 1.7). Both comprise an N-terminal secretory signal sequence, followed by the fibrinogen-binding A domain which consists of 520 aa in ClfA and 540 aa in ClfB. The A domains from ClfA and ClfB are only 27% identical. C-terminal to the A domain in both proteins is a region of repeating serine and aspartic acid dipeptide (region R), which is required for projecting the ligand-binding A domain away from the cell surface (Hartford *et al.*, 1997). The length of this region varies from strain to strain (McDevitt and Foster, 1995). Region R is followed by the typical wall and membrane spanning domains, region W and region M.

1.2.3.2.5.1 ClfA

ClfA was first identified by analysis of Tn*917* mutants that were defective in clumping in the presence of soluble fibrinogen (McDevitt *et al.*, 1994). The fibrinogen binding domain of the protein is located in the unique 520 aa residue A region (McDevitt *et al.*, 1995). This was shown through fibrinogen binding studies with the recombinant 520 aa A region. Furthermore, the full-length recombinant A region could block *S. aureus* cell clumping and adherence to fibrinogen. Anti-ClfA A region antibodies could also block these interactions (McDevitt *et al.*, 1995). Analysis of the fibrinogen-binding capacity of truncated derivatives of region A revealed that the ligand-binding domain is located between residues 220-559. Further truncation of this recombinant molecule leads to a loss in fibrinogen-binding activity

ClfA binds to the C-terminus of the γ-chain of fibrinogen. Twelve residues in this region are thought to be important in the interaction with ClfA (Hawiger *et al.*, 1982; Strong *et al.*, 1982; McDevitt *et al.*, 1997). These same twelve residues mediate the interaction between fibrinogen and the platelet integrin (Savage *et al.*, 1995). The binding of ClfA to fibrinogen is regulated by Ca^{2+}. At Ca^{2+} concentrations in the millimolar range (1-10 mM) the ClfA-fibrinogen interaction is progressively inhibited (O'Connell *et al.*, 1998). As the concentration of free Ca^{2+} in blood plasma is 1.3 mM,

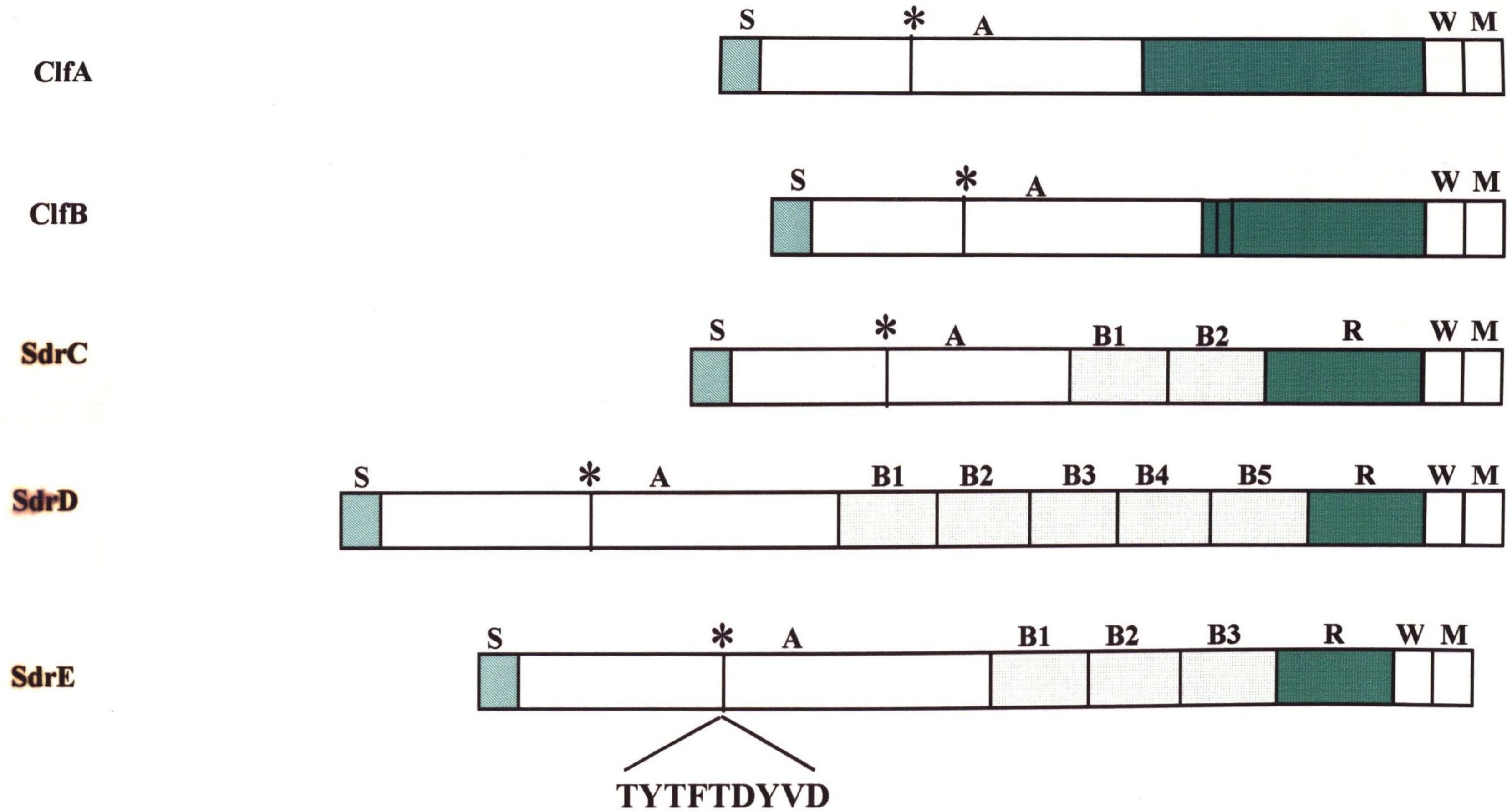

Figure 1.7 Schematic representation of the *S. aureus* Clf-Sdr protein family. * indicates the motif TYTFTDYVD.

this could have implications in the ability of ClfA to bind fibrinogen *in vivo*. However, Ca^{2+} concentrations can vary in extracellular spaces and at sites of platelet-rich thrombi and freshly implanted biomaterial (Brown *et al.*, 1995). Hence, a Ca^{2+}-dependent regulation of ligand binding could allow ClfA to remain partially unoccupied. Thus as bacteria circulate in plasma they would tend to adhere to fibrinogen/platelet-containing coagulation sites.

A Ca^{2+}-binding EF-hand motif has recently been identified within the A region of ClfA, and site specific mutations affecting this motif make mutant recombinant proteins less sensitive to Ca^{2+}-dependent regulation, but also reduce binding to fibrinogen and the synthetic γ-chain peptide (O'Connell *et al.*, 1998).

Unlike many MSCRAMMs, which are expressed maximally in the exponential phase of growth, ClfA is expressed throughout the growth cycles in all strains of *S. aureus* tested. In addition, the *clfA* gene does not appear to be regulated by any of the known regulatory loci such as *agr* and *sar*. Unlike Cna, ClfA is expressed by almost all strains of *S. aureus*, making it a more appealing candidate for the development of a staphylococcal vaccine. The role of ClfA in the pathogenicty of *S. aureus* has been investigated. When compared with a *clfA*- mutant, ClfA was shown to act as a virulence factor in a rat endocarditis model (Vaudaux *et al.*, 1995) and in the murine model of septic arthritis (Josefsson *et al.*, 2001). Indeed the virulence of ClfA probably related to its potent antiphagocytic activity. This was shown by O'Connell and co-workers (D. O'Connell, unpublished data), who demonstrated that the A domain of ClfA strongly interferes with the binding of leukocytes to fibrinogen. This reaction is important in allowing the phagocytic cells to reach sites of infection and tissue damage.

1.2.3.2.5.2 ClfB

Until recently, it was thought that ClfA was the sole fibrinogen-binding protein in *S. aureus*. However, ClfB can also adhere to fibrinogen and can promote cell clumping in the presence of soluble fibrinogen (Ní Eidhin *et al.*, 1998). Although ClfA and ClfB both bind to fibrinogen and have a similar structural organisation, they are encoded by distinct genes that are located at different regions of the chromosome. Like ClfA, the

ligand-region of ClfB is located in the A region. Recombinant ClfB region A can bind to fibrinogen and can inhibit the binding of *S. aureus* cells defective in ClfA to immobilised fibrinogen. Polyclonal antibodies raised against the A domain can also inhibit *S. aureus* *clfA-* mutants from binding to fibrinogen (Ní Eidhin *et al.*, 1998). ClfB is also inhibited by millimolar concentrations of Ca^{2+}. Unlike ClfA, ClfB is only expressed during the exponential phase of growth under conditions of aeration (Ní Eidhin *et al.*, 1998). Early studies with ClfA, which showed no fibrinogen binding in a *clfA-* mutant, were performed with stationary phase cells where ClfB was no longer expressed (McDevitt *et al.*, 1995). Negligible levels of fibrinogen binding are detected in a *S. aureus clfA-clfB-* double mutant grown to exponential and stationary phase (McAleese *et al.*, 2001; This study, Chapter 6).

Although ClfB is only expressed during the exponential phase of growth, it does not appear to be regulated by any of the known regulatory loci in *S. aureus* (McAleese *et al.*, 2001). Transcription of the *clfB* gene appears to stop as the cells enter late exponential phase and the ClfB protein is subsequently lost from the cell surface due to proteolytic cleavage mediated by the metalloprotease aureolysin and through a dilution effect as the cells divide (Mc Aleese *et al.*, 2001).

1.2.3.2.6 Sdr Proteins

ClfA and ClfB are both members of a larger family of structurally-related *S. aureus* surface proteins known as the serine-aspartate repeat protein multigene family (Josefsson *et al.*, 1998a). Members of this family of proteins are characterised by the presence of the R domain, which contains tandem repeats of serine and aspartic acid (SD) dipeptides. The number of SD repeats present varies considerably from protein to protein (Josefsson *et al.*, 1998a). Three other proteins from *S. aureus*, SdrC, SdrD and SdrE, contain the R domain (Josefsson *et al.*, 1998a). Characterisation of these proteins is the subject of this thesis.

Each of the Sdr proteins is predicted to have a similar structural organisation to ClfA and ClfB (Figure 1.7), with the exception of an additional B-repeat region that is located between the unique A region and region R. Each B-repeat consists of 110-113 aa

residues and contains a classical EF-hand motif that binds Ca^{2+} with high affinity (Josefsson *et al.*, 1998b). Occupation of these sites within the B-region by Ca^{2+} is critical for maintaining the structural integrity of the protein. When the cation is removed the structure collapses. However, structural integrity is restored when cations are added back (Josefsson *et al.*, 1998a). The number of B-repeats present in each Sdr protein varies with two, five and three repeats present in SdrC, SdrD and SdrE, respectively (Figure 1.7). It is possible that the B-repeats are also required for ligand binding. However this has not been characterised.

The N-terminal signal sequences and the C-terminal wall- and membrane-spanning domains present in ClfA, ClfB, SdrC, SdrD and SdrE are similar. However, even greater homology is seen when the signal sequences and the W and M domains of SdrC, SdrD and SdrE are compared with each other (Josefsson *et al.*, 1998a). Each Sdr protein contains the cell wall anchoring motif, LPXTG. The only area that is unique between the five proteins in this family is the N-terminal A domain, with only 21-30% homology in any pairwise combination (Josefsson *et al.*, 1998a). Only one stretch of aa residues is present in all five proteins. The consensus for this motif is TYTFTDYVD. This motif occurs in the C-terminus of the EF-hand of ClfA which is implicated in Ca^{2+} binding and cation-mediated regulation of ligand binding (O'Connell *et al.*, 1998). It is possible that the TYTFTDYVD motif present in the other four proteins plays a role in ligand binding. It is proposed that the ligand-binding activity of the Sdr proteins is present in the A-domain.

The Sdr proteins are encoded by three closely linked genes (*sdrC, sdrD* and *sdrE*) that are arranged in tandem array. The *sdrC* and *sdrD* genes are separated by 369 bp, while the *sdrD* and *sdrE* genes are separated by 397 bp. These large intergenic regions suggest that each gene is transcribed independently. In a survey of 31 *S. aureus* strains from human and bovine origin, most strains were shown to possess all three *sdr* genes. Some strains such as strain Phillips do not have a copy of *sdrC*, while 8325-4 does not have a copy of *sdrE* (Josefsson *et al.*, 1998a). In addition, strain EMRSA-16 does not possess the *sdrD* gene (this study). However, recent data from a screen of over 380 *S. aureus* clinical isolates showed that only approximately 50 % of the strains had the *sdrC*

gene and about 50% had the *sdrD* gene. The *sdrE* gene was present in over 78% of these isolates (S. Peacock, unpublished).

The function of the Sdr proteins is not understood. Recently, a protein that is homologous to SdrE was isolated from *S. aureus* strain O24 (Tung *et al.*, 2000). This protein, named Bbp, was shown to bind to bone sialoprotein (BSP) through a domain present in its A region. Although the A-regions of Bbp and SdrE are similar (Figure 1.8), studies have shown that SdrE does not bind to BSP (K. McCrea, unpublished). The function of the SdrC, SdrD and SdrE proteins is unclear.

1.2.3.2.7 Elastin-Binding Protein.

Elastin fibres are components of the mammalian extracellular matrix and are present in abundance in tissues that require elasticity, such as the skin, the lung and blood vessels (Mecham, 1993). Mature elastin is a polymer of tropoelastin, which is the monomeric form of elastin secreted from fibroblast cells (Rosenbloom, 1987). *S. aureus* can bind to soluble tropoelastin and to elastin peptides, which are chemically degraded products of mature elastin (Park *et al.*, 1991). This may have implications for skin colonisation and for establishment of infection in the lung and at sites of tissue damage by *S. aureus*.

S. aureus expresses an 83 kDa protein known as EbpS that binds to soluble tropoelasin and to soluble elastin peptides (Park *et al.*, 1996; Roche et al., 2001). The elastin-binding domain is located very close to the N-terminus of the protein between residues 14-34 (Park *et al.*, 1999). EbpS is unlike other *S. aureus* MSCRAMMs as it does not have a recognisable secretory signal sequence or the C-terminal wall-membrane anchoring structure seen in most other wall-associated proteins (Figure 1.9). Recent studies in our lab have shown that EbpS is an integral membrane protein (R. Downer, unpublished). Furthermore, EbpS is not a true MSCRAMM as it does not promote bacterial adhesion to immobilised elastin substrates.

Figure 1.8 Clustal W alignment of the aa sequences from the A regions of SdrE and Bbp. The SdrE A region (554 aa) has an additional 5 aa residues compared with the Bbp A region (549 aa). This additional region is highlighted in blue in the SdrE sequence. Identical residues are indicated by *. Similar residues are indicated by **.** or **:** and are highlighted in green. Dissimilar residues are highlighted in red. The overall sequence identity between SdrE and Bbp is approximately 71%.

```
SdrE
AENTSTENAKQDDATTSDNKEVVSETENNSTTENNSTNPIKKETNTDSQPEAKKESTSSS 60
Bbp
AENTSTENAKQDEASASDNKEVVSETENNSTQKNDLTNPIKKETNTDSHQEAKEAPTTSS 60
************:*::*************** :*: ************: ***: .*:**

SdrE
TQKQQNNVTATTETKPQNIEKENVKPSTDKTATEDTSVILEEKKAPNNTNNDVTTKPSTS 120
Bbp
TQQQQNNATTSTETKPQNIEKENVKPSTDKTATEDTSVILEEKKAPNNTNNDVTTKPSTS 120
**:****.*::*************************************************

SdrE
EPSTSEIQTKPTTPQESTNIENSQPQPTPSKVDNQVTDATNPKEPVNVSKEELKNNPEKL 180
Bbp
-----EIQTTPTTPQESTNIENSQPQPTPSKVDNQVTDTTNPKEPVNVSKEELKNNPEKL 175
*    ****.****************************:*********************

SdrE
KELVRNDSNTDHSTKPVATAPTSVAPKRVNAKMRFAVAQPAAVASNNVNDLIKVTKQTIK 240
Bbp
KELVRNDSNTDRSTKPVATAPTSVAPKRVNAKIRFAVAQPAAVASNNVNDLITVTKQMIT 235
***********:********************:*******************.**** *.

SdrE
VGDGKDNVAAAHDGKDIEYDTEFTIDNKVKKGDTMTINYDKNVIPSDLTDKNDPIDITDP 300
Bbp
EGIKDDGVIQAHDGEHIIYTSDFKIDNAVKAGDTMTVKYDKETIPSDITDDFTPVDITDP 295
 *   .*.*   ****:.* *  ::*.***  **  *****::***:.****:**.   *:*****

SdrE
SGEVIAKGTFDKATKQITYTFTDYVDKYEDIKSRLTLYSYIDKKTVPNETSLNLTFATAG 360
Bbp
SGEVIAKGTFDLNTKTITYKFTDYVDRYENVNAKLELNSYIDKKEVPNETNLNLTFATAD 355
***********  ** ***.******:**:::::* * ****** *****.********.

SdrE
KETSQNVTVDYQDPMVHGDSNIQSIFTKLDEDKQTIEQQIYVNPLKKSATNTKVDIAGSQ 420
Bbp
KETSKNVKVEYQKPIVKDESNIQSIFSHLDTTKHEVEQTIYVNPLKLNAKNTNVTIKSGG 415
****:**.*:**.*:*:.:*******::**  *: :** ******* .*.**:* *  ..

SdrE
VDDYGNIKLGNGSTIIDQNTEIKVYKVNSDQQLPQSNRIYDFSQYEDVTSQFDNKKSFSN 480
Bbp
VADNGDYYTGDGSTIIDSNTEIKVYKVASGQQLPQSNKIYDYSQYEDVTNSVTINKNYGT 475
* * *:    *:******.********* *.*******:***:*******...   :*.:..

SdrE
NVATLDFGDINSAYIIKVVSKYTPTSDGELDIAQGTSMRTTDKYGYYNYAGYSNFIVTSN 540
Bbp
NMANINFGDIDSAYIVKVVSKYTPGAEDDLAVQQGVRMTTTNKYNYSSYAGYTILFYQLL 535
*:*.::****:****:********  ::.:* : **. * **:**.* .****: ::

SdrE            DTGGGDGTVKPEEK 554
Bbp             TLVVVTVSVKPEEK 549
                       :******
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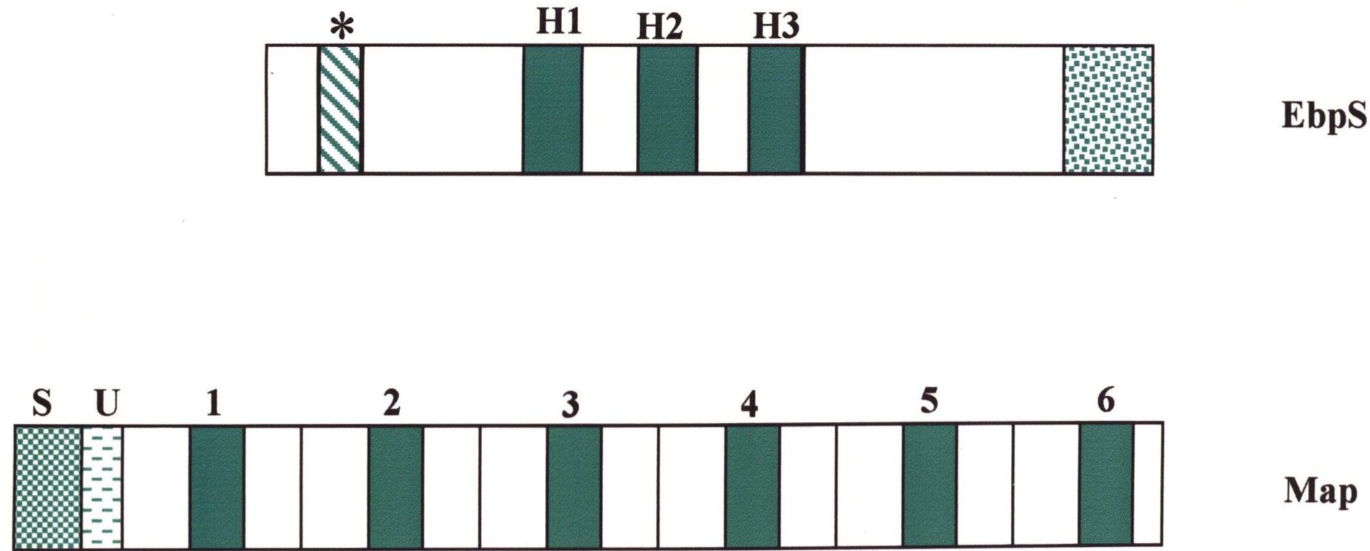


Figure 1.9 Schematic representation of the elastin-binding protein (EbpS) and the MHCII analogous protein (Map) of *S. aureus*. * indicates the ligand-binding region of EbpS. H1-H3 represent hydrophobic, putative membrane spanning domains in EbpS. Six repeated regions are present in Map. The shaded area within these repeats represents a region that is highly homologous to the MHC classII molecule. S represents the signal sequence, while U represents a unique region in Map.

1.2.3.2.8 MHC Class-II Analogous Protein (Map)

Map is an unusual surface-located protein found in *S. aureus.* It can be released from cells following LiCl treatment (McGavin *et al.*, 1993; Jonsson *et al.*, 1995). It is composed of six repeated domains of 110 residues each, with a central portion composed of a subdomain with a high homology to the peptide binding groove of the β-chain of MHCII molecules (Jönsson *et al.*, 1995) (Figure 1.9). Map contains an N-terminal signal peptide and is probably secreted through a Sec-dependent pathway. However, the C-terminal wall attachment region present in most other *S. aureus* surface proteins is absent, suggesting that it is not covalently linked to the cell wall.

One unique feature of Map is that it can bind to many different host proteins in vitro (McGavin *et al.*, 1993). Recently, an extracellular adherence protein Eap, which appears to be related to Map, has been characterised (Palma *et al.*, 1999). Both of these proteins can bind to many different proteins as well as to proteins on the surface of the bacterial cell. It has been postulated that these proteins can act as transplantable substrates for promoting bacterial attachment to mammalian cells and to surfaces of indwelling medical devices.

1.2.3.2.9 Other surface-located ligand-binding proteins

With the near-completion of the *S. aureus* genome-sequencing project, it has become apparent that there are at least six further proteins present in *S. aureus* that contain putative N-terminal signal sequences and C-terminal wall-anchoring domains. It is likely that these proteins are expressed on the surface of *S. aureus* and are capable of interacting with host factors. Indeed, *S. aureus* has been shown to interact with several mammalian proteins for which no bacterial ligand-binding proteins have been identified. *S. aureus* receptors for vitronectin (Herrmann *et al.*, 1988; Liang *et al.*,1993, 1995; Chhatwal *et al.*, 1987), laminin (Lopes *et al.*, 1985; Mota *et al.*, 1988), thrombospondin (Herrmann *et al.*, 1991) and mucin (Shutter *et al.*,1996) remain uncharacterised.

1.3 Regulation of Virulence Factor Expression

In *S. aureus*, most of the genes that encode virulence factors are coordinately regulated at the transcriptional level by a locus known as the accessory gene regulator (*agr*) (Morfeldt *et al.*, 1988; Peng *et al.*, 1988; Recsei *et al.*, 1986). It was initially proposed that *agr* was responsible for the down-regulation of surface proteins during exponential phase and the up-regulation of secreted proteins post-exponentially. However, while this is true in many cases, several other important regulatory genes have been identified and it is now clear that many accessory genes are regulated by loci other than *agr* (e.g. *sarA, sarH1, sea*, σ^B). While all of these loci can act outside of the *agr* pathway, it has been shown that *agr* expression is also influenced by other loci such as *sarA* (Chien and Cheung, 1998) and *sarH1* (Tegmark *et al.*, 2000). In addition, *sarA* expression is influenced by loci such as σ^B (Manna *et al.*, 1998) and *sarH1* (Tegmark *et al.*, 2000). Regulation of virulence factor expression in *S. aureus* appears to be far more complex than was originally hypothesised, involving interactions between many different regulatory loci.

1.3.1 The *agr* System

The *S. aureus agr* locus is a global regulatory locus that controls the expression of many different surface proteins and secreted proteins. *agr* homologues are also found in other staphylococcal strains such as *Staphylococcus epidermidis* (Van Wamel *et al.*, 1998) and *Staphylococcus lugdunensis* (Vandenesch *et al.*,1993). Several studies have shown that in a *S. aureus agr* mutant decreased levels of secreted protein synthesis and increased levels of cell surface-associated protein synthesis are detected (Janzon *et al.*, 1986; Kornblum *et al.*, 1990). The *agr* locus was identified in *S. aureus* by Tn*551* transposon mutagenesis. Mutants that had altered exoprotein expression levels were found to contain chromosomal Tn*551* insertions in what is now known as the *agr* locus (Mallonee *et al.*, 1982; Recsei *et al.*, 1986).

The *agr* regulon comprises two divergently transcribed promoters, P2 and P3, which drive expression of RNAII and RNAIII (Arvidson, 1983; Novick *et al.*, 1993;

Novick *et al.*, 1995) (Figure 1.10). The P2 operon contains four genes (*agrB, agrC, agrD* and *agrA*) all of which are required for transcriptional activation of the *agr* system (Novick *et al.*, 1995). The primary function of this four-gene unit is to activate the two major *agr* promoters P2 and P3 (Lofdahl *et al.*, 1988; Peng *et al.*, 1988; Kornblum *et al.*, 1990). A second regulatory product, SarA, is also involved in the activation of P2 and P3 (Cheung *et al.*, 1995). The P3 transcript, RNAIII, is the effector molecule of the *agr* response and it functions at a transcriptional level to regulate *agr*-dependent gene expression (Janzon and Arvidson, 1990; Kornblum *et al.*, 1990; Novick *et al.*, 1993).

AgrC and AgrA form a classic two-component signal transduction system (Stock *et al.*, 1989). AgrC is a transmembrane receptor protein that is phosphorylated at a conserved histidine residue in response to the autoinducing peptide (AIP) *in vitro* (Lina *et al.*, 1998). AgrA is the response regulator, which acts together with the SarA protein to activate transcription of the P2 and P3 promoters. AgrD is a 46 kDa protein encoded by the *agrD* gene. AgrD is post-translationally modified to produce a cyclic octapaptide (auto inducing peptide; AIP) that is responsible for the autoinduction of the *agr* system (Ji *et al.*, 1995). AgrB is a 26 kDa transmembrane protein that determines the specificity of AgrD processing and is probably responsible for secretion of the octapeptide as well as for the modification, which involves the formation of a cyclic thiolactone bond between an internal cysteine and the C-terminal carboxyl group (Ji *et al.*, 1997; Otto *et al.*, 1998; Mayville *et al.*, 1999).

The initial stimulus for switching on of the *agr* system is cell density (Balaban and Novick, 1995; Ji *et al.*, 1995). As cells grow exponentially, they presumably express a low basal level of the *agr* P2 operon. This results in the expression and processing of the AgrD octapeptide pheromone that is secreted into the medium. When the octapeptide reaches a critical concentration it is thought to bind to a part of the AgrC sensor that is exposed on the outer face of the cytoplasmic membrane. This activates the protein kinase activity of AgrC, which in turn activates the response regulator, AgrA, thus stimulating higher levels of transcription at the P2 and P3 promoters. This autoinducing system resembles the competence inducing system of *Bacillus subtilis*, which also involves peptide pheromone density-dependent activation (Magnuson *et al.*, 1994).

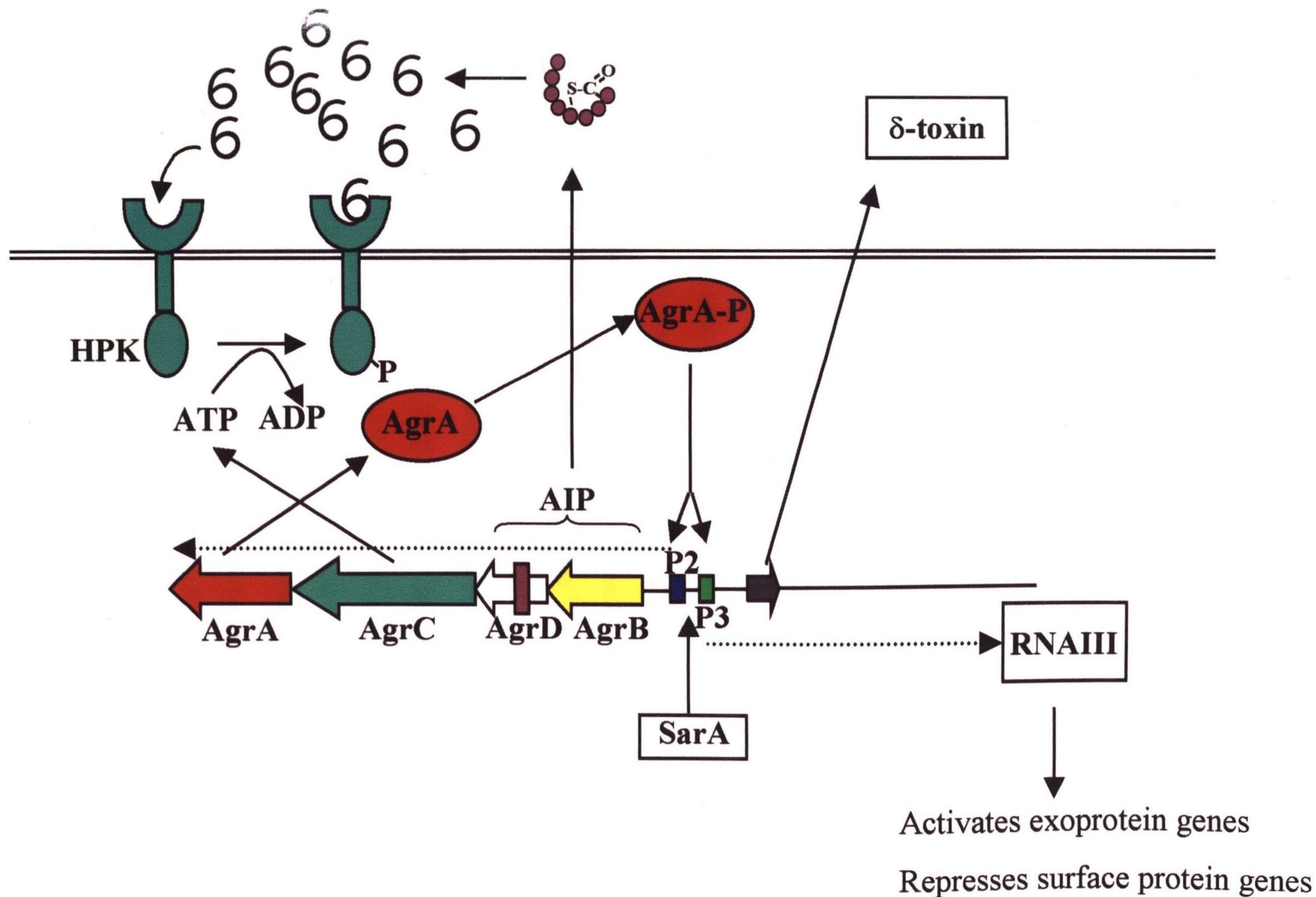


Figure 1.10 Representation of the *agr* regulon in *S. aureus*. AgrC; membrane-bound histidine-protein kinase. AgrA; Response regulator. AgrD; Encodes autoinducing peptide (AIP). AgrB; Membrane protein that is responsible for processing the AIP. RNAIII; Effector molecule of the *agr* response.

Structure-function studies of the octapeptide AIP have shown that linear derivatives are inactive and that the cyclic ring structure is essential (Mayville *et al.*, 1999). In fact most changes to the primary aa sequence of the peptide caused it to become an inhibitor rather than an activator of the *agr* response (Mayville *et al.*, 1999). Similar inhibitory properties were observed when the thiolactone bond was replaced with a lactone or amide bond, suggesting that the thiolactone bond is essential. At least four distinct groups of activator specificity have been found in different strains of *S. aureus* (Ji *et al.*, 1997; Lina *et al.*, 1998). Each activator type has a distinct octapaptide sequence and also varies in the sensor sequence of the AgrC protein. Despite differences in sequence and length of AgrD autoinducing peptide between different strains, the central cysteine residue is conserved and is thought to be involved in the formation of the cyclic thioester structure (Ji *et al.*, 1997). It is thought that octapeptides from heterologous strains can bind to native AgrC proteins without inducing the *agr* response, thus sterically preventing autoinduction by the native octapeptide (Mayville *et al.*, 1999). This represents a novel form of bacterial interference, which could have implications in nasal and mucosal colonisation.

In addition to the octapeptide autoinducing peptide, Balaban and co-workers have reported that there is a second protein present in *S. aureus* that is responsible for induction of the *agr* response (Balaban *et al.*, 1998). This is a 38 kDa extracellular protein called RAP (RNAIII activating protein) that is supposedly produced by *agr*-null as well as *agr+* strains, suggesting that is encoded outside of the *agr* locus. These results are in direct conflict with the work of Novick and co-workers who claim that there is no *agr*-induction activity in *agr*-null supernatants. This is an area of great debate and it is currently unclear whether RAP actually interacts with the *agr* system.

Autoinduction of the P2 operon results in the concomitant activation of the transcript from the *agr* P3 promoter, producing the 514 nt P3 transcript RNAIII, the effector of *agr*-specific target gene regulation (Novick *et al.*, 1993). Interestingly, the P3 operon encodes one translated product, the 26 kDa δ-toxin peptide, which has no overall role in the regulatory function of the *agr* locus (Janzon and Arvidson, 1990; Novick *et al.*, 1993). RNAIII analogs encoded by other staphylococci vary considerably in primary sequence, however they are predicted to have similar overall secondary structure (Novick

et al., 1993; Tegmark *et al.*, 1998). The primary regulatory function of RNAIII is at the level of transcription (Novick *et al.*, 1993), probably mediated through one or more regulatory proteins. However, RNAIII also appears to play a role in translational regulation. It is thought that the 5' end of RNAIII may be involved in translational regulation, while the 3' end appears to be important for both the transcriptional activation and repression functions of the molecule (Novick *et al.*, 1993; Cao *et al.*, 1995; Piriz Duran *et al.*, 1996). Deletion of the 5' end of RNAIII results in the abolition of α-toxin translation, without affecting the transcription of the *hla* gene (Novick *et al.*, 1993). While there is some sequence identity between RNAIII and the 5' end of *hla* mRNA, the exact mechanism by which RNAIII interacts with target gene promoters is unknown.

The list of virulence factors whose expression is controlled by RNAIII includes the following. Proteins that are upregulated by RNAIII are mainly secreted proteins and include; α-toxin, β-toxin, δ-toxin, TSST-1, SEB, SEC, FAME, lipase, serine protease, staphylokinase and type 5 capsular polysaccharide. Some surface-associated proteins appear to be downregualted by RNAIII. These include protein A, FnBPA, FnBPB and coagulase. Others such as Cna (Blevins *et al.*, 1999), ClfB (McAleese *et al.*, 2001) and EbpS (Roche *et al.*, 2001) are regulated independently of *agr*.

1.3.2 The *sarA* Locus

The *S. aurues sarA* locus is a global regulatory locus that was originally identified as a Tn*917* insertion that had decreased expression of several of the exoprotein genes belonging to the *agr* regulon (Cheung *et al.*, 1992). This locus contains three overlapping transcription units of 0.56, 0.8 and 1.2 kb, with a common 3' end that have been designated *sarA, sarC* and *sarB*, respectively (Figure 1.11). Each of these units contains a single gene that codes for a 14.7 kDa DNA-binding protein known as SarA, which binds to AT-rich DNA (Novick, 2000). SarA up-regulates transcription of the two *agr* operons (P2 and P3) approximately 5- to 10-fold by binding to the intergenic region (Heinrichs *et al.*, 1996; Morfeldt *et al.*, 1996; Chien and Cheung, 1998; Rechtin *et al.*, 1999). However, SarA also affects the expression of certain exoprotein genes directly, independently of its effects on *agr* (Cheung *et al.*, 1997; Chan and Foster, 1998; Blevins

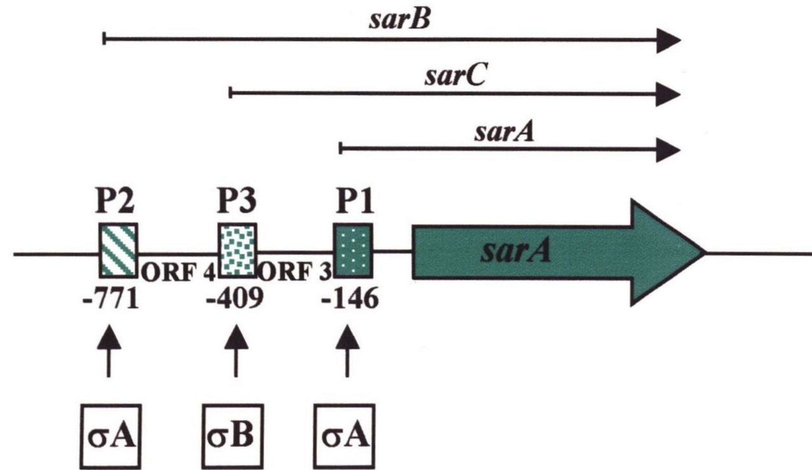


Figure 1.11 Representation of the *S. aureus sarA* locus. The *sar* locus consists of three overlapping transcripts; *sarA*, *sarB* and *sarC*, all of which encode the SarA effector protein The P2 and P1 promoters, which drive the *sarB* and *sarA* transcripts are activated by the primary sigma factor, σA. The P3 promoter, which drives the *sarC* transcript is activated in stationary phase by the alternative sigma factor, σB.

et al., 1999; Cheung *et al.*, 1999). For example, transcription of the collagen binding protein gene, *cna*, is repressed by SarA, but is unaffected by RNAIII (Blevins *et al.*, 1999).

Transcription at the *sarA* locus is complex. Three separate promoters (P1, P2 and P3) ensure that the transcript is present in both exponential and stationary phase. Both the P1 and P2 promoters are activated by the primary sigma factor (σ^A), which also activates housekeeping genes, and expression at these promoters is maximal during the exponential phase of growth (Manna *et al.*, 1998). However, the P3 *sar* promoter is activated in stationary phase by an alternative sigma factor, σ^B, which is stationary phase/stress inducible (Deora *et al.*, 1997; Manna *et al.*, 1998).

A *sar* homologue has been identified in *S. epidermidis* that complements a *sarA* mutant of *S. aureus* (Fluckiger *et al.*, 1998). The *S. epidermidis sar* locus lacks the two smaller open reading frames present in *S. aureus*, supporting the idea that their putative translation products are not important in *sar* function.

1.3.3 Sar Homologues in *S. aureus*

A new global regulator, SarH1, which is homologous to SarA, has recently been described in *S. aureus* (Tegmark *et al.*, 2000). Expression of *sarH1* is regulated by both SarA and Agr and some of the previously reported effects of SarA and Agr on target gene expression are now thought to be mediated through *sarH1* (Tegmark *et al.*, 2000). The *sarH1* locus comprises three transcripts of 952, 1549 and approximately 3000 nt, each of which are thought to encode a single product, SarH1 (Figure 1.12). The transcription start point of the smallest transcript (P1) is located 150 nt upstream of the predicted start point, while the start of the 1500 nt transcript (P2) is located 800 nt upstream of the SarH1 translation start point. A perfect σ^B promoter consensus sequence precedes the P2 transcript. Indeed the P2 promoter has been shown to be regulated by σ^B, with increased levels of the 1500 nt transcript present in stationary phase (Tegmark *et al.*, 2000). The start of the 3000 nt transcript (P3) is unmapped.

Both *sarA* and *agr* appear to have a negative regulatory effect on *sarH1*. In *agr* or *sarA* mutants, expression of *sarH1* from P1 is dramatically increased (Tegmark *et al.*,

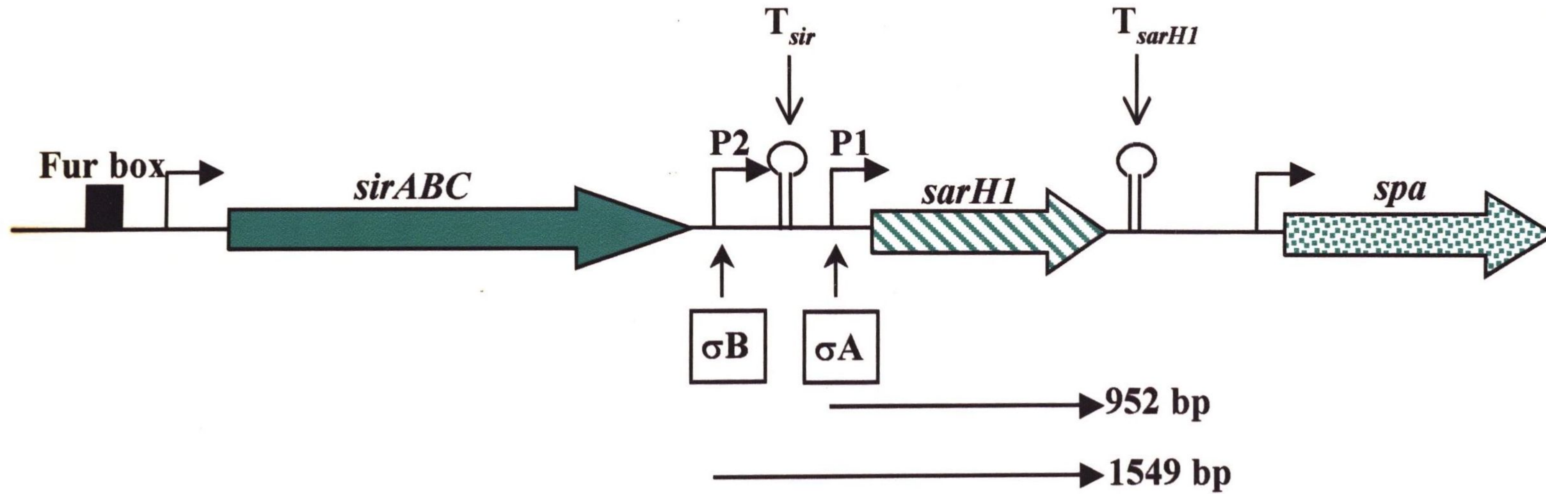


Figure 1.12 Representation of the *S. aureus sarH1* locus. *sarH1* is located between the *sirABC* and *spa* genes. The P2 promoter is activated by σB and produces a 1549 bp transcript. The P1 promoter is activated by σA and produces a 952 bp transcript. The P3 promoter is unmapped, but presumably lies within the *sirABC* gene. The *sirABC* and *sarH1* transcription termination sites are indicated (T).

2000). Under derepressed conditions, *sarH1* transcription is highest during early exponential phase, declines during late exponential phase and reaches a steady-state level during the post-exponential phase of growth. These data indicate that factors other than SarA and RNAIII are involved in the temporal control of *sarH1* expression.

The effects of SarH1 on the transcription of *hla* (α-toxin), *spa* (protein A) and *ssp* (serine protease) have been evaluated and have been shown to be effected by both RNAIII and SarA (Tegmark *et al.*, 2000). It is possible that SarH1 can bind directly to target gene promoters and regulate their transcription in a *sarA/agr* independent manner. However, this has not yet been demonstrated.

Another *sar* homologue that has been identified recently is a locus known as *rot* (McNamara *et al.*, 2000). The product of this locus (Rot), which was identified by Tn*917* transposon mutagenesis, shares homology with SarA. Rot (repressor of toxins) is thought to be involved in the negative regulation of toxin and protease production. It is thought that Rot represses the transcription of extracellular protein genes during exponential phase. In stationary phase, transcription of these genes is derepressed by RNAIII, which presumably competes with Rot, displacing it and thus allowing transcription of the extracellular protein gene to occur (McNamara *et al.*, 2000). However, these interactions remain to be characterised at the molecular level.

Analysis of *S. aureus* genome sequences has shown that there are several other *sar* homologues present. It is highly likely that these loci have a role to play in the complex interactions that control accessory gene regulation in *S. aureus*.

1.3.4 The *sae* Locus

The *sae* locus (*Staphylococcus aureus* exoprotein expression) encodes a two-component signal transduction system which stimulates the production of several exoproteins including α-toxin, β-toxin, DNase and coagulase at the transcriptional level by a pathway that does not involve *agr* or *sarA* (Giraudo *et al.*, 1994; 1997). This locus encodes an activator gene (*saeR*) and a sensor histidine kinase (*saeS*) (Figure 1.13) (Giraudo *et al.*, 1999). It is thought that SaeR exerts its effects on target gene promoters directly. A Tn*551* insertion in the *sae* locus affected the expression of several different

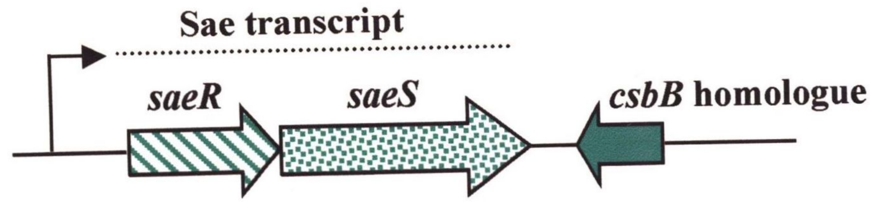


Figure 1.13 The *S. aureus sae* locus. The *saeR* and *saeS* genes are co-transcribed. The products of these genes, SaeR and SaeS form a two-component signal transduction system. SaeS is the sensor histidine-kinase and SaeR is the response regulator. An incomplete ORF that has homology to the *B. subtilis csbB* gene lies downstream of *sae*S. This ORF may be involved in the transcription termination of the *sae* locus.

exoprotein genes, with the notable exception of protein A, serine protease and lipase (Giraudo *et al.*, 1994; 1997). However, importantly, mutation of *sae* does not appear to affect *sarA* or *agr* expression, indicating that it acts downstream from or independent of these loci in the exoprotein expression pathway.

1.3.5 Sigma Factor-Dependent Regulation

In bacteria, alternative sigma factors are known to play a crucial role in regulating gene expression upon major changes in the environment. In *B. subtilis*, the alternative sigma factor (σ^B) regulates a large number of general stress genes (Boylan *et al.*, 1993; Völker *et al.*, 1994). Expression of these genes is thought to provide multiple stress resistance to starving *B. subtilis* cells in anticipation of future stress (Hecker and Völker, 1998). In *S. aureus*, the alternative sigma factor (σ^B) is induced during stationary phase and upon heat shock by the product of the *rsbU* gene (Kullik and Giachino, 1997). *rsbU* is activated by the primary sigma factor, σ^A (Figure 1.14). *S. aureus* mutants defective in σ^B production show diminished stress tolerance in comparison to wild type cells (Chan *et al.*, 1998; Kullik *et al.*, 1998; Nicholas *et al.*, 1999), indicating that σ^B is important to the survival of *S. aureus* under extreme conditions.

The exact role of σ^B in the pathogenesis of *S. aureus* is not entirely clear. While some studies have indicated that σ^B does not contribute to the infection process (Chan *et al.*, 1998; Kullik *et al.*, 1998; Nicholas *et al.*, 1999), other groups have suggested that these animal models do not reflect the natural situation of the host and that they may be misleading (Gertz *et al.*, 2000). Recently, 27 cytoplasmic proteins that are under the positive control of σ^B were identified by 2D-gel electrophoresis (Gertz *et al.*, 2000). Included in this list of σ^B-dependent proteins is SarA, the expression of which is repressed in a σ^B mutant. These data conflict with previous reports that showed elevated SarA expression in a σ^B mutant (Cheung *et al.*, 1999). However, since both σ^B and the *sar* P3 promoter are activated in stationary phase, the idea that σ^B is a positive activator of SarA is more appealing. The other 26 proteins identified by Gertz and co-workers are uncharacterised.

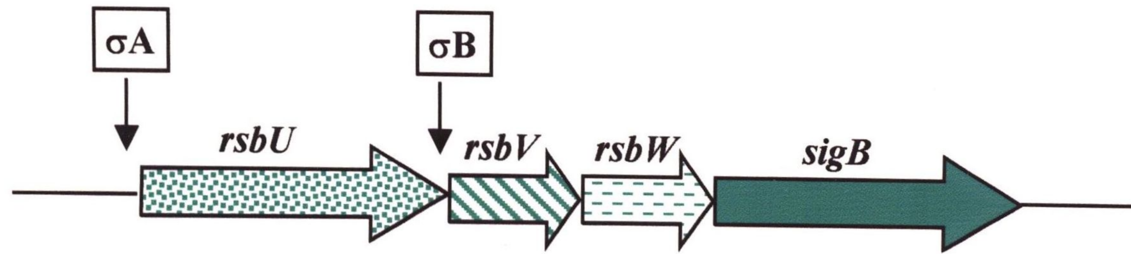


Figure 1.14 The *S. aureus sigB* operon. The function of the *rsb* genes in *S. aureus* have not been confirmed. It is thought that *rsbU* is activated by σ^A and that RsbU activates transcription at the *sigB* promoter. However these putative promoter sites have not been confirmed experimentally.

Since *agr* is expressed maximally during exponential phase, before σ^B expression is induced, it is unlikely that σ^b affects *agr* expression. However, σ^B appears to have a dramatic effect on the expression of SarA during stationary phase. Considering this, it is tempting to speculate that σ^B can exert a range of pleiotropic effects through *sarA* during stationary phase. Indeed, certain studies indicate that overproduction of α-toxin in a σ^B mutant occurs via a SarA-dependent pathway (Cheung *et al.*, 1999). Other studies show that biofilm production, which is due to expression of PIA from the i*caADBC* locus, is positively regulated by σ^B (Rachid *et al.*, 2000). It is not known if biofilm expression is regulated directly by σ^B or through the secondary effects of another regulatory locus.

1.3.6 Summary of the Interactions Between Global Regulatory Loci in *S. aureus*

It is now abundantly clear that regulation of virulence genes in *S. aureus* is an extremely complex process that involves the interaction of many different regulatory loci, allowing the bacterium to adapt and survive in changing environments. *agr* and *sarA* appear to be two of the most influential regulatory loci in exponential phase. These loci are intrinsically linked, as transcription of *agr* is positively regulated by SarA. In turn, both RNAIII and SarA are involved in the negative regulation of the newly-described *sar* homologue, SarH1. While *agr* is expressed maximally in exponential phase, *sarA* and *sarH1* appear to be expressed throughout the growth cycle from three independently transcribed promoters (P1, P2 and P3). As the cells enter stationary phase, the alternative sigma factor, σ^B, is expressed. This molecule interacts with the P3 promoters of *sarA* and *sarH1* to positively regulate their expression. Limited studies on the *sae* locus suggest that its products do not interact with other regulatory loci. It is likely that other uncharacterised regulatory loci exist in *S. aureus* and it is possible that they may interact with loci such as *agr* and *sarA*. Interactions between the known regulatory loci in *S. aureus* are described in figure 1.15.

The success of *S. aureus* as a pathogen is attributed to its excellent ability to establish infections and respond to hostile environments inside and outside of the host. This is achieved through tightly regulated temporal expression of surface proteins and other proteins that allow the organism to adapt to changing environments. The regulatory

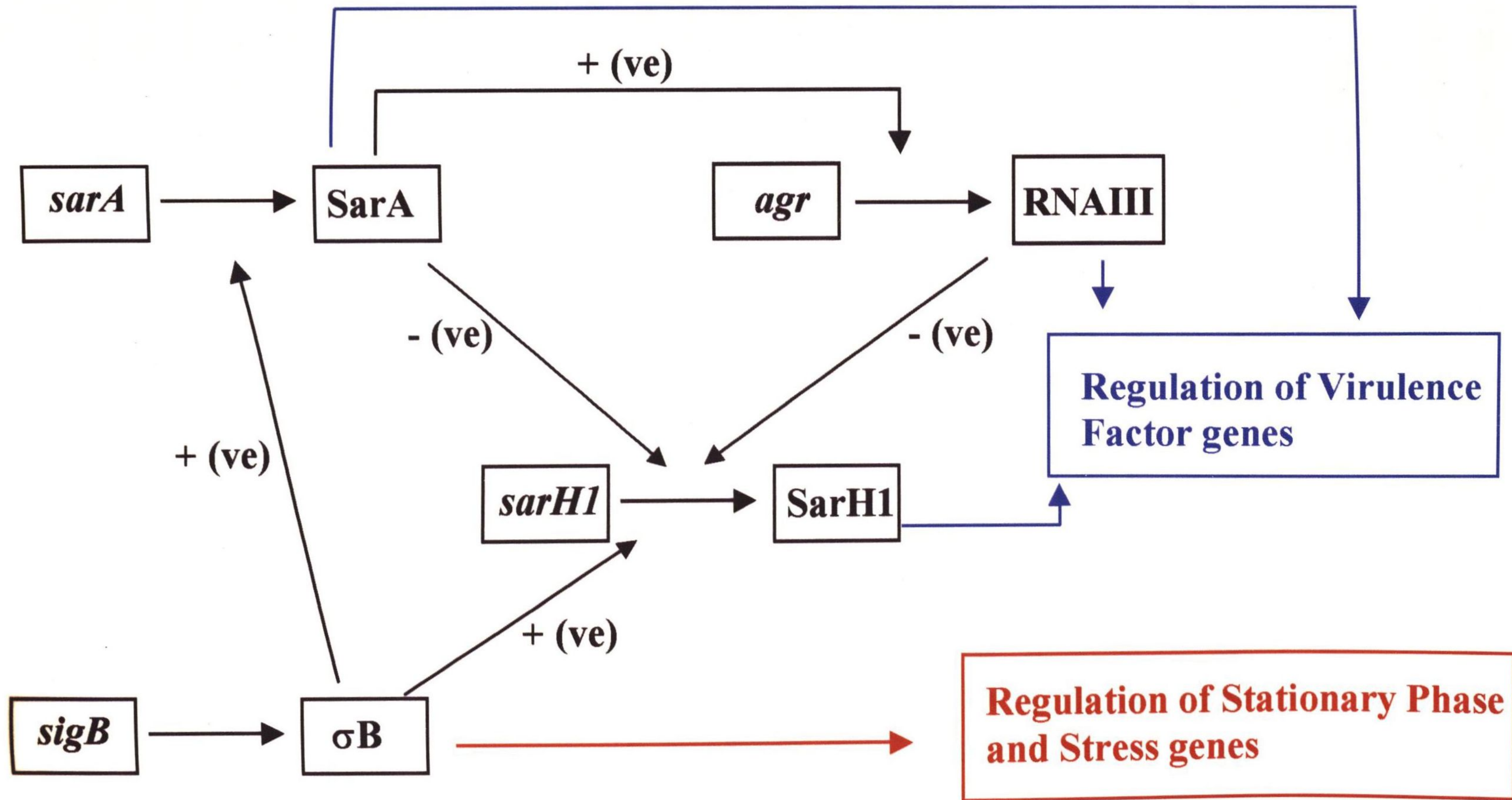


Figure 1.15 Interactions between the *agr*, *sarA*, *sarH1* and *sigB* loci in *S. aureus*.

loci that control expression of such genes are interconnected and they exert their effects on target genes either directly or secondarily through another regulatory locus. Understanding the intricacies of these regulatory pathways will provide insight into how the process of infection is controlled.

1.4 New Approaches to Combating *S. aureus* Infections

S. aureus is an important community- and nosocomially-acquired pathogen that can cause a number of illnesses. These diseases range from non-threatening manifestations such as skin abcesses and impetigo, to life-threatening conditions such as pneumonia, endocarditis, septicemia and toxic shock syndrome. Staphylococcal infections are classically treated with antibiotics, which can usually eliminate the disease. However, in recent years the emergence of multi-drug resistant strains has caused some alarm. Recent data has shown that up to 40-60% of staphylococci isolated from patients who acquired staphylococcal diseases while in hospital were resistant to multiple antibiotics, including methicillin (Lee, 1998). Glycopeptide antibiotics, such as vancomycin, remain the only drugs effective against these multi-drug resistant staphylococci. However, recent reports of *S. aureus* isolates that had reduced susceptibility to vancomycin have caused concern amongst the scientific community (Tenover, 1999) and have highlighted the importance of finding new targets for combating staphylococcal infections.

1.4.1 Unsuccessful *S. aureus* Vaccines

The ability of *S. aureus* to develop resistance to antibiotics devalues the prospect of developing novel antibiotics against staphylococcal infection. A far more appealing option would be the development of a staphylococcal vaccine. The idea of developing a vaccine against *S. aureus* is not new. Between 1977 and 1994, several unsuccessful strategies were investigated. Immunization with killed bacterial cells has in the past provided a successful vaccine against a number of different organisms. However, this approach, using killed *S. aureus* cells, did not protect laboratory animals or humans

against staphylococcal disease (Greenberg *et al.*, 1987; Poole-Warren *et al.*, 1991). Other studies showed that antibodies against the cell wall-associated *S. aureus* protein A did not protect infant rats against systemic staphylococcal infection (Greenberg *et al.*, 1989). Another approach was to immunize with α-toxin, a derivative from the secreted staphylococcal α-toxin. While this strategy reduced the severity of experimental mastitis, it was unsuccessful in preventing infection (Mamo *et al.*, 1994a).

1.4.2 Experimental *S. aureus* Vaccines that Provided Protection in Animals

In recent years, several studies have presented promising results which showed that immunizing animals with recombinant surface proteins or administration of antibodies recognising surface components provided protection against subsequent challenge with *S. aureus*. Antibodies to fibronectin binding protein (FnBPA) showed some protection in an endocarditis (Schennings *et al.*, 1993) and in a mastitis model (Mamo *et al.*, 1994a). Similarly, antibodies to the fibrinogen-binding protein Fib reduced the incidence and severity of murine mastitis (Mamo *et al.*, 1994b). While immunization with a recombinant fragment of the collagen-binding protein (Cna) did not provide protection in the mastitis model (Mamo *et al.*, 1994b), it did provide protection in a murine septic arthritis model of infection (Nilsson *et al.*, 1998). In the latter study, both active and passive immunisation was achieved. Passive protection showed that the effect was antibody-mediated. Recently, Josefsson *et al.* (2001) showed similar results for ClfA. Protection against both sepsis and arthritis was achieved passively using both polyclonal anti-ClfA antibodies and human IgG enriched for anti-ClfA antibodies.

Immunization with the *S. aureus* type 5 capsular polysaccharide (CP5), linked covalently to *Pseudomonas aeruginosa* exotoxoid A, protected mice against a lethal challenge induced by peritoneal infection, as well as sublethal staphylococcal infection that diseminated to the liver and kidneys (Fattom *et al.*, 1996). In addition, rats that were passively immunized with antibodies to CP5 showed a reduced incidence of endocarditis, bacteremia, and metastatic infection in the kidneys (Lee *et al.*, 1997).

Another strategy that yielded extremely promising results was based on the use of the capsular polysaccharide adhesin, PNSG (poly-N-succinyl β-1-6 glucosamine) as a

vaccine target. PNSG is expressed at high levels *in vivo* by *S. aureus* and most clinically important isolates of coagulase negative staphylococci and is distinct from the serologically and structurally defined *S. aureus* capsular polysaccharides (McKenny *et al.*, 1999). McKenny and co-workers showed that vaccinating mice with PNSG provided protection against subsequent *S. aureus* infection in the mouse model of renal infection. Thus, PNSG has potential to provide protection against a wide range of staphylococcal infections.

1.4.3 Novel Vaccine Targets and Anti-Staphylococcal Therapies

In addition to the surface protein and capsular polysaccharide targets that have been investigated, a number of novel *S. aureus* targets for vaccine development have also been proposed. These novel targets have not yet been investigated in full and are areas of current research.

A tantalizing prospect for the development of new anti-staphylococcal therapy involves sortase (SrtA), the enzyme that anchors surface proteins to the cell wall of *S. aureus* (Mazmanian *et al.*, 1999). Since surface protein adhesins are critical to the establishment of *S. aureus* infection, eliminating their display on the surface of the bacterium could potentially destroy its colonization abilities and prevent infections from occurring. Using the purified SrtA protein to screen for compounds that inhibit cell wall sorting of surface proteins could fulfill this possibility. However, recent analysis of the *S. aureus* genome sequence (M. Pallen, unpublished) has shown that a homologue of SrtA exists. This uncharacterised protein, known as SrtB, has sequence identity to SrtA and is predicted to perform a similar function. However, it is not currently known if SrtB is functional in *S. aureus* and surface proteins that it is likely to interact with have not been identified. Therefore, although Mazmanian and co-workers (1999) have shown that several surface proteins are not displayed on the *S. aureus* cell surface in a *srtA* mutant, perhaps SrtB should be taken into consideration in the design of potential sortase inhibitors.

Another strategy for inhibiting the establishment of *S. aureus* infections focuses on the global regulatory locus, *agr*. Since *agr* controls the expression of many different

virulence genes, several groups agree that targeting either the *agr* autoinducing peptide or the AgrC receptor-histidine kinase should lead to attenuation of *S. aureus* infection (Balaban *et al.*, 1998; Lyon *et al.*, 2000). Compelling evidence presented by Lyon and co-workers (Lyon *et al.*, 2000) indicates that AgrC is responsible for *agr* activation and inhibition. In this same study, derivatives of the AgrD octapeptide autoinducing pheromone were shown to inhibit the activation of the membrane bound AgrC receptor histidine kinase. These cyclic derivatives appear to have the potential to inhibit the expression of *S. aureus* virulence factors and could represent a novel form of anti-staphylococcal therapy. In addition, the broad specificity of these inhibitors means that they can inhibit *agr* expression in other staphylococcal strains such as *S. epidermidis* and *Staphylococcus warnerii* (Lyon *et al.*, 2000).

Another *agr*-based strategy has been presented by Balaban and co-workers (Balaban *et al.*, 1998). Balaban believes that *agr* is not only activated by the octapeptide pheromone described by Novick and co-workers (Ji *et al.*, 1995), but also by a 38 kDa protein known as RAP (RNAIII activating protein). In addition, Balaban has presented data indicating that non-pathogenic strains of *S. aureus* produce a linear peptide known as RIP (RNAIII inhibiting peptide), which competes with RAP to inhibit the Agr response (Balaban and Novick, 1995). This is an area of great debate as Balaban's work is unsupported by others who have tried to repeat what she has published. Novick suggests that the *agr*-activating effects of RAP shown by Balaban were due to contamination of the 38kDa RAP protein with the AgrD octapeptide and that the RIP peptide is actually the octapeptide AIP from *S. warnerii*, which naturally inhibits the *agr* response in other staphylococcal strains (R. Novick, unpublished). However, arguments aside, Balaban has shown that mice vaccinated with RAP or treated with purified RIP were protected from *S. aureus* pathology (Balaban *et al.*, 1998). The mechanism by which this protection is elicited has yet to be proven.

Although these potential vaccines and novel therapies against *S. aureus* infection are a long way from being commercialised, they are a promising alternative to the current strategy of antibiotic therapy and may provide a mechanism for tackling what is considered to be one of the most important human pathogens.

1.5 The Importance of Characterising the *S. aureus* Surface-Associated Proteins SdrC, SdrD and SdrE

S. aureus has the potential to express at least 16 different MSCRAMMs, which allow it to interact with the host in many different ways. Proteins such as ClfA, ClfB, FnBPA, FnBPB and Cna provide the ability to bind and adhere to host molecules such as fibrinogen, fibronectin and collagen, which allow the bacterium to establish localized infections. In addition, certain MSCRAMMs allow *S. aureus* to invade host cells (FnBPA) and to resist phagocytosis (ClfA). Understanding the molecular basis of these interactions is extremely important to understanding the pathogenesis of *S. aureus* and in the design of disease treatment. Because MSCRAMMs are displayed on the surface of the bacterium, they provide promising targets for vaccine development. Indeed, evidence shows that administering a recombinant fragment of ClfA (Josefsson *et al.*, 2001) or Cna (Nilsson *et al.*, 1998) does provide protection against subsequent *S. aureus* challenge. This type of vaccine would work by generating an immune response to one or more surface proteins of *S. aureus*, thus preventing subsequent *S. aureus* infection. Individuals who are at high risk from developing *S. aureus* infection would benefit most from vaccination. These would include patients who are undergoing continuous dialysis or those who are having elective surgery.

Considering this, characterising the expression and function of novel proteins such as SdrC, SdrD and SdrE is of prime importance. If an MSCRAMM-based vaccine is to be developed, the most effective product would probably need to be directed against one or more proteins that are expressed at high levels and by most strains of *S. aureus*. Therefore, identifying the function and expression pattern of uncharacterized proteins such as SdrC, SdrD and SdrE is essential.

Following the identification of the *S. aureus* SdrC, SdrD and SdrE proteins by Dr. Elisabet Josefsson in this laboratory in 1998 (Josefsson *et al.*, 1998a), a collaboration was established between our lab and that of Prof. Magnus Höök at the Texas A&M University in Houston, Texas. This collaboration aimed to characterise the expression of these proteins in *S. aureus* and to identify their phenotype(s).

It was decided that our part of the collaboration would focus on the molecular characterisation of Sdr protein expression. In order to fulfill this goal, several targets were set. These included the construction of mutations in the *sdr* genes, the analysis of Sdr protein expression in *S. aureus* and the heterologous expression of the proteins in a surrogate Gram-positive host such as *Staphylococcus carnosus* or *L. lactis*. The generation of *sdr* mutations aimed to provide tools for studying protein expression in *S. aureus* and to assist in the identification of novel protein phenotypes by the comparison of wild type and mutant cells. Characterising the expression profiles of the Sdr proteins in *S. aureus* was important, as knowledge of a protein's expression pattern is essential when attempting to identify its function. Finally, heterologous expression of the Sdr proteins in a surrogate host was to provide an additional tool that would overcome the problem of poor expression and functional redundancy that is associated with *S. aureus* surface proteins.

A post-doctoral researcher in Prof. M. Höök's lab in Houston, Dr. Kirk. McCrea, was assigned to the Sdr project. His goals in the collaboration were to identify novel protein interactions between the Sdr proteins and the host matrix. He was provided with the *S. aureus sdr* mutants and *L. lactis* strains that expressed SdrC, SdrD and SdrE, which were generated during the course of this study, to assist in search for host ligands. However, despite two years of work, Dr. McCrea was not successful in identifying any kind of phenotype for the Sdr proteins and he subsequently left Prof. Höök's laboratory. For this reason, identifying novel phenotype(s) for the Sdr proteins became part of this study. *L. lactis* cells that expressed SdrC, SdrD and SdrE were used to analyse their interaction with various host molecules.

The generation of mutations in the *sdr* genes, Sdr protein expression in *S. aureus*, heterologous expression of the Sdr proteins in *L. lactis* and the interaction of Sdr proteins with various host molecules are described in the results chapters of this thesis.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis and *Lactococcus lactis* strains are listed in Table 2.1. *E. coli* was routinely grown on L-agar or broth at 37°C. *S. aureus* was grown either on Trypicase soy agar (TSA, Oxoid) or broth (TSB) or on Brain-Heart Infusion (BHI, Oxoid) agar or broth at 37°C, unless otherwise stated. For expression studies and adherence assays, *S. aureus* strains were grown in 50 ml volumes in 250 ml conical flasks in an orbital shaker at 200 rpm at 37°C. *S. epidermidis* strains were grown on TSA or TSB at 37°C. For expression studies and adherence assays, *S. epidermidis* cultures were grown statically in 50 ml volumes in 250 ml conical flasks at 37°C. *L. lactis* were grown statically on M17 (Difco) agar or broth, incorporating 0.5% glucose, at 30°C. Overnight cultures were grown for approximately 16 hours. Exponential phase cultures were inoculated 1:100 from overnight starter cultures (washed in phosphate buffered saline, PBS, Oxoid), and grown to the required optical density. Stocks of bacterial strains were snap frozen in liquid nitrogen and maintained as frozen cultures at –80°C in L-broth, TSB or M17 broth containing 10% glycerol.

The following antibiotics were incorporated into the media where appropriate: ampicillin (Amp), 100 μg/ml; erythromycin (Em), 10 or 100 μg/ml; tetracycline (Tet), 2 μg/ml; chloramphenicol (Cm), 10μg/ml; kanamycin (Kan), 100 μg/ml. Antibiotics were purchased from Sigma Chemical Co. The protease inhibitor α_2-macroglobulin (Roche) was incorporated in the media where appropriate at a concentration of 1.25 U/10 ml culture.

2.2 Plasmids

All plasmids and derivatives are listed in Table 2.2.

Table 2.1. Bacterial strains

Strain	Genotype	Relevant Properties	Source/ Reference
E. coli			
XL-1 Blue	[F' *pro*AB, *lac*I^{q}ZΔM15, Tn*10*(Tc^r)], *end*A1, *gyr*A96, *hsd*R17, *lac*, *rec*A1, *rel*A1, *sup*E44, *thi*-1	Propagation of plasmids	Stratagene
T61	*repA*$^+$ (pGK12) Kan^r, *recA* Tet^r.	Propagation of pG+host9. Selects against revertants of the *rep ts* mutation.	Maguin *et al.*, 1996
Topp3	Rif^r [F' *proAB lacI^q* ZΔM15 Tn*10* (Tet^r) (Kan^r)]	Protease deficient strain. Used for the expression of recombinant proteins.	Stratagene
L. lactis			
MG1363		Plasmid free derived of strain NCDO 712	Gasson, 1983
S. epidermidis			
K28		Fibrinogen binding	McCrea *et al.*, 2000
HB		Fibrinogen binding	Nilsson *et al.*, 1998
HB *sdrG*	pG+host9::'*sdrG*'	Em^r	Hartford *et al.*, 2001
9142		Fibrinogen binding	Mack *et al.*, 1994
9142 *sdrG*	pG+host9::'*sdrG*'	Em^r	Hartford *et al.*, 2001

Table 2.1, continued

Bacterial Strain	Genotype	Relevant Properties	Source/ Reference
1457		Resistant to nor-floxacin. Recipient for plasmid mobilization from *S. aureus* RN4220	Mack *et al.*, 1994
1457 *sdrG*	pG+host9::'*sdrG*'	Em^r	Hartford *et al.*, 2001.
S. aureus			
Newman		Strong adherence to fibrinogen	Duthie and Lorenz, 1952.
Newman *spa*	*spa::*Kan^r	Kan^r	Roche, 2001.
Newman *clfA*	*clfA*::Em^r	Em^r	McDevitt *et al.*, 1994
Newman *clfAspa*	*clfA*::Em^r, *spa::*Kan^r	Em^r, Kan^r	This work
Newman *clfB*	*clfB*::Tetr	Tet^r	Ní Eidhin *et al.,* 1998
Newman *clfAclfB*	*clfA*::Em^r *clfB*::Tet^r	Em^r, Tet^r	Ní Eidhin *et al.*, 1998.
Newman *sdrC spa*	*sdrC*::pG+host9 *spa*::Tc^r	Em^r, Tc^r	This work
Newman *sdrD spa*	pG+host9::'*sdrD*' *spa::*Tc^r	Em^r, Tc^r	This work
Newman *sdrCDE*	'*sdrC*':: Tc^r::'*sdrD*'	Tc^r,	This work
Newman *clfAspa sdrCDE*	*clfA*::Em^r, *spa::*Kan^r '*sdrC*':: Tc^r::'*sdrD*'	Em^r, Kan^r, Tc^r	This work

Table 2.1, continued

Bacterial Strain	Genotype	Relevant Properties	Source/ Reference
8325-4		NCTC 8325 cured of prophages	Novick, 1967
8325-4::*sdrC-* *::spa-*	pG+host9::'*sdrC*' *spa*::Tc^r	Em^r, Tc^r	This work
8325-4::*sdrD-* *::spa-*	pG+host9::'*sdrD*' *spa*::Tc^r	Em^r, Tc^r	This work
8325-4::*sdrCD-*	*sdrC*':: Tc^r::'*sdrD*'	Tc^r,	This work
RN4220		Restriction-deficient derivative of 8325-4	Kreiswirth *et al.*, 1983.
P1		Rabbit virulent strain	Sherertz *et al.*, 1993
COL		MRSA isolate	Wilkinson *et al.*,1978
S. aureus 101		Endocarditis isolate	S. Peacock
S. aureus 102		Endocarditis isolate	S. Peacock
S. aureus 103		Endocarditis isolate	S. Peacock
S. aureus 104		Endocarditis isolate	S. Peacock
S. aureus 105		Endocarditis isolate	S. Peacock
S. aureus 106		Endocarditis isolate	S. Peacock
S. aureus 107		Endocarditis isolate	S. Peacock
S. aureus 108		Endocarditis isolate	S. Peacock
S. aureus 109		Endocarditis isolate	S. Peacock
S. aureus 110		Endocarditis isolate	S. Peacock

Table 2.2 Plasmids

Plasmid	Features	Marker(s)	Source/ Reference
pBluescript KS+	High copy number *E. coli* plasmid. Used for general cloning	Amp^r	Stratagene
pCUI	Shuttle vector derived from pC194 and pUC19, maintains high copy number in *E. coli* and *S. aureus*	Amp^r in *E. coli* Cm^r in *S. aureus*	Augustin *et al.,* 1992.
pTS2	Derived from pTV1 *ts* (Youngman *et al.*, 1987) Cm^r from pC194 linked to rep_{ts} from pE194ts, with MCS from pGEM 7Z (f)+, Ts replication in *S. aureus*	Cm^r	This laboratory (C. O' Connell, unpublished)
pG+host9	Ts shuttle plasmid, derived from pVE6004 Ts replication in *S. aureus*	Em^r	Maguin *et al.*, 1986.
pKS80	*L. lactis* plasmid with a high level expression system for heterologous surface proteins	Em^r	Hartford *et al.*, submitted

Table 2.2 Plasmids, continued

Plasmid	Features	Marker(s)	Source/ Reference
pG+host9:'*sdrC*'	pG+host9 containing a 1 kb internal fragment from the A region of *sdrC*	Em^r	This work
pG+host9:'*sdrD*'	pG+host9 containing a 1 kb internal fragment from the A region of *sdrD*	Em^r	This work
pG+host9:'*sdrE*'	pG+host9 containing a 1 kb internal fragment from the A region of *sdrE*	Em^r	This work
pQE30:'*sdrC*'	pQE30 containing the full-length A region from *sdrC*	Amp^r	E. Josefsson
pQE30:'*sdrD*'	pQE30 containing the full-length A region from *sdrD*	Amp^r	E. Josefsson
pQE30:'*sdrE*'	pQE30 containing the full-length A region from *sdrE*	Amp^r	E. Josefsson
pBluescript:*tetK*	pBluescript containing the *tetK* resistance determinant on a *Hind*III fragment. Derived from pT181. Provides Tet^r in *S. aureus*	Amp^r	S. Fitzgerald
pBluescript:'*sdrC*': *tetK*:'*sdrD*':pTS2	pBluescript containing an internal fragment of *sdrC* separated from an internal fragment of *sdrD* by *tetK* and ligated with pTS2	Amp^r in *E. coli* Cm^r, Tet^r in *S. aureus*	This work

Table 2.2 Plasmids, continued

Plasmid	Features	Marker(s)	Source/ Reference
pCU1:*sdrC*	pCU1 containing the full-length *sdrC* gene with 300 bp of upstream sequence and 200 bp of downstream sequence	Cm^r	This work
pCU1:*sdrE*	pCU1 containing the full-length *sdrE* gene with 300 bp of upstream sequence and 200 bp of downstream sequence	Cm^r	This work
pKS80:*clfA*	pKS80 containing the full-length *clfA* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	This work
pKS80:*clfB*	pKS80 containing the full-length *clfB* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	D. Litt, personal communication
pKS80:*sdrC*	pKS80 containing the full-length *sdrC* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	This work
pKS80:*sdrD*	pKS80 containing the full-length *sdrD* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	This work

Table 2.2 Plasmids, continued

Plasmid	Features	Marker(s)	Source/ Reference
pKS80:*sdrE*	pKS80 containing the full-length *sdrE* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	This work
pKS80:*sdrG* (K28)	pKS80 containing the full-length *sdrG* gene, cloned from *S. epidermidis* strain K28, in-frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	This work
pKS80:*sdrG* (HB)	pKS80 containing the full-length *sdrG* gene, cloned from *S. epidermidis* strain HB, in-frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	This work
pKS80:*spa*	pKS80 containing the full-length *spa* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon located within the *Bcl*I site of the vector.	Em^r	J. Pènedes
pKS80:*map*	pKS80 containing the full-length *map*gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon located within the *Bcl*I site of the vector.	Em^r	J. Pènedes
pKS80:*ebpS*	pKS80 containing the full-length *ebpS* gene, cloned from *S. aureus* 8325-4 in-frame with the ATG start codon located within the *Bcl*I site of the vector.	Em^r	R. Downer

2.3 DNA manipulation

All DNA manipulations were carried out by standard methods, unless otherwise stated (Sambrook *et al.*, 1989). Enzymes for DNA manipulation were purchased from Sigma Chemical Co., Roche, New England BioLabs, Stratagene and Promega and were used according to the manufacturers' instructions. Recombinant lysostaphin was obtained from AMBI, New York. Other chemicals were obtained from Sigma and BDH.

Chromosomal DNA was isolated from *S. aureus* and *S. epidermidis* using the Genomic DNA purification kit from Edge BioSystems, with the following modification; 200 μg lysostaphin was incorporated into the spheroplast buffer used to resuspend the bacterial pellet. The mixture was incubated at 37°C for 20 min until bacterial lysis had occurred. The remainder of the procedure was carried out according to the manufacturer's instructions. Plasmid DNA was isolated from *E.coli* strains using the Promega Wizard SV Plus Minipreps DNA purification system, as recommended by the manufacturer. The same kit was used to isolate DNA from *S. aureus* and *L. lactis*, with the following modifications. To isolate plasmid DNA from *S. aureus* or *L. lactis*, 200 μg recombinant lysostaphin (for *S. aureus*) or 500 U mutanolysin and 200 μg lysozyme (for *L. lactis*) were added to the resuspension buffer and incubated at 37°C for 20 min before proceding with the isolation. *E.coli* transformants were screened for the presence of chimeric plasmids using the rapid colony screening procedure developed by Le Gouill and Déry (1991).

DNA hybridisations were carried out according to the method of Southern (1975). Approximately 5 μg of genomic DNA was digested for 16 hours with 20 U enzyme in a total volume of 70 μl. Digested DNA fragments were separated for 16 hours at 15 V by agarose gel electrophoresis on a 1% agarose gel (Roche) in 1 x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) using a horizontal midi-gel electrophoresis apparatus (BioRad). Following electrophoresis, DNA fragments were depurinated (0.2 M HCl, 10 min), denatured (1.5 M NaCl, 0.5 M NaOH, 45 min) and neutralised (1.5 M NaCl, 1 M Tris-HCl, pH 7.4), and then transferred from the gel for 16 hours to a positively charged nylon membrane (Roche) by capillary transfer using 10 x SSC (1.5 M NaCl, 0.15 M Na_3Citrate). DNA fragments were fixed to the membrane by incubation at 120°C for 2

hours. The membrane was prehybridised in a standard prehybridisation solution (5 x SSC, 0.1% N- lauroylsarcosine, 0.02% SDS, 1 x Blocking reagent (Roche)) for 2 hours at 68°C and then hybridised for 16 hours with the probe (0.5 μg DIG-labelled PCR product/ml prehybridisation solution) at 68°C. Following hybridisation, the membrane was washed twice at room temperature with 2 x SSC, 0.1% SDS and twice at 68°C with 0.5 x SSC, 0.1% SDS. The membrane was then washed for 5 min with 0.1 M maleic acid, 0.15 M NaCl, pH7.5, Tween 20 (0.3% v/v). Blocking was performed for 1 hour in 1 x blocking reaget (Roche) and the membrane then incubated with anti-DIG fab fragments (Roche), as recommended by the manufacturer. Unbound antibody was removed by washing twice in 0.1 M maleic acid, 0.15 M NaCl, pH7.5, Tween 20 (0.3% v/v). The membrane was equilibrated in 100 mM Tris, pH9.5, 100 mM NaCl and then incubated with the chemiluminescent substrate CSPD (Roche), as advised by the manufacturer. Finally, hybridisation of the DNA probe was detected by exposure of the membrane to autoradiographic film (X-Omat, Kodak)

2.4 DNA sequencing

All confirmatory sequencing in the forward direction was performed at Medigene, Lochhamer Strasse, Germany.

2.5 Mutagenesis of *sdrC* and *sdrD* by directed plasmid integration

Mutations in the *sdrC* and *sdrD* genes of *S. aureus* were made by directed plasmid integration using the temperature sensitive plasmid pG+host9.

2.5.1 *sdrC*

Using the primers sdrCF1 and sdrCR1 (Table 2.3), a 1 kb internal fragment from the unique A region of *sdrC* was amplified by polymerase chain reaction (PCR) from pQE30:'*sdrC*'. In order to facilitate cloning, an *Spe*I restriction site was incorporated into the 5' end of the sdrCF1 forward primer, while an *Xma*I site was incorporated into the 5'

Table 2.3 Primers

Primer name	Sequence	5' Restriction Site
SdrCF1:	GGACTAGTGCTACAGCTAATCCATCTAG	*Spe*I
SdrCR1:	CCCCCCGGGATCGAATTGATCAGTAACATC	*Xma*I
SdrDF1:	CGGAATTCGTGATAATCAATCGAGTG	*Eco*RI
SdrDR1:	AACTGCAGCTCATTTACAACATAATCTG	*Pst*I
SdrEF1:	CGGAATTCAGATGATGCAACGACTAG	*Eco*RI
SdrER1:	AACTGCAGTATCTACATAGTCTGTAAATG	*Pst*I
SdrCF2:	CGGGATCCAAGCTTAGATTAAAAGTGAG	*Bam*HI
SdrCR2:	GCTCTAGACTGGGAATTTCTAAACAG	*Xba*I
SdrDF2:	CGGGATCCTGTAATTGTCTATGAATTAG	*Bam*HI
SdrDR2:	GCTCTAGAGCACTATTTAATCTCAAAC	*Xba*I
SdrEF2:	CGGGATCCGTACAAACGAATAAGAAAAAGC	*Bam*HI
SdrER2:	GCTCTAGAGTAATTCATATTATCGCCTC	*Xba*I
SdrCF3:	GCCGAATTCAGTGAATACTGTTGCAGC	*Eco*RI
SdrCR3:	GCCAAGCTTCCAACTATATTTAGTTTTGTC	*Hind*III
SdrDF3:	GGAAATAAAGTTGAAGTTTC	
SdrDR3:	GCCGGATCCGCATTTGTTAAAGAATTTTCC	*Bam*HI
M13F1:	GTAAAACGACGGCCAGT	
M13R1:	GGAAACAGCTATGACCAT	

Restriction sites are underlined.

Table 2.3 Primers, continued

Primer name	Sequence	5' Restriction Site
ClfAF4	CGCGGATCCATATGAAGAAAAAGAAAAAC	*Bam*HI
ClfAR4	CGCGGATCCTTATTTCTTATCTTTATTTC	*Bam*HI
SdrCF4	CCGGGATCCTGAATAATAAAAAGACAGC	*Bam*HI
SdrCR4	CCGGGATCCTTATTTATTTTGTTTTTACG	*Bam*HI
SdrDF4	CCGGGATCCTAAACAGAGAAAATAAAACGGC	*Bam*HI
SdrDR4	CCGGGATCCTTATTTATTTTGTTTTTACGACG	*Bam*HI
SdrEF4	CCGGGATCCTGATTAACAGGCATAATAAAAAG	*Bam*HI
SdrER4	CCGGGATCCTTATTTGTTTTGTTTTTGCGACG	*Bam*HI
SdrGF4	CGCGGATCCTGATTAATAAAAAAAATAATTT ACTAAC	*Bam*HI
SdrGR4	CGCGGATCCTTAATTTTATTTTTTCTATTTTTG CGAC	*Bam*HI
SdrCF5	CCCGGATCCGCAGAACATACGAATGGAG	*Bam*HI
SdrCR5	CCCAAGCTTACTTTTGGTCGCCATTAGCAG	*Hind*III
SdrDF5	CGCGGATCCCAGGCAGAAAGTACTAATAA AGAATTG	*Bam*HI
SdrDR5	CCGCGGTCGACTTCTTGACCAGCTCCGCCA	*Aac*I
SdrEF5	CGCGGGATCCCAGGCTGAAAACACTAGTAC AGAAAATGCA	*Bam*HI
SdrER5	CGCAAGCTTCTTTCTTCAGGTTTAACAGTACC	*Hind*III

Restriction sites are underlined.

end of the sdrCR1 reverse primer. Reaction mixtures (100 μl) contained 100 pmoles of each of the forward and reverse primers, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, 10 ng plasmid DNA template (pQE30:'*sdrC*') and 1.25 U *Taq* DNA polymerase in a standard *Taq* reaction buffer (Roche). Amplification was allowed to proceed in a Perkin Elmer Cetus thermocycler with an initial denaturation at 94°C for 5 min, followed by 30 cycles with denaturation at 94°C, annealing at 50°C and extension at 72°C for 1 min. The final extension was for 10 min. A single product was obtained, purified using the Wizard PCR purification kit (Promega), cloned between the *Spe*I and *Xma*I sites of pBluescript KS+ and then sub-cloned into the temperature sensitive shuttle plasmid, pG+host9, at the same restriction sites. (*E.coli* strain T61 prevents selection of revertants of the *rep ts* mutation of pG+host9 and was used to propagate all pG+host9 constructs.) The construct was isolated from *E. coli* T61, electroporated into *S. aureus* RN4220, and then transduced into *S. aureus* strains Newman and 8325-4, as described previously (Augustin and Gotz, 1990; Foster, 1998). (Following transformation to *S. aureus*, the temperature sensitive pG+host9 construct was maintained extrachromosomally at temperatures below 30°C.) Both Newman and 8325-4 plasmid-containing strains were initially grown overnight at the permissive temperature (30°C). Serial dilutions of these cultures were plated in duplicate on TSA containing 10 μg/ml Em and incubated at the restrictive temperature (42°C) and the permissive temperature (30°C). The efficiency of plating (eop) was calculated by expressing the number of colonies obtained at the restrictive temperature as a function of the number of colonies obtained at the permissive temperature. Insertion mutants obtained at the restrictive temperature were confirmed by DNA hybridisation, as described in section 2.3.

A DIG labelled probe specific to the A region of *sdrC* was constructed by PCR. The following reaction mixture was set up in a standard 1 x Taq polymerase reaction buffer (Roche); 10 ng pQE30:'sdrC', 100 pmoles sdrCF1, 100 pmoles sdrCR1, 10 μl DIG-dUTPs (Roche), 1.5 mM $MgCl_2$, 1.25 U Taq polymerase (Roche). Amplification was allowed to proceed in a Perkin Elmer Cetus thermocycler with an initial denaturation at 94°C for 5 min, followed by 30 cycles with denaturation at 94°C, annealing at 50°C and extension at 72°C for 1 min. The final extension was for 10 min.

2.5.2 *sdrD*

Mutations in *sdrD* were constructed by a similar method. A 1 kb internal fragment was amplified by PCR from region A of *sdrD* using the primer pair sdrDF1 and sdrDR1, with pQE30:'*sdrD*' as a template, as described above. An *Eco*RI restriction site was incorporated into the 5' end of the forward primer (sdrDF1) and a *Pst*I site was incorporated into the 5' end of the reverse primer (sdrDR1) in order to facilitate cloning. Otherwise, mutations in the *sdrD* genes of strains Newman and 8325-4 were constructed in a manner identical to that for *sdrC*. Mutations were confirmed as before, by Southern hybridisation, using a DIG-labeled probe specific to the A region of *sdrD*, that had been amplified from pQE30:'*sdrD*' using primer pair sdrDF1 and sdrDR1.

2.5.3 *sdrE*

A 1 kb internal fragment from the unique A region of *sdrE* was amplified by PCR using the primers sdrEF1 and sdrER1 with pQE30:'*sdrE*' as a template, as described above. An *Eco*RI site was incorporated into the 5' end of the forward primer (sdrEF1), while a *Pst*I site was incorporated into the 5' end of the reverse primer (sdrER1). The resulting fragment was purified, cloned into pBluescript KS+ between the *Eco*RI and the *Pst*I sites and then sub-cloned into pG+host9. The resulting construct, pG+host9:'*sdrE*', was transformed into *S. aureus* RN4220 by electroporation and then transduced into strains Newman, COL and P1, as before. These strains were grown at 30°C in the presence of Em and then plated at 42°C on TSA containing Em.

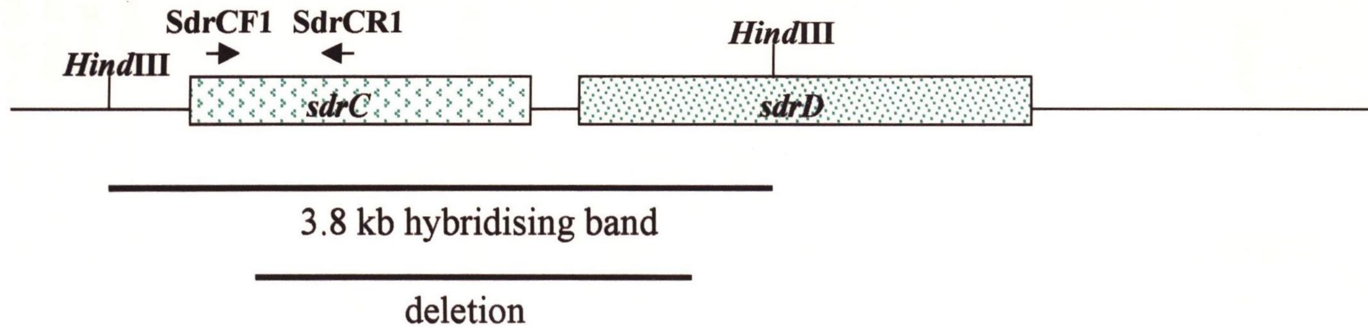
2.5.4 Stability of the mutations

Four independent 8325-4 mutants were grown in TSB in the absence of selective antibiotic for approximately 70 generations. Chromosomal DNA was isolated for each of the strains and digested overnight with *Hind*III. Chromosomal DNA of the wild type strain was also digested. These digests were analysed by Southern hybridisation, using a

DIG-labeled probe specific to the A region of *sdrC*, as above, to determine if the pG+host9 construct had remained integrated in the chromosome.

2.6 Construction of a double mutation in the *sdrC* and *sdrD* genes of strain 8325-4 by allele replacement

A double mutation in both the *sdrC* and *sdrD* genes of strain 8325-4 was generated by allele replacement. This was carried out by deleting a 2.1 kb fragment of DNA from the chromosome of 8325-4, which included the 3' two thirds of the *sdrC* gene and part of the 5' end of the *sdrD* gene, including it's promoter region (Figure 2.1). An 814 bp fragment from the unique A region of *sdrC* was amplified by PCR, using the primers sdrCF3 and sdrCR3, with 100 ng 8325-4 chromosomal DNA as a template. The resulting product contained an *Eco*RI site at the 5' end and a *Hind*III site at the 3' end. A 916 bp fragment from region A of *sdrD* was also amplified from the same template, using the primers sdrDF3 and sdrDR3. The primer sdrDF3 does not contain any restriction site at the 5' end. However, a natural *Hind*III site is present in the DNA fragment generated by PCR with primers sdrDF3 and sdrDR3. Therefore, following cleavage, the resulting product contained a *Hind*III site at the 5' end and a *Bam*HI site at the 3' end. The *tetK* drug resistance determinant had previously been cloned into the *Hind*III site of pBluescript KS+ (S. Fitzgerald, unpublished data). This construct was used as a template for the PCR amplification of the *tetK* fragment, using the primers M13F1 and M13R1, that hybridise outside the multiple cloning site of the vector. The high fidelity polymerase *pfu* (Stratagene) was used to amplify the product, according to manufacturer's instructions. The *sdrC* and *sdrD* PCR products were ligated at the 3' and 5' *HindIII* sites and cloned into pBluescript KS+ at the *Eco*RI and *Bam*HI sites (Figure 2.2). The resulting plasmid was linearised by digestion with *Hind*III and ligated with the 1.5 kb *tetK* PCR product that had been cleaved with *Hind*III. A shuttle plasmid was constructed by combining the resulting plasmid with the *S. aureus* temperature sensitive plasmid, pTS2 (Figure 2.2). This shuttle plasmid was electroporated into the restriction negative host RN4220 and then transduced into strain 8325-4. Standard temperature shift experiments were performed (Foster, 1998) resulting in the isolation of an allele



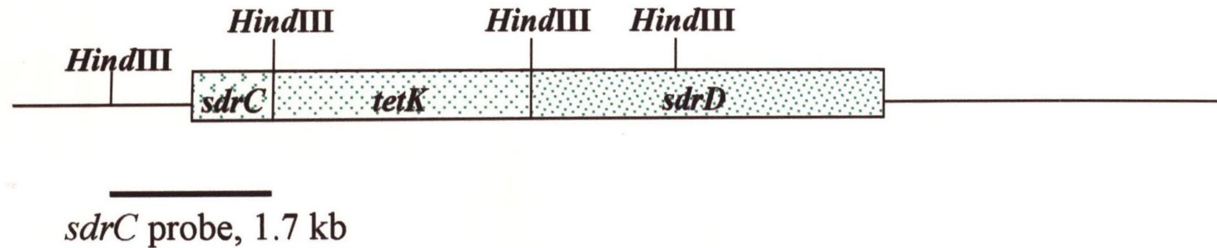


Figure 2.1 Schematic representation of the *sdr* locus in *S. aureus* 8325-4 wild type and the *sdrCD* double mutant, showing the region of DNA that the *sdrC* DIG-labeled probe hybridises to during Southern blotting. In the wild type, the *sdrC* probe hybridises to a 3.8 kb DNA fragment. In the *sdrCD* double mutant, the *sdrC* probe hybridises to a 1.7 kb fragment. "Deletion" represents the area of DNA that is eliminated from the chromosome and replaced by the *tetK* gene during allele replacement.

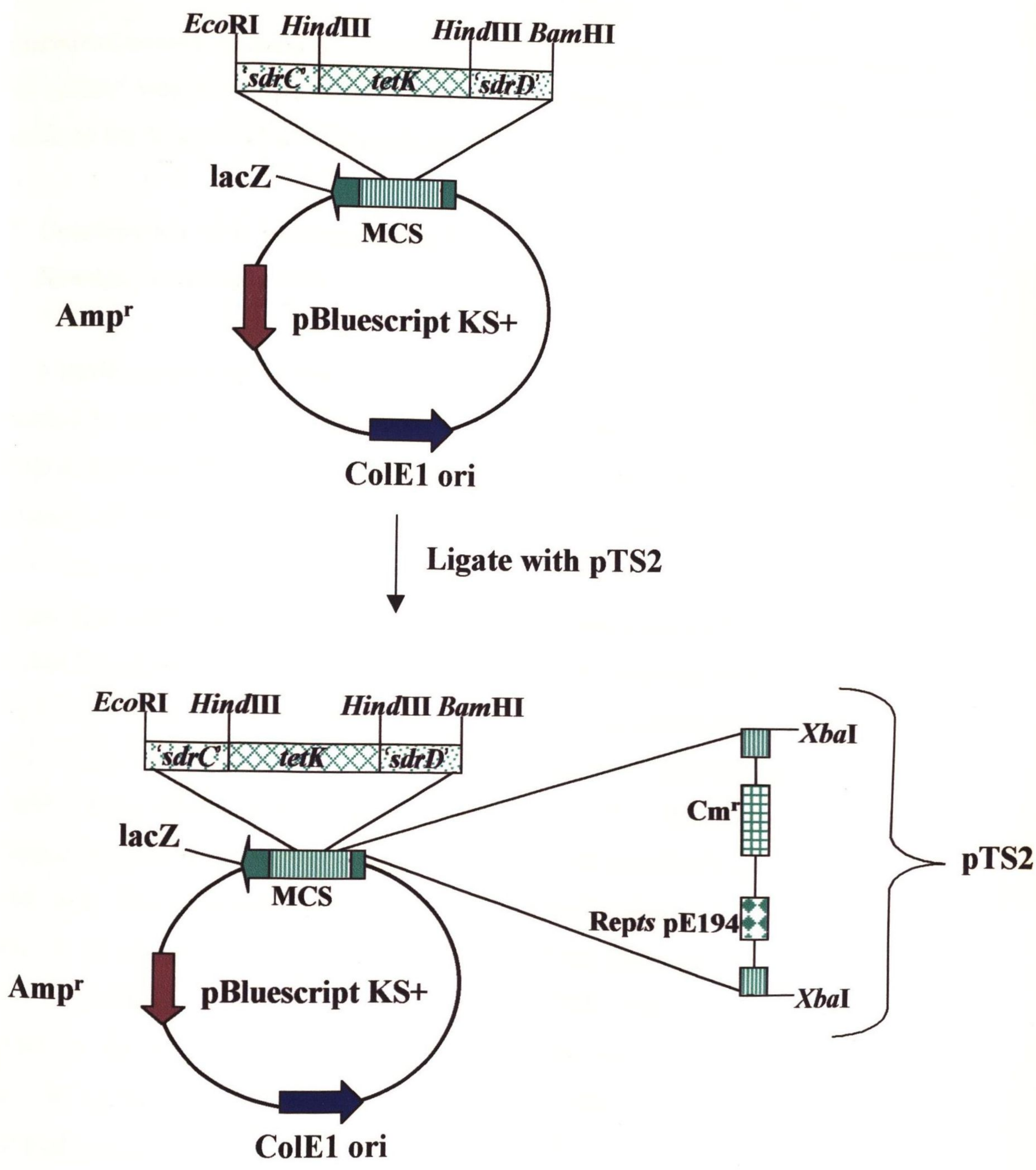


Figure 2.2 Schematic representation of the construction of the pBluescript:'*sdrC*':*tetK*:'*sdrD*': pTS2 plasmid. An internal fragment from *sdrC* was ligated together in pBluescript with an internal fragment of *sdrD* through a *Hind*III site. The *tetK* drug resistance fragment was inserted into this *Hind*III site. This construct was made temperature sensitive for replication in *S. aureus* by ligation with the plasmid pTS2 at the unique *Xba*I site, present in each of the plasmids.

replacement mutant deficient in both the *sdrC* and *sdrD* genes (*sdrCD* double mutation). This mutant was confirmed using Southern hybridisation, with a DIG-labeled probe specific to the A region of *sdrC* (amplified using primers sdrCF1 and sdrCR1).

2.7 Construction of a triple mutation in the *sdrC*, *sdrD* and *sdrE* genes of strain Newman by transduction.

A triple mutation in the *sdrC*, *sdrD* and *sdrE* genes of *S. aureus* strain Newman was generated by transduction (See Figure 3.12). An overnight culture of the 8325-4 *sdrCD* double mutant was diluted 1:100 into phage broth (20 g/L Nutrient broth No. 2 (Oxoid)), containing 10 mM $CaCl_2$, and grown for 4 hours at 37°C with shaking. 300 µl of this culture was infected with 200 µl of a phage 85 stock, diluted to the highest dilution that still provided confluent lysis, and incubated at room temperature for 30 min. The culture was then mixed with 10 ml molten phage top agar (20 g/L Nutrient broth No. 2 (Oxoid), 3.5 g/L Agar No.1 (Oxoid)), containing 10 mM $CaCl_2$, and spread over two phage base plates (20 g/L Nutrient broth No. 2 (Oxoid), 7 g/L Agar No.1 (Oxoid)), which contained 10 mM $CaCl_2$. Plates were incubated overnight at 37°C. Phage plate stocks were harvested by transferring the top agar to an Oakridge tube and centrifuging for 10 min at 15,000 rpm. The supernatant was removed and filtered to provide a sterile phage plate stock. A 20 ml stationary phase culture of the recipient strain was grown in a 250 ml flask. Cells were harvested and resuspended in 1 ml TSB. The following mixtures were then set up; (A) 1 ml L-broth (containing 10 mM $CaCl_2$), 500 µl cells, 500 µl phage plate stock, (B) 1.5 ml L- broth (containing 10 mM $CaCl_2$), 500 µl cells. The mixtures were incubated statically at 37°C for 25 min and then at 37°C with shaking for 15 min. 1 ml ice cold 0.02M Na_2Citrate was added and the mixtures centrifuged. Cells were finally resuspended in 1 ml 0.02 M Na_2Citrate and incubated on ice for 2 hours. The transduction mixture was plated on TSA containing 0.05% Na_2Citrate and 2 µg/ml Tet, and then incubated overnight at 37°C. Colonies obtained were restreaked twice on TSA containing 2 µg/ml Tet and 0.05% Na_2Citrate and then analysed for the introduction of the *sdrCD* double mutation from 8325-4, coupled with the loss of *sdrE* from the chromosome. This was carried out by Southern hybridisation, using DIG-labeled probes

specific to the A region of *sdrC* (amplified using sdrCF1 and sdrCR1) and to the A region of *sdrE* (amplified using sdrEF1 and sdrER1).

2.8 Construction of mutations in the *sdrC, sdrD, sdrE, clfA* and *spa* genes of *S. aureus* strain Newman by transduction.

A mutation in the *sdrC, sdrD, sdrE, clfA* and *spa* genes of *S. aureus* strain Newman was constructed by transduction. Phage plate stocks containing the *sdrCD*::Tet^r mutation and the *clfA*::Em^r mutation were generated by propagating phage 85 on *S. aureus* 8325-4::*sdrCD-* and 8325-4::*clfA-* mutant strains, as described in section 2.7. A culture of Newman::*spa-*::Kan^r (recipient strain) was grown for 16 hours. Cells were harvested and resuspended in 1 ml TSB. The 8325-4::*sdrCD-* phage plate stock was used to infect the NewmanΔ*spa* recipient strain, as described in section 2.7. Transductants selected and restreaked twice on TSA plates containing 2 μg/ml Tet and 0.05% Na_2Citrate. The introduction of the *sdrCD* mutation, coupled with the loss of the *sdrE* gene from Newman::*spa-* was confirmed by PCR. The resulting Newman::*spa-*::*sdrCD-* mutant was grown for 16 hours and the cells then harvested and resuspended in 1 ml TSB. The 8325-4::*clfA-* phage plate stock was used to infect this recipient strain as described in section 2.7. Transductants were selected and restreaked twice on TSA containing 10 μg/ml Em and 0.05% Na_2Citrate. The introduction of the *clfA* mutation was demonstrated by testing transductants for loss of the ability to clump in 1 mg/ml fibrinogen.

2.9 Complementation of the *sdrC* and *sdrE* mutations

The entire open reading frames of the *sdrC* and *sdrE* genes, including 300 bp of upstream sequence and 200 bp of downstream sequence, were amplified from the chromosome of strain Newman using primers sdrCF2 and sdrCR2, and sdrEF2 and sdrER2, respectively. A *Bam*HI site was incorporated into the 5' end of both forward primers, while an *Xba*I site was incorporated into the 5' end of both reverse primers, in order to facilitate cloning. Following restriction digestion, both of these fragments were

ligated with the shuttle plasmid, pCU1, which had been cleaved with *Bam*HI and *Xba*I. The resulting constructs, pCU1:*sdrC* and pCU1:*sdrE*, were electroporated into the restriction negative host RN4220 and then transduced into the relevant mutant strains. Western immunoblotting was used to confirm that protein synthesis was restored in the corresponding mutants.

2.10 Purification of recombinant SdrC, SdrD and SdrE proteins by immobilised metal chelate affinity chromatography

Recombinant proteins of SdrC region A (aa 51-496), SdrD region A (aa 53-569) and SdrE region A (aa 53-607) were purified by immobilised metal chelate affinity chromatography. The DNA sequence coding for the unique A region of each of protein was amplified by PCR and cloned into the expression vector pQE30 by E. Josefsson and K. McCrea. PQE30 contains an IPTG-inducible promoter, which allows for the control of target gene expression. In strain Topp3, the pQE30 target gene promoter is repressed by the LacIQ repressor, which prevents transcription of the target gene. IPTG binds to LacIQ, resulting in derepression of the promoter and thus induction of target gene expression. The primers (SdrCF5/R5, SdrDF5/R5, SdrEF5/R5) used to amplify the *sdr* gene fragments are shown in Table 2.3. These constructs expressed the unique A region of each protein as a recombinant molecule with a hexa-histidine affinity tag at the N-terminus. pQE30:$sdrC_{51\text{-}496}$, pQE30:$sdrD_{53\text{-}569}$ and pQE30:$sdrE_{53\text{-}607}$ were transformed into the protease-deficient *E. coli* strain Topp3 and grown to an OD_{600nm} of 0.7-0.9. Each culture was then induced for 4 hours at 37°C with 1.5 mM IPTG (Biosynth) and the cells harvested. Cells were lysed in a French pressure cell and the lysate centifuged and filtered to remove bacterial cell debris. A iminodiacetic acid-Sepharose 6B Fast Flow column (10 x 1cm) was charged with 150 mM Ni^{2+} and equilibrated with binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH7.9). The cleared lysed cell supernatant was applied to the column and washed with binding buffer until the absorbance at 280 nm of the eluate was <0.001. Bound protein was eluted with a continuous linear gradient of imidazole (5-100 mM; total volume of 200 ml) in 0.5 M NaCl and 20 mM Tris-HCl (pH 7.9). Eluted fractions were monitored by absorbance at

280 nm and peak fractions were analysed by SDS-PAGE and Western immunoblotting. Protein fractions that contained the recombinant protein were pooled and dialysed against 50 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH7.4).

2.11 Anti-SdrC antibodies

Antibodies to recombinant SdrC region A were raised in two young New Zealand white rabbits. Recombinant SdrC region A was purified from the protease-deficient *E. coli* strain Topp3 (pQE30:$sdrC_{51\text{-}496}$) as described in section 2.10. 500 μl of the recombinant protein (25 μg, diluted in sterile PBS) was emulsified in an equal volume of adjuvant and administered subcutaneously. The first injection contained Freund's complete adjuvant, while the following two (given at two-week intervals) contained Freund's incomplete adjuvant. Following a test bleed, where the sera was observed to react with the recombinant protein, the animals were bled out. The serum was recovered as described previously (McDevitt *et al.*, 1994).

2.12 Anti SdrD and SdrE antibodies

Antibodies to recombinant SdrD region A were raised in two young New Zealand white rabbits by E. Josefsson using a procedure similar to that described in section 2.11. SdrD region A recombinant protein was purified from *E. coli* Topp3 cells carrying pQE30:$sdrD_{53\text{-}569}$. Antibodies to recombinant SdrE region A were also raised by K. McCrea using a similar method. SdrE region A recombinant protein was purified from *E. coli* Topp3 cells carrying pQE30:$sdrE_{53\text{-}607}$.

2.13 Purification of anti-SdrC and anti-SdrD sera

Recombinant SdrC region A and recombinant SdrD region A (expressed by pQE30:$sdrC_{51\text{-}496}$ and pQE30:$sdrD_{53\text{-}569}$, respectively) were purified from *E. coli* Topp3, as described in section 2.11. In both cases 4 mg of the recombinant protein was coupled

to cyanogen bromide activated sepharose (Pharmacia), according to the manufacturer's instructions. The antisera was applied to the column and washed with 50 mM Tris, 0.5 M NaCl, pH 8.0 until the absorbance at 280 nm of the eluate was <0.001. Bound antibodies were eluted with a continuous linear gradient of 50 mM glycine, 0.5 M NaCl pH 2.7. Eluted 2 ml fractions were collected in 20 μl Tris, pH 9.0 and were monitored by absorbance at 280 nm. Peak fractions were pooled and concentrated by pressure diaflow. Finally, the purified antibodies were dialysed in PBS, concentrated, and stored in 0.15% sodium azide.

2.14 Isolation of cell wall-associated proteins from *S. aureus*

Cell wall-associated proteins were isolated from *S. aureus* wild type, mutant and complemented mutant strains by the following method. Cells were grown to the required growth phase and harvested by centrifugation. In some cases, the protease inhibitor α_2-macroglobulin (Roche) was included in the growth media at a concentration of 1.25 U/10 ml culture in an attempt to limit proteolytic degradation of the surface-expressed proteins. Cells were washed twice in PBS and adjusted to an OD_{600nm} of 40 in PBS. This fixed number of cells were centrifuged and resuspended in 1 ml 30% (w/v) raffinose (Sigma), constituted in 20 mM Tris (pH 8.0), 10m M $MgCl_2$. The following components were then added; 400 μg lysostaphin, 5 mM EDTA, 75 μl complete® mini protease inhibitor cocktail (Roche), 1 mM PMSF, and the reactions incubated in a 37°C water bath for 20 min, with occasional shaking. During this incubation, protoplasts were formed and cell wall-associated proteins were released into the supernatant. Finally, samples were centrifuged at 8,000 rpm for 10 min to pellet the protoplasts. The supernatant, which contained solubilised cell wall-associated proteins, was removed for further analysis.

2.15 SDS-PAGE and Western immunoblotting

Samples were analysed by SDS-PAGE and Western immunoblotting. Cell wall-associated proteins were first boiled for 10 min in 2 x final sample buffer (10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 3% [w/v] SDS, 0.01% [w/v] bromophenol blue in

62.5 mM Tris-HCl, pH 6.8), and 20 µl aliquots were separated by SDS-PAGE (Leammli, 1970), using 4.5% stacking and either 7.5% or 10% separating acrylamide gels, where appropriate. Two sets of protein molecular weight size standards were separated alongside the protein samples. These included prestained molecular weight markers (New England Biolabs) which included protein bands in the range of 175 kDa, 83 kDa, 62 kDa and 48 kDa and BlueRanger™ prestained molecular weight markers (Pierce) which included protein bands in the range of 200 kDa, 97 kDa, 67 kDa and 43 kDa. Using both of these sets of markers to judge the size of unknown protein bands allowed for accurate determination of sizes. Following separation, proteins were either stained with Coomassie blue or transferred for 2 hours at 70 V onto a PVDF membrane (Roche), using a wet transfer cell (BioRad). After protein transfer, membranes were incubated overnight at 4°C in Marvel (10% in TS buffer) to block any non-specific interactions. Primary antibodies were then suitably diluted (generally 1:1000) in TS buffer containing 10% Marvel and incubated with the membrane for 1.5-2 hours. Excess unbound antibody was removed by washing the membranes three times for 10 min in TS buffer, 0.1% Tween 20. Protein A coupled to horse radish peroxidase (Sigma), diluted 1:500 in TS buffer containing 10% Marvel was used routinely to detect bound primary antibody by incubating with the membrane for 1.5-2 hours. Excess protein A-peroxidase was removed by washing three times for 10 min in TS buffer, 0.1% Tween 20. The blot was then developed in the dark using the chemiluminescent substrate "Lumiglo" (New England BioLabs), and finally exposed to X-Omat autoradiographic film (Kodak).

2.16 Expression of *S. aureus* surface proteins in *L. lactis*

S. aureus surface proteins ClfA, SdrC, SdrD and SdrE and the *S. epidermidis* protein SdrG, were expressed in the heterologous host, *L. lactis* strain MG1363. The open reading frames of *clfA*, *sdrC*, *sdrD* and *sdrE* were amplified from *S. aureus* strain Newman chromosomal DNA using the following primer pairs; ClfAF4 and ClfAR4, SdrCF4 and SdrCR4, SdrDF4 and SdrDR4, SdrEF4 and SdrER4. Each of the forward and reverse primers contained an engineered *Bam*HI site at their 5' end, which was designed to make an in-frame fusion of each individual gene with a start codon (ATG), located

within the *Bcl*I site of the *L. lactis* plasmid, pKS80. The open reading frame of *sdrG* was amplified from chromosomal DNA of *S. epidermidis* strains K28 and HB using primer pair SdrGF4 and SdrGR4. In each case PCR mixtures were set up, as before, using 100 ng of chromosomal DNA template and 5 U *pfu* polymerase in a standard *pfu* reaction buffer, as described by the manufacturer. Extension times were varied according to the length of the amplified product, with 2 min extension allowed for every 1 kb of sequence to be amplified. PCR products were purified, using the Wizard PCR purification kit (Promega) and digested overnight with 20U *Bam*HI. The *L. lactis* plasmid, pKS80, was cut overnight with 10 U *Bcl*I and dephosphorylated using shrimp alkaline phosphatase, as described by the manufacturer (Roche). The cleaved PCR products were then ligated with pKS80 and concentrated by ethanol precipitation. DNA pellets were resuspended in 5 µl nuclease-free H_2O and mixed with 100µl *L. lactis* MG1363 cells, made competent by washing in 0.5 M Sucrose, 10% glycerol (Wells *et al.*, 1993). Cells were electroporated in a 2 cm gap cuvette (Flowgen) with the following parameters; 2.5 kV, 400 Ω, 25 µF. 960 µl recovery broth (M17, 0.5M sucrose, 2 mM $CaCl_2$, 20 mM $MgCl_2$) was immediately added and the cells were incubated on ice for 10 min. The mixture was then transferred to a microfuge tube and incubated for 2 hours at 30°C. The cells were harvested by centrifugation, resuspended in 100 µl recovery broth and plated on M17 agar containing 0.5% glucose and 5 µg/ml Em. Plates were incubated overnight at 30°C. Positive clones were identified by whole cell dot blot. Individual colonies were inoculated into M17 broth containing 0.5% glucose and 5 µg/ml Em and grown overnight statically at 30°C. 10 µl from each culture was transferred to a nitrocellulose membrane (Protran, Schleicher & Scheuell). The host strain containing pKS80 with no insert was included as a negative control. Membranes were allowed to dry and were then blocked for 2 hours in TS buffer containing 10% Marvel. Primary antibodies, specific to the corresponding *S. aureus* or *S. epidermidis* protein, were diluted to a suitable concentration in TS buffer containing 10% Marvel and incubated with the membrane for 2 hours. Membranes were washed three times in TS buffer to remove any unbound antibody. The membrane was then incubated with protein A-peroxidase for 2 hours and washed again, three times in TS buffer, to remove any unbound protein A-peroxidase present. Blots were detected, as described in section 2.15

2.17 Isolation of cell wall-associated proteins in *L. lactis*

Heterologously expressed proteins were isolated from the cell wall of *L. lactis* in a manner similar to that described for *S. aureus* in section 2.14, with the following differences. Cells were grown for 16 hours in 50 ml volumes of M17 broth containing 0.5% glucose and 5 μg/ml Em at 30°C with no shaking. Cells were harvested by centrifugation and washed twice with 5 mM EDTA and concentrated to an OD_{600nm} of 40 in 1 ml 26% raffinose (20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$). The following components were then added; 75 μl complete® mini protease inhibitor cocktail, 1 mM PMSF, 5 mM EDTA, 500 U mutanolysin (Sigma) and 200 μg lysozyme (Sigma). The remainder of the procedure was carried out as described in section 2.14. Proteins were analysed by Western immunoblotting as described in section 2.15.

2.18 N-terminal sequencing of *L lactis*-expressed ClfA and SdrE

Cell wall-associated proteins were isolated from *L.lactis* cells expressing (a) ClfA and (b) SdrE, as described in section 2.17. Following isolation, proteins were dialysed into 5 L PBS for 2 hours in order to remove the raffinose. Proteins were concentrated by adding 1:10 volumes of 100% trichloroacetic acid and incubating on ice for 30 min. Proteins were then harvested by centrifugation at 4°C and washed in acetone. Following a second centrifugation step, protein pellets were air-dried for 30 min and dissolved in 20 μl 1 x SDS-PAGE final sample buffer. Samples were separated on a 7.5% acrylamide gel and transferred onto PVDF membrane. The N-terminal sequences were determined by Edman degradation at the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge, U.K.

2.19 Adherence assays

2.19.1 Plate assays for bacterial adherence to fibrinogen, fibronectin, collagen, mucin and von Willebrand factor

The adherence of bacterial cells to several different imobilised matrix and plasma proteins was measured by an assay that had been developed previously (Wolz *et al.*, 1996), and modified in this laboratory (O. Hartford, personal communication). Human fibrinogen (Calbiochem), human fibronectin (Sigma), human collagen (Sigma), bovine mucin (Calbiochem) or human von Willebrand factor (Calbiochem) were diluted to a specific concentration in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3.2 mM NaN_3, pH 9.6), and 100 μl used to coat a 96-well flat-bottomed plate (Sarstedt or Maxisorb, Nunc) overnight at 4°C. Control wells contained carbonate buffer only. After washing three times in PBS, the plate was blocked for 1 hour at 37°C with 2% (w/v) bovine serum albumin (BSA, Sigma), that had been sterilized using a 0.45 μM Sartorius filter. Plates were washed again three times in PBS and 100 μl of a specific density of stationary or exponential phase cells was added. In most cases, an OD_{600nm} of 1.0 was used. However, in the case of *S. epidermidis*, an OD_{600nm} of 4.0 was used. After the addition of the cells, plates were incubated for 2 hours at 37°C. Wells were washed three times in PBS and adherent cells fixed by the addition of 100 μl 25% (v/v) formaldehyde. Plates were incubated at room temperature for 30 min with the formaldehyde and washed three times in PBS. 100 μl of crystal violet (0.5% w/v) was added to each well for one minute and the wells washed three times in PBS. For most cases, plates were read immediately by an ELISA reader at 570 nm. However, for some of the weaker interactions, a more sensitive method for detecting results was required. In these cases, crystal violet was released from adherent cells by the addition of 100 μl/well of 10% acetic acid. Plates were then read after 5 min by an ELISA plate reader at 570 nm.

Antibody and Ca^{2+} inhibition assays were also performed to show that reactions could be inhibited by specific antibodies and to determine if a given reaction was Ca^{2+} dependent. To study the inhibition of binding by a specific antibody, serial two-fold dilutions of the antibody were made, with a starting concentration of 300 μg/ml-500

μg/ml, depending on the antibody used. A fixed number of cells was incubated with the different antibody dilutions for 1 hour at 37°C and 100 μl was added to the wells of a plate that had been coated and blocked as described above. The remainder of the assay was performed as described above. The effect of Ca^{2+} ions on ligand binding was also studied. A fixed number of cells were incubated for 1 hour at 37°C with different concentrations of $CaCl_2$, ranging from 0-100 mM and then added to the wells of a plate that had been coated and blocked as described above. Wells were washed with saline, rather than PBS, as PBS causes precipitation with $CaCl_2$. The rest of the assay was carried out as described above.

2.19.2 Adherence of cells to immobilised elastin peptides.

Adherence of cells to immobilised elastin was measured in a fluorometric assay developed in this laboratory (F. Roche, personal communication). This assay works on the following basis. Bacterial cells are fluorescently labeled using a fluorescent probe (SYTO 13, Molecular Probes) that can permeate cells and bind specifically to nucleic acid. This results in the production of a fluorescent signal by labeled cells, allowing cells that have adhered to an immobilised substrate coated on a mulit-well plate to be detected in a luminescence spectrometer.

Serial dilutions of kappa-elastin (Elastin Product Company) ranging from 50 μg/well to 3.125 μg/well in 0.1 M sodium bicarbonate buffer pH8.0, were made in a 96-well flat bottomed plate (Porvair). The κ-elastin peptides were coated onto the plate surface by incubation at room temperature under UV light (366 nm) for 18 hours (Hinek *et al.*, 1999). Plates were then washed three times in 10 mM Tris-HCl pH 7.5 and blocked for 2 hours at room temperature in 5% BSA that had been sterilised using a 0.45 μM Sartorious filter. Stationary phase cells were washed in 10 mM Tris pH 7.5 and adjusted to an OD_{600nm} of 1.0. Cells were then labeled in the dark with a nucleic acid binding probe (SYTO 13, Molecular Probes), as recommended by the manufacturer, and

100 μl added to the wells. Plates were inubated for 2 hours at 37°C in the dark and then washed three times in 10mM Tris pH 7.5. Results were read in a LS-50B Luminescence spectrometer, with an excitation wavelength of 509 nm and emission wavelength of 488 nm.

2.19.3 Clumping assays

Clumping assays were performed in 96-well flat-bottomed plates (Sarstedt) using 50 μl volumes of human fibrinogen (Calbiochem) which was serially diluted 2-fold in PBS from a starting concentration of 1 mg/ml. Cells were prepared by washing once in PBS and adjusting to an OD_{600nm} of 6. 20 μl of cells were added to each well and the plates agitated briskly for 5 min. Results were read visually, where the clumping titre was the lowest dilution of fibrinogen at which clumping still occurred.

2.20 Ligand affinity blotting

The interaction between *L. lactis* cells expressing SdrC, SdrD and SdrE and human vitronectin was measured by ligand affinity blotting. 100 μg of human vitronectin (Calbiochem) was biotinylated as recommended by the manufacturer, using the EZ™-Link Sulfo-NHS-LC-Biotinylation kit (Pierce). Incorporation of the biotin was measured using the avidin-HABA reagent provided. Cell wall-associated proteins were isolated as described in section 2.17. Protoplasts were also recovered and were boiled in SDS-PAGE final sample buffer in order to induce lysis and release of membrane and cytoplasmic components. Cell wall and protoplast fractions were separated on a 10% polyacrylamide gel and transferred to a PVDF membrane as described in section 2.15. The membrane was blocked overnight at 4°C in 5% BSA. Biotinylated vitronectin was diluted 1:500 in 5% BSA and incubated with the membrane for 2 hours at room temperature. The membrane was then washed three times for 10 min in TS buffer, 0.1% Tween 20 in order to remove any unbound vitronectin. Strepavidin-HRP (Roche) was diluted 1:2000, according to the manufacturer's instructions, and incubated with the membrane for 2 hours at room temperature. Unbound strepavidin was removed by

washing three times for 10 min in TS buffer, 0.1% Tween 20. Blots were developed and exposed to autoradiographic film as described in section 2.15.

2.21 Activation of platelet aggregation.

Human blood was recovered in 3.8% sodium citrate and centrifuged at 700 rpm to remove erythrocytes. The platelet rich plasma (PRP), which remained in the supernatant, was recovered. Tubes were centrifuged again at 3000 rpm and the supernatant, which contained platelet poor plasma (PPP), was recovered. Both exponential and stationary phase *S. aureus* and *L. lactis* cells were washed once in PBS and adjusted to an OD_{600nm} of 1.6. Aggregations were measured in a platelet aggregometer at 37°C. The aggregometer was first blanked using PPP. 450 μl PRP was added to a siliconised 1 ml tube and placed into the aggregometer. 50 μl of bacterial cells were then added and the reaction allowed to proceed. If aggregation had not occurred in < 25 min, then the result was considered negative.

Chapter 3

Construction of Mutations in the *sdrC*, *sdrD* and *sdrE* genes of *Staphylococcus aureus*

3.1 Introduction

Modern molecular genetic analysis has led to a greater understanding of microbial pathogenicity and virulence. While the genetics of *Bacillus subtilis* and *Streptococcus pneumoniae* are arguably well advanced, progress in the molecular analysis of other Gram positive pathogens such as *Staphylococcus aureus* has been slower than that of Gram negative organisms such as *Escherischia coli* and *Salmonella typhimurium*. This is due mainly to the difficulties encountered in the genetic manipulation of Gram positive bacteria. However, this situation has begun to change and recent years have seen rapid advances in the dissection of the pathogenicity of *S. aureus*. These advances owe much to the development of techniques for generating mutations in *S. aureus*.

In order to study the phenotypic effects of a particular bacterial protein and to investigate its role in virulence, it is necessary to construct a strain that is defective in the corresponding gene. Mutations in many different putative surface protein virulence factors have been constructed in *S. aureus* (Table 3.1). Testing the virulence of specific isogenic mutants with respect to the corresponding wild type strain in animal models has shown that certain staphylococcal proteins are major virulence factors in animal models of infection, while others are not. Specifically, comparing mutant and wild type strains, Patti *et al.* were able to show that the staphylococcal collagen adhesin is a major virulence factor in experimental septic arthritis (Patti *et al.* 1994). In contrast, using similar methods, Phonimdaeng *et al.* (1990) showed that staphylococcal coagulase is not a major virulence factor in the mouse mastitis model of staphylococcal infection.

Several methods for the construction of isogenic mutations in *S. aureus* are available. (1) Transposon mutagenesis has been used to isolate insertion mutations in chromosomal genes encoding virulence factors and other chromosomal genes (Foster, 1998). Transposons carried on temperature sensitive plasmids, allow the selection of transposon insertions into the chromosome at the restrictive temperature. Both the closely related class II elements, Tn*551* and Tn*917*, and the closely related conjugative transposons Tn*916* and Tn*918* have been used successfully to generate mutations in *S. aureus* and *S. epidermidis* (Novick *et al.*, 1979; Clewell *et al.*, 1985; Lee *et al.*, 1987; Heilman *et al.*, 1996a). Success depends on a sufficiently high transposition frequency

Table 3.1 Mutations in surface protein genes of *S. aureus*

Gene	Protein	Reference
spa	Protein A	Patel *et al.*, 1987
clfA	Clumping factor A (ClfA)	McDevitt *et al.*, 1994
clfB	Clumpling factor B (ClfB)	Ní Eidhin *et al.*, 1998
cna	Collagen binding protein (Cna)	Patti *et al.*, 1992
fnbA	Fibronectin binding protein A (FnBPA)	Greene *et al.*, 1995
fnbB	Fibronectin binding protein B (FnBPB)	Greene *et al.*, 1995
ebpS	Elastin binding protein (EbpS)	Roche, 2001
sdrC	SdrC (function unknown)	This study
sdrD	SdrD (function unknown)	This study
sdrE	SdrE (Function unknown)	This study

and upon the randomness with which the transposon inserts into the target genome. Although insertions with transposons Tn*551*, Tn*917* and Tn*916* have been isolated and mapped at different sites in the *S. aureus* chromosome (Pattee, 1981; Jones *et al.*, 1987; Yost *et al.*, 1988; Pattee *et al.*, 1990), it appears that not all transposons insert entirely at random and that hot-spots for insertion occur. Therefore, it is often necessary to carry out several rounds of enrichment in order to isolate the required mutation (McDevitt *et al.*, 1994). One of the main advantages of transposon mutagenesis is that sequence knowledge of the gene is not required. A simple phenotypic screen is all that is required to select specific mutations.

(2) Allele replacement by recombination allows a genetically altered cloned copy of a gene to be introduced into the corresponding chromosomal allele by homologous recombination. While allele replacement is a more precise method than transposon mutagenesis, it requires that the target gene has been cloned and preferably sequenced. Typically, the target gene is cloned on a multicopy plasmid in *E. coli* and inactivated by insertion of a large DNA fragment (usually a drug resistance determinant) into a specific restriction site in the coding sequence. These insertions can be accompanied by a deletion in the coding sequence to form substitution mutations. The genetically modified copy of the gene is subsequently integrated into the chromosome by homologous recombination. A number of different drug resistance markers have been used successfully to generate mutations in *S. aureus*. These include erythromycin resistance (*ermC*; O' Reilly *et al.*, 1986; Phonimdeang *et al.*, 1990), tetracycline resistance (*tetK*; O' Connell *et al.*, 1993; Greene *et al.*, 1995; Ní Eidhin *et al.*, 1998), tetracycline-minocycline resistance (*tetM*; Sloane *et al.*, 1991; Novick *et al.*, 1993), kanamycin-neomycin resistance (Patel *et al.*, 1989), gentamicin-kanamycin resistance (Patti *et al.*, 1994) and ethidium bromide resistance (Patel *et al.*, 1987). It is also possible to use allele replacement to return unmarked mutations to the chromosome. Point mutations have been introduced into the chromosome of several Gram-positive pathogens by plasmid integration and excision. These include *S. aureus* (Ouyang *et al.*, 1999) *Listeria monocytogenes* (Michel *et al.*, 1990; Guzman *et al.*, 1995) and *Lactococcus lactis* (Biswas *et al.*, 1993).

Several different plasmid systems have been used for allele replacement. Plasmid incompatibility was employed to generate mutations in the alpha toxin gene (O' Reilly *et al.*, 1986), the coagulase gene (Phonimdaeng *et al.*, 1990) and the protein A gene of *S. aureus* (Patel *et al.*, 1987). This was the first method described for generating allele replacement mutants in *S. aureus*. It utilized the phenomenon of plasmid incompatibility to eliminate a shuttle plasmid carrying a mutated copy of a gene, allowing subsequent selection for allelic exchange between mutated and wild type copies of the target gene.

Temperature-sensitive plasmids are also used for allele replacement. The plasmid carrying the genetically altered cloned copy of the target gene is first propagated at the permissive temperature (usually 30°C) and subsequently grown at the restrictive temperature (usually 42°C). This allows for selection of cells where the plasmid has integrated into the chromosome at the site of shared homology. Cells are then grown once more at the permissive temperature, which activates plasmid rolling circle replication generating single stranded DNA. This is thought to stimulate recombination and plasmid excision. Following several temperature shifts, the plasmid becomes eliminated from the bacterial population, allowing one to identify recombinants (Figure 3.1). Three different temperature sensitive plasmids have been used to generate allele replacement mutants in *S. aureus*. pTS1 and pTS2 (derived from pE194 *ts:* Gryczan *et al.*, 1982) were used to generate mutants in the *fnb* genes (Greene *et al.*, 1995), the *clfB* gene (Ní, Eidhin *et al.*, 1998) and the *sdrC* and *sdrD* genes (this study) of *S. aureus*. pRN8103, a derivative of pT181 (Novick *et al.*, 1986), has also been used in *S. aureus*, while the broad host range pG+host vectors (Maguin *et al*, 1992, 1996) have been used with limited success (Supersac *et al.*, 1997).

Suicide plasmids can be used to generate marked mutations in *S. aureus*. Strain RN4220 is electrotransformed with a plasmid construct that is lacking a replicon for *S. aureus*. The only way that the marker can survive is by inserting into the chromosome at the site of shared homology (i.e. at the target gene). Most integrants are single cross-overs. However, double cross-overs may also be isolated at a lower frequency (McDevitt *et al.*, 1993). Approximately 1:50-1:100 integrants are double cross-overs. The mutation can then be moved to related *S. aureus* strains by generalised transduction with bacteriophages ϕ11, $\phi 80\alpha$ or ϕ85. Mutations in the coagulase gene (McDevitt *et al.*,

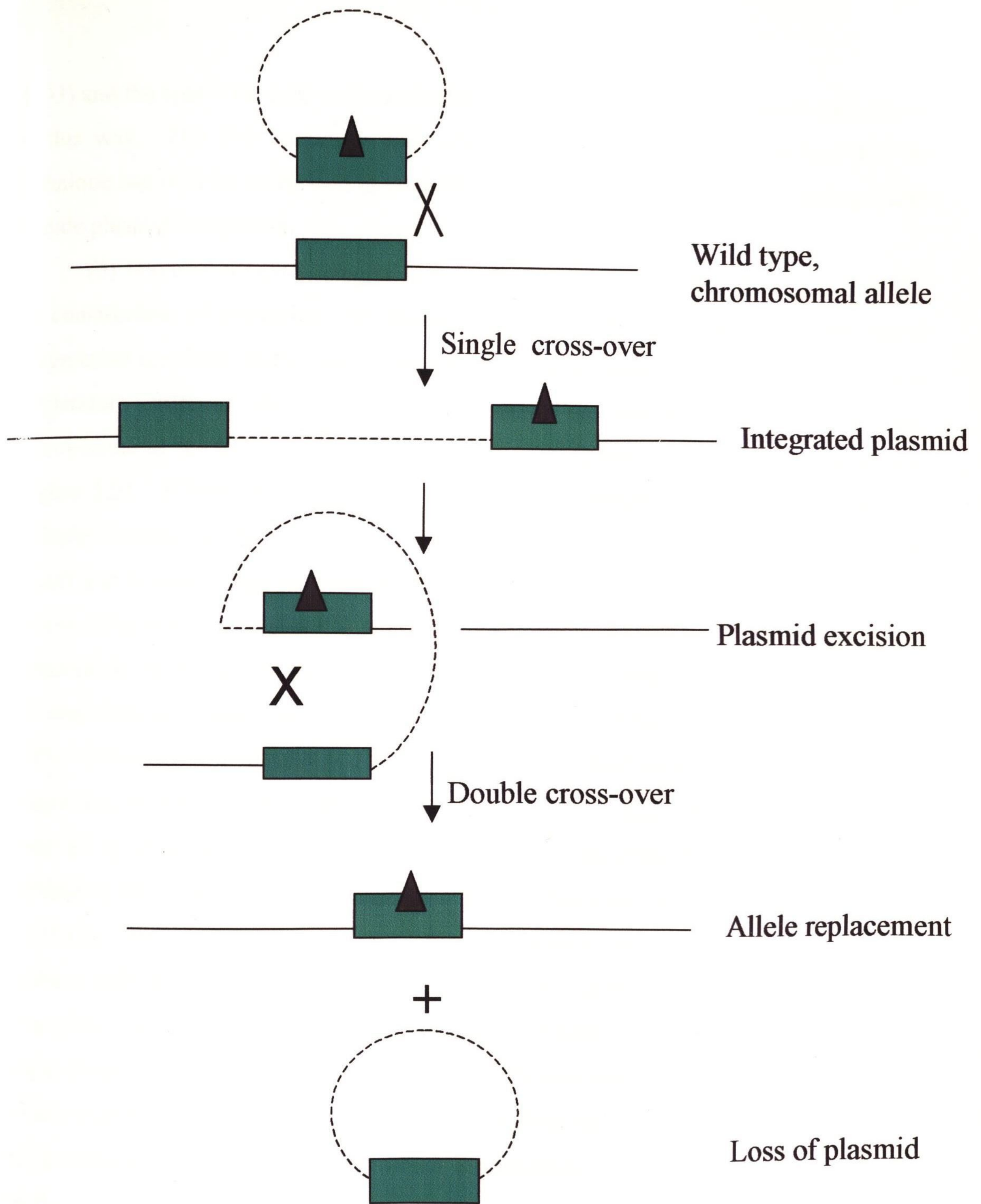


Figure 3.1 Schematic representation of plasmid integration and excision. The plasmid integrates into the chromosome by a single cross-over event on the right of the mutation carried by the plasmid. Subsequent excision by a recombination event on the oppsite side of the chromosomal locus results in the exchange of mutant and wild type alleles.

1993) and the type 5 capsular polysaccharide operon (Sau *et al.*, 1997) have been isolated in this way. The low frequency with which integration occurs and the fact that the technique can only be performed in strain RN4220 are two disadvantages associated with suicide plasmid integration.

(3) Directed plasmid integration provides a rapid and more versatile method for the construction of mutations. An internal fragment of the target gene is cloned into a temperature sensitive vector and propagated at the permissive temperature. Following a temperature shift to the restrictive temperature, the plasmid integrates into the chromosome at the site of the target gene, disrupting it and thus generating a mutation (Figure 3.2). pG+host9 is a streamlined temperature sensitive shuttle plasmid with a multiple cloning site (MCS), a single (defective) replication system that functions both in *E. coli* and a broad range of Gram positive hosts, and with a single antibiotic resistance marker (Em) that is expressed in both types of bacteria (Figure 3.3). Its use for allele replacement in *S. aureus* has been limited due to the difficulty to promote plasmid excision following single cross-over integration into the chromosome (Supersac *et al.*, 1997). However, this phenomenon makes pG+host an ideal vector for directed plasmid integration, as single cross-over integrants can be grown stably at 37°C. It has been observed by other workers and in this study, that plasmids often form tandem dimers or multimeric arrays when they integrate by a single cross-over in *S. aureus* (McDevitt *et al.*, 1993). Differentiation of a single cross-over integrant from an amplified integrant by Southern hybridisation is straightforward. In principle, if the genomic DNA is cut with an enzyme that cleaves once within the plasmid backbone and not in the gene itself, the single cross-over integrant gives two bands of different size from that of the wild type (which is missing). An amplified integrant gives three bands, the two bands seen in the single cross-over, plus a third band which corresponds to the size of the plasmid and insert.

A number of different methods are used for transferring plasmids between strains in *S. aureus*. Protoplast transformation (O'Reilly and Foster, 1988) is a rather laborious method that is now seldom used in *S. aureus*. However, it is still used for transformation of other staphylococcal species such as *S. epidermidis* and *S. carnosus*, which are not easily electrotransformed (Heilmann *et. al*, 1996a; 1996b). Electrotransformation is the

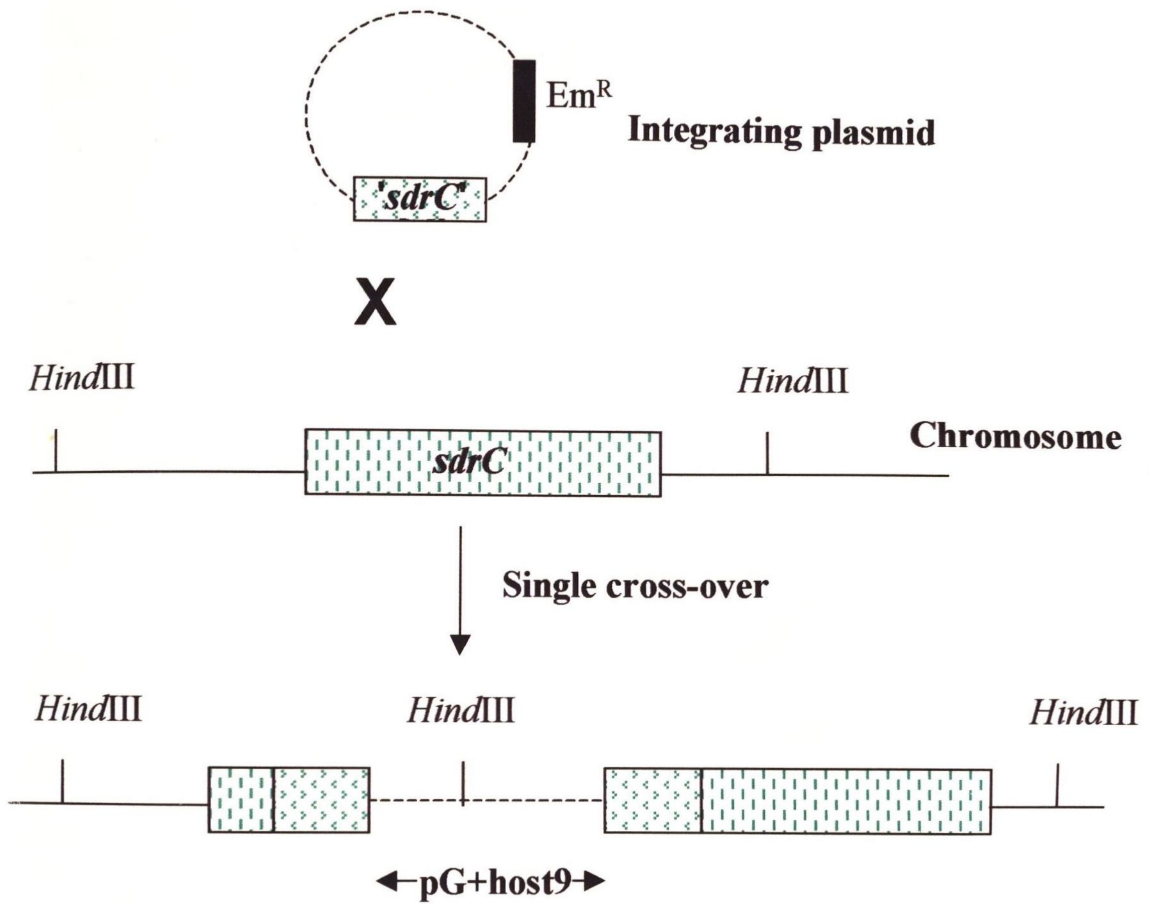
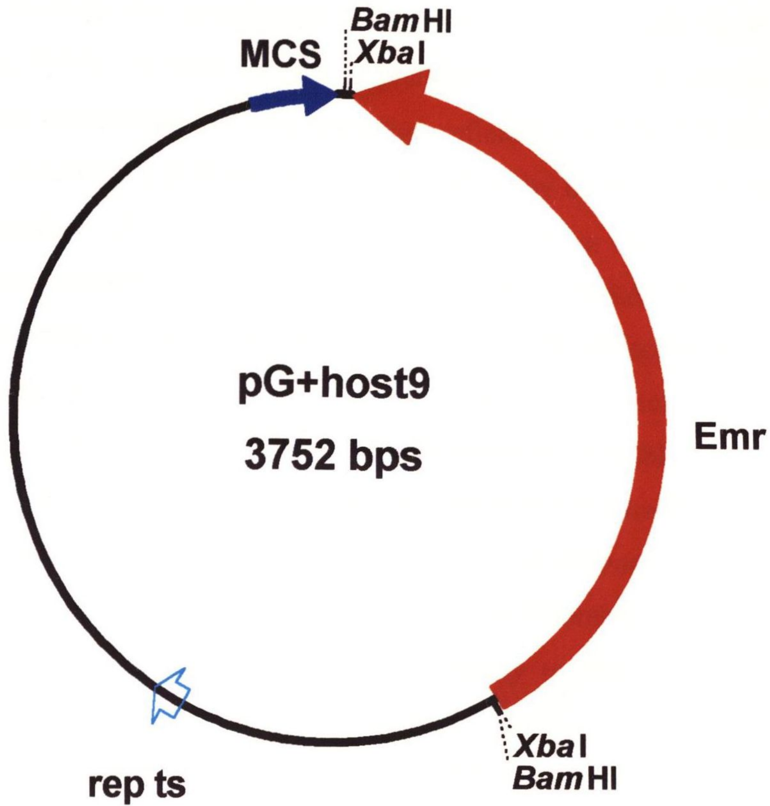


Figure 3.2 Disruption of the *sdrC* gene by directed plasmid integration using the temperature sensitive shuttle vector, pG+host9. The integrating plasmid carries an internal fragment from the unique A region of *sdrC*. A single cross-over event results in the integration of the plasmid and disruption of the *sdrC* gene. Once integrated, the plasmid is flanked by a direct duplication of the *sdrC* gene fragment it carried.



MCS

BstXI, SacII, NotI, XmaI, XbaI, SpeI, BamHI, SmaI, PstI, EcoRI,
EcoRV,HindIII, ClaI, AccI, SalI, XhoI, ApaI,m DraI, KpnI

Figure 3.3 Map of the temperature sensitive shuttle plasmid pG+host9, showing multiple cloning site (MCS), temperature sensitive replication system (rep ts) and erythromycin resistance determinant (Emr). pG+host9 is a broad host range lactococcal plasmid derived from pVE6004.

most widely used method for introducing recombinant plasmids into staphylococci (Augustin and Gotz, 1990). In most cases it is preferable to protoplast transformation. Plasmids must first be transformed into the restriction-deficient host RN4220 (Kreiswirth *et al.*, 1983). Once established in RN4220, plasmids can easily be transferred into other strains by electroporation or transduction. Transduction is an extremely efficient mechanism for moving recombinant plasmids and chromosomal markers between strains of *S. aureus.* The transducing phage packages up to 40 kb of chromosomal and plasmid DNA and can transfer it to another strain at high frequency (Novick *et al.*, 1986). Recombinant plasmids are often transduced at higher frequencies, possibly due to the fact that multimers of >40 kb are the substrate for the transducing phage (Novick *et al.*, 1981). When chromosomal mutations are transduced, they can be maintained in the recipient strain only if a double cross-over event occurs, whereby the incoming gene replaces the resident allele. Conjugational mobilisation is used mainly to transfer plasmids into strains of *S. epidermidis* (Mack *et al.*, 1994).

The open reading frames of *sdrC*, *sdrD* and *sdrE* are closely linked in tandem array in most strains of *S. aureus* (Figure 3.4). However, Southern hybridisation analysis revealed that some *S. aureus* strains carry only two *sdr* genes. Strains 8325-4 and RN4220 do not carry a copy of the *sdrE* gene, while strain Phillips does not contain *sdrC* (Josefsson *et al.*, 1998a). Also, strain EMRSA-16 does not have a copy of the *sdrD* gene (this study, data not shown).

In this study, single isogenic mutants were generated in the *sdrC* and *sdrD* genes of several *S. aureus* strains by directed plasmid integration, using pG+host9. Attempts to generate a mutation in the *sdrE* gene by this method were unsuccessful. A double mutant in both the *sdrC* and *sdrD* genes (*sdrCD* mutation) of strain 8325-4 was constructed by allele replacement. This mutation was subsequently transduced into strain Newman, resulting in the deletion of the *sdrE* gene from strain Newman and the generation of a triple mutation in the *sdrC, sdrD* and *sdrE* genes (*sdrCDE* mutation). These mutations, where appropriate, were complemented with either the *sdrC*, *sdrD* or *sdrE* gene, together with approximately 300 b.p. of upstream sequence and 200 b.p. of downstream sequence, on the multicopy plasmid pCU1. These constructs were made to demonstrate that the effects of each of the mutations could be reversed by supplying the intact structural gene

Newman

Phillips

8325-4

EMRSA-16

Figure 3.4 Distribution of *sdr* genes amongst different strains of *S. aureus*. In strain Newman, the three *sdr* genes are closely linked in tandem array. Strain Phillips does not have a copy of *sdrC*, strain 8325-4 does not have a copy of the *sdrE* gene, while strain EMRSA-16 does not carry a copy of the *sdrD* gene.

in *trans*, and that any effects of the mutations were due to the loss of the structural gene, and not to downstream effects resulting from its interuption.

3.2 Results

3.2.1 Construction of single mutations in the *sdrC* gene by directed plasmid integration.

Single mutants in *sdrC* were constructed by directed plasmid integration in two different strains of *S. aureus* (Newman and 8325-4), as described in section 2.5.1 by cloning an internal fragment of *sdrC* into pG+host9 and directing integration of the plasmid construct into the chromosome at the *sdrC* locus. In strain Newman (the strain from which the internal fragment of *sdrC* was cloned), following a temperature shift to the restrictive temperature, the efficiency of plating (eop) was 1.2 x 10^{-3}. For the non-homologous strain, 8325-4, the eop was 1.5 x 10^{-4}. Chromosomal DNA was isolated from several different mutant colonies in both of these strains and analysed by Southern hybridisation (Figure 3.5, 3.6). In both cases, DNA was digested with *Hind*III and probed with a DIG-labeled probe directed towards the unique A region of *sdrC*. The probe was amplified from pQE30:'*sdrC*'using primer pair sdrCF1 and sdrCR1 (Table 2.3). Figure 3.5 shows a Southern blot of *Hind*III digested DNA from four independently isolated putative mutants of strain Newman. *Hind*III cut outside the *sdrC* locus, resulting in a single band of 3.8 kb in the wild type (lane 5). Because *Hind*III cut once within the pG+host backbone and outside the *sdrC* gene, one would expect to observe two bands where a single cross-over had occurred and three bands where a single cross-over with tandem duplication of the plasmid had occurred. In lanes 1 and 4, two bands of 12 kb and 1.5 kb were observed. These corresponded to single cross-over insertions in the *sdrC* gene. Lanes 2 and 3 showed the same 12 kb and 1.5 kb bands, with an additional band of 4.7 kb. This additional band correspondsed to the size of the pG+host backbone plus the *sdrC* insert and is presumed to represent amplification of the plasmid within the chromosome. A similar pattern was observed for the four putative 8325-4 *sdrC* mutants that were tested (Figure 3.6). *Hind*III cut DNA was analysed by Southern hybridisation.

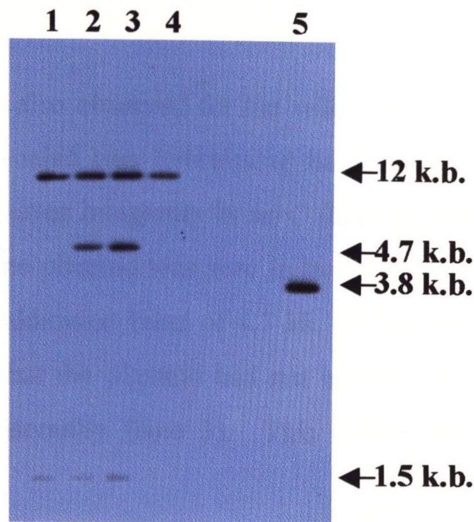


Figure 3.5 Southern blot of Newman *sdrC* mutants generated by directed plasmid integration. Lanes 1 & 4; single cross-over with no amplification. Lanes 2 & 3; single cross-over with tandem duplication of the plasmid, Lane 5; Newman wild type. Chromosomal DNA (5µg) was cut with 20U *Hind*III and the blot probed with a DIG-labelled probe, specific to the A region of *sdrC*.

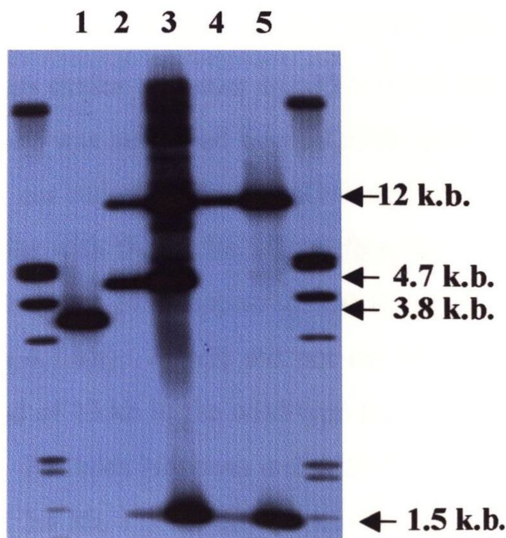


Figure 3.6 Southern blot of 8325-4 *sdrC* mutants generated by directed plasmid integration. Lane 1; 8325-4 wild type. Lane 2; single cross-over with tandem duplication of the plasmid. Lane 3; non-integrated plasmid, replicating extrachromosomally. Lanes 4 &5; single cross-over integration with no amplification of the plasmid. Chromosomal DNA (5µg) was cut with 20U *Hind*III and the blot probed with a DIG-labelled probe, specific to the A region of *sdrC*.

A single band of 3.8 kb was also observed for the wild type in 8325-4 (Figure 3.6, lane 1). Two of the isolates revealed two hybridising bands of 12 kb and 1.5 kb, which corresponded to single cross-over integrants in *sdrC* (lanes 4 and 5). Single cross-over with tandem duplication of the plasmid was seen in lane 2, which showed the 12 kb and 1.5 kb bands as well as an additional band of 4.7 kb. In the case of one of the 8325-4 integrants, it was observed that the plasmid had not inserted into the chromosome and was replicating extrachromosomally (lane 3). This isolate was not used for further studies.

3.2.2 Construction of single mutants in the *sdrD* gene by directed plasmid integration.

Single *sdrD* mutants were also constructed in *S. aureus* strains Newman and 8325-4 by directed plasmid integration, as described in section 2.5.2, by cloning an internal fragment from the unique A region of *sdrD* into pG+host9, and integrating the construct into the *sdrD* locus in the *S. aureus* chromosome. The eop for strain Newman was 7.5×10^{-4}, while the eop for strain 8325-4 was 1.1×10^{-4}. DNA was isolated from two putative mutants each from strains Newman and 8325-4 and digested overnight with *Hinc*II. The DIG-labeled probe was amplified from pQE30:'*sdrD*' using the primer pair sdrDF1 and sdrDR1. *Hinc*II cut twice within the pG+host9 vector, releasing a 550 bp fragment that did not hybridise with the probe. Therefore, the amplified band in both Newman and 8325-4 *sdrD* Southern blots appeared 550 bp smaller than the calculated value (i.e. 4.2 kb rather than 4.7 kb). *Hinc*II did not cut within the *sdrD* gene itself, thereby releasing a single band of 13 kb in the wild type for strains Newman and 8325-4 (Figures 3.7 and 3.8, lane 1). For both Newman and 8325-4, three bands were observed (lanes 2 and 3), with sizes of 3.8 kb, 1.3 kb and 4.2 kb for the putative mutant isolates. As the wild type band is not present, these isolates were thought to represent single cross-over integrants where tandem duplication of the plasmid had occurred. The 4.2 kb band was presumed to represent the amplified plasmid.

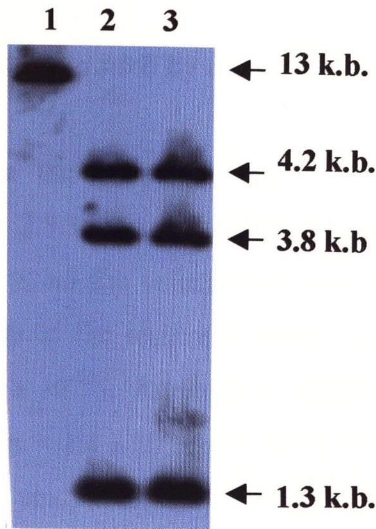


Figure 3.7 Southern blot of Newman *sdrD* mutants generated by directed plasmid integration. Lane 1; Newman wild type. Lanes 2 & 3; single cross-over with tandem duplication of the plasmid. Chromosomal DNA (5µg) was cut with 20U *Hinc*II and the blot probed with a DIG-labelled probe specific to the A region of *sdrD*.

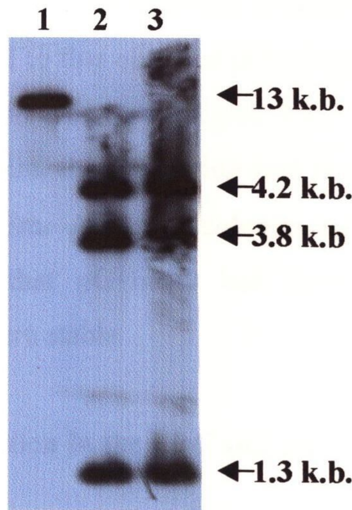


Figure 3.8 Southern blot of 8325-4 *sdrD* mutants generated by directed plasmid integration. Lane 1; 8325-4 wild type, lane 2 & 3, single cross-over with tandem duplication of the plasmid. Chromosomal DNA (5µg) was cut overnight with 20U *Hinc*II and the blot probed with a DIG-labelled probe specific to the A region of *sdrD*.

3.2.3. Construction of a single mutant in *sdrE* by directed plasmid integration.

An internal fragment from the unique A region of *sdrE* was cloned into pG+host9, as described in section 2.5.3, and the construct was transduced into strain Newman. Despite repeated attempts, no Em resistant colonies were obtained following plating the plasmid-containing cells at the restrictive temperature (42°C), even at the lowest dilutions plated (e.o.p. <5.4×10^{-10}). Attempts to isolate mutants in strains COL and P1 were also unsuccessful (e.o.p. < 1.4×10^{-9} and < 8.9×10^{-10}, respectively). DNA sequencing, PCR and Southern blot analysis of the cloned *sdrE* fragment confirmed that it was correct (data not shown).

3.2.4. Investigation of the stability of the pG+host9 integrants.

To determine if the pG+host9 integration mutants were stable following repeated growth at 37°C in the absence of selective antibiotic, four independently isolated 8325-4 *sdrC* mutant colonies were grown in TSB for approximately 70 generations in the absence of Em, and analysed by Southern hybridisation, as before. Results showed that the Southern blot pattern was identical to that obtained prior to growth in the absence of antibiotic, with no evidence of the wild type band (Figure 3.9). Also, following growth for 70 generations in the absence of Em, the population was plated on TSA and replica plated onto TSA containing 10μg/ml Em. All of the colonies tested (>5000) were found to be resistant to Em, indicating that pG+host9 had remained integrated in the chromosome and that the mutations were stable.

3.2.5. Construction of a double mutation in the *sdrC* and *sdrD* genes of 8325-4.

A double mutation in the *sdrC* and *sdrD* genes of strain 8325-4 was constructed by allele replacement, as described in section 2.6. A large 2178 bp deletion in the *sdr* locus was made by ligating together two PCR fragments from the unique A regions of *sdrC* (814 bp) and *sdrD* (916 bp), and cloning in the correct orientation into the *E. coli* plasmid, pBluescript KS+. The *tetK* tetracycline (Tet) resistance determinant was

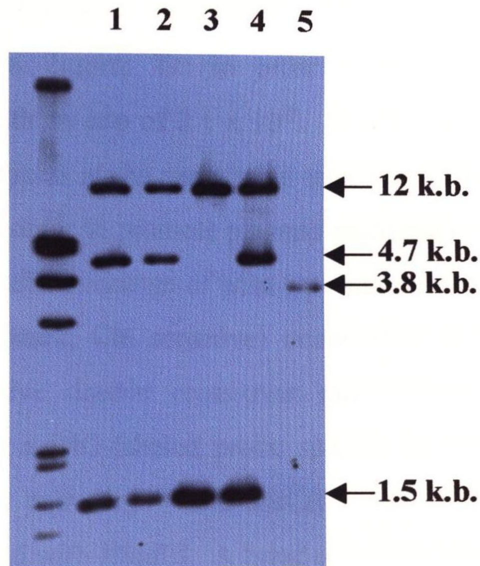


Figure 3.9 Southern blot of stability test of 8325-4 *sdrC* mutants. Mutants were grown for 70 generations in the absence of selective antibiotic and then analysed by Southern blotting for plasmid rearrangements. Lanes 1, 2 &4; 8325-4 *sdrC* mutants with tandem duplication. Lane 3; 8235-4 *sdrC* mutant with no amplification. Lane 5; 8325-4 wild type.

inserted into the unique *Hind*III site that separated the *sdrC* and *sdrD* gene fragments. This construct was converted to a temperature sensitive (*ts*) shuttle plasmid by ligation with the *S. aureus ts* plasmid pTS2, which encodes choramphenicol (Cm) resistance, and transferred to *S. aureus*. Following a temperature shift to the restrictive temperature (42°C), cells were plated on 2μg/ml Tet in order to select for single cross-over integration. This occurred with an eop of 2.1 x 10^{-6}. Single cross-over integrants were then grown through several cycles 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 (Figure 3.10). Double cross-overs (Tet resistant, Cm sensitive) occurred at a rate of 1:500. Four independently isolated putative double cross-over recombinants were analysed by Southern hybridisation, using a DIG-labeled probe specific to the unique A region of *sdrC* (amplified using primer pair sdrCF1 and sdrCR1, section 2.5.1). Chromosomal DNA was isolated and digested with *Hind*III. A band of 3.8 kb was observed in the wild type (Figure 3.11, lane 5). A smaller band of 1.7 kb was expected if a double cross-over had occurred and this was observed in lanes 1-3. However, one of the isolates tested had not undergone a double cross-over (lane 4), and is presumed to represent a single cross-over integrant. This isolate was not used for further studies.

3.2.6. Construction of a triple mutation in the *sdrC*, *sdrD* and *sdrE* genes of strain Newman.

Having isolated a double mutation in the *sdrC* and *sdrD* genes of strain 8325-4, this strain was used to generate a triple mutation in the *sdrC*, *sdrD* and *sdrE* genes in strain Newman by transduction, as described in section 2.7. Because 8325-4 does not carry a copy of the *sdrE* gene and considering that the three *sdr* genes are closely linked in strain Newman, it was possible to introduce the *sdrCD* double mutation from 8325-4 into Newman, while deleting the *sdrE* gene, and thus generating a triple mutation (Figure 3.12). Transductants were analysed by Southern hybridisation using probes specific to *sdrC* region A and *sdrE* region A. Probes were DIG-labeled using sdrCF1/sdrCR1 and sdrEF1/sdrER1 primer pairs (Table 2.3) with pQE30:'*sdrC*' and pQE30:'*sdrE*' as

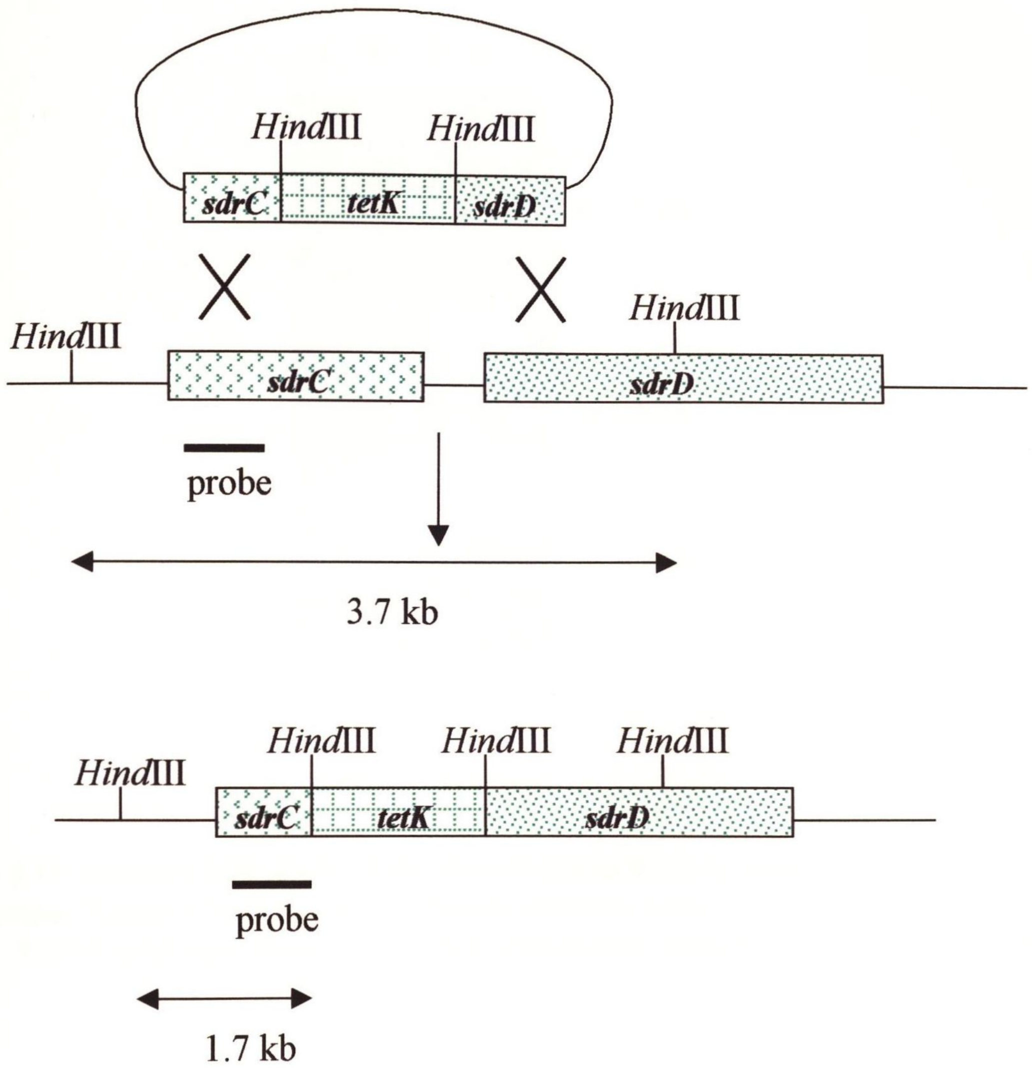


Figure 3.10 Schematic representation of the generation of a double mutant in the *sdrC* and *sdrD* genes of strain 8325-4 by allele replacement. An 814 bp 5' fragment of the *sdrC* gene was ligated together with a 916 bp 5' fragment of the *sdrD* gene, separated by the *tetK* drug resistance determinant. This construct was introduced into strain 8325-4. A double cross-over resulted in homologous recombination brtween the 5' ends of the *sdrC* genes (cloned and chromosomal alleles) and between the 5' ends of the *sdrD* genes. This allows for insertion of the mutant casette into the chromosome and deletion of the 3' end of the *sdrC* gene and the 5' end of the *sdrD* gene. This resulted in the generation of mutations in both the *sdrC* and *sdrD* genes.

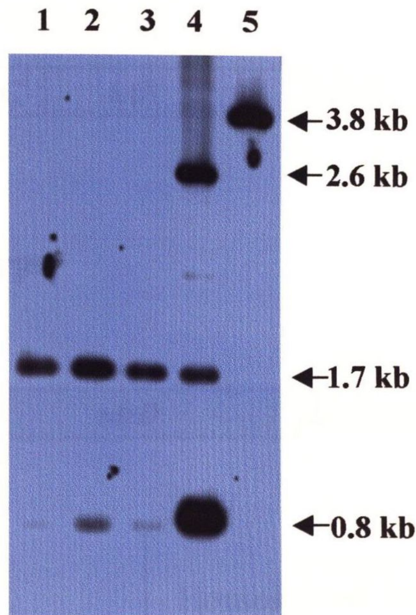


Figure 3.11 Southern blot of *sdrCD* double mutants in 8325-4, generated by allele replacement. Lanes 1-3; 8325-4 *sdrCD* double mutants. Lane 4; single cross over. Lane 5; 8325-4 wild type. Blots were probed with an *sdrC* region A DIG-labelled probe

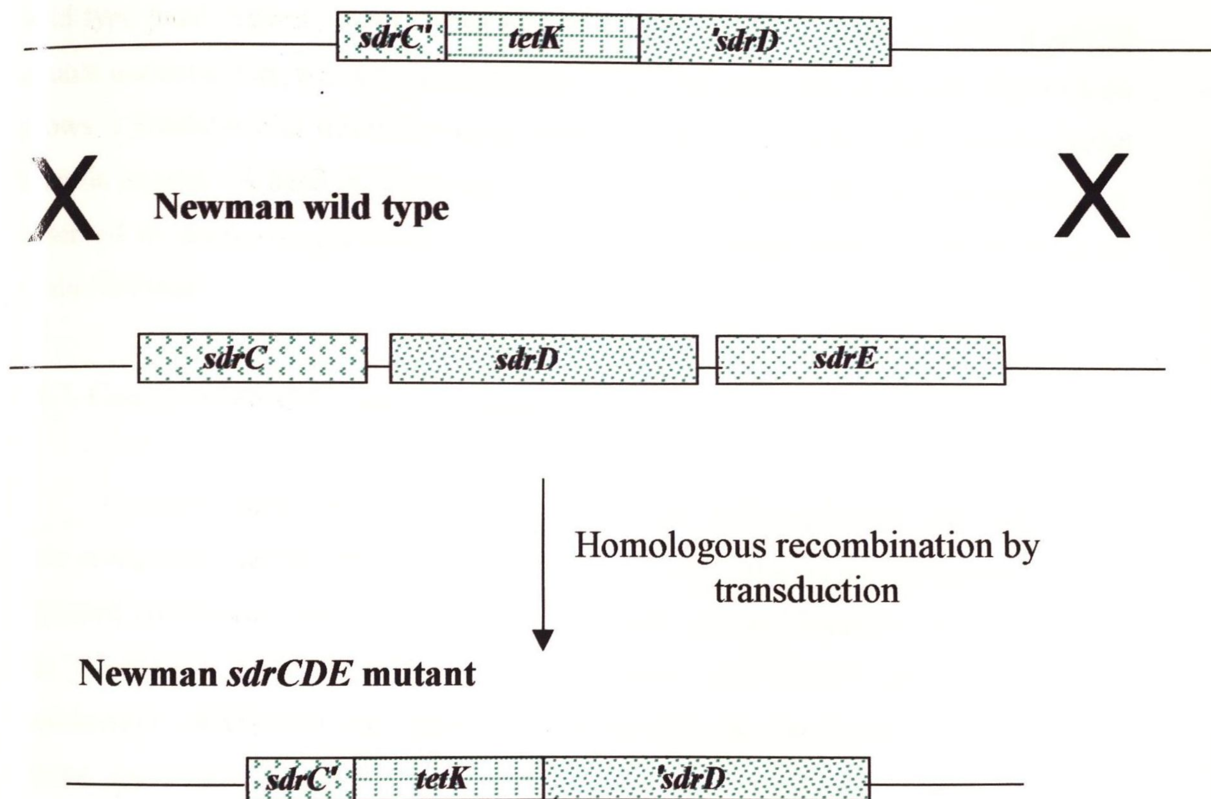


Figure 3.12 Schematic representation of the generation of a triple mutation in strain Newman by transduction from an *sdrCD* double mutation in strain 8325-4. A transducing phage was propagated on 8325-4 containing a double mutation in the *sdrC* and *sdrD* genes. This lysate was then used to infect Newman wild type cells. A double cross-over homologous recombination event occurred at sites upstream and downstream of the *sdr* locus. This resulted in allelic exchange between the mutant 8325-4 *sdr* locus and the wild type Newman *sdr* locus, with deletion of the *sdrE* gene from strain Newman

templates, respectively. DNA was isolated from three separate transductants and digested with *Hind*III. Figure 3.13 shows a Southern blot using the *sdrC* probe. Lane 4 showed a wild type band of 3.8 kb. Lanes 1-3 showed a single band of 1.7 kb with no wild type band present. Lane 5 represented *Hind*III cut DNA from the 8325-4 *sdrCD* double mutant donor, where a single band of 1.7 kb was observed, as before. Figure 3.14 shows a Southern blot where the same samples were probed with a DIG-labeled probe specific to *sdrE*. A band of 18 kb was observed in the wild type (lane 4). No bands were observed in lanes 1-3, indicating that *sdrE* had been deleted from the chromosome of strain Newman.

3.2.7. Complementation of the *sdr* mutants.

Complementing plasmids, which expressed the full-length *sdrC* and *sdrE* genes were constructed as described in section 2.9 (pCU1:*sdrC* and pCU1:*sdrE*, respectively). Repeated attempts to clone the full-length *sdrD* gene proved unsuccessful. pCU1:*sdrC* was transferred from RN4220 into the Newman and 8325-4 *sdrC* mutants by transduction. pCU1:*sdrE* was transferred from RN4220 into the Newman *sdrCDE* triple mutant, also by transduction.

3.3. Discussion.

Single mutations were generated in both the *sdrC* and *sdrD* genes in strains Newman and 8325-4 by directed plasmid integration using the temperature sensitive shuttle plasmid pG+host9. pG+host9, which is a broad host range lactococcal plasmid derived from pVE6004 (Maguin *et al.*, 1992), was originally developed as an allele replacement vector and has been used effectively to generate allele replacement mutants in lactococci (Maguin *et al.*, 1992; Biswas *et al.*, 1993) and streptococci (Perez-Casal *et al.*, 1993). However, its use as an allele replacement vector in staphylococci is rather limited due to the failure of the plasmid to excise readily from the staphylococcal chromosome following single cross-over integration (Supersac *et al.*, 1997). While this is a serious disadvantage for allele replacement, the failure to excise from the

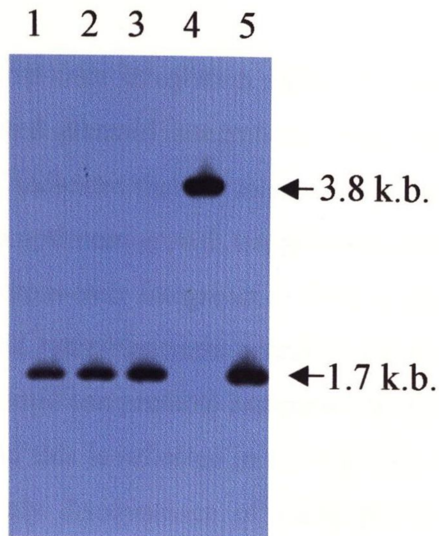


Figure 3.13 Southern blot of *sdrCDE* triple mutations in strain Newman, generated by transduction. Lanes 1-3; Newman *sdrCDE* triple mutants. Lane 4; Newman wild type. Lane 5; 8325-4 *sdrCD* double mutant. Blots were probed with an *sdrC* region A DIG-labelled probe

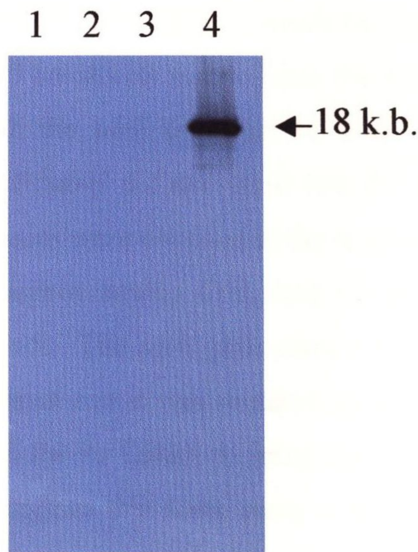


Figure 3.14 Southern blot of sdrCDE triple mutants in strain Newman, generated by transduction. Lanes 1-3; Newman *sdrCDE* triple mutants. Lane 4; Newman wild type. Blots were probed with an *sdrE* region A DIG-labelled probe

chromosome following single cross-over integration makes pG+host9 an ideal vector for constructing mutations by directed plasmid integration. Also, the fact that the *ts* rep mutation is effective below 37°C indicates that the integrants should be sufficiently stable for experiments performed at the optimum growth temperature of the bacterium. Indeed, prolonged growth of the single cross-over integrants at 37°C in the absence of selective antibiotic showed that no plasmid rearrangements occurred and that the mutations were stable. The rate at which the plasmid can promote integration into the chromosome at the site of the target gene is high and this is reflected in the eop values, which ranged from 1.3×10^{-3}-7.5×10^{-4}. The only disadvantage of using pG+host9 is the difficulty experienced in cloning fragments into the plasmid. During the course of this study, it was observed that pG+host9 did not appear to accept PCR products readily. To overcome this problem, PCR products were first cloned into an *E. coli* vector such as pBluescript KS+ and then subcloned into pG+host9. Inexplicably, pG+host9 appeared to ligate much more efficiently with inserts that had been initially cloned in another vector.

The reason for the failure of pG+host9:'*sdrE*' to integrate into the chromosome of *S. aureus* strains Newman, COL and P1 is not clear. Considering the high efficiency with which the *sdrC* and *sdrD* pG+host9 constructs inserted into the chromosome, one would have expected similar results with the *sdrE* construct in strain Newman. However, despite repeated attempts, pG+host9:'*sdrE*' did not insert into the chromosome of strain Newman, and no Em resistant colonies were obtained at the restrictive temperature. The plasmid was transduced into *S. aureus* strains COL and P1 and Em^r colonies were selected at 42°C with the same result. The *sdr*E gene cannot be essential because it is missing from several *S. aureus* strains and it was mutated by transduction (this study). There must be another explanation for its failure to integrate. Anecdotal evidence (S. Foster, personal communication) suggests that some areas of the *S. aureus* chromosome are resistant to homologous recombination, possibly due to DNA structure. This could be an explanation for the failure of pG+host9:'*sdrE*' to insert into the chromosome at the *sdrE* locus.

Southern hybridisation analysis of the *sdrC* and *sdrD* pG+host9 integrants in strains Newman and 8325-4 proved conclusively that mutations had been generated by gene disruption. It is not known how closely related the *sdrC* and *sdrD* genes of strains

8325-4 and Newman are. However, both the *sdrC* and *sdrD* genes are contained on the same sized *Hind*III and *Hinc*II restriction fragments, respectively. Where integration had occurred, the wild type band disappeared and was replaced by either two bands, where a single cross-over had occurred, or three bands, where a single cross-over with tandem duplication of the plasmid had occurred. The phenomenon of tandem duplication of the plasmid has been reported before and does not affect the integrity of the mutation.

Because the *sdr* genes are closely related, it is possible that they have similar functions. It was therefore considered necessary to construct, by allele replacement, a double mutation in the *sdrC* and *sdrD* genes in strain 8325-4 (which naturally lacks *sdrE*) and a triple mutation in the *sdrC*, *sdrD* and *sdrE* genes of strain Newman. Southern hybridisation analysis of putative double cross-overs in strain 8325-4 confirmed that part of the *sdrC* gene had been disrupted as the 3.8 kb wild type band was eliminated and replaced by a truncated band of 1.7 kb PCR analysis also confirmed the presence of the *tetK* marker in the chromosome (data not shown). This double cross over also resulted in the mutation of the *sdrD* gene, as the 5' end of *sdrD*, including the promoter region, was deleted in the construction of the allele replacement cassette (Figure 3.11).

The triple mutation was constructed by transduction. During transduction, the transducing phage can package up to 40 kb of chromosomal DNA. In strain Newman, the 3.5 kb *sdrE* gene lies 403 bp downstream of *sdrD*. The likelihood was high that the phage would package enough DNA downstream from the *sdrCsdrD::tetK* mutation when propagated on the 8325-4 *sdrCD* double mutant. As a result, when this packaged DNA was transduced into strain Newman, the DNA that lies downstream of the *sdrD* locus in 8325-4 was co-transduced and the *sdrE* gene was eliminated from strain Newman by homologous recombination. This was confirmed by Southern hybridisation.

Chapter 4

Expression of SdrC, SdrD and SdrE Proteins by *Staphylococcus aureus*

4.1 Introduction

S. aureus is primarily an extracellular pathogen. In order to colonise the host, it adheres to components of the extracellular matrix (ECM) by surface protein adhesins called MSCRAMMs (microbial surface components recognising adhesive matrix molecules) (Patti *et al.*, 1994; Foster and Höök, 1998). Several MSCRAMMs have been characterised at the molecular level, which include protein A, which binds to von Willebrand factor as well as to IgG (Hartlieb *et al.*, 1998), the collagen binding protein Cna (Switalski *et al.*, 1993), the fibronectin binding proteins FnBPA and FnBPB (Signas *et al.*, 1989; Jönsson *et al.*, 1991) and the fibrinogen binding proteins or clumping factors ClfA and ClfB (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998). With the exception of the elastin binding protein EbpS, each of the MSCRAMMs are members of a family of surface-anchored proteins which are covalently joined to peptidoglycan at the LPXTG motif present in the C-terminus of the protein by a membrane-associated enzyme called sortase (Navarre and Schneewind, 1999).

The *sdrC*, *sdrD* and *sdrE* genes encode proteins of 947 aa, 1315 aa and 1166 aa, respectively. Each of these proteins have all of the characteristic features of *S. aureus* MSCRAMMs, including (i) an N-terminal secretory signal sequence, (ii) a wall spanning domain, (iii) a C-terminal LPXTG motif, and (iv) a hydrophobic membrane-spanning domain, followed by several positively charged residues. Furthermore, the SdrC, SdrD and SdrE proteins, together with the fibrinogen binding proteins, ClfA and ClfB, are members of a family of structurally related surface proteins known as the serine-aspartate repeat (Sdr) protein multigene family (Josefsson *et al.*, 1998a). These proteins are characterised by the presence of the R domain, which contains the serine and aspartic acid dipeptide repeats. The Sdr proteins are also predicted to have similar structural organisation to ClfA and ClfB, with the exception of an additional B repeat region located between region A and region R (see Figure 1.7). Each B repeat contains a classical EF-hand motif which binds Ca^{2+} with high affinity. Occupation of these sites by Ca^{2+} results in a rigid rod-like structure and is critical for maintaining the structural integrity of the protein (Josefsson *et al.*, 1998b).

Most *S. aureus* MSCRAMMs are transcribed during the exponential phase of growth (Table 4.1). However, while proteins such as ClfB disappear from the cell surface as the cell enters stationary phase (Ní Eidihin *et al.*, 1998; McAleese *et al.*, 2001), other proteins such as ClfA remain anchored to the cell wall and are displayed on the cell surface during stationary phase (McDevitt *et al.*, 1994). It is thought that ClfB is eliminated from the cell surface during post-exponential phase by proteolysis and a dilution effect as the cells divide (McAleese *et al.*, 2001). However, it is not understood why ClfA remains displayed on the cell surface during stationary phase. Clearly, different *S. aureus* MSCRAMMs have different expression patterns and are not all expressed on the bacterial cell surface at the same time during the growth cycle. Therefore, in order to identify the phenotype of a novel surface protein, knowledge of its expression pattern on the bacterial cell surface is essential. As a prelude to determining the functions of the SdrC, SdrD and SdrE proteins, it was necessary to show that they were expressed by the bacterium (and were thus not pseudogenes). It was important to show that they were located on the cell surface, to determine the optimum growth conditions for expression and to determine whether they were present on cells from the exponential or stationary phase of growth. Identifying the correct size of each of the expressed proteins was important for future ligand-interaction studies. Because surface proteins are frequently cleaved and thus often inactivated by staphylococcal proteases, it was important to identify the growth conditions required for expression of the full-length protein.

ClfB is arguably one of the most well-characterised MSCRAMMs in terms of bacterial expression (Ní Eidhin *et al.*, 1998, McAleese *et al.*, 2001). ClfB is expressed in the exponential phase of growth by most *S. aureus* strains. In strain Newman, ClfB is expressed as a 150 kDa molecule that is partly degraded to a truncated 120 kDa protein in late exponential phase. This proteolytic degradation is mediated by the *S. aureus* metalloprotease (aureolysin), which cleaves between the S and L residues and the A and V residues of the SLAVA motif located within the ligand binding region A of the protein (McAleese *et al.*, 2001). Region A of ClfB is composed of three independently-folded domains known as N1, N2 and N3. Cleavage at the SLAVA motif is thought to release the N1 domain from the cell wall-anchored protein (Figure 4.1). A similar pattern is

Table 4.1 Transcription Patterns of *S. aureus* Surface Proteins

Gene	Transcribed	Protein Activity	Reference
spa	exponential phase	Maximal during exponential phase, but still present in stationary phase	Cheung *et al.*, 1997
clfA	ND	Activity present during exponential and stationary phase	McDevitt *et al.*, 1995
clfB	exponential phase	Only active during exponential phase	McAleese *et al.*, 2001
cna	exponential phase-	Activity maximal during exponential phase	Blevins *et al.*, 1999
fnbA	exponential phase	Activity maximal during exponential phase	Wolz *et al.*, 2000
fnbB	exponential phase	Activity maximal during exponential phase	Wolz *et al.*, 2000
ebpS	exponential phase	Activity present during exponential and stationary phase	Roche, 2001

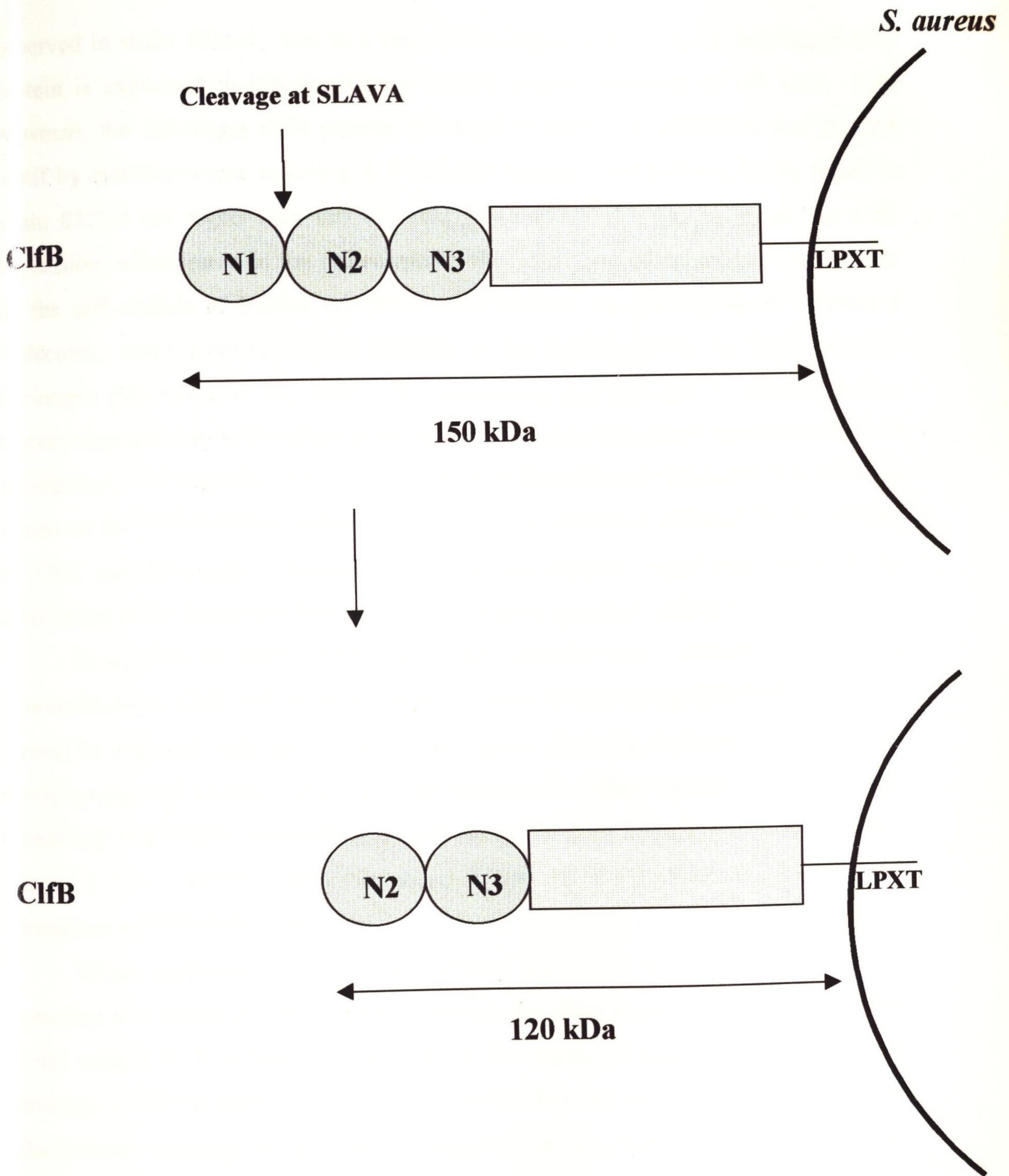


Figure 4.1 Cleavage of the *S. aureus* cell wall-associated ClfB protein at the SLAVA motif (located between the N1 and N2 domains of region A). Cleavage of ClfB at SLAVA results in the release of the N1 domain. The full-length ClfB protein is 150 kDa, while the truncated ClfB protein (missing the N1 domain) is 120 kDa.

observed in strain 8325-4. Due to a truncated R region in 8325-4, the full-length ClfB protein is expressed in this strain as a slightly smaller molecule of 140 kDa. Like Newman, the full-length ClfB protein expressed by 8325-4 is cleaved at the SLAVA motif by metalloprotease resulting in a 110 kDa protein. The protease levels found in strain 8325-4 are higher than that of strain Newman and this has an impact on ClfB expression, where more of the proteolytically degraded form of the molecule is present on the cell surface of 8325-4 compared with Newman. In both cases, the truncated molecules, which have undergone cleavage at the SLAVA motif, no longer bind to fibrinogen (McAleese *et al.*, 2001). We hypothesise that this type of specific surface protein cleavage may be involved in the detachment of the bacterium from a specific site of infection. For example, if *S. aureus* is bound to immobilized fibrinogen (e.g. which is coated on the surface of an biomedical device) via an interaction between the N1 domain of ClfB and fibrinogen, cleavage of ClfB at the SLAVA motif may result in the detachment of the bacterium from that site, facilitating spread of infection.

Expression of SdrC, SdrD and SdrE proteins was analysed by Western immunoblotting. Cell wall-associated proteins were released from stabilised protoplasts formed by digestion with lysostaphin, which cleaves at the pentaglycine cross-bridge of peptidoglycan in *S. aureus*. The solubilised proteins were then separated by SDS-PAGE, transferred to a PVDF membrane and analysed by Western imunoblotting (described in section 2.15). Specific antibodies directed towards the unique A region of the appropriate protein were used.

When antibodies to *S. aureus* proteins are raised in rabbits, contaminating antibodies that recognise other *S. aureus* proteins are often present due to prior exposure of the animal to *S. aureus*. Depending on the degree of contamination, it is often necessary to affinity-purify these antibodies before they can be used in Western blotting. This involves coupling the appropriate antigen to cyanogen bromide-activated sepharose to form an affinity matrix and loading the sera. Contaminating antibodies pass through the column, while specific antibodies bind to the corresponding antigen and are retained on the column. They can then be eluted with low pH buffer. The eluate can then be concentrated to provide high titre, specific antibodies against a cognate antigen. Affinity purification of SdrC and SdrD antibodies was performed.

This chapter describes the surface expression of SdrC and SdrD in strains Newman and 8325-4 and of SdrE in strain Newman.

4.2 Results

4.2.1 Expression of recombinant region A from SdrC, SdrD and SdrE proteins in *E. coli* strain Topp3

In order to generate antibodies that reacted with the SdrC, SdrD and SdrE proteins, the unique A region of each protein was expressed as a recombinant molecule with a hexa-histidine affinity tag at the N-terminus. The DNA sequence coding for the unique A region of each protein was amplified by PCR and cloned into the expression vector pQE30 (these plasmids were constructed by Dr. E. Josefsson and Dr. K. McCrea). The plasmids pQE30:$sdrC_{51\text{-}496}$, pQE30:$sdrD_{53\text{-}569}$ and pQE30:$sdrE_{53\text{-}607}$ were transformed into the protease deficient *E. coli* strain Topp3. Cultures were grown to exponential phase, induced with IPTG, lysed and the fusion proteins purified by Ni^{2+}-chelate chromatography as described in section 2.10. The SdrC A region protein that was used to immunise rabbits had an apparent molecular weight of 58 kDa (its size was predicted to be 49 kDa). Some breakdown was apparent in the fractions that eluted from the Ni^{2+}-chelate column (Figure 4.2). The SdrD protein was also purified and was used for affinity purification of sera raised by Dr. E. Josefsson. The SdrE protein was purified for platelet activation experiments (Chapter 7).

4.2.2 Purification of Sdr Protein antibodies

4.2.2.1 Anti-SdrC antibodies

Anti-SdrC region A antibodies were raised in two New Zealand white rabbits as described in section 2.11. The antibodies were used to probe Western blots where either the cognate SdrC antigen (recombinant SdrC region A) or cell wall-associated proteins isolated from stationary phase *S. aureus* strain Newman cells had been separated by SDS-

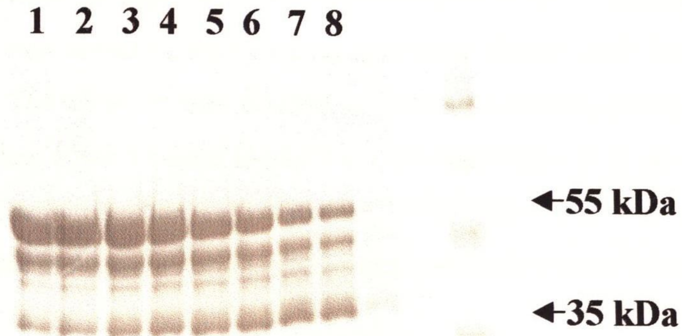


Figure 4.2 Coomassie stained SDS-PAGE gel of fractions collected following purification of SdrC region A from *E. coli* strain Topp3. Fractions shown in lanes 1-8 (2 ml each) were pooled and concentrated to a final volume of 4 ml (4-fold concentration). The final concentration of recombinant protein was 4.5 mg/ml.

PAGE and transferred to PVDF membranes. The titre of SdrC-specific antibodies present in the serum was high; a 1:10,000 dilution of the sera reacted with 20 μg of SdrC recombinant protein in a Western immunoblot. However, when tested against *S. aureus* cell wall-associated proteins, a high level of cross-reaction with *S. aureus* surface proteins other than SdrC was observed, making it impossible to discern individual protein bands (Figure 4.3 (a)). Therefore, it was deemed necessary to affinity-purify the anti-SdrC antibodies before they could be used to study protein expression in *S. aureus*. This was performed as described in section 2.13. Results in the following sections of this chapter showed that the contaminating anti-staphylococcal antibodies were removed.

4.2.2.2 Anti-SdrD antibodies

Anti-SdrD antibodies were raised by Dr. E. Josefsson. This antiserum was tested as described in section 4.2.2.1 and was found to be highly cross-reactive with many other proteins in the cell wall fraction of *S. aureus* strain Newman cells from stationary phase (Figure 4.3 (b)). Therefore, it was considered necessary to affinity-purify the anti-SdrD antibodies. This was performed as described in section 2.13. The process succeeded in removing the cross-reactive antibodies and resulted in the recovery of specific, high titre antibodies, where a dilution of 1:10,000 reacted strongly with 20 μg of recombinant SdrC region A.

4.2.2.3 Anti-SdrE antibodies

Anti-SdrE antibodies were provided by Dr. K. McCrea, Texas A & M, Houston. These antibodies were tested by Western immunoblotting for cross-reaction with wall-associated proteins from stationary phase *S. aureus* Newman cells. Some cross-reaction occurred, but this was substantially less than that observed for the anti-SdrC and SdrD antibodies. Importantly, much of the cross-reaction was observed with proteins of a relatively low molecular weight (< 60-80 kDa). The full-length SdrE protein has a predicted molecular weight of 117 kDa and was likely to migrate more slowly than

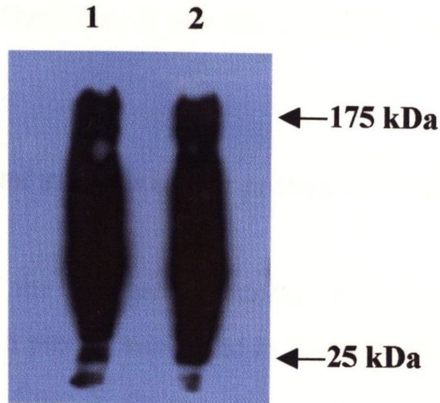


Figure 4.3 (a) Western immunoblot of *S. aureus* cell wall-associated proteins isolated from strain Newman stationary phase cells (lanes 1 & 2). Non-affinity purified antibodies directed towards the A region of SdrC were used. A large degree of cross-reaction with *S. aureus* surface factors was detected.

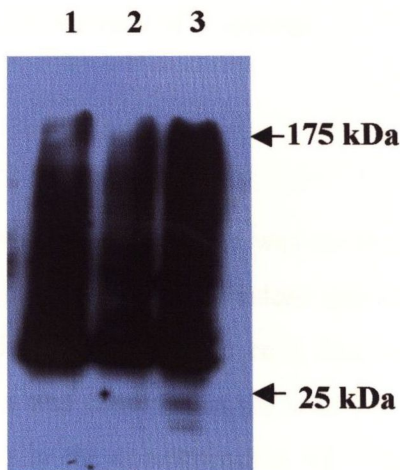


Figure 4.3 (b) Western immunoblot of *S. aureus* cell wall-associated proteins isolated from strain Newman stationary phase cells (lanes 1-3). Non-affinity purified antibodies directed towards the A region of SdrD were used. A large degree of cross-reaction with *S. aureus* surface factors was detected.

predicted in SDS-PAGE. Therefore, it was not considered necessary to affinity-purify the anti-SdrE antibodies.

4.2.3 Western Immunoblot analysis of Sdr protein expression in *S. aureus*.

In order to identify the *sdr* gene products, mono-specific antibodies directed towards the A domain of each protein were used in Western immunoblot analysis of wall-associated proteins released from *S. aureus* cells during protoplast formation. Where affinity-purified antibodies were used, a single immunoreactive band was seen. This was presumed to be the native wall-associated protein. Identification of the proteins was confirmed by comparing the wild type with the appropriate mutant and complemented mutant. In each case, cells were grown to mid exponential phase (OD600nm = 0.6-0.8) and stationary phase (16 hours) and the cell wall-associated proteins released as described in section 2.14. Western immunoblotting was carried out as described in section 2.15

4.2.3.1 Expression of SdrC on the surface of *S. aureus*

4.2.3.1.1 Strain Newman

Expression of SdrC by wild type Newman was compared to the Newman *sdrC* mutant and complemented mutant (Chapter 3). Western immunoblots were probed with specific affinity-purified anti-SdrC antibodies. In cells from stationary phase, a single immunoreactive band of 155 kDa was observed in the wild type sample (Figure 4.4, lane 1). This band was not observed in the corresponding *sdrC* mutant in stationary phase (lane 4), suggesting that the 155 kDa immunoreactive band, present in the wild type sample was the SdrC protein. Protein expression was restored in stationary phase when the *sdrC* mutation was complemented with the full-length *sdrC* gene cloned on a multicopy plasmid (lane 3). When α_2-macroglubulin (1.25 U/10 ml culture) was included in the growth media a 155 kDa protein was detected (lane 2). In exponential phase, a 155 kDa protein was observed in the wild type lane (lane 6), but not in the corresponding *sdrC* mutant lane (lane 5). These data suggested that SdrC was expressed

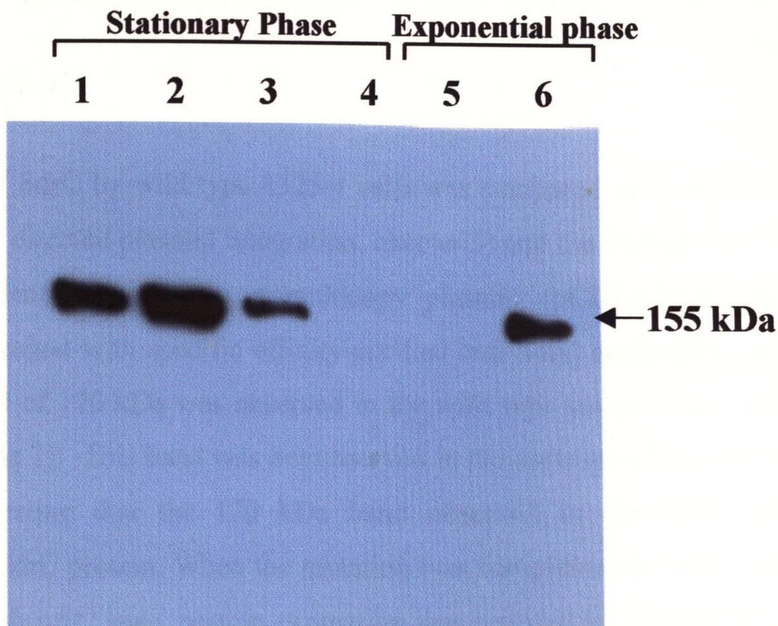


Figure 4.4 Western immounblot of SdrC expression in *S. aureus* strain Newman. Specific affinity-purified antibodies recognising the A region of SdrC were used. Lane 1; cell wall-associated proteins isolated from Newman wild type cells grown to stationary phase. Lane 2; cell wall-associated proteins isolated from Newman wild type cells grown to stationary phase in the presence of α_2-macroglobulin. Lane 3; cell wall-associated proteins isolated from a stationary phase culture of a Newman *sdrC* mutant complemented with pCU1:*sdrC*. Lane 4; cell wall-associated proteins isolated from a Newman *sdrC* mutant grown to stationary phase. Lane 5; cell wall-associated proteins isolated from Newman *sdrC* mutant cells grown to exponential phase. Lane 6; cell wall-associated proteins isolated from Newman wild type cells grown to exponential phase. This is a representative of 2 different experiments.

on the cell surface, throughout the growth cycle in strain Newman. No difference in the size of SdrC was observed when α_2-macroglobulin was included in the growth media, suggesting that SdrC was not proteolytically cleaved in strain Newman.

4.2.3.1.2 Strain 8325-4

Expression of SdrC by wild type 8325-4 cells was compared to the 8325-4 *sdrC* mutant (generated by directed plasmid integration, chapter 3) and the 8325-4 *sdrC* mutant expressing the full-length gene from a multicopy plasmid (pCU1:*sdrC*). Western immunoblots were probed with specific affinity-purified anti-SdrC antibodies. A single immunoreactive band of 120 kDa was observed in the wild type sample from stationary phase (Figure 4.5, lane 1). This band was not observed in the corresponding *sdrC* mutant lane (lane 3), suggesting that the 120 kDa band observed in the wild type lane corresponded to the SdrC protein. When the mutation was complemented with a plasmid carrying the full-length *sdrC* gene, protein expression was restored at a higher level (lane 4). The size of the SdrC protein present in the complemented mutant (lane 4) was slightly smaller than the 120 kDa band observed in the wild type (lane 1). The reason for this is unclear, but is probably due to additional proteolytic cleavage. Wild type cells were also grown to stationary phase in the presence of the protease inhibitor α_2-macroglobulin (1.25 U/10 ml of culture, Roche). When this protease inhibitor was included in the growth media, a 155 kDa band was observed (lane 2). The 155 kDa moiety is presumed to represent the full-length SdrC protein, while the 120 kDa band represents a proteolytically cleaved form of the protein. SdrC was not detected in samples from wild type cells or in the corresponding *sdrC* mutant grown to exponential phase (lanes 5 & 6). An immunoreactive band was observed for the complemented mutant, grown to exponential phase (lane 7). These data suggested that in 8325-4 SdrC was only expressed at a detectable level on the cell surface in cells from stationary phase. Furthermore, SdrC appeared to be proteolytically cleaved because a high molecular weight immunoreactive protein was detected in samples grown in the presence of α_2-macroglogulin.

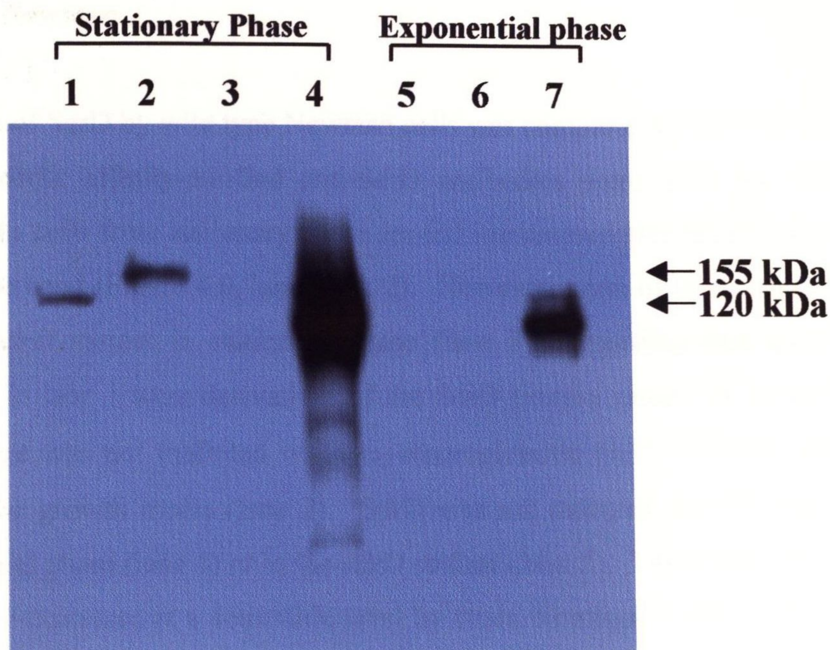


Figure 4.5 Western immunoblot of SdrC expression in strain 8325-4. Specific affinity-purified antibodies recognising the A region of SdrC were used. Lane 1; cell wall-associated proteins isolated from 8325-4 wild type cells grown to stationary phase. Lane 2; cell wall-associated proteins isolated from 8325-4 wild type cells grown to stationary phase in the presence of α_2-macroglobulin. Lane 3; cell wall-associated proteins isolated from an 8325-4 *sdrC* mutant grown to stationary. Lane 4; cell wall-associated proteins isolated from a stationary phase culture of an 8325-4 *sdrC* mutant complemented with pCU1:*sdrC*. Lane 5; cell wall-associated proteins isolated from 8325-4 wild type cells grown to exponential phase. Lane 6; cell wall-associated proteins isolated from 8325-4 *sdrC* mutant cells grown to exponential phase. Lane 7; cell wall-associated proteins isolated from an exponential phase culture of an 8325-4 *sdrC* mutant complemented with pCU1:*sdrC*. This is a representative of 2 different experiments.

4.2.3.2 Expression of SdrD on the surface of *S. aureus*

4.2.3.2.1 Strain Newman

Expression of SdrD by wild type Newman cells was compared to the *sdrD* mutant (Chapter 3). Specific affinity-purified anti-SdrD antibodies were used for Western immunoblotting. In cells from stationary phase several immunoreactive bands were seen rather than a single band (Figure 4.6, lanes 1 & 2). However, none of these bands were observed in the *sdrD* mutant in stationary phase (lane 3), suggesting that all of the proteins observed in lane 1 were derivatives of the SdrD protein caused by proteolysis. Proteolytic cleavage was not inhibited when α_2-macroglobulin (1.25 U/10 ml culture) was included in the growth media (lane 2). SdrD was not detected in wild type cells grown to exponential phase (lane 4) or in the *sdrD* mutant (lane 5). These data suggested that SdrD was only expressed at a detectable level by strain Newman in stationary phase. A large amount of proteolytic degradation that was not inhibited by α_2-macroglobulin occurred. It is likely that this degradation occurred during the isolation of the cell surface-associated proteins and not during cell growth. The small bands of <50-60 kDa that were observed in figure 4.6, lanes 1 &2 could not have been attached to the cell wall and still react with antibodies that recognise the N-terminal A domain.

4.2.3.2.2 Strain 8325-4

Expression of SdrD by wild type *S. aureus* 8325-4 cells was compared to the corresponding 8325-4 *sdrD* mutant (generated by directed plasmid integration, Chapter 3). Western immunoblots were probed with specific affinity-purified anti-SdrD antibodies. A single immunoreactive band of 140kDa was observed in wild type cells from stationary phase (Figure 4.7, lane 1). This band was not observed in the *sdrD* mutant (lane 3), suggesting that the 140 kDa band observed in the wild type represented the SdrD protein. When the protease inhibitor α_2-macroglobulin (1.25 U/10 ml culture) was included in the growth media, a single immunoreactive band of 160 kDa was

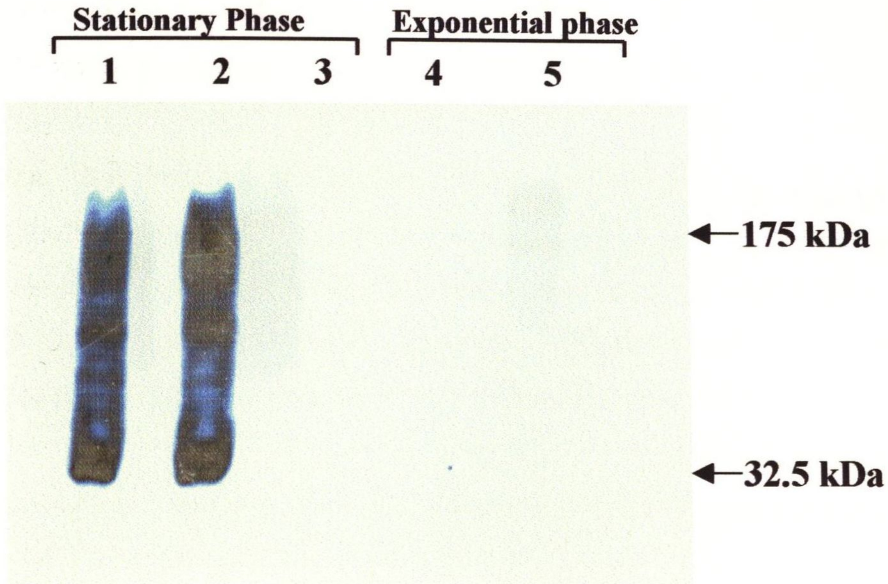


Figure 4.6 Western immunoblot of SdrD expression in *S. aureus* strain Newman. Specific affinity-purified antibodies recognising the A region of SdrD were used. Lane 1; cell wall-associated proteins isolated from stationary phase wild type Newman cells. Lane 2; cell wall-associated proteins isolated from stationary phase wild type Newman cells grown in the presence of α_2-macroglobulin. Lane 3; cell wall-associated proteins isolated from stationary phase Newman *sdrD* mutant cells Lane 4; cell wall-associated proteins isolated from exponential phase wild type Newman cells. Lane 5; cell wall-associated proteins isolated from exponential phase Newman *sdrD* mutant cells. This is a representative of 4 different experiments.

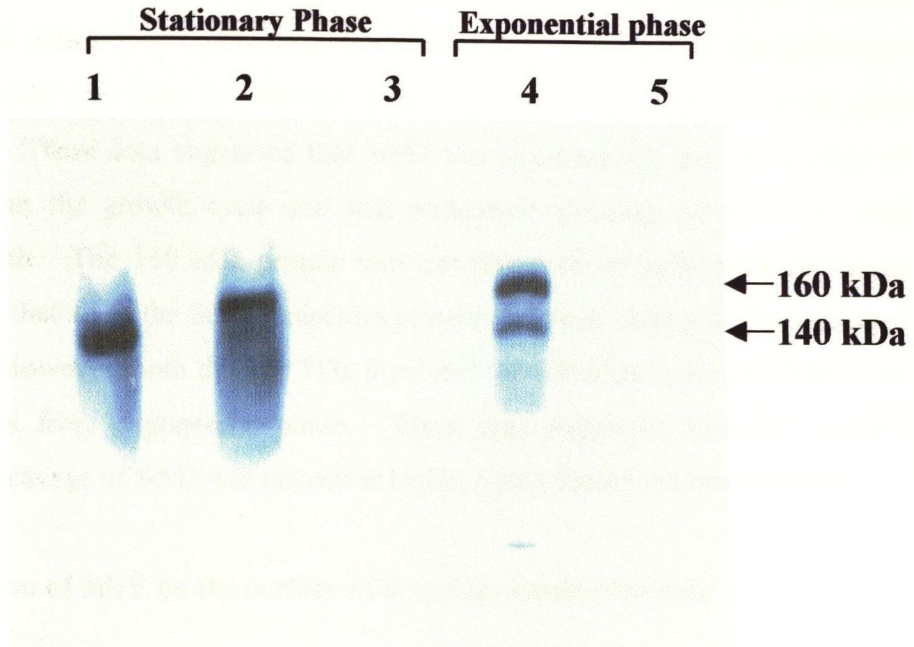


Figure 4.7 Western immunoblot of SdrD protein expression in *S. aureus* strain 8325-4. Specific affinity-purified antibodies recognising the A region of SdrD were used. Lane 1; cell wall-associated proteins isolated from stationary phase wild type 8325-4 cells. Lane 2; cell wall-associated proteins isolated from stationary phase wild type 8325-4 cells grown in the presence of α_2-macroglobulin. Lane 3; cell wall-associated proteins isolated from stationary phase 8325-4 *sdrD* mutant cells. Lane 4; cell wall-associated proteins isolated from exponential phase wild type 8325-4 cells. Lane 5; cell wall-associated proteins isolated from exponential phase 8325-4 *sdrD* mutant cells. This is a representative of 2 different experiments.

observed (lane 2), indicating that the 140 kDa protein observed in the stationary phase was a cleavage product and that cleavage was inhibited by α_2-macroglobulin. In cells from exponential phase, two immunoreactive proteins of 140 kDa and 160 kDa were observed in the wild type (lane 4). Neither of these proteins were observed in the *sdrD* mutant (lane 5). These data suggested that SdrD was expressed on the cell surface of 8325-4 throughout the growth cycle and that proteolytic cleavage of SdrD occurred during cell growth. The 160 kDa protein was not observed on cells from stationary phase, indicating that all of the SdrD molecules present had been cleaved to the truncated 140 kDa form. However, both the 160 kDa form and the 140 kDa truncated form were observed in cells from exponential phase. These data suggested that the protease responsible for cleavage of SdrD was present at higher levels during stationary phase.

4.2.3.3 Expression of SdrE on the surface of *S. aureus* strain Newman

Expression of SdrE by wild type Newman cells was compared to that of the Newman*sdrCDE*- mutant (generated by allele replacement, Chapter 3) and the Newman*sdrCDE*- mutant complemented with the full-length Newman *sdrE* gene cloned on a multicopy plasmid (pCU1:*sdrE*). Western immunoblots were probed with antibodies directed towards the unique A region of SdrE. Because antibodies had not been affinity purified, single immunoreactive bands were not observed. SdrE proteins were identified by comparing the wild type sample with the *sdrE* mutant. A 130 kDa band was detected in the protein sample from cells grown to stationary phase (Figure 4.8, lane 1). This protein was missing in the Newman *sdrCDE*⁻ mutant. Expression of this protein was restored in the complemented mutant (lane 3). In the exponential phase sample, a band of 180 kDa was present in the wild type lane (lane 4), but not in the corresponding mutant lane (lane 5). Expression of the 180 kDa protein was observed in the complemented mutant (lane 6) in exponential phase. These data suggested that SdrE was expressed throughout the growth cycle in strain Newman. It was presumed that the 180 kDa band observed in cells from exponential phase was the full-length form of the SdrE molecule and that the 130 kDa band observed in stationary phase samples represented a proteolytically cleaved product.

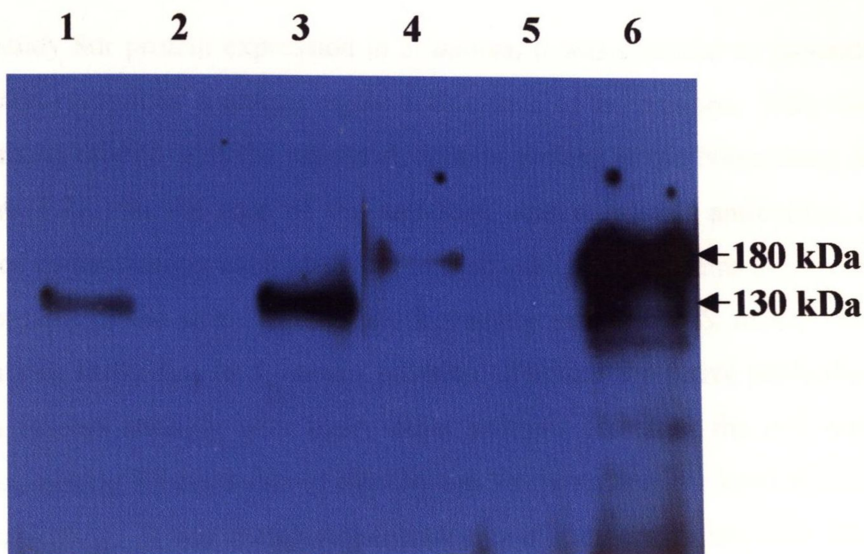


Figure 4.8 Western immunoblot of SdrE expression in *S. aureus* strain Newman. Antibodies specific to the A region of SdrE were used. Lane 1; cell wall-associated proteins isolated from wild type Newman cells grown to stationary phase. Lane 2; cell wall-associated proteins isolated from Newman *sdrCDE* mutant cells grown to stationary phase. Lane 3; cell wall-associated proteins isolated from stationary phase Newman *sdrCDE* mutant cells complemented with pCU1:*sdrE*. Lane 4; cell wall-associated proteins isolated from wild type Newman cells grown to exponential phase. Lane 5; cell wall-associated proteins isolated from Newman *sdrCDE* mutant cells grown to exponential phase. Lane 6; cell wall-associated proteins isolated from exponential phase Newman *sdrCDE* mutant cells complemented with pCU1:*sdrE*. This is a representative of two different experiments.

4.3 Discussion

In order to study Sdr protein expression in *S. aureus*, it was essential to generate specific antibodies that recognised a unique region within each of the proteins. This was achieved by immunising rabbits with the unique A domain present at the N-terminus of each protein (Figure 1.7). In the case of the anti-SdrC and anti-SdrD antibodies, a substantial amount of contaminating antibodies that reacted with many proteins in the cell wall extract were present in the sera. Rabbits are frequently exposed to *S. aureus* and their sera often contains antibodies to *S. aureus* proteins. The cross-reactive antibodies present in the sera reacted strongly with many other proteins found in the cell wall fraction of *S. aureus*, making it impossible to identify the Sdr protein under investigation by Western immunoblotting. It was therefore necessary to affinity-purify both the anti-SdrC and anti-SdrD antibodies using their cognate antigen. Affinity purification was successful and provided high titre, specific antibodies, that reacted with a single band in Western immunoblot analysis of *S. aureus* Newman and 8325-4. The level of cross-reactive antibodies present in the anti-SdrE antisera was much lower and did not necessitate affinity purification.

SdrC and SdrD protein expression was studied in two different strains of *S. aureus*, Newman and 8325-4. As the *sdrE* gene is not found in strain 8325-4, SdrE protein expression was only studied in strain Newman. Single immunoreactive bands were identified for both SdrC and SdrD in strain 8325-4, but the expression pattern for these proteins in the two hosts was quite different. While SdrC was only detected in the stationary phase of growth in strain 8325-4, SdrD was seen throughout the growth cycle. These data suggested that SdrC and SdrD were controlled by different regulatory factors. It was apparent that both of the proteins were subject to proteolytic cleavage during cell growth. In stationary phase, SdrC was detected as a 120 kDa immunoreactive band in strain 8325-4. However, when the protease inhibitor α_2-macrolobulin was included in the growth media, a 155 kDa band was detected, suggesting that proteolytic cleavage of the SdrC molecule occured during cell growth. α_2-macroglobulin is a 680 kDa molecule that cannot permeate bacterial cells and is washed away from the cells prior to surface

protein isolation. Therefore, any effects seen with this protease inhibitor occur during cell growth. It was assumed that the 155 kDa band observed when cells were grown in the presence of α_2-macroglobulin was the full-length mature form of the SdrC protein of strain 8325-4.

The predicted size of the mature SdrC molecule is approximately 94 kDa. However many *S. aureus* surface proteins migrate aberrantly on SDS-PAGE. This has been observed with ClfA (Hartford *et al.*, 1997), ClfB (Ní Eidhin *et al.*, 1998) and FnBPA and FnBPB (McGavin *et al.*, 1997). This is due to the serine-aspartate dipeptide repeats and to the hydrophilic N-terminal sub-domain 1 of region A in the case of the Clf/Sdr proteins.

The predicted molecular weight of SdrD is 134 kDa. However, in 8325-4 stationary phase cells, SdrD was detected as a single 140 kDa immunoreactive band, while in exponential phase, two immunoreactive bands of 140 kDa and 160 kDa were observed. Furthermore, when α_2-macroglobulin was included in the growth media, a single immunoreactive band of 160 kDa was released from stationary phase cells. These data indicated that proteolytic degradation of SdrD occurred during cell growth. In the case of SdrC and SdrD in strain 8325-4, the protease responsible for cleavage of the proteins was inhibited in the presence of α_2-macroglobulin. This protease(s) appeared to be more abundant in the stationary phase of growth where complete degradation of both SdrC and SdrD was observed. In exponential phase, partial degradation of SdrD was seen, indicating that less of the protease responsible for the cleavage event was present. Clearly, the 140 kDa SdrD protein is a truncated version of the full-length molecule. However, it is not certain whether the 160 kDa SdrD protein represents the mature full-length SdrD protein. Evidence presented in chapter 5 suggests that the 160 kDa SdrD band seen in 8325-4 may also be a cleavage product of a higher molecular weight form (see Figure 6.8 (a)).

In strain Newman a different pattern of expression for SdrC and SdrD was observed. While SdrC was only detected in 8325-4 cells from the stationary phase of growth, a single 155 kDa immunoreactive band for SdrC was detected throughout the growth cycle in strain Newman. This 155 kDa SdrC protein is thought to represent the mature full-length form of SdrC, suggesting that SdrC is not cleaved by proteases in

strain Newman. This differs from the pattern of expression for SdrC observed in strain 8325-4, where the protein was expressed as a truncated molecule and is only detected in cells grown to stationary phase.

Expression of SdrD in Newman also differed from that of 8325-4. While SdrD was detected in 8325-4 throughout the growth cycle, the protein was only seen in Newman cells from stationary phase. Furthermore, while α_2-macroglobulin could inhibit the proteolytic cleavage of SdrD in strain 8325-4, no inhibition of SdrD protein degradation in Newman was observed when cells were grown in the presence of α_2-macroglobulin. While it was clear that SdrD was proteolytically cleaved during cell growth in strain 8325-4, it appeared that the large degree of proteolytic degradation that occurred with SdrD in strain Newman happened during cell-surface protein isolation. The presence of low molecular weight (20-60 kDa) SdrD immunoreactive protein bands in the protein fraction isolated from wild type Newman cells suggested that these fragments were N-terminal truncates that were not attached to the cell wall. This truncation must have occurred during cell surface protein isolation. If N-terminal degradation occurs during cell growth, N-terminal fragments are released into the medium, leaving the remaining C-terminal region attached to the cell wall. Thus, the low molecular weight proteins that reacted with anti-SdrD region A antibodies in Western immunoblotting must have been formed during protein isolation and not whilst the protein was attached to the cell wall. It is perplexing why this type of degradation occurred with SdrD in strain Newman and not for other strains and other proteins. However the fact that it occurs is indisputable, as this experiment was repeated on four separate occasions. Each time the surface proteins were isolated freshly and analysed by Western blotting and on each occasion, the same result was yielded.

Expression of *S. aureus* cell wall-associated proteins in stationary phase, but not in exponential phase is contrary to the behavior of other *S. aureus* surface proteins. Since most MSCRAMMs are anchored to the pentaglycine cross-bridge of peptidoglycan during cell wall biosynthesis, it was not thought that surface proteins could be expressed and correctly sorted to the cell wall when cell division had stopped during stationary phase. It is possible that SdrC is present in strain 8325-4 during exponential phase and that SdrD is present in strain Newman during exponential phase at levels below the limit

of detection for Western immunoblotting and that protein expression only reaches detectable levels during stationary phase. Transcript and *lacZ* promoter fusion analysis is required in order to confirm the possibility that *sdrC* and *sdrD* gene expression is switched on in stationary phase in strains 8325-4 and Newman, respectively.

The differences in expression of SdrC and SdrD proteins in 8325-4 and Newman could be due to transcriptional control by different regulators, different susceptibility to one or more proteases and different expression of proteases in the two hosts. The genetic regulatory system of *S. aureus* is complicated, involving the interaction of several different loci (reviewed in detail in chapter 1). Both *agr* and *sar* are the best characterised global regulatory loci in *S. aureus* (Peng *et al.*, 1988; Cheung *et al.*, 1992; Morfeldt *et al.*, 1996). The *agr* locus is known to control transcription of the *fnb* genes (Saravia-Otten *et al.*, 1997), while *sarA* is known to control transcriptional regulation of *cna* (Blevins *et al.*, 1999) However, other regulatory loci may also play a role in the regulation of virulence factors. SarH1 is now known to play a role in the transcriptional regulation of the *hla* (Tegmark *et al.*, 2000), while the *spa* locus is controlled by *agr* and *sarH1* (Tegmark *et al.*, 2000; Patel *et al.*, 1992). Other loci such as *sigB*, *xpr* and *sae* may also play a role in the transcriptional regulation of surface protein and other virulence genes. SigB is an alternative sigma factor, recently identified in *S. aureus*. It is expressed during stationary phase and upon heat shock (Kullik and Giachino, 1997) and is known to effect transcription of *sar* P3 promoter. Because 8325-4 produces a defective SigB protein, it is possible that the difference in expression patterns for SdrC and SdrD between strains 8325-4 and Newman is due to a direct or indirect effect of SigB. Analysis of the *sdrC* and *sdrD* transcripts in *sigB* and *sar* mutants could be used to explore this possibility. A difference in the levels of proteases produced by strains Newman and 8325-4 has been observed previously in this laboratory. The level of proteases found in the culture supernatant of strain 8325-4 is higher than that of strain Newman (McAleese *et al.*, 2001). Since the expression of proteases is controlled largely by the *sar* locus (Chan and Foster, 1999), it is possible that the difference in the protease profiles of strains 8325-4 and Newman is caused by indirect effects of the 8325-4 *sigB* mutation.

To identify the protease(s) responsible for cleavage of the SdrC and SdrD proteins, a number of experiments could be performed. Incubating Newman cells

expressing SdrC with concentrated culture supernatant from 8325-4 could show that the protease responsible for the cleavage of SdrC is present in strain 8325-4 but not in strain Newman. If this is true, cleavage of the 155 kDa SdrC protein observed in Newman cells to the truncated 120 kDa form, normally only seen in 8325-4 should occur. Incubating recombinant SdrC and SdrD A domains with different purified proteases could identify the protease responsible for proteolytic cleavage of these proteins. This may be a more efficient method for identifying proteases responsible for cleavage of specific proteins than studying protein expression in protease-deficient mutants as certain proteases have pleiotropic effects. For example, aureolysin activates the serine protease (Drapeau *et al.*, 1978).

SdrE was expressed throughout the growth cycle in strain Newman. In exponential phase, a 180 kDa protein was observed, while a 130 kDa protein was detected in stationary phase. Data presented in chapter 5 shows that the full-length mature SdrE protein is 180 kDa. The 130 kDa protein is thought to be a proteolytically cleaved truncate of the full-length molecule.

Each of the Sdr proteins was shown to be susceptible to proteolytic cleavage. It is known that the A regions of ClfA and ClfB are composed of at least three independently folded domains. Proteolytic cleavage of ClfB by the metalloprotease aureolysin occurs at a motif (SLAVA) located between domains N1 and N2 (Figure 4.1). A similar motif (SLAAVA) is also found in the A region of ClfA. Cleavage at the SLAVA motif in ClfB results in the release of the N1 domain and eliminates the ligand binding activity of the remaining cell wall-associated protein (McAleese *et al.*, 2001). This is one of many cleavage events in domain N1. The intact N1 domain cannot be resolved after aureolysin treatment, while several short peptides are (McAleese *et al.*, 2001). It is likely that the truncation of the Sdr proteins is also due to release of a sub-domain from region A. This requires N-terminal sequencing of proteolytically cleaved recombinant proteins. The SLA(A)VA motif found in ClfA and ClfB is not present in any of the Sdr proteins. However, metalloprotease is known to cleave N-terminal of hydrophobic residues, so other sites may occur in the N-termini of the Sdr proteins. In addition, serine protease cleaves FnBPA and FnBPB (McGavin *et al.*, 1997) and may also be responsible for the cleavage of Sdr proteins.

It was important to determine if each of the proteins was expressed in exponential or stationary phase, as well as the level of proteolytic degradation, before proceding to functional studies. In strain Newman, the binding properties of SdrC and SdrE can be studied at any stage in the growth cycle. However, for SdrD, stationary phase Newman cells are required to study its effects. Similarly, for strain 8325-4, effects of SdrC must be analysed in stationary phase, whereas the properties of SdrD can be studied throughout the growth cycle. However, the poor expression of the Sdr proteins and the propensity to be degraded by proteases secreted by the host strain makes expression in a heterologous host such as *Staphylococcus carnosus* or *L. lactis* an attractive alternative strategy for studying novel phenotypes of Sdr proteins.

Further studies on Sdr protein expression could focus on regulation. The three *sdr* genes are closely linked in tandem array. However they are likely to be independently expressed because of (i) the length of the intergenic regions between *sdrC* and *sdrD,* and *sdrD* and *sdrE* and (ii) the different expression patterns observed. The difference in expression patterns for each of the Sdr proteins suggests that transcriptional control may be different in each case. Transcript analysis and the study of *lacZ* promoter fusions in different regulatory mutants (e.g. *agr*, *sar*, *sarH1*, *sigB*) could be used in future studies to determine the factors involved in the transcriptional control of *sdr* gene expression.

Chapter 5

Expression of the SdrG protein by *Staphylococcus epidermidis*

5.1 Introduction

Staphylococcus epidermidis is a member of the coagulase negative staphylococci (CoNS) and is considered to be an important pathogen. It is a common inhabitant of human skin and is a frequent etiological agent for infections associated with implanted medical devices such as catheters and artificial joints (Wong *et al.*, 1996). *S. epidermidis* is also associated with cases of peritonitis in patients undergoing peritoneal dialysis (von Graevenitz and Amsterdam, 1992) and with neonatal infections (Neumeister *et al.*, 1995). Pathogenesis is facilitated by the ability of the organism to first adhere to and subsequently form biofilms on indwelling medical devices.

Adherence of *S. epidermidis* to synthetic surfaces has been correlated with both surface hydrophobicity and cell-surface proteins (Fleer and Verhoef, 1989; Martin *et al.*, 1989). Polysaccharide expressed by the *ica* operon is crucial for biofilm formation. One group have suggested that the polysaccharide adhesion (P/SA) is sufficient for both the adhesion and cell-cell interactions associated with the accumulation phase of biofilm formation (McKenney *et al.*, 1998). Another view is that adherence is mediated by a surface-associated protein whilst the polysaccharide is responsible only for the accumulation phase (Heilmann *et al.*, 1996, 1997). Like *S. aureus*, *S.epidermidis* can adhere to many serum and tissue proteins, including fibrinogen, fibronectin, collagen, laminin and vitronectin (Herrmann *et al.*, 1988; Paulsson *et al.*, 1992, Switalski *et al.*, 1983). It is thought that these interactions are mediated by MSCRAMM-like surface proteins. However, most of the proteins responsible for this adherence in *S. epidermidis* remain uncharacterised.

Recently a protein that is produced by *S. epidermidis* termed Fbe was shown to have fibrinogen binding properties (Nilsson *et al.*, 1998; Pei *et al.*, 1999). In addition, three serine-aspartic acid dipeptide repeat-containing proteins have been identified in *S. epidermidis* (McCrea *et al.*, 2000). SdrG and SdrF are structurally related to the Sdr proteins of *S. aureus* and are thought to be anchored to the cell wall at the LPXTG motif present in the C-terminus (Figure 5.1). SdrH does not contain this common cell wall anchoring motif and is thought to be attached to the cell wall non-covalently (Figure 5.1). The SdrG protein, which is expressed from a gene originally cloned from *S. epidermidis*

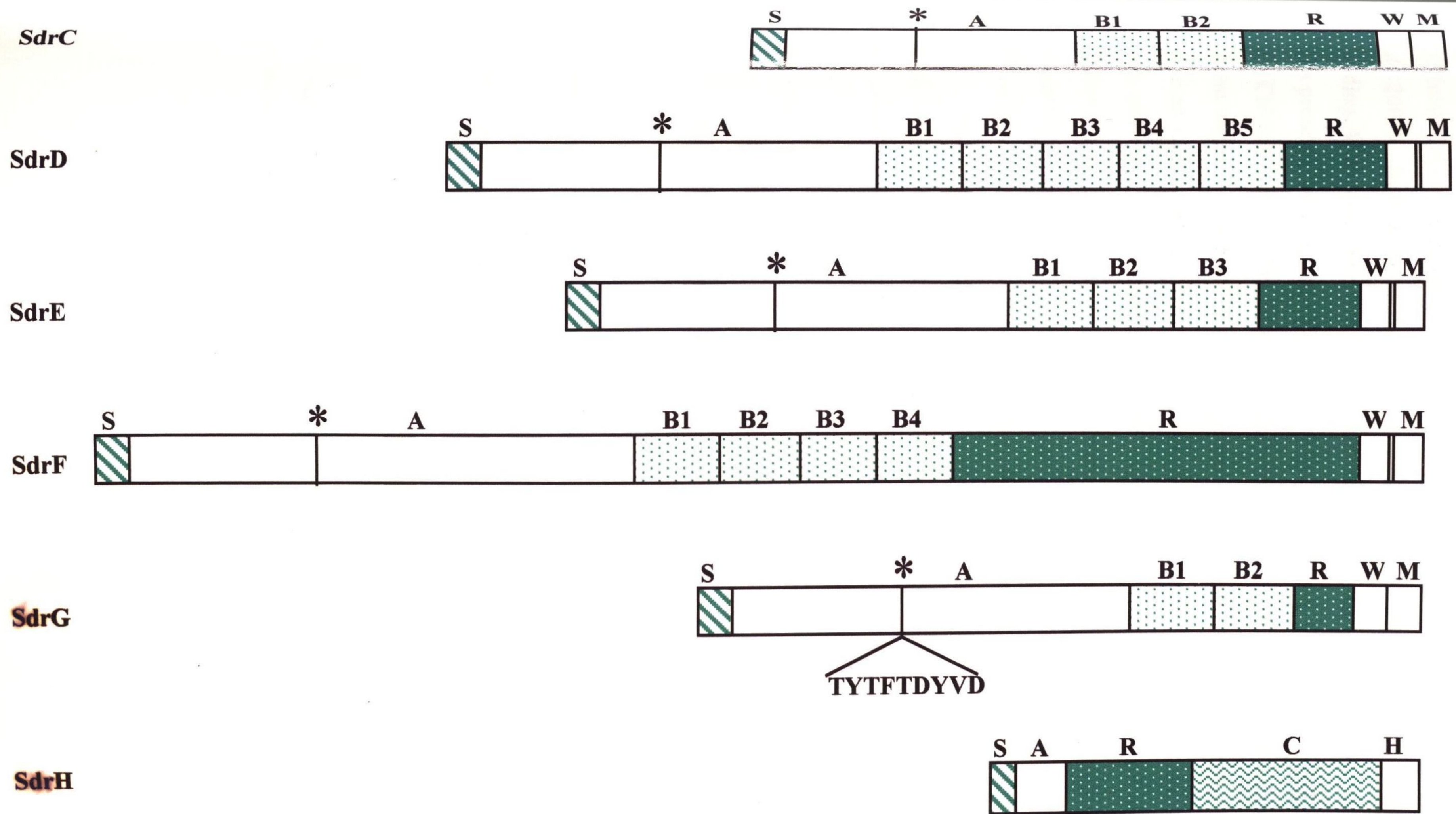


Figure 5.1 Schematic representation of the SdrC, SdrD and SdrE proteins from *S. aureus* strain Newman and the SdrF, SdrG and SdrH proteins from *S. epidermidis* strain K28.

strain K28 (McCrea *et al.*, 2000), is closely related to Fbe. In fact, it has recently been postulated that Fbe and SdrG are the same protein, but from different strains (Hartford *et al.*, 2001). Both proteins have a 50 residue signal sequence, a 548 residue fibrinogen-binding A domain (96% identical) followed by two B repeats of 113 and 111 residues. The proteins have repeats of the serine-aspartic acid dipeptide which are characteristic of the Clf-Sdr family. The SdrG protein from strain K28 has 56 residues in the dipeptide repeat region, while Fbe from strain HB has 216 residues. At the C-terminus the proteins have an LPXTG motif, a hydrophobic domain and positively charged residues asociated with anchoring the protein to the cell surface (Navarre & Schneewind, 1999). The fibrinogen binding properties of Fbe were demonstrated using a recombinant protein comprising the unique A region (Pei *et al.*, 1999). The ability of Fbe to bind to fibrinogen when expressed on the surface of *S. epidermidis* was not assessed.

This chapter describes the expression of the SdrG protein by three different *S. epidermidis* strains (HB, 9142 and 1457) and shows that SdrG is both necesssary and sufficient for fibrinogen binding in *S. epidermidis*.

5.2 Results

5.2.1 Anti-SdrG antibodies

Antibodies specific to the unique A region of SdrG were raised previously in New Zealand white rabbits (McCrea *et al.*, 2000).

5.2.2 Isolation of mutations in the *sdrG* gene of *S. epidermidis* strains HB, 9142 and 1457

Mutations in the *sdrG* gene of *S. epidermidis* strains HB, 9142 and 1457 were isolated by directed plasmid integration by Dr. Orla Hartford, using the temperature sensitive shuttle plasmid pG+host9 (Hartford *et al.*, 2001).

5.2.3 Expression of SdrG by *S. epidermidis* strain HB.

In order to determine the optimal growth conditions necessary for the expression of SdrG, strain HB was grown under different conditions before cell wall-associated proteins were isolated by lysostaphin digestion and analysed by Western immunoblotting using antibodies specific to the A region of SdrG. Wild type and HB*sdrG*- mutant cells were grown in 50 ml of either TBS or BHI broth to exponential (OD_{600nm}=0.8) or stationary phase (16 hours) in 250 ml flasks, with or without shaking at 37°C. Cell wall associated proteins were released from stabilised protoplasts by lysostaphin digest as described in section 2.14 and separated by SDS-PAGE, transferred to a PVDF membrane and probed with antibodies recognising the unique A domain of SdrG, as described in section 2.15. Results summarised in Table 5.1, show that the SdrG protein was only detected on wild type cells grown to exponential phase in TSB with no shaking. These conditions were used for further protein expression studies.

5.2.4 Expression of SdrG protein by *S.epidermidis* HB, 9142 and 1457

S. epidermidis wild type strains HB, 9142 and 1457 and their corresponding *sdrG* mutants were grown to exponential phase in 50 ml TBS, with no shaking at 37°C. Cell wall-associated proteins were isolated and analysed by Western immunoblotting as described above. A single immunoreactive band of 190 kDa was detected in the wild type sample from strain HB (Figure 5.2, lane 1). This band was not observed in the corresponding HB*sdrG*- mutant sample (lane 2). In 9142 wild type cells a 195 kDa band was observed (lane 3), which was not present in the corresponding 9142*sdrG*- mutant (lane 4). No immunoreactive protein was detected in either the wild type or mutant samples for strain 1457 (lanes 5 & 6). These data suggested that the protein detected in the wild type samples for strains HB and 9142 was SdrG. Strain 1457 did not appear to express SdrG under these conditions. The small difference in size between the SdrG

Table 5.1 Expression of SdrG in *S. epidermidis* strain HB

Strain	Media	Growth phase	Shaking/Static	SdrG Expression
Wild type	TSB	Exponential	Shaking	No
Wild type	TSB	Exponential	Static	**Yes**
Wild type	TSB	Stationary	Shaking	No
Wild type	TSB	Stationary	Static	No
Wild type	BHI	Exponential	Shaking	No
Wild type	BHI	Exponential	Static	No
Wild type	BHI	Stationary	Shaking	No
Wild type	BHI	Stationary	Static	No
HB*sdrG-*	TSB	Exponential	Shaking	No
HB*sdrG-*	TSB	Exponential	Static	No
HB*sdrG-*	TSB	Stationary	Shaking	No
HB*sdrG-*	TSB	Stationary	Static	No
HB*sdrG-*	BHI	Exponential	Shaking	No
HB*sdrG-*	BHI	Exponential	Static	No
HB*sdrG-*	BHI	Stationary	Shaking	No
HB*sdrG-*	BHI	Stationary	Static	No

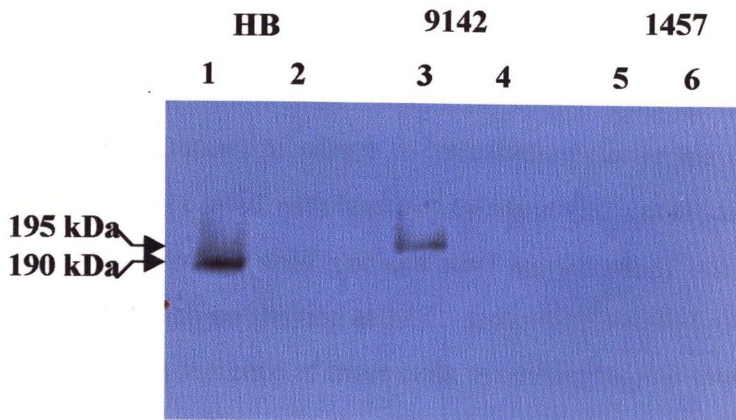


Figure 5.2 Expression of SdrG in *S.epidermidis* strains HB, 9142 and 1457. *S. epidermidis* wild type and *sdrG* mutant cells were grown to exponential phase in TSB at 37°C without shaking. Cell wall-associated proteins were isolated and analysed by Western immunblotting using antibodies directed towards the unique A region of SdrG. Lane 1; HB wild type. Lane 2; HBΔ*sdrG*. Lane 3; 9142 wild type. Lane 4; 9142Δ*sdrG*. Lane 5; 1457 wild type. Lane 6; 1457Δ*sdrG*. This is a representative of 2 separate experiments.

proteins expresed by strains HB and 9124 may be explained by a difference in the size of the serine-aspartic acide dipeptide repeat region.

5.2.5 Adherence of *S. epidermidis* wild type and *sdrG* mutant strains to immobilised fibrinogen

The ability of *S. epidermidis* wild type strains HB, 9142 and 1457 and the corresponding *sdrG* mutants to adhere to immobilised fibrinogen was measured. A 96-well ELISA plate was coated with human fibrinogen (20 μg/ml), as described in section 2.19.1. Each *S. epidermidis* wild type and *sdrG* mutant strain was grown to exponential phase in 50 ml TSB with no shaking at 37°C. Cells were washed in PBS and resuspended to an OD_{600nm} of 4. Adherence of these cells to fibrinogen was measured as described in section 2.19.1. Due to the weak interaction between *S. epidermidis* and fibrinogen, an alternative approach to reading the results was made. Following the staining of adherent cells with crystal violet, 100 μl 10% acetic acid was added to the wells. This resulted in the release of crystal violet from adherent cells and provided a more uniform and reproducible reading. Results showed that *S. epidermidis* strains HB and 9142 adhered to immobilised fibrinogen (Figure 5.3). No binding was detected with *S. epidermdis* strain 1457 wild type or *sdrG* mutant cells. In the HB*sdrG-* mutant binding was eliminated, suggesting that SdrG is the sole fibrinogen-binding moiety in *S. epidermidis* strain HB. However, only a 50% reduction in fibrinogen-binding was observed with the 9142Δ*sdrG* mutant in comparison to the wild type. These data suggested that a second fibrinogen binding protein may be expressed by strain 9142.

5.3 Discussion

In order to determine the conditions under which SdrG was expressed by *S. epidermidis*, Western immunoblotting was carried out on surface proteins isolated from strain HB grown under a number of different conditions. Antibodies had been raised against the recombinant A region of SdrG (McCrea *et al.*, 2000). Results showed that SdrG was only detected in protein samples isolated from strain HB when the cells were

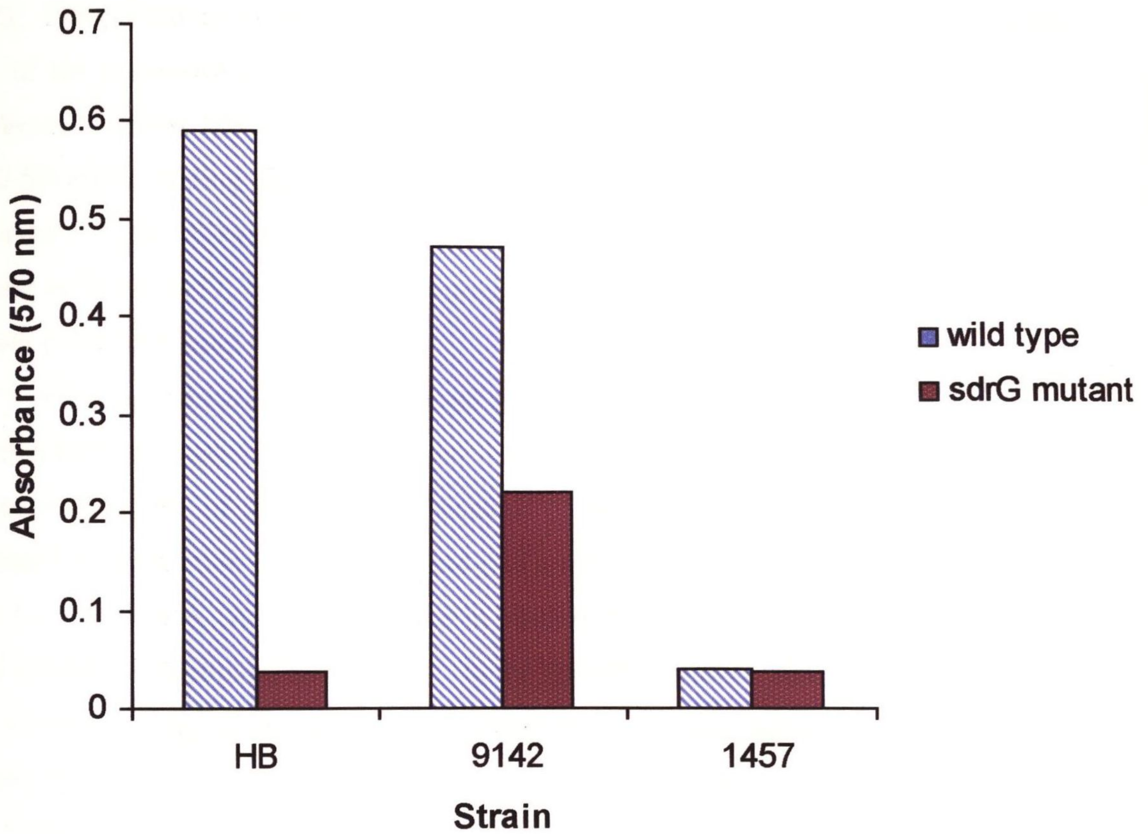


Figure 5.3 Adherence of *S. epidermidis* strains HB, 9142, 1457 and the corresponding *sdrG* mutant strains to immobilised fibrinogen. 10 μg/ml fibrinogen was immobilised on a 96-well ELISA plate and the ability of the *S. epidermidis* wild type and *sdrG* mutants to adhere was tested. This experiment was performed in triplicate with similar results.

grown to exponential phase in TSB with no shaking at 37°C. In strains HB and 9142 single immunoreactive bands of 190 kDa and 195 kDa were detected in the wild type samples, but not in the *sdrG* mutant samples, suggesting that this protein corresponded to SdrG. The difference in the sizes of these proteins is probably due to a difference in the size of the serine-aspartic acid dipeptide repeat region. DNA sequence data from *S. epidermidis* strains HB and K28 show that there is a large difference in the size of the *sdrG* SD repeat coding region present in the *sdrG* genes. Similar differences have been observed for other SD repeat containing proteins in *S. aureus*. Although the *sdrG* gene is present in strain 1457, SdrG protein expression was not detected in this strain under the growth conditions used.

Fibrinogen binding studies showed that both *S. epidermidis* strains HB and 9142 could adhere to immobilised fibrinogen. However, the level of binding was low in comparison to that of *S. aureus* and required (i) an increased number of cells, (ii) an increased concentration of fibrinogen and (iii) a more sensitive detection procedure than with *S. aureus* cells. A clear difference in binding was seen between the wild type and *sdrG* mutants with strains HB and 9142. In the HB*sdrG*- mutant, adherence to fibrinogen was eliminated, suggesting that SdrG was both necessary and sufficient for fibrinogen-binding in strain HB. However, in the 9142*sdrG*- mutant, adherence to fibrinogen was only reduced by 50%, suggesting that SdrG is not the only fibrinogen-binding protein that is expressed by this strain. The fact that strain 1457 did not adhere to fibrinogen agrees with expression data, which showed that SdrG was not expressed by this strain under the conditions used.

Chapter 6

Expression of ClfA, ClfB, SdrC, SdrD, SdrE and SdrG by *Lactococcus lactis*

6.1 Introduction

Phenotypic studies in *S. aureus* and other bacteria are often carried out by comparing the properties of a given strain with a defined isogenic mutant. However, identifying the phenotype of a surface protein in *S. aureus* can be difficult due to poor expression and the problem of redundancy that is often encountered. For example, there are at least two fibrinogen-binding proteins (ClfA and ClfB) and two fibronectin-binding proteins (FnBPA and FnBPB) present in most strains of *S. aureus* (Ni Eidhin *et al.*, 1998; Jonsson *et al.*, 1991). Recently, the situation was further complicated when it was discovered that the A domain of FnBPA could bind fibrinogen (Wann et al., 2000).

Surface presentation of heterologous molecules in Gram-positive bacteria has generated interest in the past number of years, with antigens (Medaglini *et al.*, 1995; Samuelson *et al.*, 1995), immunoglobulins (Gunneriusson *et al.*, 1996) and enzymes (Strauss and Gotz, 1996) being successfully displayed on the surface of different Gram-positive hosts. Heterologous expression of antigenic determinants on the surface of Gram-positive bacterial cells was first described in by Hansson *et al.* (1992) for *Plasmodium falciparum* and streptococcal antigens expressed on the surface of the coagulase negative bacterium *Staphylococcus xylosus* (Hansson *et al.*, 1992). *Staphylococcus carnosus* has also been used for expression of surface molecules (Samuelson *et al.*, 1995, Sinha *et al.*, 2000). Heterologous expression in the non-invasive bacterium, *Listeria innocua*, was used to show that the surface associated internalin molecule of *Listeria monocytogenes*, InlB, was both necessary and sufficient to promote bacterial internalisation (Braun *et al.*, 1999). However, the most widely used Gram-positive heterologous expression system is the food grade organism *Lactococcus lactis*. Several different surface proteins have been successfully expressed on the surface of this bacterium. These include the tetanus toxin fragment C (Wells, *et al.*, 1993), the *Streptococcus pyogenes* M6 protein (Piard *et al.*, 1997), the *Streptococcus gordonii* polypeptides, SspA and SspB (Holmes *et al.*, 1998), the *S. aureus* ClfA molecule (Que *et al.*, 2000) and the *S. aureus* FnBPA and FnBPB proteins (Sinha *et al.*, 2000). .

Most of these surrogate expression systems involve the use of multicopy plasmids. However, recently Stutzamann-Meier and co-workers showed heterologous

expression of the *S. aureus* proteins ClfA and coagulase, following single copy integration of the genes into the chromosome of *Streptococcus gordonii* (Stutzamann-Meier *et al.*, 2001). Here, the *clfA* and *coa* genes were placed under the control of a constituative *S. gordonii* promoter that provided expression of the heterologous proteins at levels similar to the native host. This was both novel and useful, as it provided controlled expression of the target genes from a regulated promoter. However, because *S. gordonii* is a pathogenic organism with ligand-binding activities, it is not an ideal surrogate host. The development of similar integration systems in other non-pathogenic bacteria such as *L. lactis* would provide a more useful method for studying the properties of heterologous proteins where over-expression of the protein is undesirable.

Lactococcus lactis subsp. *cremoris* MG1363 is a plasmid-free Gram-positive food grade organism, which is employed as a starter culture for the fermentation of food and as a food preservative and flavour enhancer (Gasson, 1983). The genetic systems of this bacterium are well-developed (Wells *et al.*, 1993), making *Lactococcus lactis* an appealing host for heterologous expression of surface proteins from other Gram-positive bacteria. In this study, the Sdr proteins of *S. aureus* (ClfA, ClfB, SdrC, SdrD, SdrE) and the SdrG protein from *S. epidermidis* were expressed on the surface of *L. lactis* MG1363. The object of this study was (i) to identify the size of each full-length mature staphylococcal protein by comparing expression data from the native and heterologous host and (ii) to assist in functional studies by overcoming the problem of redundancy encountered in *S. aureus* and *S. epidermidis*.

The *L. lactis* expression vector, pTREX1, was developed for heterologous expression of Gram-positive surface proteins (Wells & Schofield, 1996). It contains the *L. lactis* P1 promoter, which determines high-level constitutive expression of a target gene that is cloned in-frame with the expression cassette (Wells & Schofield, 1996). The vector used in this study, pKS80 (Hartford *et al.*, 2001), is a derivative of pTREX1, designed to increase the expression level of target genes. It contains the translation initiation region and the beginning of an ORF linked to a lactococcal phage promoter P11. This is translationally coupled to the start of the target gene in order to increase its translation efficiency. It also replaces the promoter, P1, with a stronger phage promoter, LPS2 (Figure 6.1). pKS80 exhibited a 7.5-fold increase in the expression level of a

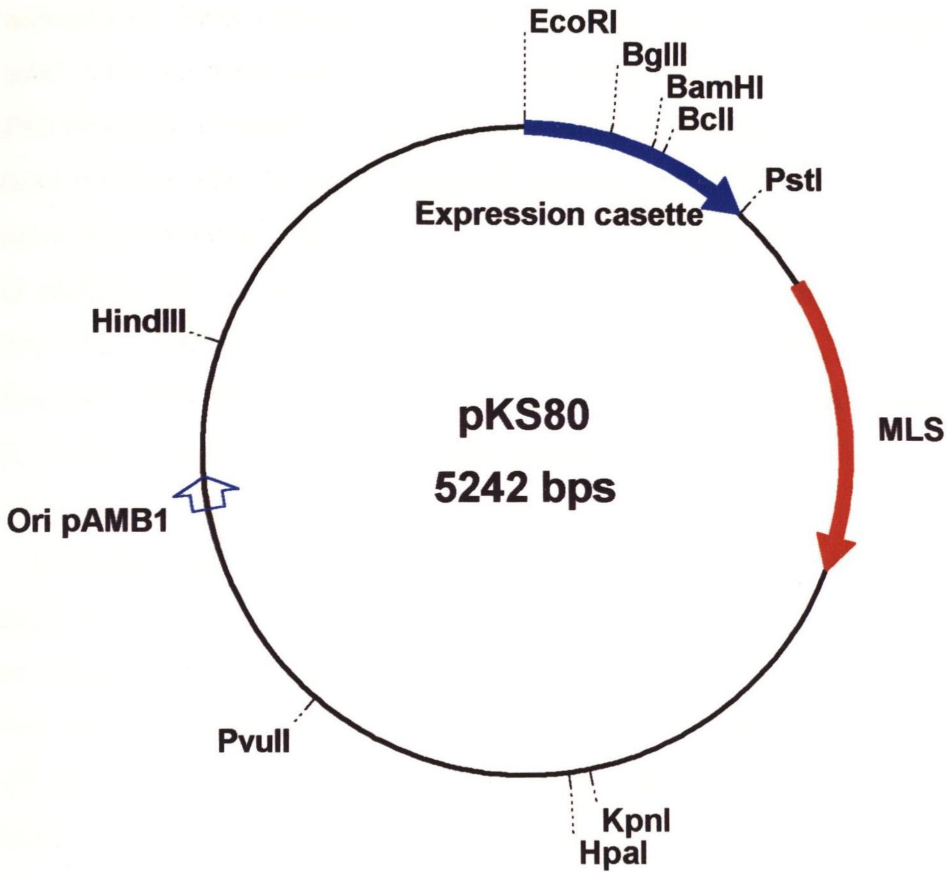


Figure 6.1 Map of the *L. lactis* expression plasmid pKS80, showing the expression casette, the MLS resistance determinant (which provides resistance to aminoglycoside antibiotics, including erythromycin) and the pAMB1 replication system. pKS80 is derived from the *L. lactis* plasmid pTREX-1. Details of the pKS80 expression casette are shown in Figure 6.2.

reporter gene, compared to pTREX1, in *L. lactis* (D. Litt, unpublished data). These vectors contain the pAMβ1 replicon (Simon and Chopin, 1988) which is not active in *E. coli*, necessitating direct cloning in *L. lactis*. The entire open reading frames of the *clfA, clfB, sdrC, sdrD, sdrE* and *sdrG* genes were each cloned into pKS80, under the control of the LPS2 promoter, and transformed, separately, into *L. lactis* MG1363 (Figure 6.2). The translated proteins were secreted, presumably through a Sec-dependent pathway, using the native staphylococcal signal sequence. *L. lactis* is known to express a number of surface proteins that are anchored to the cell wall peptidoglycan via the LPXTG motif, including the extracellular lactococcal protease, PrtP (Buist *et al.*, 1998). It was therefore likely that heterologous staphylococcal proteins, which also contain LPXTG motifs, would be anchored to the lactococcal cell wall.

Expression of each protein on the lactococcal cell surface was examined and compared to the profiles observed in the native staphylococcal host. Proteolysis of surface-expressed *S. aureus* proteins occurred in *L. lactis*, despite the fact that *L. lactis* MG1363 had been cured of the plasmid which encodes the major lactococcal protease, PrtP (Buist *et al.*, 1998). This was evident from previous studies, where ClfA was reported as a 130 kDa molecule when isolated from ClfA-expressing *L. lactis* cells and analysed by Western immunoblotting (Que *et al.*, 2000). The full-length ClfA molecule migrates with an apparent molecular weight of approximately 200 kDa (Hartford *et al.*, 1997), implying that the 130 kDa molecule observed by Que and co-workers is a proteolytic cleavage product of the mature ClfA protein. This cleavage must be due to another lactococcal protease, such as the extracellular serine protease, SK11, which is produced by *L. lactis* MG1363 (de Vos *et al.*, 1989). This was a consideration that was taken into account during this study when analysing expression of the staphylococcal proteins in *L. lactis*. N-terminal sequencing of *L. lactis*-expressed ClfA and SdrE proteins was performed to determine if proteolytic cleavage had occurred while the protein was expressed on the lactococcal cell surface. In addition, functional analysis of the heterologously expressed ClfA, ClfB and SdrG proteins was performed in order to confirm that these proteins behaved in the same manner as in their native hosts. As expected, ClfA, ClfB and SdrG each conferred fibrinogen-binding properties to *L. lactis*

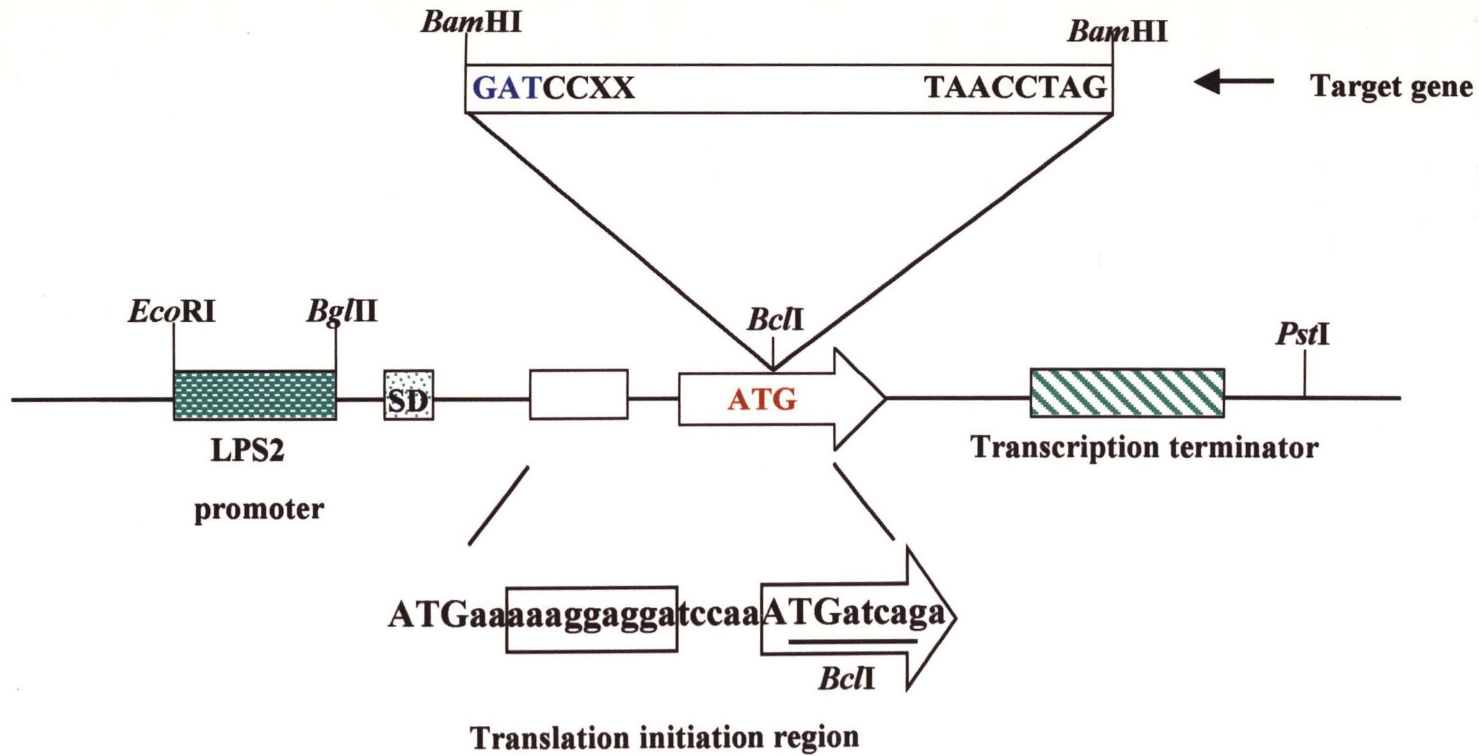


Figure 6.2 In-frame fusion of a target gene with the ATG codon located within the *Bcl*I site of the *L. lactis* plasmid pKS80 (indicated in red). When a *Bam*HI-cut PCR product is cloned into the *Bcl*I site of pKS80, the first 9 bases at the 5' end of the target gene sequence are ATGGATCCX. The resulting protein will contain 1 extra amino acid and 1 substituted amino acid at its N-terminus. Expression of the heterologous gene is driven by the lactococcal phage promoter LPS2. Translation initiation and transcription termination signals are provided by the vector

MG1363. Similar studies showed that SdrC, SdrD and SdrE did not bind to fibrinogen when expressed on the surface of *L. lactis*.

L. lactis cells expressing ClfA, ClfB, SdrC, SdrD, SdrE and SdrG were also tested for their ability to bind to a variety of matrix and plasma proteins (described in Chapter 8). In addition, the ability of *L. lactis*-expressed ClfA, ClfB, SdrC, SdrD and SdrE proteins to promote the aggregation of human platelets was investigated. This data is described in Chapter 7. This chapter describes the expression of ClfA, ClfB, SdrC, SdrD, SdrE and SdrG in *L. lactis* MG1363, functional analysis of *L. lactis*-expressed ClfA, ClfB and SdrG and N-terminal sequencing of *L. lactis*-expressed ClfA and SdrE.

6.2 Results

6.2.1. Cloning the *clfA*, *clfB*, *sdrC*, *sdrD*, *sdrE* and *sdrG* genes into pKS80 in *L. lactis* MG1363

6.2.1.1. *clfA*

To express the ClfA protein in *L. lactis*, the coding sequence of the *clfA* gene was amplified from *S. aureus* strain Newman chromosomal DNA by PCR, using *pfu* polymerase and the primers ClfAF4 and ClfAR4. This PCR product was then fused through the engineered *Bam*HI sites, present at both the 3' and 5' ends, to the *L. lactis* expression vector pKS80, as described in section 2.16. Insertion of the *clfA* gene at the *Bcl*I site of pKS80 resulted in an in-frame fusion between *clfA* and the ATG codon and initiation signals in the vector. Translational coupling was also provided (Figure 6.2). The entire ligation mixture (2 μg of the *Bam*HI digested PCR product ligated with 500 ng of the *Bcl*I digested vector) was transformed directly into *L. lactis* MG1363. The transformation efficiencies in *L. lactis* were substantially lower than that obtained when transforming electrocompetent *E. coli* cells, but higher than with *S. aureus*, resulting in approximately 20-50 colonies per transformation.

A phenotypic screening procedure was used to identify clones that contained the *clfA* gene inserted into the vector in the correct orientation. Each individual transformant

was grown for 18 hours in M17 broth containing 0.5% glucose and 5 μg/ml Em, and 10 μl of the culture was transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with antibodies specific to the unique A domain of ClfA and detected as described in section 2.15. Approximately 1:3 clones reacted strongly with the anti-ClfA antibodies, indicating that ClfA was expressed at high levels on the surface of *L. lactis*. *L. lactis* cells carrying the empty pKS80 vector or pKS80 containing the insert in the opposite orientation did not react with the anti-ClfA antibodies (Figure 6.3, (a)).

6.2.1.2 *clfB*

The full-length *clfB* gene was amplified from *S. aureus* Newman chromosomal DNA by PCR, cloned into the *Bcl*I site of pKS80, and transformed into *L. lactis* MG1363 by our collaborators (David Litt, University of Cambridge, UK.). This clone was provided and was analysed as part of this study.

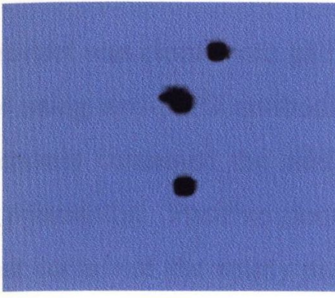
6.2.1.3 *sdrC*

The open reading frame of *sdrC* was amplified from the chromosome of *S. aureus* strain Newman using *pfu* polymerase and the primers sdrCF4 and sdrCR4, incorporating a *Bam*HI site into the 3' and 5' ends of the PCR product. The PCR product was cloned into pKS80 as described in section 5.2.1.1. Clones that expressed the SdrC protein on their cell surface (approx. 1:3) were identified by whole cell dot blot using antibodies specific to the A region of SdrC. This was carried out as described for ClfA in section 5.2.1.1. The anti-SdrC antibodies did not react with *L. lactis* MG1363 carrying the empty pKS80 plasmid or the plasmid with the insert in the opposite orientation(Figure 6.3, (b)).

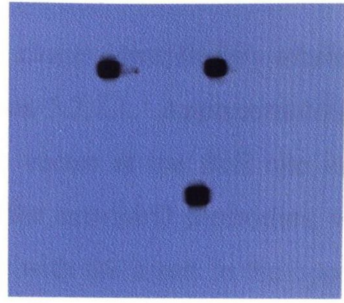
6.2 1 4 *sdrD*

The full-length *sdrD* gene was amplified by PCR, using *pfu* polymerase, with *S. aureus* Newman chromosomal DNA as a template. Engineered *Bam*HI sites were incorporated into the 3' and 5' ends of the primers sdrDF4 and sdrDR4. The resulting

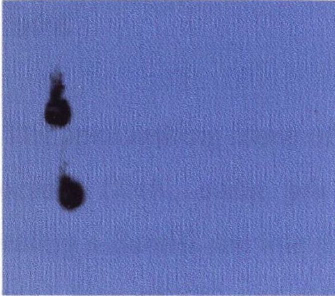
6.3 (a) ClfA



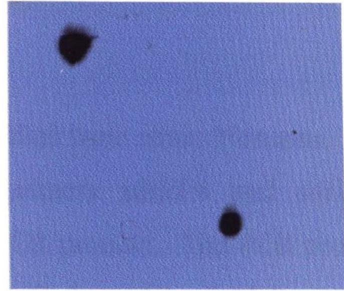
6.3 (b) SdrC



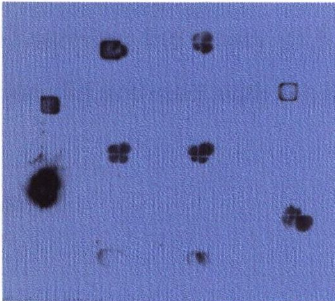
6.3 (c) SdrD



6.3 (d) SdrE



6.3 (e) SdrG (HB)



6.3 (f) SdrG (K28)

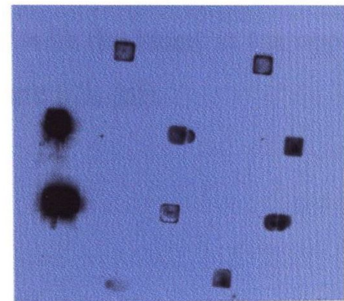


Figure 6.3 (a)-(f) Identification of *S. aureus* proteins expressed on the surface of *L. lactis* MG1363 by whole cell immunoblotting. Transformants from each cloning experiment were immobilised on a nitrocellulose membrane and the membrane incubated with antibodies recognising the unique A region of each protein. Interactions between *L. lactis* cells expressing heterologous proteins and the corresponding antibody were detected by incubation with protein A peroxidase followed by chemiluminescence.

PCR product was cloned into pKS80 and SdrD-expressing clones identified by whole cell dot blot using anti-SdrD antibodies, as described in section 5.2.1.1. Approximately 1:5 transformants contained the *sdrD* gene inserted into the vector at the *Bcl*I site in the correct orientation. Positive clones reacted strongly with the anti-SdrD antibodies, while cells that contained the empty pKS80 vector or the vector with the insert in the opposite orientation showed no reaction (Figure 6.3, (c)).

6.2.1.5 *sdrE*

The open reading frame of the *sdrE* gene was amplified from strain Newman chromosomal DNA, using *pfu* polymerase and the primers sdrEF4 and sdrER4, incorporating a *Bam*HI site into the 3' and 5' ends of the PCR product. The PCR product was cloned into pKS80 and SdrE-expressing cells identified as described in section 5.2.1.1. Positive clones were obtained at a rate of 1:5. A strong reaction was observed for the clones that expressed the SdrE protein on their cell surface, while *L. lactis* MG1363 carrying the empty pKS80 plasmid or the vector with the insert in the opposite orientation did not react with the anti-SdrE antibodies (Figure 6.3, (d)).

6.2.1.6 *sdrG*

The open reading frame of the *sdrG* gene was amplified from *S. epidermidis* strains HB and K28 chromosomal DNA, using *pfu* polymerase and the primers sdrGF4 and sdrGR4, incorporating *Bam*HI sites at the 3' and 5' ends of the PCR product. The PCR products were cloned into pKS80 and SdrG-expressing cells identified as described in section 5.2.1.1. Positive clones were obtained at a rate of 1:5 for strain K28 and 1:10 for strain HB. These clones were identified by whole cell dot blot using antibodies specific to the A domain of SdrG. A small amount of cross-reaction was observed with these anti-SdrG antibodies against the wild type *L. lactis* cells carrying the empty pKS80 vector. However, a clear difference between positive and negative clones was distinguishable (Figures 6.3, (e) & (f)), allowing unambiguous identification of SdrG-expressing clones.

6.2.2 Quantification of heterologous protein expression in *L. lactis* MG1363 by Western whole cell immunoblotting

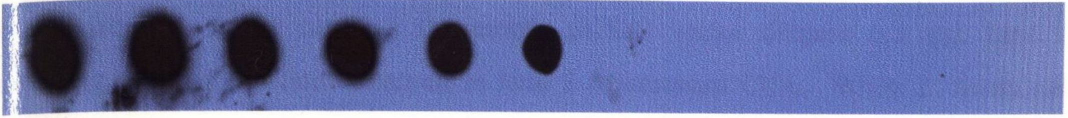
In order to determine the approximate level of protein expression provided by pKS80 in *L. lactis* MG1363, a series of whole cell Western immunoblots were carried out. *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD, and SdrE were grown to stationary phase, washed in PBS and adjusted to an OD_{600nm} of 1.0. Each suspension of cells was then serially diluted two-fold and 10 µl of each dilution immobilised on a nitrocellulose membrane. Each membrane was blocked and incubated with antibodies that recognised the unique A domain of each individual protein. Reactions with these antibodies were detected as described in section 2.15. It was observed that native surface proteins, such as ClfA, SdrC and SdrD cannot be detected on the surface of *S. aureus* by whole cell dot-blot, presumably, due to low levels of expression. Scatchard analysis has shown that certain surface protein molecules, such as the uncharacterised proteins responsible for binding to human virtonectin, are displayed on the surface of *S. aureus* strain V8 with a copy number of between 260-5240 (Liang *et al.*, 1993). This is clearly below the limit of detection for cell immunoblotting. However, in *L. lactis*, heterologous proteins were detected when cells were diluted between 1:32 and 1:512 (Figure 6.4). This suggested that expression levels of heterologous *S. aureus* proteins in *L. lactis,* from within pKS80, was 30- to 500-fold greater than in *S. aureus*.

6.2.3 Expression of *S. aureus* proteins on the surface of *L. lactis* MG1363.

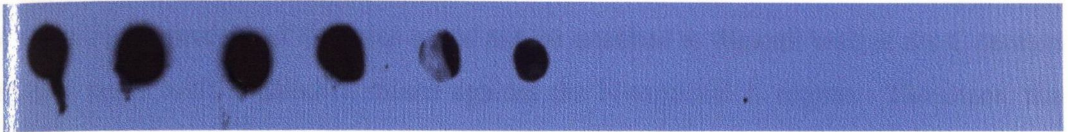
6.2.3.1 ClfA

Cell surface-associated proteins were released from stabililsed *L. lactis* protoplasts carrying (i) pKS80:*clfA* and (ii) pKS80, by lysozyme/mutanolysin digestion, as described in section 2.17. These proteins were separated by SDS-PAGE and analysed by Western immunoblotting, as described in section 2.15, using specific affinity-purified antibodies recognising the A domain of ClfA. The protein fraction isolated from *L. lactis*

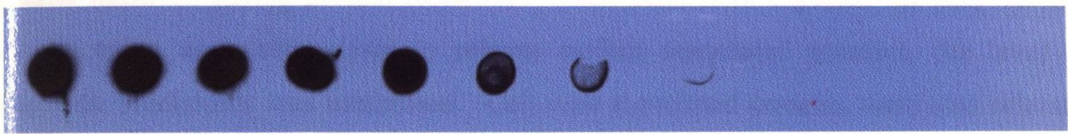
ClfA



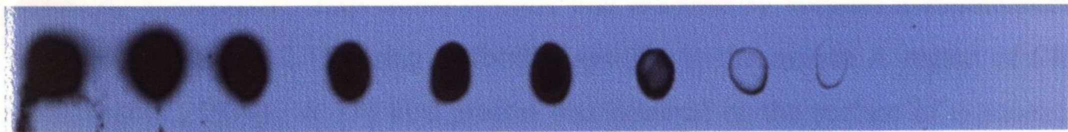
ClfB



SdrC



SdrD



SdrE

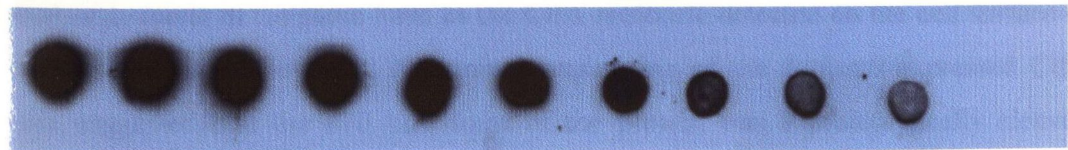


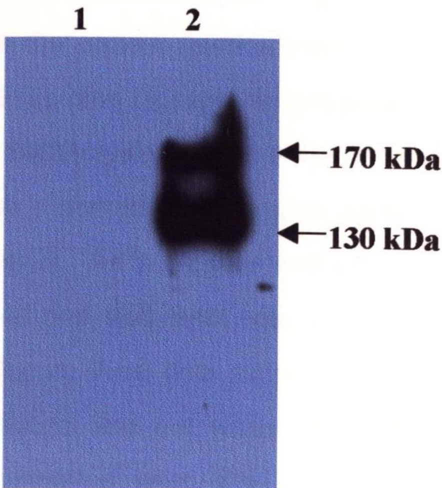
Figure 6.4 Quantification of *S. aureus* protein expression in *L. lactis* MG1363. A fixed number of cells were serially diluted 2-fold up to a dilution of 1/1024 and immobilised on a nitrocellulose membrane. Membranes were incubated with antibodies specific to the unique A region of each *S. aureus* protein and reactions detected by incubation with protein A-peroxidase and chemiluminescence.

carrying pKS80:*clfA* contained two immunoreactive proteins of 170 kDa and 130 kDa (Figure 6.5 (a), lane 2). No immunoreactive band was observed in the protein fraction isolated from *L. lactis* carrying the empty pKS80 vector (Figure 6.5 (a), lane 1), suggesting that the proteins observed in lane 2 represented ClfA. When *L. lactis* cells expressing ClfA were digested with mutanolysin/lysozyme for 60 min, the 170 kDa and 130 kDa bands were not observed. Instead, a band of 40 kDa was detected (Figure 6.5 (b), lane 2). A protein of this size could not be attached to the cell wall at the C-terminus and still react with antibodies raised against the N-terminal A region. Therefore, these data suggested that proteolytic cleavage of *S. aureus* proteins expressed on the surface of *L. lactis* occurred during protein isolation. When cells were digested for only 15 min with lysozyme and mutanolysin to release surface associated proteins, the level of proteolytic breakdown was minimised. Cell wall associated proteins were also released from *S. aureus* strain Newman protoplasts generated by lysostaphin digestion and separated by SDS-PAGE. Expression of ClfA was analysed by Western immunoblotting as described in section 2.15, using antibodies specific to the unique A region of ClfA (McDevitt *et al.*, 1994). A 170 kDa protein was detected on the surface of exponential phase cells (Figure 6.5 (c), lane 2). These data suggested that ClfA was expressed on the surface of *L. lactis* in the same form as the ClfA molecule detected on the cell surface of *S. aureus* Newman. However, N-terminal sequencing of the *L. lactis*-expressed ClfA protein suggested that the 170 kDa form of the protein was a proteolytically cleaved truncate of the full-length mature molecule (this data is described in detail in section 6.2.5).

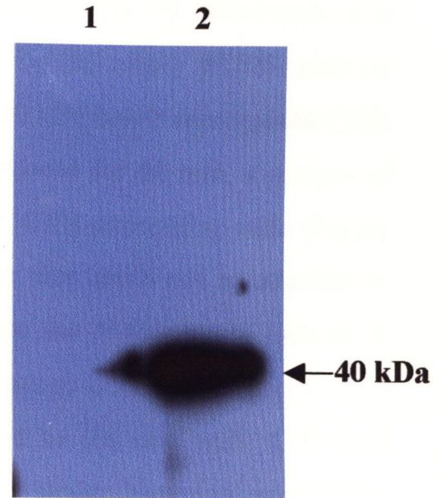
6.2.3.2 ClfB

Cell surface associated proteins were released from stabilised *L. lactis* protoplasts carrying (i) pKS80:*clfB* and (ii) pKS80, by mutanolysin/lysozyme digestion, as described in section 2.17. The released proteins were separated by SDS-PAGE and analysed by Western immunoblotting, as described in section 2.15, using polyclonal antibodies specific to the unique A region of ClfB. Following mutanolysin/lysozyme digestion for 15 min, two immunoreactive bands of 150 kDa and 120 kDa were observed in the protein

6.5 (a)



6.5 (b)



6.5 (c)

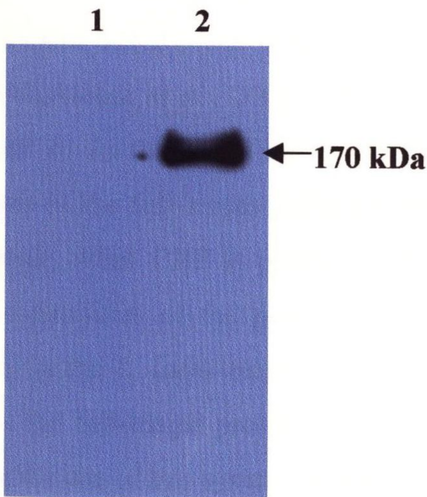


Figure 6.5 Expression of ClfA on the surfce of *L. lactis* MG1363. 6.5 (a); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*clfA* (lane 2), following a 15 min digest with mutanolysin/lysozyme. 6.5 (b); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*clfA* (lane 2), following a 60 min digest with mutanolysin/lysozyme. 6.5 (c); lane 1; cell wall associated proeins isolated from Newman *clfA* mutant cells grown to exponential phase. Lane 2; cell wall-associated proteins isolated from wild type Newman cells grown to exponential phase. Blots were probed with affinity-purified antibodies recognising the unique A region of ClfA. This is a representative of 2 different experiments.

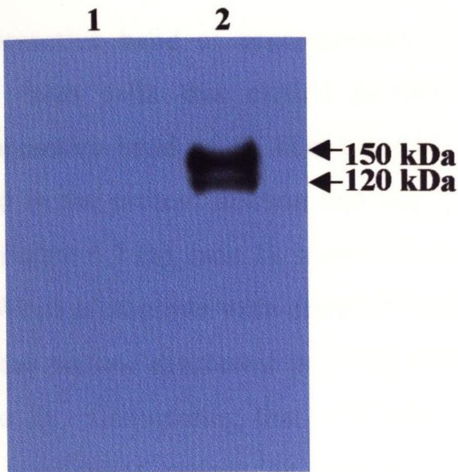
fraction released from cells carrying pKS80:*clfB* (Figure 6.6 (a), lane 2). No bands were detected in the protein fraction released from cells carrying the empty pKS80 plasmid (Figure 6.6 (a), lane 1), suggesting that the 150 kDa and 120 kDa bands represented ClfB. When the mutanolysin/lysozyme digest was allowed to proceed for 60 min, a protein of 45 kDa was observed in the protein fraction isolated from ClfB-expressing cells (Figure 6.6 (b), lane 2). As is the case with ClfA, a protein of this size could not be attached to the cell wall and still react with antibodies that recognise the N-terminally located A domain. Again, these data suggested that proteolytic cleavage occurred during surface protein isolation and not whilst the protein was attached to the cell wall. Cell wall associated proteins were also released from *S. aureus* strain Newman protoplasts generated by lysostaphin digestion and separated by SDS-PAGE. Expression of ClfB was analysed by Western immunoblotting as described in section 2.15, using antibodies specfic to the unique A region of ClfB (Ní Eidhin *et al.*, 1998). ClfB was expressed on the surface of *S. aureus* Newman as a 150 kDa protein during the exponential phase of growth (Figure 6.6 (c), lane 2) and this is thought to represent the full-length form of the protein (McAleese *et al.*, 2001). These data suggested that ClfB was expressed on the surface of *L. lactis* in its full-length form (150 kDa molecule). Some proteolytic breakdown of the full-length molecule occurred, where a 120 kDa protein was detected. In *S. aureus*, when ClfB is proteolytically cleaved to the 120 kDa form, the fibrinogen binding capabilities of the protein are eliminated (McAleese *et al.*, 2001). A similar truncation in the *L. lactis*-expressed protein probably leads to a similar loss in function. However, the full-length protein is present in large quantities on the surface of *L. lactis*, justifying the use of this system for functional studies,

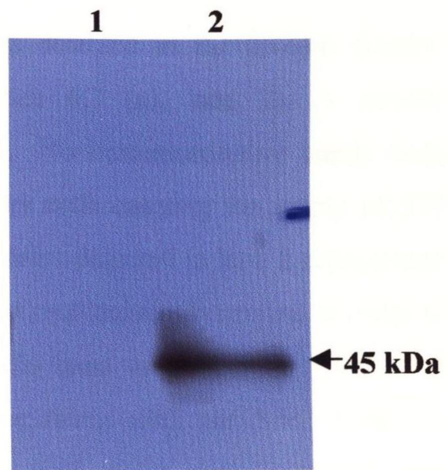
6.2.3.3 SdrC

Cell surface associated proteins were released from stabilised *L. lactis* protoplasts carrying (i) pKS80:*sdrC* and (ii) pKS80, by mutanolysin/lysozyme digestion, as described in section 2.17. The released proteins were separated by SDS-PAGE and analysed by Western immunoblotting, as described in section 2.15, using specific affinity-purified antibodies directed towards the unique A region of SdrC. A dominant

6.6 (a)

6.6 (b)





6.6 (c)

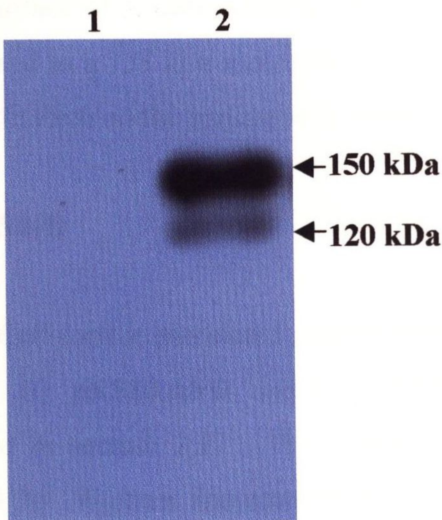


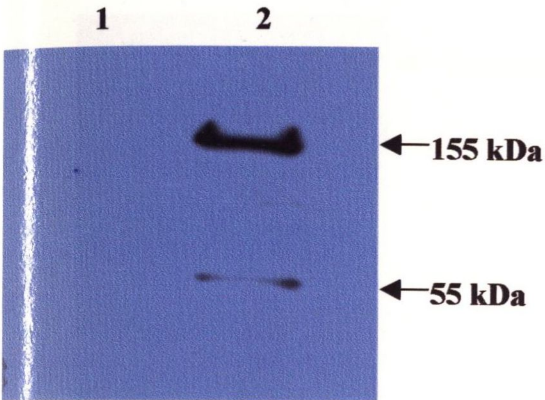
Figure 6.6 Expression of ClfB on the surface of *L. lactis* MG1363. 6.6 (a); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*clfB* (lane 2), following a 15 min digest with mutanolysin/lysozyme. 6.6 (b); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*clfB* (lane 2), following a 60 min digest with mutanolysin/lysozyme. 6.6 (c); lane 1; cell wall-associated proteins isolated from Newman *clfB* mutant cells grown to exponential phase. Lane 2; cell-wall associated proteins isolated from wild type Newman cells grown to exponential phase. Blots were detected using antibodies recognising the unique A domain of ClfB. This is a representative of 2 different experiments.

immunoreactive band of approximately 155kDa was detected in the protein fraction isolated from cells that carried pKS80:*sdrC* (Figure 6.7 (a), lane 2). A smaller immunoreactive band of 55 kDa was also observed. No immunoreactive bands were observed in the protein fraction that was isolated from cells carrying the empty pKS80 vector (Figure 6.7 (a), lane 1), suggesting that the proteins detected in lane 2 represented SdrC. When protoplasts were digested for 60 min with mutanolysin/lysozyme in order to release the surface associated proteins, only the 55 kDa band was observed (Figure 6.7 (b), lane 2). Considering that a 55 kDa protein that reacts with anti-SdrC A region specific antibodies cannot be attached to the cell wall, these data suggested that proteolytic cleavage of the full-length molecule occurred during isolation of the cell wall-associated proteins and not whilst they were attached to the cell wall. Expression of SdrC on the surface of *S. aureus* is described in Chapter 4, where the full-length SdrC protein is proposed as a 155 kDa molecule. These data suggested that SdrC was expressed in its full-length form on the surface of *L. lactis*.

6.2.3.4 SdrD

Cell surface associated proteins were released from stabilised *L. lactis* protoplasts carrying (i) pKS80:*sdrD* and (ii) pKS80, by mutanolysin/lysozyme digestion, as described in section 2.17. The released proteins were separated by SDS-PAGE and analysed by Western immunoblotting, as described in section 2.15, using specific affinity-purified antibodies directed towards the unique A region of SdrD. A single immunoreactive protein of 205 kDa was detected in the cell wall-associated protein fraction isolated from cells carrying pKS80:*sdrD* (Figure 6.8 (a), lane 2). No band was detected in the protein fraction isolated from cells that carried the empty pKS80 vector (Figure 6.8 (a), lane 1), suggesting that the 205 kDa band observed in lane 2 represented SdrD. When the mutanolysin/lysozyme digest was allowed to proceed for 60 min, two proteins of 52 kDa and 40 kDa were observed in the protein fraction isolated from SdrD-expressing cells (Figure 6.8 (b), lane 2). As is the case with ClfA, ClfB and SdrC, proteins of this size could not be attached to the cell wall and still react with antibodies that recognise the N-terminally located A domain of SdrD. Again, these data suggested

6.7 (a)



6.7 (b)

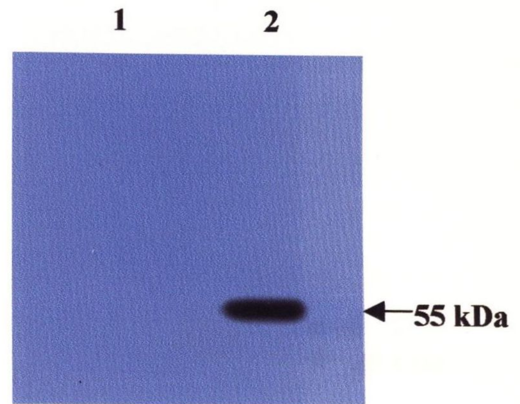
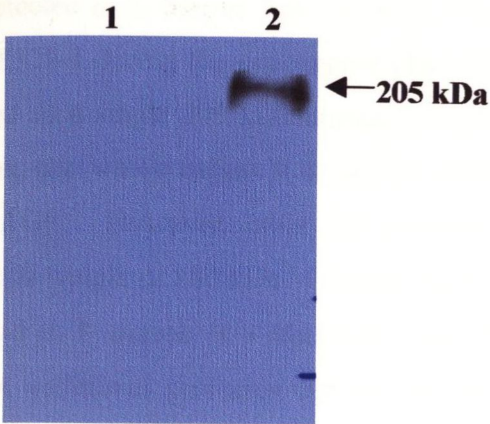


Figure 6.7 Expression of SdrC on the surface of *L. lactis* MG1363. 6.7 (a); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*sdrC* (lane 2), following a 15 min digest with mutanolysin/lysozyme. 6.7 (b); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*sdrC* (lane 2), following a 1hour digest with mutanolysin/lysozyme. This is a representative of 2 different experiments.



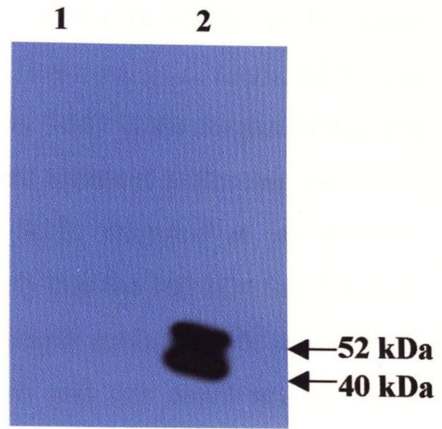


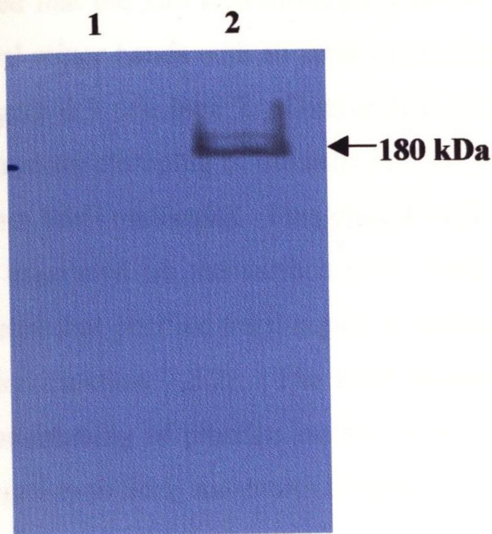
Figure 6.8 Expression of SdrD on the surfce of *L. lactis* MG1363. 6.8 (a); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*sdrD* (lane 2), following a 15 min digest with mutanolysin/lysozyme. 6.8 (b); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*sdrD* (lane 2), following a 1hour digest with mutanolysin/lysozyme. Blots were detected using affinity-purified antibodies recognising the unique A domain of SdrD. This is a representative of 2 different experiments.

that proteolytic cleavage of heterologous proteins occurred during surface protein isolation and not whilst the protein was attached to the cell wall. Expression of SdrD on the surface of *S. aureus* cells is described in Chapter 4. The largest form of SdrD that was detected in *S. aureus* was 160 kDa, which was present on the surface of *S. aureus* strain 8325-4 during the exponential phase of growth. However, in *L. lactis*, SdrD was detected as a single 205 kDa immunoreactive band. As SdrD is the largest of the Sdr proteins, one would expect it to migrate with the highest apparent molecular weight on SDS-PAGE. However, other Sdr proteins such as SdrE, migrated at an apparent molecular weight of 180 kDa. Considering this, it is likely that the 160 kDa SdrD protein observed in *S. aureus* is a truncated from of the full-length molecule. *S. aureus* may produce additional proteases that are absent in *L. lactis* and this could account for a proteolytic cleavage event that occurs in *S. aureus*, but not in *L. lactis*. These data suggested that SdrD was expressed on the surface of *L. lactis* in its full-length form.

6.2.3.5 SdrE

Cell surface associated proteins were released from stabilised *L. lactis* protoplasts carrying (i) pKS80:*sdrE* and (ii) pKS80, by mutanolysin/lysozyme digestion, as described in section 2.17. The released proteins were separated by SDS-PAGE and analysed by Western immunoblotting, as described in section 2.15, using polyclonal antibodies specific to the unique A region of SdrE. A 180 kDa immunoreactive protein was released from *L. lactis* cells that carried pKS80:*sdrE* (Figure 6.9 (a), lane 2). This band was not observed in the cell wall-associated protein fraction isolated from cells carrying the empty pKS80 vector (Figure 6.9 (a), lane 1), suggesting that the 180 kDa band seen in lane 2 represented SdrE. Following 60 min digestion with mutanolysin/ lysozyme, two bands of 70 kDa and 50 kDa were detected in the cell wall-associated protein fraction isolated from SdrE-expressing cells (Figure 6.9 (b), lane 2). Again, these data suggested that proteolytic cleavage of heterologous proteins occurred in *L. lactis* during protein isolation, and not whilst the protein was attached to the cell wall. Expression of SdrE in *S. aureus* strain Newman is described in Chapter 4. SdrE was

6.9 (a)



6.9 (b)

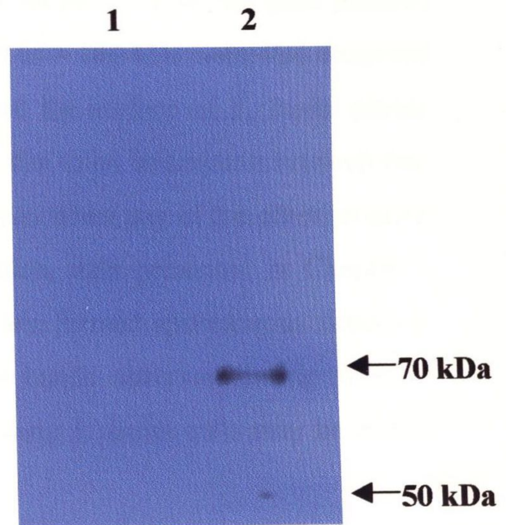


Figure 6.9 Expression of SdrE on the surfce of *L. lactis* MG1363. 6.9 (a); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*sdrE* (lane 2), following a 15 min digest with mutanolysin/lysozyme. 6.9 (b); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*sdrE* (lane 2), following a 1hour digest with mutanolysin/lysozyme. Blots were detected using antibodies specific to the A domain of SdrE. This is a representative of 2 different experiments.

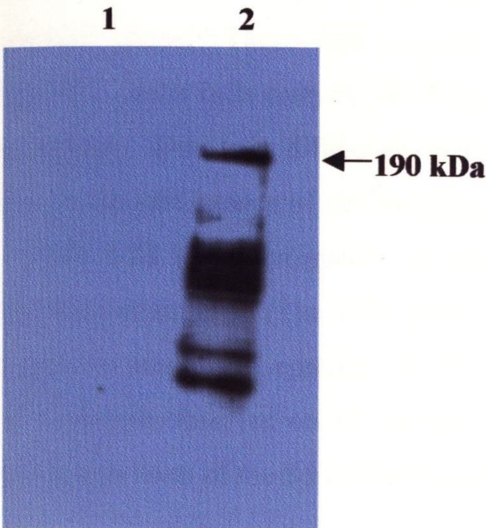
expressed on the surface of *S. aureus* Newman as a 180 kDa molecule during exponential phase. N-terminal sequencing of the *L. lactis*-expressed SdrE protein (section 6.2.5) showed that the 180 kDa molecule was the full-length mature form of the SdrE protein. Several minor bands with an apparent molecular weight of > 180 kDa were also observed in Figure 6.9 (a), lane 2. Over-expression of SdrE on the surface of *L. lactis* causes spontaneous clumping of the cells in PBS, presumably due to an interaction between two or more SdrE molecules. This phenomenon was not seen when any of the other proteins were expressed on the surface of *L. lactis*. In addition, data presented in Chapter 7 indicated that purified SdrE region A recombinant protein formed spontaneous dimers in solution (section 7.2.2). The high molecular weight bands observed during Western immunoblotting of proteins isolated from SdrE-expressing *L. lactis* cells may be due to aggregation of SdrE molecules in solution.

6.2.3.6 SdrG

Cell surface associated proteins were released from stabilised *L. lactis* protoplasts carrying (i) pKS80:*sdrG* cloned from strain HB, (ii) pKS80:*sdrG* cloned from strain K28 and (iii) pKS80, by mutanolysin/lysozyme digestion, as described in section 2.17. The released proteins were separated by SDS-PAGE and analysed by Western immunoblotting, as described in section 2.15, using polyclonal antibodies specific to the unique A region of SdrG. A 190 kDa immunoreactive protein was detected in the cell wall-associated protein fraction isolated from *L. lactis* cells carrying pKS80:*sdrG* (HB) (Figure 6.10 (a), lane 2). In addition, a substantial amount of proteolytic breakdown was observed with the SdrG (HB) protein, despite a short incubation with mutanolysin/lysozyme during isolation of surface proteins. A 170 kDa immunoreactive protein was present in the cell wall-associated proteins isolated from *L. lactis* cells carrying pKS80:*sdrG* (K28) (Figure 6.10 (b), lane 2). As seen with the SdrG (HB) protein, a large amount of proteolytic degradation was observed with the SdrG (K28) protein. However, no immunoreactive bands were detected in the protein fractions isolated from *L. lactis* cells carrying the empty pKS80 vector (Figures 6.10 (a) & (b), lane 1), suggesting that the proteins observed in figure 6.10 (a) lane 2 and figure 6.10 (b) lane

6.10 (a)

6.10 (b)



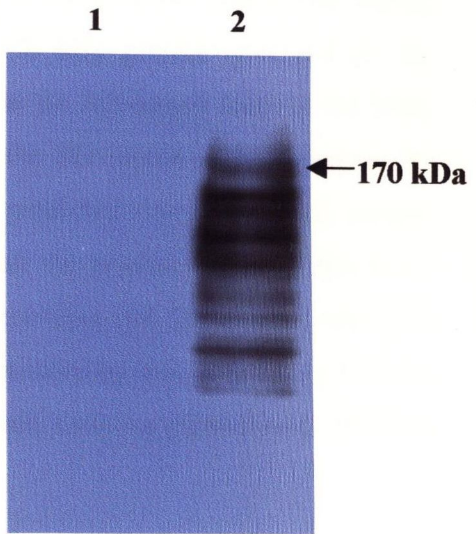


Figure 6.10 Expression of SdrG on the surfce of *L. lactis* MG1363. 6.10 (a); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*sdrG* cloned from strain HB (lane 2), following a 15 min digest with mutanolysin/lysozyme. 6.10 (b); cell wall-associated proteins were isolated from *L. lactis* cells carrying pKS80 (lane 1) and pKS80:*sdrG* cloned from strain K28 (lane 2), following a 15 min digest with mutanolysin/lysozyme. Blots were detected with antibodies recognising the unique A domain of SdrG. This is a representative of two different experiments.

2 represented SdrG. Expression of SdrG on the surface of *S. epidermidis* strain HB is described in Chapter 5. Because no *sdrG* mutant in *S. epidermidis* strain K28 was available, expression of SdrG on the surface of strain K28 was not studied. SdrG was expressed as a 190 kDa protein on the surface of *S. epidermidis* strain HB during exponential phase. These data suggested that the 190 kDa protein observed on the surface of *L. lactis* cells carrying pKS80:*sdrG* (HB) was the full-length form of the SdrG (HB) protein. Due to a difference in size between the SD repeat regions (region R) present in the *sdrG* genes of strains HB and K28, the predicted size of the SdrG protein from strain K28 is approximately 19 kDa smaller than the predicted size of the SdrG protein from strain HB. (The *sdrG* gene in strain HB contains 108 SD repeats, while the *sdrG* gene in strain K28 contains 28 SD repeats). Considering this, it is likely that the 170 kDa protein observed on the surface of *L. lactis* cells carrying pKS80:*sdrG* (K28) is the full-length form of the SdrG (K28) protein.

6.2.4 Fibrinogen binding properties of *L. lactis* and *S. aureus*/*S. epidermidis*-expressed ClfA, ClfB, SdrC, SdrD, SdrE and SdrG proteins

6.2.4.1 Adherence of *L. lactis* and *S. aureus* expressing ClfA or ClfB proteins to immobilised fibrinogen

It has been well documented that both ClfA and ClfB can promote bacterial adherence to immobilised fibrinogen (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998). This test was designed to determine if the *L. lactis*-expressed ClfA and ClfB proteins could bind to fibrinogen in the same manner. *L. lactis* strains carrying the empty pKS80 vector or the ClfA- and ClfB-expressing plasmids were grown to stationary phase. *S. aureus* Newman cells containing mutations in (i) the *clfB* gene (expressing ClfA alone), (ii) the *clfA* gene (expressing ClfB alone) and (iii) the *clfA* and *clfB* genes (expressing neither ClfA nor ClfB) were grown to exponential phase. Human fibrinogen was serially diluted 2-fold from a starting concentration of 10 μg/ml and used to coat a 96-well ELISA plate. Adherence of the above strains to immobilised fibrinogen was measured as described in section 2.19.1. The Newman*clfA*- and Newman*clfB*- mutant strains and the *L. lactis* ClfA

and ClfB-expressing strains bound to fibrinogen in a dose-dependent manner (Figure 6.11). No adherence to fibrinogen was observed with the Newman*clfA−clfB-* double mutant strain or with the *L. lactis* strain carrying the empty pKS80 vector. ClfA-dependent fibrinogen binding was higher than ClfB-dependent fibrinogen binding at fibrinogen concentrations > 1 mg/ml. This was true for both *L. lactis* and *S. aureus*. However, *L. lactis*-expressed ClfA and ClfB proteins demonstrated a higher affinity for fibrinogen than the corresponding *S. aureus*-expressed proteins. This is probably due to the higher levels of protein expression in *L. lactis*, compared with *S. aureus*. This assay showed that both ClfA and ClfB could promote binding to fibrinogen when expressed on the surface of *L. lactis*.

6.2.4.2 Adherence of the *L. lactis*-expressed SdrG proteins to immobilised fibrinogen.

Data presented in Chapter 5 showed that SdrG was necessary for fibrinogen binding in *S. epidermidis* strain HB. This assay was designed to demonstrate that SdrG was sufficient for promoting fibrinogen-binding when expressed in *L. lactis*. It was observed that SdrG-mediated binding of *S. epidermidis* cells to immobilised fibrinogen was weaker than ClfA-mediated adherence of *S. aureus* cells to immobilised fibrinogen. It was unclear if this was due to a lower affinity of the SdrG protein for fibrinogen of to other factors, such as lower protein expression levels or antigenic masking by capsular polysaccharide/slime in *S. epidermidis*. Because SdrG was expressed in the same host and from the same pKS80 promoter in *L. lactis*, it was likely that both proteins were expressed at similar levels. This was substantiated by whole cell dot blot analysis where similar expression levels were observed for both ClfA and SdrG in *L. lactis*. It was therefore possible to compare the affinity of SdrG for fibrinogen with that of ClfA when expressed on the lactococcal cell surface. *L. lactis* strains carrying the empty pKS80 vector and the ClfA and SdrG-expressing plasmids were grown to stationary phase. Human fibrinogen was serially diluted 2-fold from a starting concentration of 10 μg/ml and used to coat a 96-well ELISA plate. Adherence of *L.lactis* cells expressing ClfA, SdrG(HB) and SdrG(K28) to immobilised fibrinogen was measured as described in

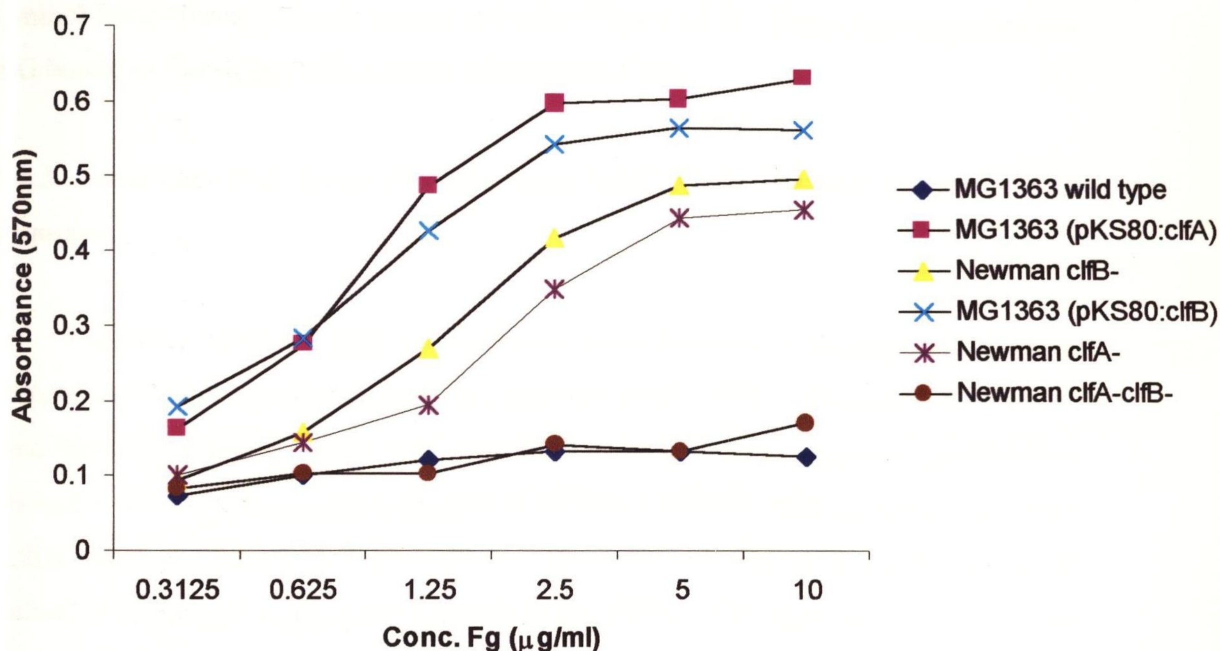


Figure 6.11 Adherence of ClfA and ClfB-expressing *L. lactis* and *S. aureus* cells to immobilised fibrinogen. A 96- well ELISA plate was coated with serial 2-fold dilutions of fibrinogen with a starting concentration of 10 μg/ml. A fixed number of bacterial cells were incubated with the plates, stained with crystal violet and adherence measured in an ELISA plate reader at 570 nm. This is a representative of 2 different experiments.

section 2.19.1. Figure 6.12 shows that both the ClfA and SdrG-expressing cells bound to fibrinogen in a dose-dependent manner, while the *L. lactis* cells carrying the pKS80 vector did not adhere. ClfA-mediated adherence to fibrinogen was approximately 3-fold higher than SdrG-mediated fibrinogen adherence at fibrinogen concentrations > 1 mg/ml. However, when expressed on the surface of *L. lactis*, SdrG (HB) and SdrG (K28) bound to immobilised fibrinogen with similar affinities (Figure 6.12). These data suggested that SdrG bound to fibrinogen with a lower affinity than ClfA.

6.2.4.3 Adherence of *L. lactis* cells expressing SdrC, SdrD and SdrE to immobilised fibrinogen.

The SdrC, SdrD and SdrE proteins have some sequence homology and a similar structural organisation to ClfA and ClfB (Josefsson *et al.*, 1998). However, the putative ligand binding A regions share only 21-30% homology in any pairwise combination. Only one stretch of amino acids is present in all five A regions. The consensus sequence for this motif is T/IY/FT/VFTD/NYVD/N. However, this region is not thought to be involved in fibrinogen binding (O' Connell *et al.*, 1998). This experiment was designed to show whether any of the Sdr proteins could bind to immobilised fibrinogen. ClfA, SdrC, SdrD and SdrE-expressing *L. lactis* cells and *L. lactis* carrying pKS80 were grown to stationary phase. Human fibrinogen, 10 μg/ml was used to coat a 96-well ELISA plate. Adherence of *L.lactis* cells expressing ClfA, SdrC, SdrD and SdrE to immobilised fibrinogen was measured as described in section 2.19.1. While the ClfA-expressing *L. lactis* strain adhered to fibrinogen, no adherence was observed with any of the other strains (Figure 6.13). These data indicated that SdrC, SdrD and SdrE did not bind to fibrinogen.

6.2.4.4. Cation inhibition of ClfA and ClfB-mediated adherence to immobilised fibrinogen

The ability of both ClfA and ClfB to bind to fibrinogen is dependent on Ca^{2+} concentration (O'Connell *et al.*, 1998, Ní Eidhin *et al.*, 1998). In *S. aureus*, the binding

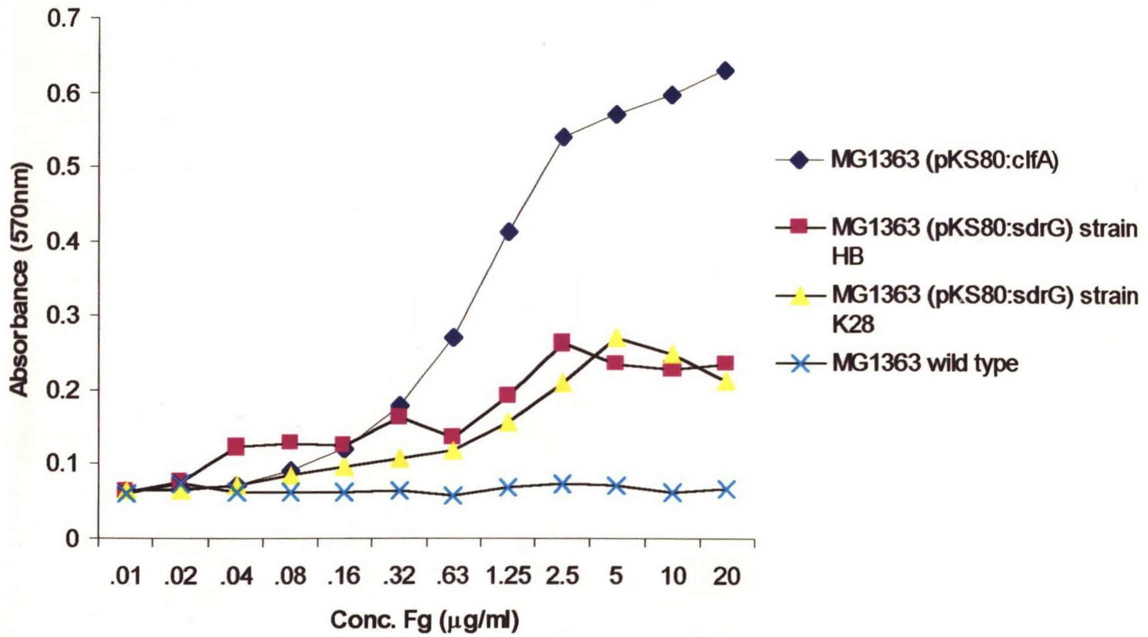


Figure 6.12 Adherence of SdrG(HB), SdrG(K28) and ClfA-expressing *L. lactis* cells to immobilised fibrinogen. A 96- well ELISA plate was coated with serial 2-fold dilutions of fibrinogen with a starting concentration of 20 µg/ml. A fixed number of bacterial cells were incubated with the plates, stained with crystal violet and adherence measured in an ELISA plate reader at 570 nm. This is a representative of 2 different experiments.

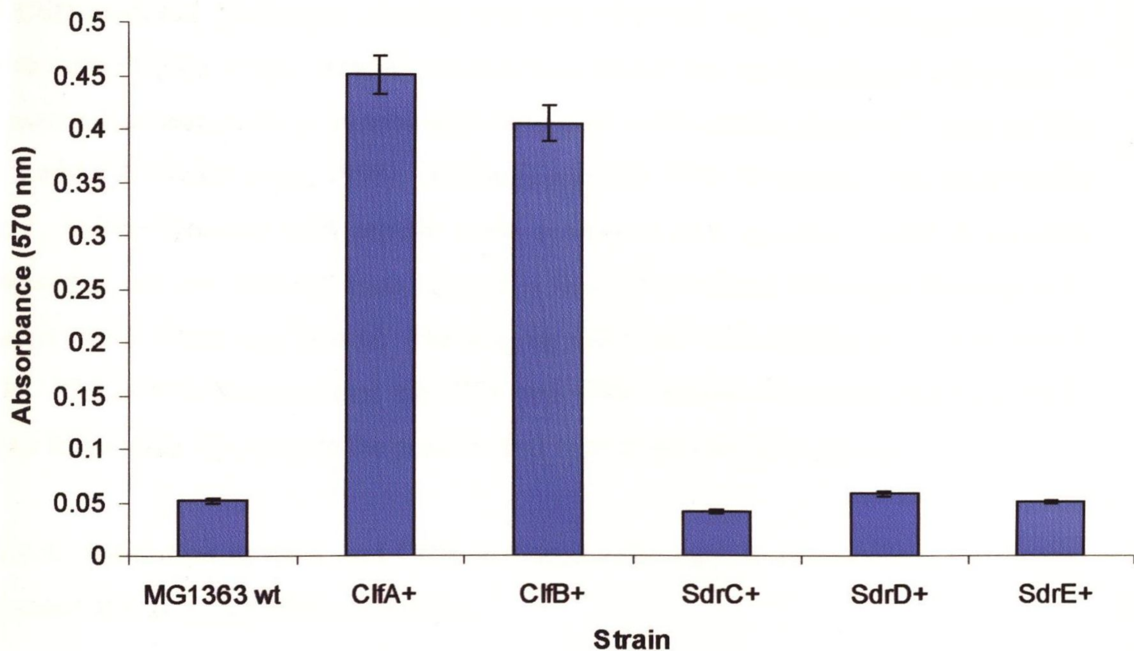


Figure 6.13 Adherence of SdrC, SdrD, SdrE, ClfA and ClfB-expressing *L. lactis* strains to immobilised fibrinogen. A 96- well ELISA plate was coated human fibrinogen, 10 μg/ml. A fixed number of bacterial cells were incubated with the plates, stained with crystal violet and adherence read in an ELISA plate reader at 570 nm. This is a representative of 2 different experiments.

of both of these proteins to fibrinogen is inhibited in the presence of Ca^{2+}. The aim of his test was to determine if Ca^{2+} could inhibit the ClfA- and ClfB-promoted adherence of *L. lactis* cells to immobilised fibrinogen. ClfA- and ClfB-expressing *L. lactis* cells and *S. aureus* Newman*clfA*- and Newman*clfB*- mutant strains were pre-incubated with different concentrations of Ca^{2+}, ranging from 0-200mM, and tested for their ability to bind to immobilised fibrinogen (5 μg/ml), as described in section 2.19.1. Inhibition of fibrinogen binding was observed for ClfA in both *L. lactis* and *S. aureus* (Figures 6.14). Inhibition of ClfB-mediated fibrinogen binding was also observed with the ClfB-expressing *L. lactis* cells (Figure 6.15). Previous studies have shown that ClfB-mediated adherence of *S. aureus* Newman cells to immobilised fibrinogen is also inhibited by Ca^{2+}, with an IC_{50} of 2 mM (Ní Eidhin *et al.*, 1998). Although a higher level of binding was observed for the *L. lactis*-expressed ClfA protein when compared to *S. aureus*, similar IC_{50}s were obtained in the two different hosts. The IC_{50} for ClfA-mediated fibrinogen binding in *S. aureus* and *L. lactis* was 14 mM. The IC_{50} for ClfB-mediated binding in *L. lactis* was 2 mM. These data indicated that the ClfA and ClfB proteins that were expressed in *L. lactis* had similar functions to the proteins that were expressed in *S. aureus*.

6.2.4.5. Inhibition of ClfA and ClfB-mediated adherence to immobilised fibrinogen by anti-ClfA and anti-ClfB antibodies.

The ability of polyclonal antibodies recognising the unique A region of ClfA and ClfB to inhibit the binding of the corresponding *L. lactis* and *S. aureus*-expressed proteins to immobilised fibrinogen was tested. *L. lactis* cells expressing ClfA and ClfB and *S. aureus* Newman*clfA*- and Newman*clfB*- mutant strains were pre-incubated with different concentrations of the corresponding antibody, as described in section 2.19.1. 96-well ELISA plates were coated with human fibrinogen (5 μg/ml) and the ability of the above strains to adhere to immobilised fibrinogen was tested. Complete inhibition of ClfA-mediated fibrinogen binding in *S. aureus* was achieved following incubation of 1.0 OD_{600nm} unit of cells with 300 pg/ml of affinity-purified anti-ClfA antibodies. A 3-fold higher concentration of anti-ClfA antibodies was required to inhibit the binding of *L. lactis* cells expressing ClfA (Figure 6.16). A dose-dependent reduction of ClfB-mediated

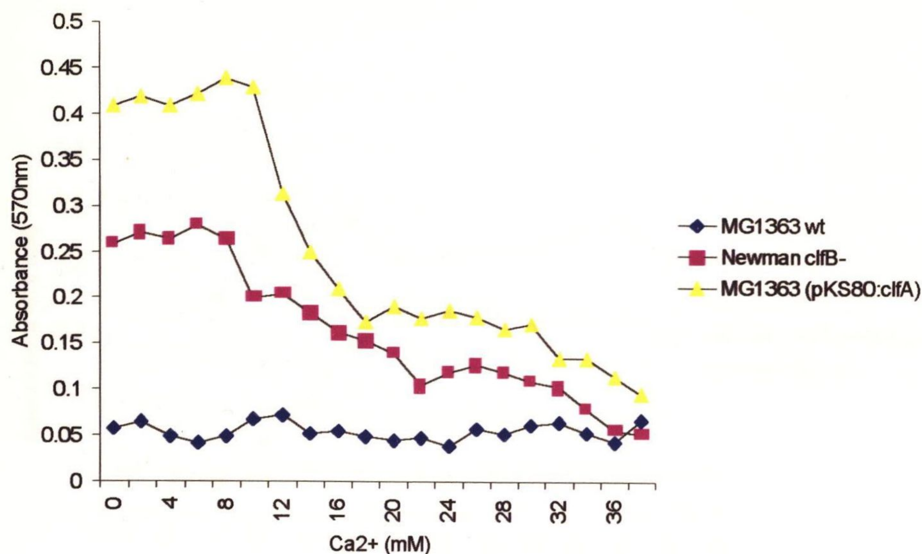


Figure 6.14 Ca^{2+} inhibition of ClfA-mediated adherence to fibrinogen *L. lactis* and *S. aureus.* Cells were preincubated with different concentrations of Ca^{2+} and tested for their ability to adhere to immobilised fibrinogen. This is a representative of 2 different experiments.

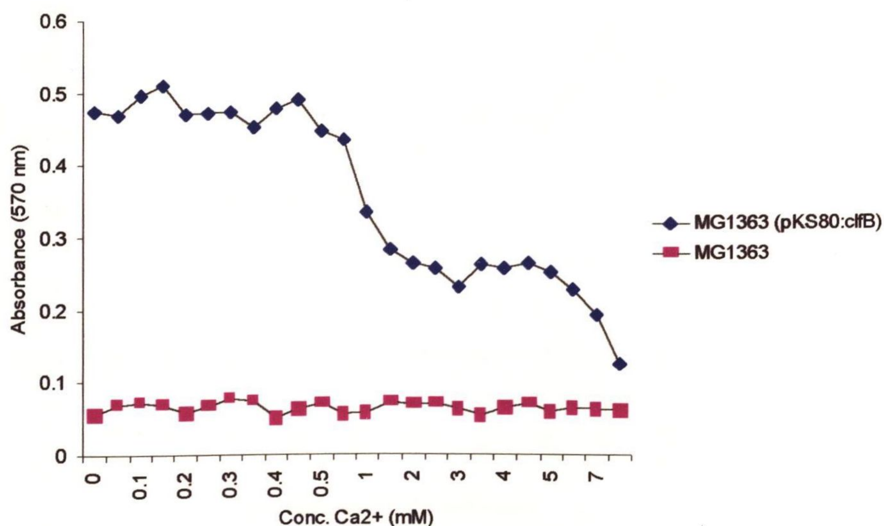


Figure 6.15 Ca^{2+} inhibition of ClfB-mediated adherence to fibrinogen *L. lactis.* Cells were preincubated with different concentrations of Ca^{2+} and tested for their ability to adhere to immobilised fibrinogen. This is a representative of 2 different experiments.

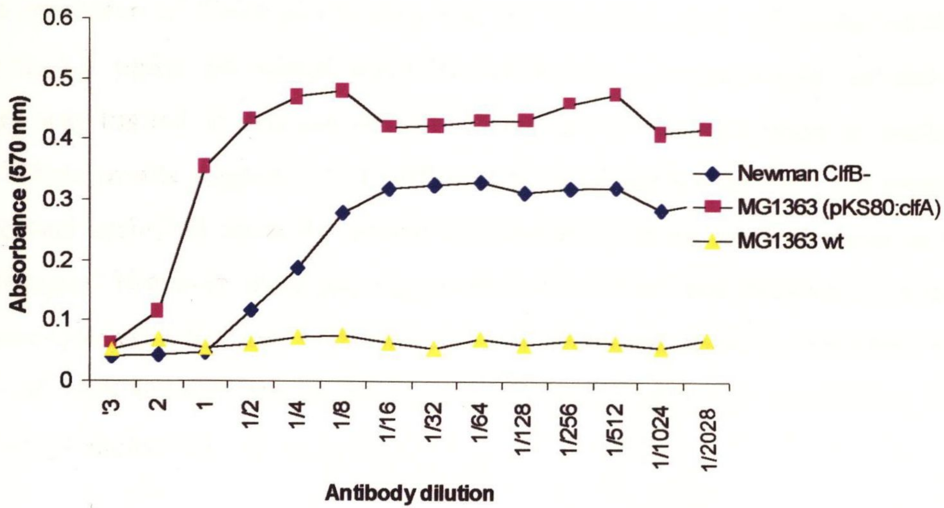


Figure 6.16 Inhibition of ClfA-mediated adherence to immobilised fibrinogen in *L. lactis* and *S. aureus* by polyclonal anti-ClfA antibodies. Cells were preincubated with different dilutions of antibody specific to the unique A region of ClfA and tested for their ability to adhere to immobilised fibrinogen. This is a representative of 2 different experiments

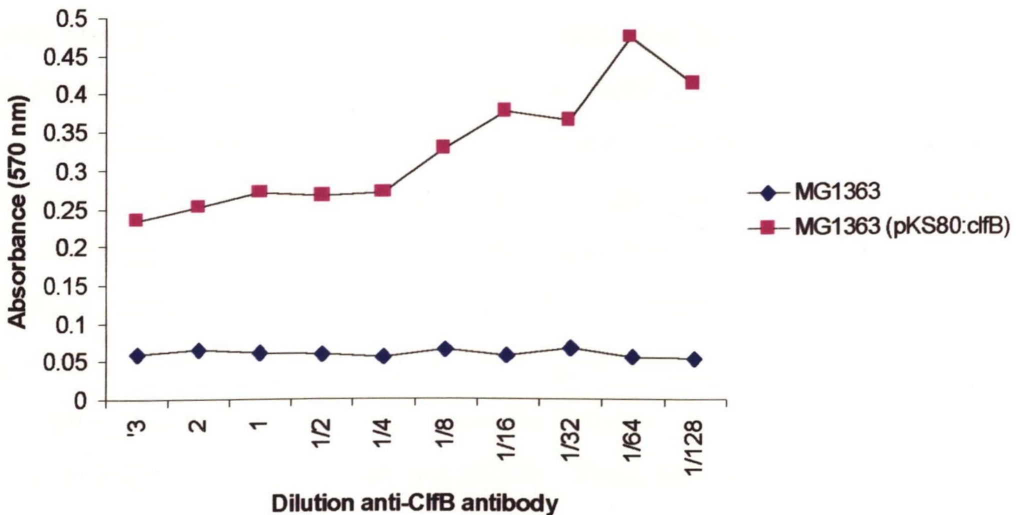


Figure 6.17 Inhibition of ClfB-mediated adherence to immobilised fibrinogen in *L. lactis* by polyclonal anti-ClfB antibodies. Cells were preincubated with different dilutions of antibody specific to the unique A region of ClfB and tested for their ability to adhere to immobilised fibrinogen. This is a representative of 2 different experiments.

fibrinogen binding was observed in both *S. aureus* and *L. lactis* (Figure 6.17). However, complete inhibition of fibrinogen binding was not observed, even following incubation with up to 1.5 μg/ml polyclonal anti-ClfB antibodies. As the supply of anti-ClfB antibodies was limited, it was not possible to increase the concentration of antibodies further. These results suggested that sufficient blocking antibodies were not present in the polyclonal anti-ClfB antibody sample to completely inhibit the interaction of ClfB with fibrinogen. However, these data suggested that the interaction between *L. lactis* and *S. aureus*-expressed ClfA and ClfB proteins and fibrinogen could be inhibited in the presence of high enough concentrations of polyclonal antibodies recognising the A domains of the molecules.

6.2.4.6 Clumping of ClfA and ClfB-expressing *L.lactis* cells in the presence of soluble fibrinogen.

Both ClfA and ClfB have been shown previously to promote clumping of *S. aureus* cells in a solution of fibrinogen (Hartford *et al.*, 1997; Ní Eidhin *et al.*, 1998). This test was designed to show that ClfA and ClfB could promote clumping of *L. lactis* cells when expressed on the *L. lactis* cell surface. *L. lactis* cells expressing ClfA and ClfB and *L. lactis* carrying the empty pKS80 vector were grown to stationary phase. *S. aureus* Newman*clfA*- and Newman*clfB*- mutant strains were also grown to exponential phase and a clumping assay carried out as described in section 2.19.1. A clumping titre of 1μg/ml fibrinogen was observed with both the pKS80:*clfA* carrying *L. lactis* cells and the Newman*clfB*- cells, while a clumping titre of 4 μg/ml fibrinogen was observed with both the pKS80:*clfB* carrying *L. lactis* cells and the Newman*clfA*- cells. No clumping was observed with the *L. lactis* cells carrying pKS80. These data showed that. *L. lactis*-expressed ClfA and ClfB proteins promoted clumping in the presence of soluble fibrinogen.

6.2.5 N-terminal sequencing of *L. lactis*-expressed ClfA and SdrE proteins.

In order to determine if the *S. aureus* proteins expressed by *L. lactis* were present in their full-length mature form, N-terminal sequencing of the *L. lactis*-expressed ClfA and SdrE proteins was performed. ClfA and SdrE proteins were isolated from *L. lactis* cells carrying pKS80:*clfA* and pKS80:*sdrE*, concentrated and transferred to a PVDF membrane, as described in section 2.20. N-terminal sequencing was performed by Edman degradation at the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge, U.K. Following cleavage of the N-terminal signal sequence, the expected N-terminal amino acid sequence of the mature ClfA protein is SNESK. This corresponds to the first five amino acids at the N-terminus of region A. However, sequence results showed that the first six amino acids present at the N-terminus of the 170 kDa ClfA molecule expressed by *L.lactis* were SLAAVA. This motif, which is known to act as a recognition sequence for proteolytic cleavage by the staphylococcal metalloprotease (McAleese *et al.*, 2001), is present in the A domain of ClfA. Therefore, it appeared likely that the ClfA molecule that was expressed by *L. lactis* was a truncated protein that had been cleaved at the SLAAVA motif. It is known that this truncated form of ClfA (residues 220-559) can bind to fibrinogen with the same affinity as the full-length protein (O'Connell *et al.*, 1998). Furthermore, this 170 kDa form of ClfA is also expressed by *S. aureus* Newman (Figure 6.5 (c)). Although ClfA may not be present in its full-length mature form on the surface of *L. lactis*, this truncated protein appears to represent the form of ClfA that is present on the surface of *S. aureus* cells.

Following cleavage of the predicted N-terminal signal sequence, the expected N-terminal amino acid sequence of the mature SdrE protein is AENTST. This corresponds to the first six amino acids at the N-terminus of SdrE region A. Sequence results showed a perfect match for this sequence, indicating that the 180 kDa SdrE protein expressed on the surface of *L. lactis* is the full-length mature form of the molecule and therefore, suggests strongly that the 180 kDa molecule released from *S. aureus* Newman cells is the full-length form of the protein.

6.3 Discussion

The reasons for expressing the Clf/Sdr proteins in *L. lactis* were two-fold. (1) To identify the correct size of the proteins, and (2) to overcome the problem of redundancy experienced in *S. aureus* when attempting to identify a novel phenotype for a surface protein. Identifying the correct size of a surface protein in *S. aureus* can be difficult due to poor expression and proteolytic degradation by endogenous proteases. Over-expression of *S. aureus* proteins in the heterologous host *L. lactis* allowed unambiguous identification of the highest molecular weight form of the protein that was expressed. Each protein was then compared, in terms of size, with the protein that was detected on the surface of *S. aureus*. In the case of ClfA, ClfB, SdrC, SdrE and SdrG the proteins that were expressed by *L. lactis* were the same size as the largest immunoreactive form of the proteins expressed by the native staphylococcal host.

When cell wall-associated proteins are released by lysostaphin digestion of staphylococcal cells, the released protein no longer has peptidoglycan residues attached to it. This is because lysostaphin cleaves at the pentaglycine cross-bridge of peptidoglycan, the point at which the surface protein is anchored (Figure 6.18). When cell wall-associated proteins are released by mutanolysin/lysozyme digestion of lactococcal cells, the released protein still has some peptidoglycan attached to it. This is because mutanolysin and lysozyme cleave between the N-acetyl muramic acid and N-acetyl glucosamine residues of peptidoglycan (Figure 6.18). It was therefore possible that the proteins released from *L. lactis* would have a slightly higher molecular weight than the native proteins released from *S. aureus* or *S. epidermidis*. This was not observed by Western immunoblot analysis because the predicted size differences of such proteins would not be resolved by SDS-PAGE. Presumably a more sensitive technique, such as mass spectrometry, would show a difference in size between the solubilised forms of the native and heterologously expressed proteins.

It was shown that prolonged incubation of *L. lactis* cells expressing each of the heterologous staphylococcal proteins with mutanolysin/lysozyme resulted in proteolytic degradation of the protein. This is possibly due to the release of one or more lactococcal proteases during protoplast formation and occurs despite the presence of protease

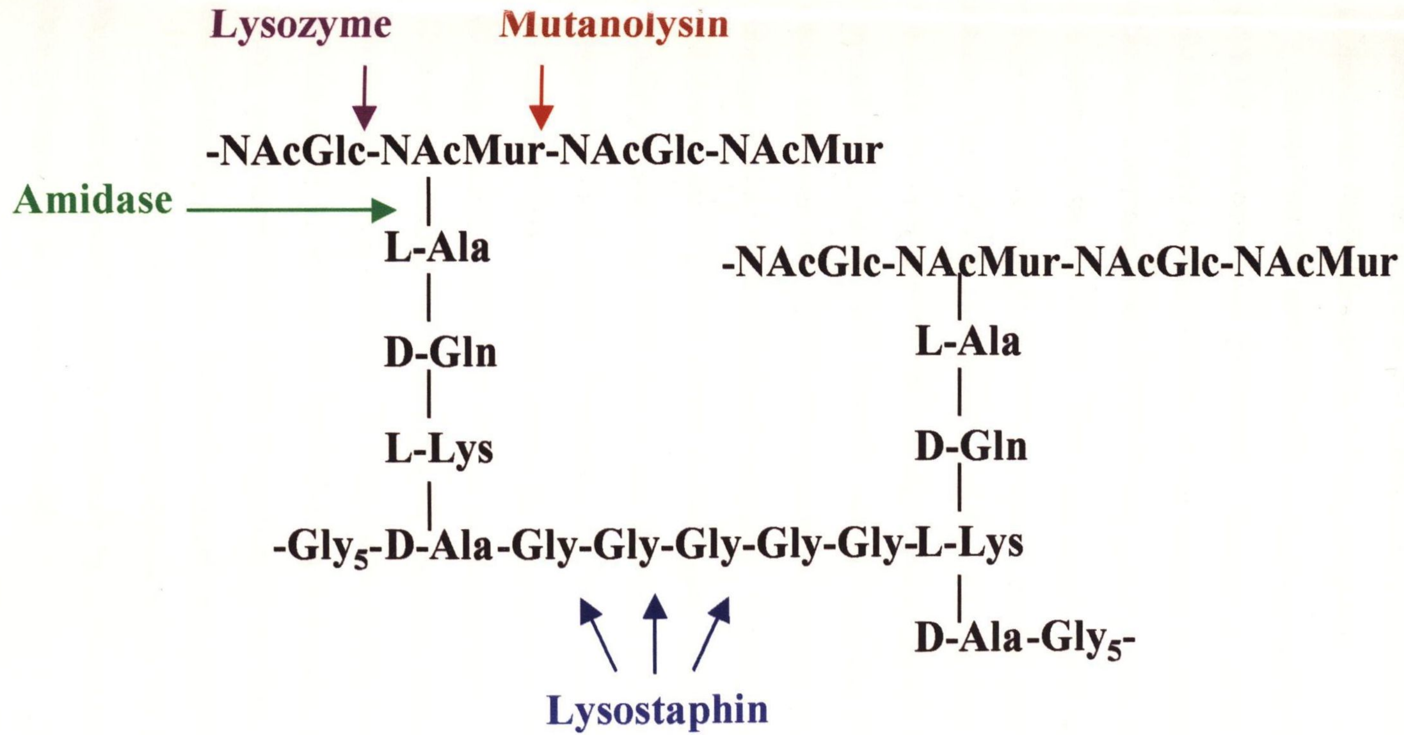


Figure 6.18 Cleavage of *S. aureus* peptidoglycan by the lysostaphin, lysozyme, mutanolysin and amidase

inhibitors. Limited incubation of the cells with mutanolysin/lysozyme allowed the detection of high molecular weight proteins by Western immunoblotting. This suggested that proteolytic cleavage of heterologous proteins did not occur whilst the protein was attached to the lactococcal cell wall, but during cell surface protein isolation. The fact the ClfA, ClfB, SdrC, SdrE and SdrG proteins detected on the surface of *L. lactis* were the same molecular weight as the corresponding native staphylococcal proteins suggested that these were the full length forms of the molecules.

To confirm this possibility, N-terminal sequencing of the *L. lactis*-expressed ClfA and SdrE molecules was performed. Because *L. lactis* does not express any high molecular weight surface proteins, it was possible to identify the ClfA and SdrE protein bands on a Coomassie-stained SDS-PAGE gel of surface proteins isolated from *L. lactis* cells expressing these proteins. This is not possible in *S. aureus* due to the large number of high molecular weight proteins expressed and the poor expression levels of surface proteins. The 170 kDa ClfA protein and the 180 kDa SdrE protein expressed in *L. lactis* were subjected to N-terminal sequencing. Results showed that the 180 kDa SdrE protein expressed by *L. lactis* (and hence *S. aureus*) was the full-length mature form of the protein. However, the 170 kDa ClfA protein expressed by *L. lactis* and was shown to be a cleavage product of the full-length molecule. Cleavage of ClfA in *L. lactis* occurred at the SLAAVA motif present in the unique ligand-binding A region. As this *L. lactis*-expressed protein is the same size as that expressed by *S. aureus*, it is likely that similar cleavage occurs in *S. aureus*. Hartford *et al*. (1997) reported that the full-length form of ClfA was >200 kDa. This was shown by expression of the full-length *clfA* gene from a multi-copy plasmid (pCU1) in *S. aureus* strain Newman. However, this >200 kDa form of ClfA is not observed in *S. aureus* wild type cells, presumably due to low expression levels and proteolytic cleavage. A ClfA molecule that has been cleaved at or near the SLAAVA motif, located in the ligand-binding A-domain, still binds to fibrinogen. It is currently not understood if further truncation of the ClfA A-domain leads to a loss in fibrinogen-binding function. Further investigation is required to resolve this issue.

To establish that the 150 kDa ClfB protein, the 155 kDa SdrC protein and the 170 kDa and 190 kDa SdrG proteins expressed by *L. lactis* are the full-length forms of the proteins, N-terminal sequencing is required.

The 205 kDa SdrD protein expressed by *L. lactis* had a higher molecular weight that the 160 kDa SdrD protein expressed by *S. aureus* strain 8325-4. This suggested that the SdrD protein detected in *S. aureus* was a proteolytically degraded product, while the SdrD protein expressed on the surface of *L. lactis* was the full-length mature form of the molecule. Confirming this would also require N-terminal sequencing.

These data showed that with the exception of SdrD, the proteins expressed by *L. lactis* were the same size as the proteins expressed in the native staphylococcal host. This had important implications for functional studies using *L. lactis* expressing heterologous staphylococcal proteins. One of the major problems encountered when studying the function of a given protein in *S. aureus* is redundancy. For example, there are at least two cell wall-associated fibrinogen-binding proteins and at least two fibronectin-binding proteins in *S. aureus*. Comparing mutant and wild type cells when looking for a novel phenotype can be rewardless if more than one protein is performing that same function. Expressing staphylococcal proteins in the heterologous host *L. lactis* provided an efficient mechanism to study novel functions of the Sdr proteins. To ensure that staphylococcal proteins could function normally when expressed on the surface of *L. lactis*, functional studies with *L. lactis*–expressed ClfA, ClfB and SdrG proteins were performed. It was known that ClfA and ClfB could bind to fibrinogen when expressed on the surface of *S aureus* and that SdrG could bind to fibrinogen when expressed on the surface of *S. epidermidis* Bacterial adherence assays showed that ClfA, ClfB and SdrG could bind to fibrinogen in a similar dose-dependent manner when expressed on the surface of *L. lactis*. Furthermore, the interaction between ClfA and ClfB and fibrinogen was inhibited by Ca^{2+} and polyclonal antibodies recognising the unique A region of the corresponding protein. ClfA and ClfB also induced cell-clumping in the presence of soluble fibrinogen. These data showed that ClfA, ClfB and SdrG behaved the same when expressed on the surface of *L. lactis* as when expressed on the surface of *S. aureus* and *S. epidermidis*.

Considering this and the fact that SdrC, SdrD and SdrE appeared to be expressed in their full-length form on the surface of *L. lactis*, the Sdr protein-expressing *L. lactis* cells could be used with confidence in functional studies. The use of ClfA, ClfB, SdrC, SdrD and SdrE-expressing *L. lactis* cells for functional studies is described in chapters 7 and 8.

Chapter 7

Interactions Between *Staphylococcus aureus* and Human Platelets

7.1 Introduction

Platelets are non-nucleated cells, 2-4 μm in diameter, with a characteristic lentiform shape that resembles a discus (Longenecker *et al.*, 1985). They are formed by the partial fragmentation of large megakaryocyte cells. The internal morphology of the platelet can be divided into four major sections (White, 1971).

The *peripheral zone* comprises the platelet membranes and the structures that provide the surface and walls of the open canalicular system (OCS), which is a system that provides a communication network between the interior and exterior of the platelet. The exterior coat or glycocalyx contains the receptors necessary for platelet function.

The *sol-gel zone* is the matrix of the cytoplasm, commonly known as the cytoskeleton. This region comprises many different fiber types. Microfilaments assist in shape change and allow the formation of pseudopods, internal contraction and the secretion of platelet granules (Gerard, 1976). Microtubules play an important role in maintaining the discoid shape during the resting state.

The *organelle zone* consists of two different types of granules, the contents of which are destined for secretion. α-granules contain fibrinogen, fibronectin thrombospondin and von Willebrand factor and release their contents through the OCS (White, 1971). Dense granules contain adenosine, nucleotides, serotonin and calcium and these contents are released by a mechanism distinct from that of α-granules. This occurs through the formation of multivesicular structures close to the platelet surface, which eventually dissolve resulting in granule release (Polasek *et al.*, 1987)

The *dense tubular system* (DST) is dispersed randomly in the platelet cytoplasm and is the site of calcium sequestration, important in triggering contractile events (Cutler and Feinstein 1978).

Platelets form an intrinsic part of the human haemostatic system, which is designed to maintain blood in a free flowing state under normal healthy physiological conditions. The coagulation system is also involved in maintaining haemostasis. This is an enzyme cascade which leads to the proteolytic cleavage of specific factors and the formation of thrombin. The platelet and coagulation systems are designed to respond to vascular

injury by very rapidly "plugging" damaged areas with platelet-fibrin thrombi in order to minimise blood loss.

Platelets express a number of different surface components which allow them to interact with the extracellular environment. These include; (1) the GPIb-IX-V complex, which is responsible for initiating thrombus formation; (2) GPIIb/IIIa, which serves as a receptor for fibrinogen, fibronectin and von Willebrand factor on activated platelets; (3) the platelet glycoprotein $\alpha_v\beta_3$, which binds to a number of different plasma proteins including fibrinogen and vitronectin (Pytela *et al.*, 1985; Charo *et al.*, 1990); and (4) the Fc receptor family of proteins which bind to the Fc region of immunoglubulin molecules.

Adhesive interactions between platelets and damaged endothelial cells are essential for maintaining the structural and functional integrity of the vascular system. The initial attachment of the platelet to the damaged endothelium is followed by platelet spreading and then the interaction of that platelet with other platelets to form an aggregated clot around the damaged area (Sakariassen *et al.*, 1989). The initial attachment of platelets to the endothelium is dependent on an interaction between the platelet glycoprotein GPIb-IX-V complex and the immobilised extracellular matrix protein von Willebrand factor (Baumgartner, 1977). Following attachment, platelet activation is initiated due to an active conformational change in the GP IIb/IIIa platelet receptor. This is coupled with granule secretion and subsequent spreading of the platelet. The secretion of platelet granules leads to the local availability of more adhesive proteins, which potentiate adhesion.

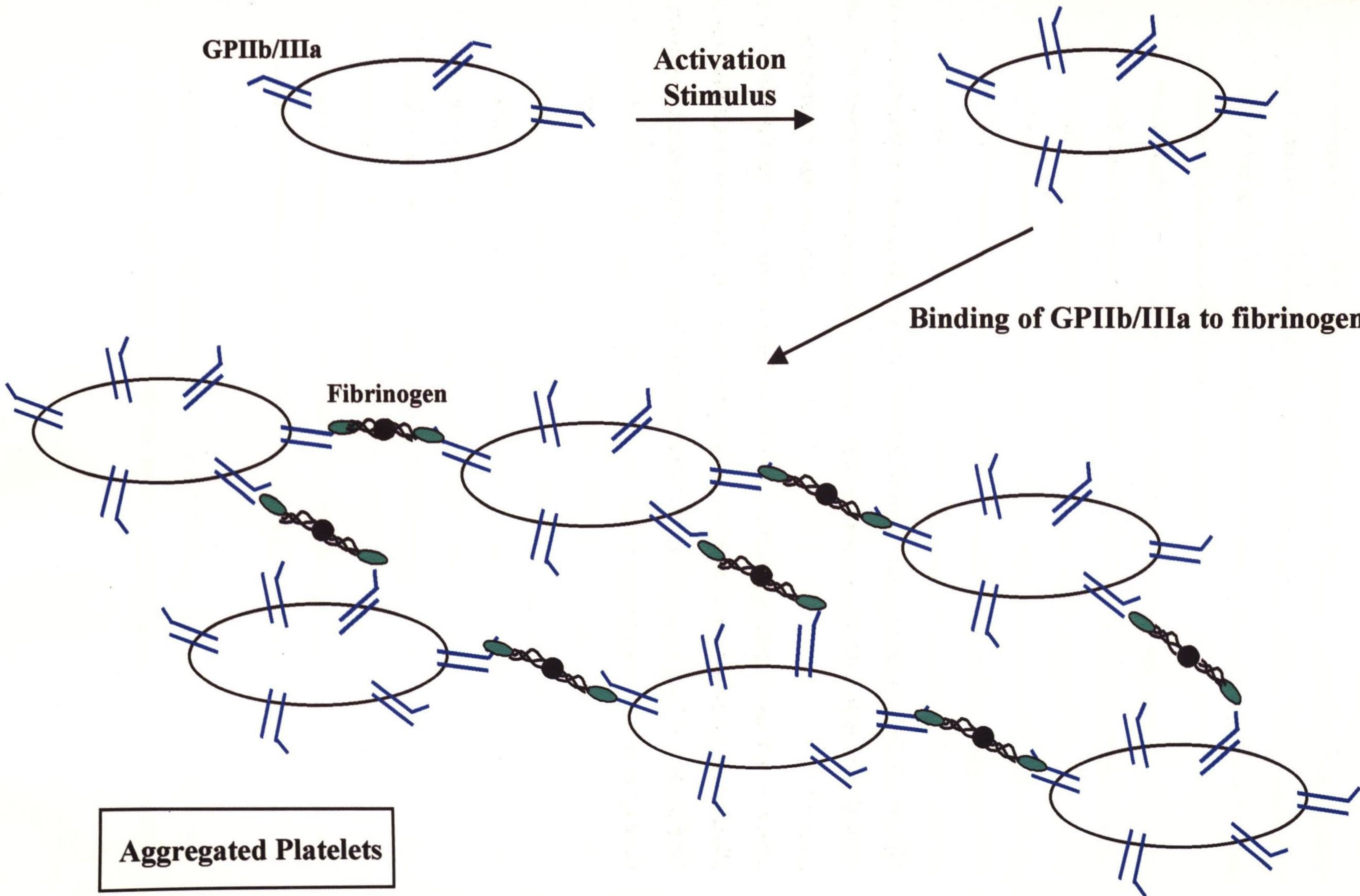
Platelet aggregation is the process by which platelets interact with each other to form a haemostatic thrombus (Born, 1972). Following aggregation, platelets change from the normal disc shape to a compact sphere with long dendritic extensions that facilitate adhesion. A number of different agonists can stimulate the activation of platelet aggregation. These include arachidonic acid, adrenaline, ADP, thrombin and collagen, to name but a few. Aggregation of platelets occurs through an interaction between the platelet GPIIb/IIIa receptor and fibrinogen (Niewiarowski *et al.*, 1971). Following the activation stimulus, a conformational change in the structure of the GPIIb/IIIa molecule occurs, coupled with an up-regulation in the number of GPIIb/IIIa molecules expressed by the platelet. These molecules can bind to the bivalent plasma protein fibrinogen,

resulting in the cross-linking of many different platelets to form a haemostatic thrombus (Figure 7.1).

In addition to their role in thrombosis and haemostasis, platelets also play an important role in protecting the host against microbial infection, which often occurs at the site of vascular damage. Platelets represent the initial and most abundant blood cells to arrive at these sites (Yeaman *et al.*, 1997). Although platelets can internalise bacteria, it is the release of antimicrobial (poly)peptides by the platelet in response to various stimuli present at the site of vascular damage that provides the most significant platelet defense mechanism against microorganisms (Yeaman *et al.*, 1997). Platelet products released during degranulation include proteins that are directly cytotoxic to Gram-positive bacteria, such as platelet microbiocidal proteins (PMPs) (Yeaman *et al.*, 1997) and group IIA phospholipase A2 (PLA2) (Kudo *et al.*, 1993; Weinrauch *et al.*, 1998). Molecules that are chemotactic to PMNs are also released, further enhancing the local mobilisation of host defenses at endovascular sites of infection (Yeaman *et al.*, 1997). It is thought that bacteria that are resistant to platelet-induced killing can effectively use the platelet-fibrin thrombus as an adhesive surface upon which the organism can dock and initiate infection. In fact, bacteria that are resistant to PMPs are associated with enhanced progression and haematogenous dissemination (Dhawan *et al.*, 1998).

Infective endocarditis (IE) is a serious complication that is associated with bacteremia, particularly that caused by Gram-positive organisms such as staphylococci, streptococci and enterococci, and fungi such as *Candida albicans*. If untreated, IE is usually fatal and even with aggressive therapy mortality rates can be as high as 20-40%. It is characterised by the formation of a vegetative lesion on the surface of a valve in the heart, which consists of platelets, leukocytes, erythrocytes and dense clusters of dividing bacteria. The development of IE is usually associated with prior valvular damage, although certain bacteria such as *S. aureus* do not appear to require a damaged valvular surface to induce IE (Bansal, 1995). This is probably the reason for the high incidence of *S. aureus* IE in intravenous drug users who have no history of valvular damage (Levine *et al.*, 1986). The ability of *S. aureus* to survive in hostile, nutrient-deficient environments may also contribute to the success of this bacterium in surviving inside platelet thrombi.

Figure 7.1 Schematic representation of the activation of platelet aggregation. Following an activation stimulus, the resting platelet undergoes a signal transduction event, resulting in an up-regulation of the number of GPIIb/IIIa molecules expressed on its surface. This is accompanied by a conformational change in the structure of the GPIIb/IIIa molecule, allowing it to bind to the bivalent molecule fibrinogen. Following GPIIb/IIIa-mediated binding to fibrinogen, the platelets become cross-linked to each other, resulting in the development of a platelet thrombus.



Resting Platelet
Activated Platelet
GPIIb/IIIa
Activation
Stimulus
Binding of GPIIb/IIIa to fibrinogen
Fibrinogen
Aggregated Platelets

S. aureus can interact with platelets (Yeaman *et al.*, 1992) and can activate subsequent platelet aggregation in the presence of plasma via a fibrinogen-dependent mechanism (Bayer *et al.*, 1995). It is thought that the adherence to and the activation of platelets by *S. aureus* are mechanistically distinct (Bayer *et al.*, 1995). The formation of a platelet-fibrin thrombus around the staphylococcal cells following platelet activation could contribute to evasion of the host immune system.

The mechanism by which *S. aureus* activates platelet aggregation is poorly understood and several reports in the literature conflict with data that is presented in this chapter. Results published by Hawiger and co-workers (1979) suggest that *S. aureus*-induced platelet aggregation is mediated by protein A. The basis of this conclusion came from the fact that purified protein A could inhibit *S. aureus*-induced platelet aggregation. However, this does not account for the possibility that protein A may bind to the platelet without activating aggregation and sterically inhibit subsequent aggregation by the bacteria. Results presented in this chapter suggest that protein A is not sufficient for activating platelet aggregation.

The work of D. Cox and colleagues (RCSI, Dublin) suggested that the *S. aureus* surface-associated proteins ClfA and ClfB were involved in the activation of platelet aggregation (S. Kerrigan, D. Cox, unpublished). They showed that *S. aureus* strain Newman induced platelet aggregation with a lag time of 7±1 min. The delay in time from the point at which the bacterial cells are added to the point at which the platelet begin to aggregate is known as the lag time. Different agonists will active platelet aggregation with different lag times, depending on the strength of their interaction with the appropriate platelet receptor. The lag time seen with *S. aureus* Newman cells is similar to that produced by *S. sanguis* strain 133-79 (5±1 min), but significantly shorter than that observed with *S. sanguis* strain NCTC 7863 (16±3 min). In general, the lag times seen with bacterial induction of platelet aggregation are significantly longer that that observed with agonists such as arachionic acid, which has a lag time of 0.12±0.01 min.

S. aureus-induced platelet aggregation was inhibited by the GPIIb/IIIa antagonists abciximab, eptifibatide and RGDS as well as by the cyclooxygenase inhibitor aspirin.

This showed that the effect of *S. aureus* on the platelets was true aggregation rather than platelet agglutination, which occurs independently of GPIIb/IIIa and fibrinogen.

In calcium free buffer, Cox and co-workers found that single *clfA* and *clfB* mutants still induced platelet aggregation. However, a *clfAclfB* double mutant of strain Newman failed to induce aggregation, suggesting that both ClfA and ClfB were involved in activating *S. aureus*-induced platelet aggregation in the absence of calcium. In contrast, when the experiment was repeated in platelet rich plasma (PRP), which contains 60 μM calcium, aggregation still occurred in the both the single and double *clfAclfB* mutants. A concentration of 60 μM Ca^{2+} is obtained when the blood is treated with 3.8% Na_2citrate and is more reflective of the *in vivo* situation. These data suggested that while ClfA and ClfB appeared to induce platelet aggregation under certain conditions, additional *S. aureus* factors were involved.

At the time, Cox and co-workers hypothesised that ClfA and ClfB were responsible for activating *S. aureus*-induced platelet aggregation in calcium-depleted environments and that other proteins were responsible for activating aggregation in the presence of calcium. This lead them to test a number of different *S. aureus* strain Newman single mutants for their ability to induce platelet aggregation in PRP (calcium replete). These included strains that were deficient in FnBPA, FnBPB, Cna, Spa, EbpS, SdrC and SdrD production. However, all of these strains could activate platelet aggregation as well as the wild type strain. These data suggested that a large redundancy in function for the induction of *S. aureus*-induced platelet aggregation existed. A more in depth view on this is presented in the discussion of this chapter.

A collaboration was established with D. Cox's group in order to identify novel *S. aureus* proteins that were involved in activating platelet aggregation. This chapter describes the involvement of ClfA, ClfB, SdrE and Protein A in the activation of *S. aureus*-induced platelet aggregation.

7.2 Results

7.2.1 Induction of Human Platelet Aggregation by *Lactococcus lactis* Cells Expressing *Staphylococcus aureus* Proteins.

A large redundancy in function appears to exist for the activation of platelet aggregation by *S. aureus*, making the identification of factors involved in the process difficult. Therefore, the ability of *S. aureus* surface proteins to activate platelet aggregation when expressed in the heterologous host *L. lactis* was studied. *L. lactis* strain MG1363 does not activate platelet aggregation. Thus, if a *L. lactis* strain expressing a specific *S. aureus* surface protein can activate platelet aggregation, that protein must be involved in the process.

L. lactis strains expressing the *S. aureus* cell wall-associated proteins ClfA, ClfB, SdrC, SdrD and SdrE had been generated previously (Chapter 6). Results presented in Chapter 6 showed that each of these proteins were expressed at high levels by *L. lactis*, that they were functional and that they were likely to be expressed in their full-length form. In addition, *L. lactis* strains expressing Protein A, Map (J. Pènedes, unpublished) and EbpS (R. Downer, unpublished) were provided. Each of these proteins appeared to be expressed at high levels in their full-length form also. Each strain was grown to stationary phase (16 hours) in M17 broth containing 0.5% glucose and 5 μg/ml Em with no shaking at 30°C. Cells were then harvested, washed in PBS and resuspended to an OD_{600nm} of 1.6. The *L. lactis* wild type strain, MG1363, was also grown under the same conditions but with no Em, washed in PBS and resuspended to an OD_{600nm} of 1.6.

The ability of each of these strains to activate human platelet aggregation in PRP (60 μM Ca^{2+}) was tested, as described in section 2.21. Results were read in a platelet aggregometer. No aggregation was observed with *L. lactis* strains expressing SdrC, SdrD, EbpS, Map or protein A (Figure 7.2) (results were considered negative if no aggregation had occurred following >25 min incubation of the bacterial cells with PRP at 37°C). The *L. lactis* wild type strain, MG1363, also failed to induce platelet aggregation. These data were partly surprising as previous reports by Hawiger and co-workers

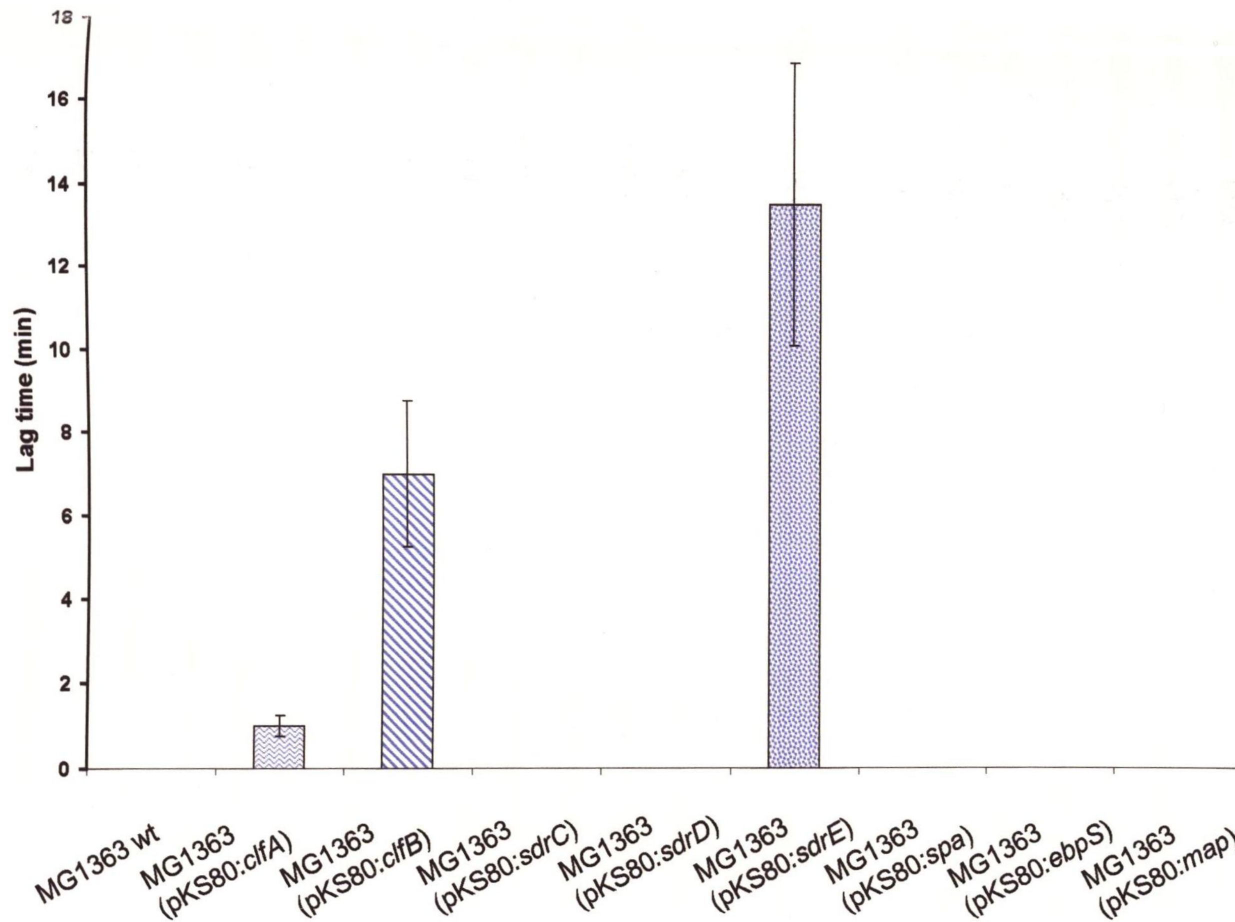


Figure 7.2 Activation of human platelet aggregation by *L. lactis* strain expressing *S. aureus* proteins ClfA, ClfB, SdrC, SdrD, SdrE, Protein A, EbpS and Map. This experiment was performed in triplicate, with three different PRP samples on three different occasions.

suggested that protein A was responsible for activating platelet aggregation. This paradox was further investigated in section 7.2.4.

In contrast, ClfA, ClfB and SdrE-expressing cells were found to activate platelet aggregation in the presence of 60 μM Ca^{2+}, with lag times of 1.5±0.5 min, 7±2 min and 13.5±3.5 min, respectively (Figure 7.2). The ability of SdrE to activate platelet aggregation represents a novel mechanism for the activation of platelet aggregation by *S. aureus*. In contrast to ClfA and ClfB-dependent platelet aggregation, SdrE only activated platelet aggregation in the presence of 60 μM Ca^{2+}. No aggregation was observed with the SdrE-expressing *L. lactis* cells in the absence of Ca^{2+} (S. Kerrigan, personal communication), suggesting that Ca^{2+} was required for this interaction. The difference in lag times and Ca^{2+}-dependence suggest that each of these interactions may occur by independent mechanisms.

7.2.2 Inhibition of platelet aggregation induced by *L. lactis* cells expressing SdrE

To further investigate the platelet activation that was observed with the *L. lactis* cells expressing SdrE, a number of inhibition experiments were performed. It was important to confirm that the effects seen were indeed platelet aggregation and not agglutination. Therefore, the ability of the GPIIb/IIIa antagonist "abciximab" to inhibit *L. lactis* (pKS80:*sdrE*)-induced platelet aggregation was tested. This monoclonal antibody inhibits the interaction between GPIIb/IIIa and fibrinogen, preventing aggregation from occurring, but does not inhibit platelet agglutination. 2 μl abciximab were pre-incubated with 450μl PRP for 15 min at 37°C, and the ability of *L. lactis* (pKS80:*sdrE*) to induce aggregation of these platelets tested. Results showed that aggregation failed to occur following pre-incubation of the platelets with abciximab, indicating that SdrE induced true platelet aggregation when expressed heterologously in *L. lactis* (Figure 7.3).

The ability of the recombinant A-region of SdrE to inhibit *L. lactis* (pKS80:*sdrE*)-induced platelet aggregation was also tested. Recombinant SdrE region A was purified as described in section 2.10. Different concentrations of SdrE region A (ranging from 25 μg-200μg) were added to 450 μl PRP and pre-incubated for 15 min at 37°C. The ability

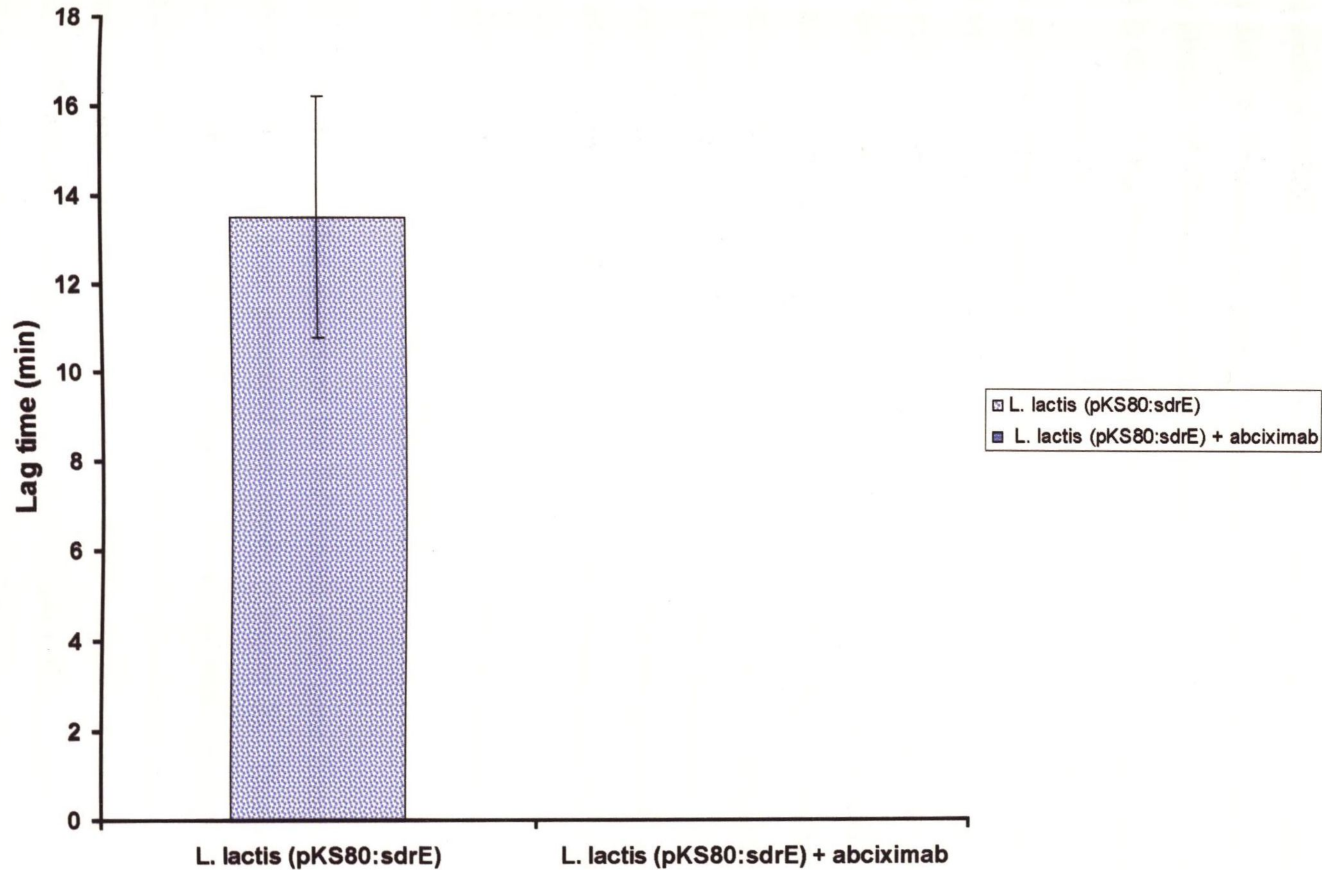


Figure 7.3 Inhibition of *L. lactis* (pKS80:*sdrE*)-induced activation of platelet aggregation following pre-incubation of platelets with the GPIIb/IIIa antagonist abciximab. This experiment was performed in triplicate

of *L. lactis* (pKS80:*sdrE*) to inhibit the aggregation of these platelets was then tested. Although SdrE region A did not itself induce platelet aggregation, when the protein was pre-incubated with platelets, it inhibited subsequent aggregation by *L. lactis* cells expressing SdrE in a dose-dependent manner (Figure 7.4). A significant increase in the lag time to aggregation was seen when platelets were pre-incubated with $\geq$ 50 μg recombinant SdrE region A. When the concentration of recombinant SdrE region A added was increased to 200μg, no aggregation was observed, indicating that complete inhibition of *L. lactis* (pKS80:*sdrE*)-induced platelet aggregation had occurred. These data showed that SdrE activated platelet aggregation in a specific manner.

Inhibition of platelet aggregation induced by *L. lactis* cells expressing SdrE, using antibodies raised against the A region of SdrE was also studied. *L. lactis* cells expressing SdrE were pre-incubated with a 1:250, a 1:125 and a 1:83 dilution of anti-SdrE region A antibodies for 30 min at 37°C. These bacterial cells were then added to PRP and their ability to induce platelet aggregation measured. Results showed a dose dependent inhibition of SdrE-mediated platelet aggregation following pre-incubation of the *L. lactis* cells with increasing concentrations of the anti-SdrE antibody (Figure 7.5), indicating that the region of SdrE responsible for activating platelet aggregation was located within the unique A domain. However, it should be noted that this particular experiment was only performed once and therefore, the statistical significance cannot be evaluated.

7.2.3 Expression of SdrE in the SdrE-negative *S. aureus* strain 8325-4 and its ability to induce platelet aggregation.

A plasmid that expressed the full-length *sdrE* gene from the mulitcopy shuttle plasmid pCU1 had been generated previously (Chapter 3). This plasmid (pCU1:*sdrE*) was transformed into the SdrE-negative *S. aureus* strain 8325-4 by transduction, as described in section 2.7. The ability of this strain to activate platelet aggregation in PRP was compared with that of the wild type 8325-4 strain. 8325-4 wild type and 8325-4 (pCU1:*sdrE*) cells were grown to exponential phase (OD_{600nm} of 0.8) and stationary phase (16 hours) with shaking at 37°C in 50 ml BHI. Cells were harvested, washed in PBS and resuspended to an OD_{600nm} of 1.6. The ability of these cells to activate platelet

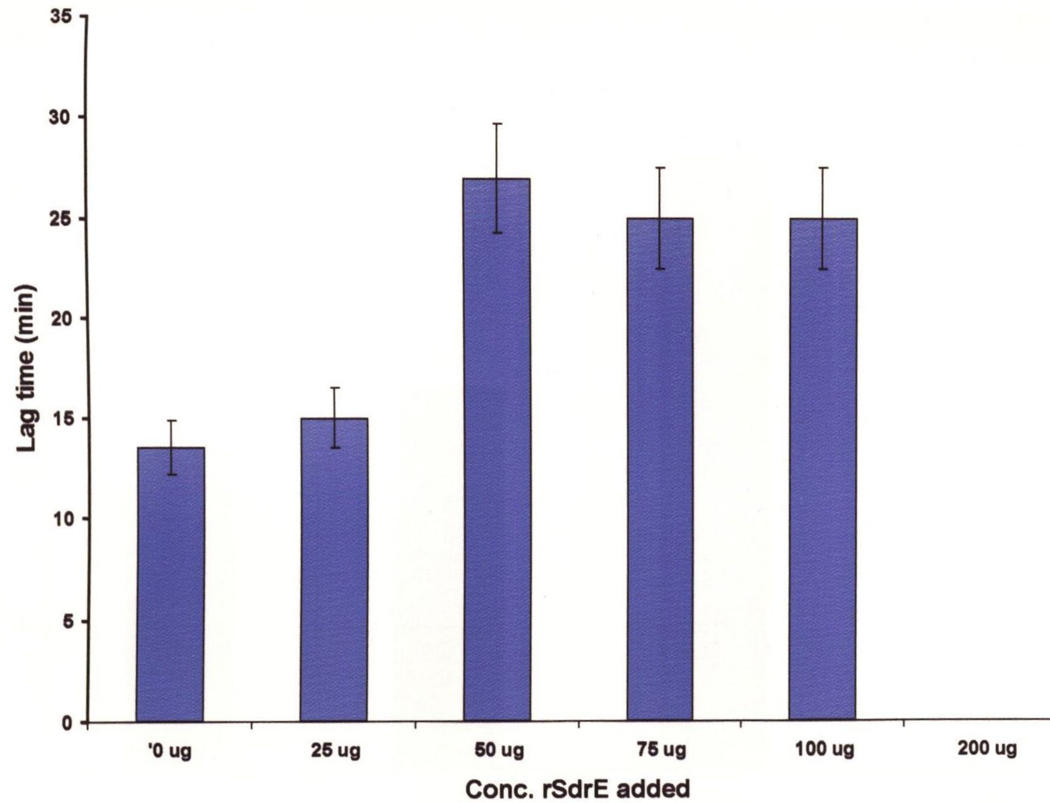


Figure 7.4 Inhibition of *L. lactis* (pKS80:*sdrE*)-induced platelet aggregation following pre-incubation of the platelets with different concentrations of the recombinant sdrE A domain (rSdrEA). This experiment was performed in triplicate.

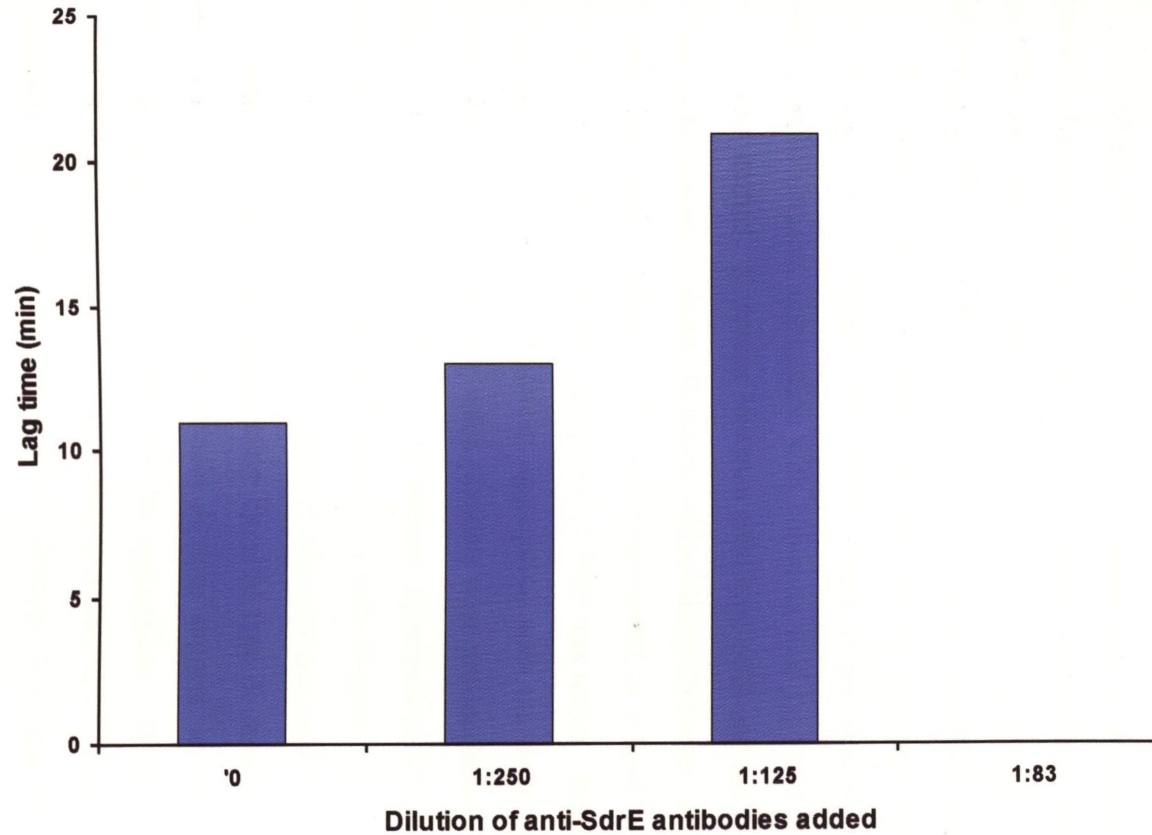


Figure 7.5 Inhibition of *L. lactis* (pKS80:*sdrE*)-mediated activation of platelet aggregation following pre-incubation of the bacterial cells with different concentrations of antibodies raised against the A domain of SdrE. This experiment was performed once and may not be statistically significant.

aggregation was studied. A significant decrease in lag time for the 8325-4 cells that expressed SdrE when compared to the wild type cells, both in exponential phase and in stationary phase (Figure 7.6). These data showed that SdrE was involved in activating platelet aggregation when expressed in *S. aureus* 8325-4.

7.2.4 Investigation of the role of protein A in the activation of platelet aggregation

It had previously been reported by Hawiger and co-workers that protein A was responsible for activating *S. aureus*-induced platelet aggregation. However, this did not agree with the observation that *L. lactis* cells expressing protein A failed to activate platelet aggregtion (section 7.2.1). Therefore, further investigation was required.

The ability of *S. aureus* strain Newman and the Newman *spa* mutant to activate platelet aggregation was tested. The effects of platelet pre-incubation with recombinant protein A on subsequent aggregation with Newman wild type and Newman *spa* mutant cells was also studied. *S. aureus* Newman wild type cells and the Newman *spa* mutant were grown to stationary phase (16 hours) with shaking (200 rpm) at 37°C in 50 ml BHI. Cells were harvested, washed in PBS and resuspended to a final OD_{600nm} of 1.6. The ability of these cells to activate aggregation of PRP was tested. Results showed that the Newman *spa* mutant activated platelet aggregation with the same lag time as the wild type cells (Figure 7.7). However, when platelets were pre-incubated with 200 μg protein A (Sigma) for 15 min at 37°C, no subsequent aggregation was seen with either the Newman wild type cells or with the Newman *spa* cells (Figure 7.7).

These data may explain the results of Hawiger and co-workers (1979). The fact that inhibition of subsequent aggregation by wild type *S. aureus* cells occurred when platelets were pre-incubated with protein A does suggest, as Hawiger proposed, that protein A is involved in activating platelet aggregation. However, the fact that this inhibition is also observed following subsequent incubation with a *spa* mutant strain indicates that this is not a specific type of inhibition. Hawiger and co-workers did not account for the fact that a redundancy in function for the activation of platelet aggregation might occur in *S. aureus* and therefore, did not account for the possibility that protein A may bind to a receptor on the platelet without inducing activation. It

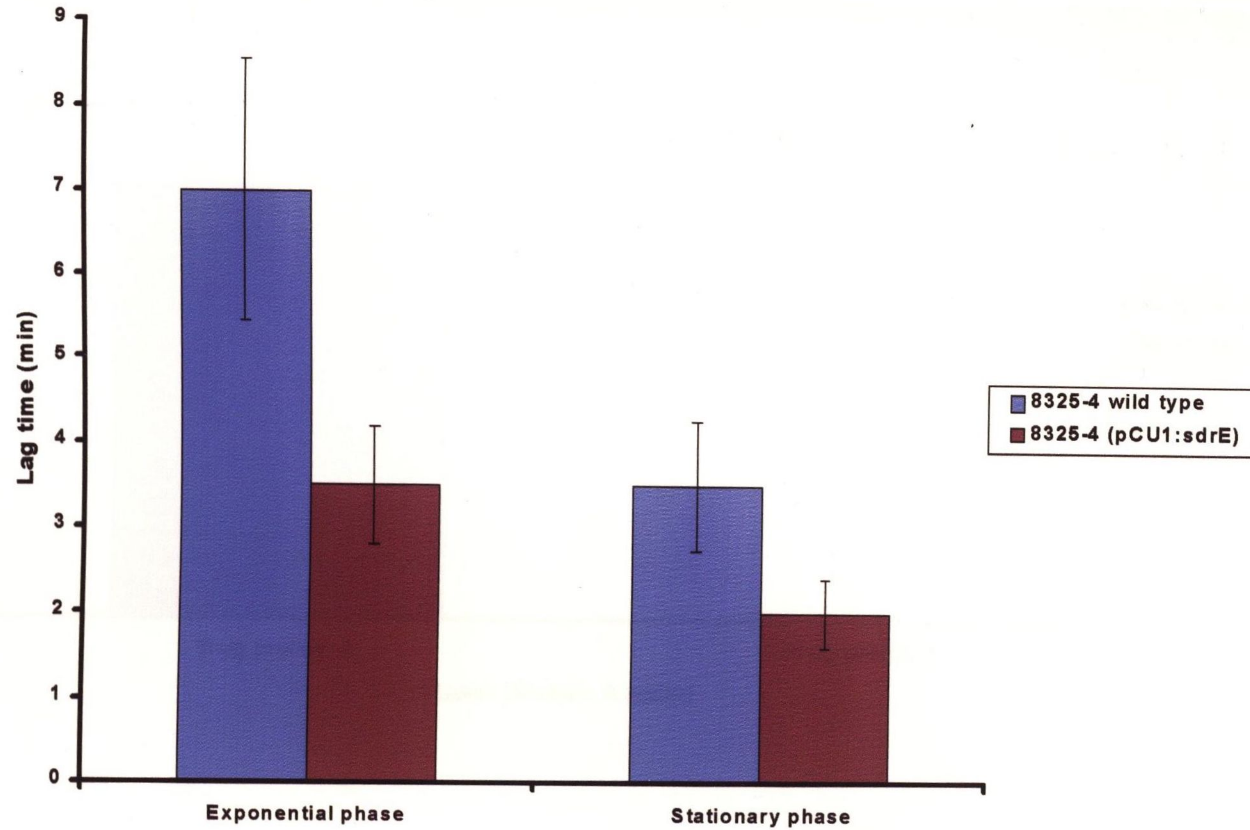


Figure 7.6 Effect of the over-expression of SdrE in the SdrE-negative *S. aureus* strain 8325-4. This experiment was performed in triplicate.

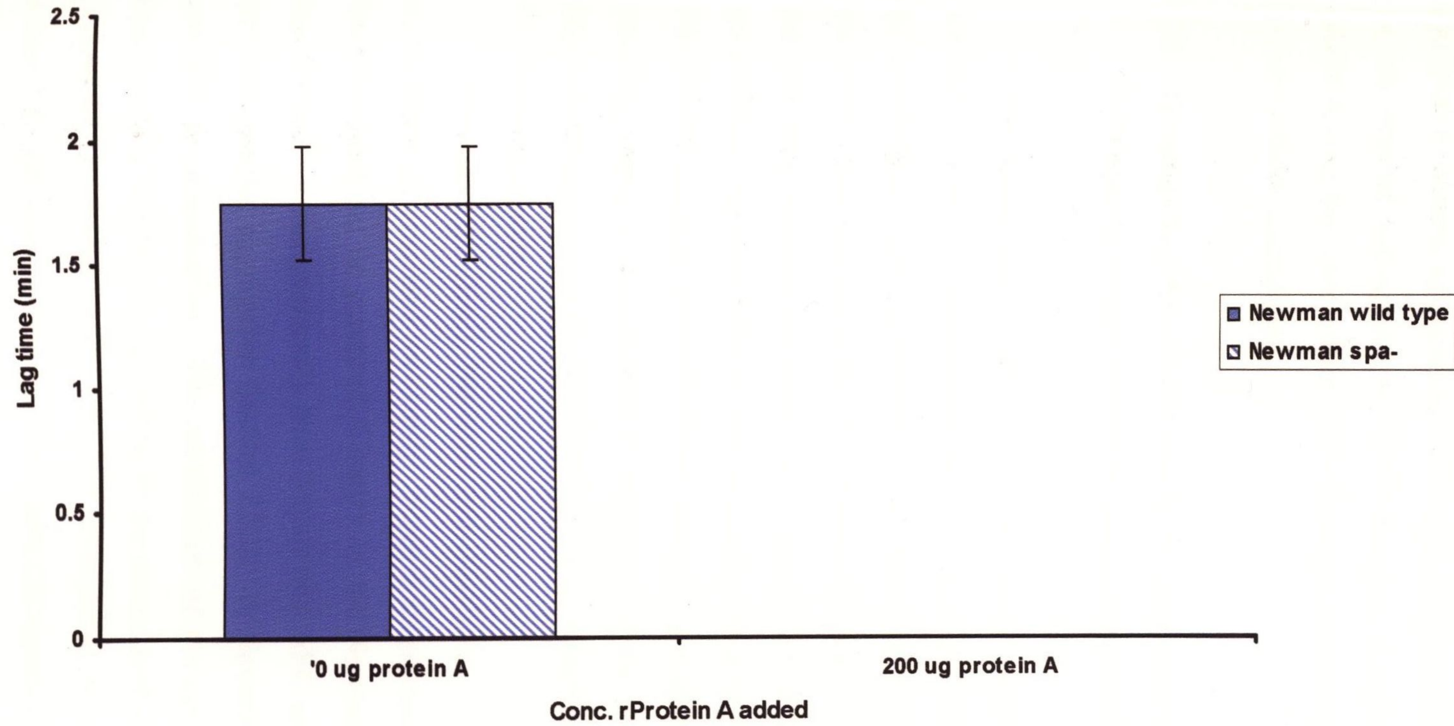


Figure 7.7 Comparison of lag times to aggregation for stationary phase Newman wild type and Newman *spa* mutant cells and inhibition of aggregation in both strains following preincubation of the platelets with 200 μg of recombinant protein A.

appears that protein A can bind to a receptor on the platelet, resulting in steric inhibition of further binding by other *S. aureus* surface proteins and thus, inhibition of platelet aggregation. This does not rule out the possibility that protein A is involved in the activation of platelet aggregation. However, coupled with the fact that protein A does not activate platelet aggregation when expressed in *L. lactis*, these data do suggest that protein A does not have a direct role and is not sufficient for the activation of *S. aureus*-induced platelet aggregation.

7.2.5 Investigating the role of ClfA, SdrE and protein A in the activation of platelet aggregation using *S. aureus* mutant strains defective in *clfA*, *sdrE* and *spa*.

Due to the large number of proteins that appeared to be involved in *S. aureus*-mediated activation of platelet aggregation, it was not possible to generate a mutant strain that was deficient in the production of all four candidate proteins (ClfA, ClfB, SdrE and protein A). This was due to the fact that the *clfB* and the *sdrE* mutant strains that were available both contained the same Tet^r marker and therefore, could not be combined by transduction. Because ClfB is only expressed in exponential phase (Ní Eidhin *et al.*, 1998; McAleese *et al.*, 2001) and ClfA, SdrE and protein A are known to be expressed in stationary phase, it was decided to analyse only the *clfA*, *sdrE* and *spa* mutants in stationary phase in order to establish if additional factors were involved in the activation of platelet aggregation.

Newman *clfA* (Em^r), Newman *spa* (Kan^r) and Newman *sdrCDE* (Tet^r) mutant strains had been generated previously. Since a single mutant in the *sdrE* gene of strain Newman could not be generated (Chapter 3), the Newman *sdrCDE*- triple mutant was used instead. It had previously been shown that SdrC and SdrD were not involved in activating platelet aggregation (section 7.2.1). A Newman *clfAspa* double mutant was generated by transduction. The introduction of the *clfA* mutation was demonstrated through a loss of cell-clumping ability in the presence of soluble fibrinogen. A Newman *clfAspasdrCDE* mutant strain was also constructed by transduction, as described in section 2.8. Introduction of the *clfA* and *sdrCDE* mutations was demonstrated by lack of cell clumping and PCR (data not shown).

Newman wild type, Newman *sdrCDE*, Newman *spa*, Newman *clfA*, Newman *clfAspa* and Newman *clfAspasdrCDE* strains were grown to stationary phase (16 hours) with shaking at 37°C in 50 ml BHI. Cells were harvested, washed in PBS and resuspended to an OD_{600nm} of 1.6. The ability of each of these strains to activate platelet aggregation in PRP was tested. Results showed that Newman wild type, Newman *sdrCDE* and Newman *spa*- cells all activated platelet aggregation with very similar lag times of approximately 1.8±0.1 min. In contrast, a significant delay in lag time to aggregation was observed with the Newman *clfA* mutant cells, where a lag time of 9±1 min was observed. A further delay in lag time was observed with the Newman *clfAspa* mutant cells, which showed a lag time of 21±9min. No aggregation was observed with the Newman *clfAspasdrCDE* mutant cells (Figure 7.8). These data suggest a number of things. (1) ClfA appears to be the most important factor involved in activating platelet aggregation with stationary phase cells and seems to function independently of other *S. aureus* molecules, (2) protein A appears to work co-operatively with SdrE, enhancing its ability to induce platelet aggregation, (3) ClfA, SdrE and protein A appear to be the only factors involved in activating *S. aureus*-induced platelet aggregation during stationary phase. However, these data do not rule out the possibility that factors other than ClfA, ClfB, SdrE and protein A are involved in activating *S. aureus*-induced platelet aggregation during exponential phase.

7.2.6 The ability of *S. aureus* clinical isolates to activate platelet aggregation

Ten *S. aureus* clinical isolates (*S. aureus* 101-110, Table 2.1), which had been isolated from patients with infective endocarditis (S. Peacock, John Radcliffe hospital, Oxford) were tested for their ability to induce platelet aggregation in PRP. Each strain was grown to stationary phase (16 hours), with shaking at 37°C in 50 ml BHI. Cells were harvested, washed in PBS and resuspended to an OD_{600nm} of 1.6. These cells were then added to PRP and the effects monitored. 7/10 strains were capable of inducing platelet aggregation with lag times that varied from 0.75-8 min (Figure 7.9). The results showed that of the clinical strains tested, most were capable of inducing platelet aggregation, thus indicating the clinical relevance of *S. aureus*-induced platelet aggregation. However, as

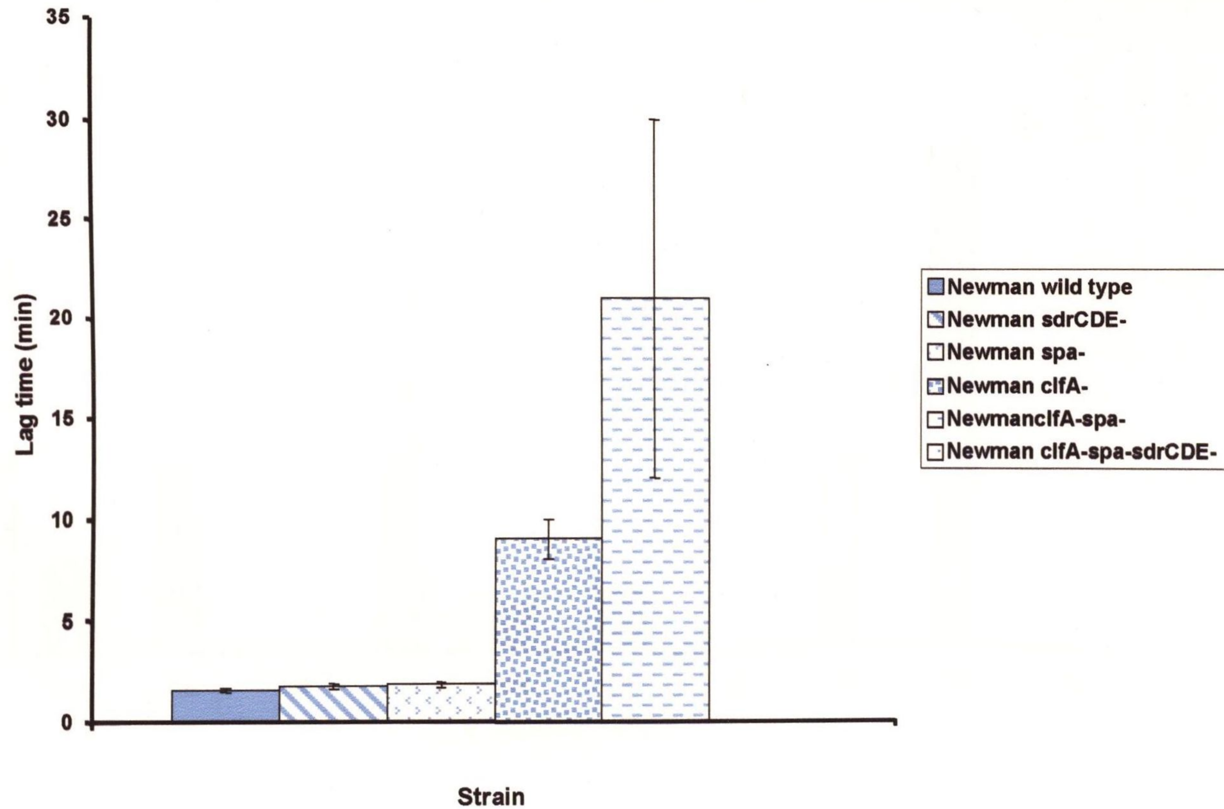


Figure 7.8 Lag time to aggregation for Newman wild type and *clfA-*, *spa-* and *sdrCDE-* mutant strains, grown to stationary phase. This experiment was performed in triplicate.

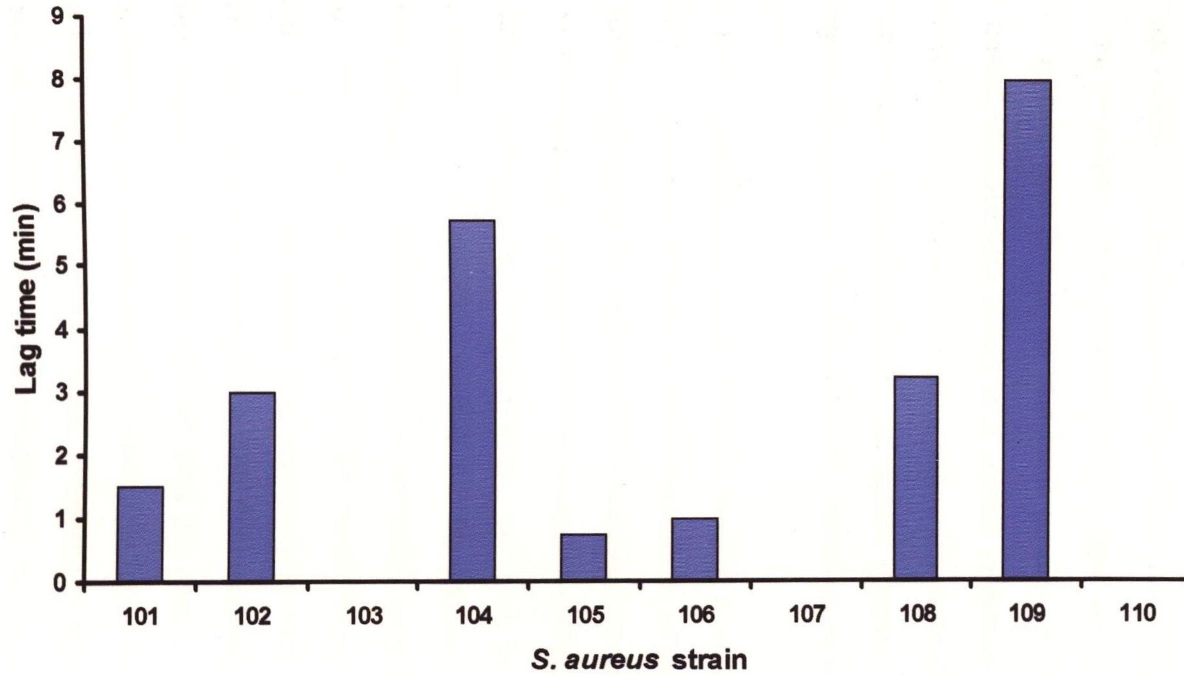


Figure 7.9 Activation of human platelet aggregation by *S. aureus* infective endocarditis isolates 101-110. This experiment was performed once.

this experiment was only performed once, the statistical significance of these data cannot be calculated.

7.3 Discussion

S. aureus-induced activation of human platelet aggregation is clearly a complicated process that involves the interaction of several *S. aureus* surface proteins with the platelet. Data presented in this chapter shows that these include ClfA, ClfB, SdrE and protein A. The redundancy in function that is associated with *S. aureus*-induced platelet aggregation suggests that this is an important process in the pathogenicity of the organism. Indeed, the excellent ability of *S. aureus* to induce platelet aggregation may contribute to the capacity of the organism to induce IE, especially in individuals without prior valvular damage, where the activation of platelet aggregtion by *S. aureus* could lead to the generation of a platelet thrombus and the formation of a vegetative lesion on the heart valve.

Because of the multi-factorial mechanisms present in *S. aureus* for activating platelet aggregation, it is extremely difficult to study the effects of individual proteins on the process. Studying the ability of single bacterial mutants to induce platelet aggregation is futile due to the presence of additional compensatory mechanisms on the bacterium. Therefore, using heterologous expression systems, such as *L. lactis*, to express individual proteins in isolation is an excellent method for identifying surface proteins that are involved in complicated processes such as the activation of platelet aggregation. Using this system, a novel Ca^{2+}-dependent mechanism for activating platelet aggregation was demonstrated. This novel mechanism was mediated by SdrE. Inhibition of *L. lactis* (pKS80:*sdrE*)-mediated platelet aggregation with the recombinant A-region of SdrE and with anti-SdrE region A antibodies showed that this was a specific reaction. These experiments also showed that the domain responsible for activating platelet aggregation in SdrE was localised in the unique A-region. Expressing ClfA and ClfB in *L. lactis* also confirmed that both of these proteins were involved in the process, as both could activate platelet aggregation in the absence of Ca^{2+} and in the presence of low concentrations (60 μM) of Ca^{2+}. Furthermore, heterologous expression of ClfA, ClfB and SdrE in *L. lactis*

showed that each of these *S. aureus* surface proteins were capable of independently activating platelet aggregation.

However, using heterologous expression systems does have its drawbacks. When protein A was expressed in *L. lactis*, these cells were not capable of inducing platelet aggregation. However, further experiments with *S. aureus* mutants showed that although protein A did not appear to be sufficient for activating platelet aggregation, it did appear to play an auxiliary role in the process. Protein A appeared to enhance the ability of SdrE to activate platelet aggregation. When ClfA was absent from stationary phase *S. aureus* cells (in a Newman *clfA* mutant) and SdrE and protein A were still present, a delay in lag time was observed when compared with the wild type cells. The delay in lag time was further enhanced when both ClfA and protein A were absent (in a Newman *clfAspa* double mutant) but when SdrE was still present. This indicated that although SdrE was capable of activating platelet aggregation on its own, its effects were enhanced in the presence of protein A. It is possible that attachment of the *S. aureus* cell to the platelet via a protein A-dependent mechanism allows closer contact between the two cells, enhancing weaker interactions (i.e. between SdrE and its receptor on the platelet). Stronger interactions such as that between ClfA and its platelet receptor probably do not require the assistance of auxiliary molecules. Ancillary functions such as these cannot be identified through heterologous expression systems.

SdrE was also expressed from a multi-copy plasmid vector in *S. aureus* strain 8325-4, a strain that does not carry a copy of the *sdrE* gene and therefore does not express the SdrE protein. 8325-4 does not activate platelet aggregation as well as strain Newman does. This could be due to a number of factors, including a difference in the expression levels of proteins such as ClfA and ClfB, as well as the absence of SdrE in strain 8325-4. However, when SdrE was expressed from a multi-copy plasmid in strain 8325-4, the lag time was significantly decreased compared to the wild type, indicating that SdrE could activate platelet aggregation when expressed in *S. aureus*.

It was difficult to show the effects of individual proteins on the activation of platelet aggregation directly in *S. aureus*, using specific mutants, due to the redundancy in function. Because a role for ClfA and ClfB had previously been demonstrated directly in *S. aureus* by Cox's group, it was decided to concentrate on demonstrating a direct role for

SdrE in stationary phase *S. aureus* strain Newman cells. Identifying each and every protein involved in *S. aureus*-mediated activation of platelet aggregation was beyond the scope of this project. Using Newman *clfA, spa* and *sdrE* mutants, it was shown that SdrE was involved in activating platelet aggregation by *S. aureus* strain Newman. This appears to be a rather weak interaction, which reflects the long lag time to aggregation seen with *L. lactis* cells expressing SdrE. However, the ability of SdrE to activate platelet aggregation appears to be enhanced when protein A is present. In addition, the effects of SdrE on platelet aggregation were overshadowed when ClfA is expressed by the bacterium. ClfA appears to be one of the primary factors present on the surface of *S. aureus* that induces platelet aggregation. This is seen in the very short lag time to aggregation with *L. lactis* cells expressing ClfA and in the significant delay in lag time observed in the Newman *clfA* mutant. The interaction between ClfA and the platelet is probably the most biologically relevant interaction. Other interactions probably only come into play in environments where ClfA is not expressed, or perhaps in situations where ClfA is already bound to another host molecule such as fibrinogen and is not accessible. ClfB appears to have an "intermediate" ability to activate platelet aggregation. The lag time to aggregation with *L. lactis* cells expressing ClfB was longer than with *L. lactis* cells expressing ClfA but significantly shorter than with the SdrE-expressing cells. However, the role of ClfB in activating platelet aggregation has not been examined in detail.

The mechanism by which ClfA, ClfB, SdrE and protein A interact with the platelet to induce aggregation is not understood. A recent report indicated that protein A can interact directly with the platelet gC1qR/p33 molecule (Nguyen *et al.*, 2000). *S. aureus* also interacts with the platelet Fc receptor molecule (Yeaman *et al.*, 1992). This interaction is thought to occur through an indirect attachment of protein A to the Fc receptor, with an IgG molecule forming a bridge between the two. Protein A can also bind to von Willebrand factor (Hartlieb *et al.*, 2000), a protein that is also bound by the platelet GPIb-IX-V complex and the GPIIb/IIIa receptor. Therefore, it is possible that protein A could interact with the platelet via a von Willebrand factor bridge. However, the relevance of these interactions to the ancillary role that protein A plays in activating platelet aggregation is not known.

Since both ClfA and ClfB molecules and the platelet GPIIb/IIIa receptor can bind to fibrinogen, it has been speculated that ClfA and ClfB may induce platelet aggregation by an indirect interaction with the GPIIb/IIIa receptor via a fibrinogen bridge. However this has not been shown and the possibility that ClfA and ClfB may stimulate platelet aggregation via a direct mechanism cannot be ruled out.

The mechanism by which SdrE stimulates platelet aggregation is unknown. Since no matrix or plasma protein ligand has been identified for SdrE, it is not possible to speculate on whether the interaction between SdrE and the platelet is direct or indirect. However, platelets are known to bind a number of different plasma proteins, which could conceivably form a cross-bridge between SdrE and a platelet receptor. In fact, preliminary experiments performed by D. Cox's group indicate that SdrE only interacts with platelets in the presence of plasma (unpublished). Further experiments are required to identify the platelet receptors that CfA, ClfB and SdrE bind to and to determine if the reaction is direct or indirect.

These experiments have clearly shown that the *S. aureus* cell wall-associated proteins ClfA, ClfB and SdrE can activate the aggregation of human platelets and that protein A has an ancillary role in the process. However, the possibility that other *S. aureus* proteins are also involved cannot be ruled out. To identify this possibility, further experiments are required. These should focus on the ability of exponential phase cells to induce platelet aggregation and would require the construction of a *S. aureus* Newman mutant that is deficient in ClfA, ClfB, SdrE and protein A production. Combining these four mutations in a single strain is possible, but technically difficult and would require further allele replacements, using alternative antibiotic resistance markers. If the *clfAclfBsdrEspa* mutant still activates platelet aggreagtion in exponential phase, then further factors are involved in the process. However, if no aggregation occurs with this mutant, then it would be clear that ClfA, ClfB, SdrE and protein A were the only proteins required for activating *S. aureus*-induced platelet aggregation.

Chapter 8

Interactions Between ClfA, ClfB, SdrC, SdrD and SdrE with Host Matrix and Plasma Proteins.

8.1 Introduction

The interactions between *S. aureus* cell wall-associated proteins and various host matrix and plasma proteins have been studied in detail over the past number of years. This reflects the importance of these surface protein-mediated interactions to the pathogenicity of *S. aureus,* in initiating colonisation and interacting with the host during infection. To date, several interactions have been characterised in detail. *S. aureus* binds to fibrinogen via ClfA and ClfB (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998), collagen via Cna (Patti *et al.*, 1992), fibronectin via FnBPA and FnBPB (Signas *et al.*, 1989; Jönsson *et al.*, 1991), elastin via EbpS (Park *et al.*, 1996) and to von Willebrand factor via protein A (Hartlieb *et al.*, 2000). However, *S. aureus* is also known to interact with a number of different host matrix and plasma proteins for which no bacterial receptor has been identified. For example, although *S. aureus* interacts with vitronectin (Liang *et al.*, 1992, 1994; Chhatwal *et al.*, 1987), laminin (Herrmann *et al.*, 1988; Lopes *et al.*, 1985; Mota *et al.*, 1988), thrombospondin (Herrmann *et al.*, 1991) and mucin (Shutter *et al.*, 1996), no bacterial receptors for these ligands have been identified.

One of the problems associated with identifying phenotypes for novel *S. aureus* proteins is the redundancy in function that exists for many interactions between the organism and the host matrix. This is evident from the existence of at least two different fibrinogen-binding proteins (ClfA and ClfB) and two distinct fibronectin-binding proteins (FnBPA and FnBPB) in most *S. aureus* strains. In addition, it is now becoming apparent that individual *S. aureus* proteins can bind to more than one ligand. For example, protein A is known to bind to IgG molecules (Sojdahl *et al.*, 1977; Moks *et al.*, 1986), to platelets (Herrmann *et al.*, 1993; Nguyen *et al.*, 2000) and to von Willebrand factor (Hartlieb *et al.*, 2000). In addition, FnBPA binds to fibrinogen as well as to fibronectin (Wann *et al.*, 2000). These factors all contribute to making the identification of novel interactions difficult.

The *S. aureus* Sdr proteins were identified in 1998 (Josefsson *et al.*, 1998a). However, despite efforts by this laboratory and the laboratory of M. Höök (Texas A & M, Houston), the host protein ligands for SdrC, SdrD and SdrE remain unidentified. Testing direct binding of various host proteins to the recombinant A domains of SdrC, SdrD and

SdrE showed that these *S. aureus* proteins did not appear to bind to vitronectin, laminin, thrombospondin, fibrinogen, fibronectin, osteopontin, decorin or bone sialoprotein (Dr. K. McCrea, unpublished). While it is possible that the Sdr proteins bind to none of these ligands, it also must be taken into consideration that using recombinant proteins can sometimes be misleading. Proteolytic cleavage of recombinant proteins often occurs during storage, even at –80°C, and this could lead to a loss of protein function. In addition, expressing a sub-domain of a Gram-positive protein in the Gram-negative bacterium *E. coli* may lead to the formation of an alternative conformational structure in the protein that could eliminate ligand-binding potential. Therefore, analysing the function of recombinant *S. aureus* surface proteins does not necessarily reflect the interactions that occur when the protein is expressed on the surface of the bacterium.

Considering these factors, the use of heterologous expression systems where the native cell wall-associated protein is expressed on the surface of a Gram-positive surrogate host is an attractive method for studying host-ligand interactions. Expression of ClfA, ClfB, SdrC, SdrD and SdrE in the Gram-positive host *L. lactis* is described in Chapter 6. Data presented in Chapter 6 showed that these proteins were expressed on the surface of *L. lactis* in their full-length form and that they were functional. Studying the interactions between *L. lactis* cells expressing *S. aureus* surface proteins overcomes the problem of redundancy experienced in *S. aureus* and presents a more precise method for investigating these interactions than with recombinant proteins.

In this chapter, *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD and SdrE were used to study interactions with the host proteins collagen, vitronectin, mucin, elastin, fibronectin and von Willebrand factor. The interaction between these proteins and fibrinogen is described in Chapter 6. When ClfA and ClfB were expressed on the surface of *L. lactis*, these cells adhered to immobilised fibrinogen. No adherence to fibrinogen was observed with *L. lactis* cells that expressed SdrC, SdrD or SdrE.

8.2 Results

8.2.1 Interactions Between ClfA, ClfB, SdrC, SdrD and SdrE and Human Collagen

S. aureus binds to type I, II, III and IV collagen (Switalski *et al.*, 1993). This reaction is thought to be solely mediated by the *S. aureus* protein Cna (Patti *et al.*, 1992). However, the possibility that other bacterial proteins are involved in this interaction cannot be ruled out completely. The ability of the Clf-Sdr proteins to mediate this interaction was tested. Type II human collagen was immobilised on a 96-well ELISA plate at a concentration of 10 μg/ml, overnight at 4°C, as described in section 2.19.1. *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD and SdrE were grown to stationary phase (16 hours) at 30°C in M17 broth containing 0.5% glucose and 5 μg/ml Em, with no shaking. The *L. lactis* host strain MG1363 was also grown under similar conditions, but with no Em. Cells were harvested, washed in PBS and resuspended to an OD_{600nm} of 1.0. Following blocking with 2 % (w/v) BSA, 100μl of cells were added to each well and their ability to adhere to immobilised collagen tested as described in section 2.19.1. Results showed that none of the cells adhered to immobilised type II collagen (data not shown).

8.2.2 Interactions Between ClfA, ClfB, SdrC, SdrD and SdrE and Bovine Mucin

Previous reports have shown that *S. aureus* binds to mucin and as yet, the bacterial receptor for this protein remains unidentified. The ability of *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD and SdrE to adhere to bovine mucin was tested. 96-well ELISA plates were coated overnight at 4°C with 20 μg/ml bovine mucin, as described in section 2.19.1. *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD or SdrE and the *L. lactis* MG1363 wild type cells were grown and prepared as described in section 8.2.1. Following blocking of the plates with 2% (w/v) BSA, 100 μl of cells were added to each well and their ability to adhere to immobilised bovine mucin measured, as

described in section 2.19.1. Results indicated that none of the proteins tested bound to bovine mucin (data not shown).

8.2.3 Interactions Between SdrC, SdrD and SdrE and Human Vitronectin

The bacterial receptor responsible for the interaction between *S. aureus* and vitronectin has not yet been identified. *S. aureus* cells bind to vitronectin in solution (Liang *et al.*, 1992, 1994; Chhatwal *et al.*, 1987). However, because *S. aureus* does not adhere to immobilised vitronectin (This study; S. Peacock, personal communication), the interaction between the Sdr proteins and vitronectin could not be measured by adherence assay. Instead, ligand affinity blotting was performed. 100 μg human vitronectin was biotinylated as described in section 2.20. Cell wall-associated proteins and protoplast fractions were isolated from *L. lactis* cells expressing SdrC, SdrD and SdrE, as described in section 2.20. The cell wall-associated proteins and protoplasts were separated by SDS-PAGE and transferred to a PVDF membrane. Following overnight blocking in 5% (w/v) BSA, membranes were incubated with biotinylated vitronectin, were washed and were incubated with Strepavidin-HRP, as described in section 2.20. Blots were then developed and exposed to autoradiographic film, as described in section 2.15. No reaction between proteins isolated from the cell wall of the *L. lactis* strains expressing SdrC, SdrD or SdrE and the biotinylated vitronectin was detected (Figure 8.1, lanes 1-3) However, a reaction was observed with a protein from the protoplast fraction of the SdrC, SdrD and SdrE-expressing cells (Figure 8.1, lanes 4-6) As this protein was the same size in each fraction (145 kDa) and because it did not correlate with the predicted size of any of the Sdr proteins, it was assumed that this vitronectin-binding protein was a *L. lactis* protein that is membrane-bound or located in the cytoplasm. SdrC, SdrD and SdrE do not appear to interact with human vitronectin. However, studying this interaction by ligand affinity blotting does not completely rule out possibility that whole *L. lactis* cells can bind to vitronectin.

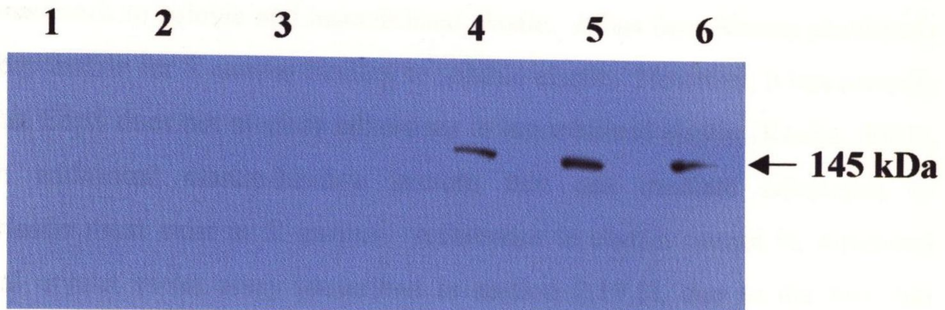


Figure 8.1 Western ligand affinity blot showing the interaction between *L. lactis* cells expressing SdrC, SdrD or SdrE and biotinylated human vitronectin. Lane 1, cell wall-associated proteins isolated by mutanolysin/lysozyme digestion of *L. lactis* cells expressing SdrC; Lane 2, cell wall-associated proteins isolated by mutanolysin/lysozyme digestion of *L. lactis* cells expressing SdrD; Lane 3, cell wall-associated proteins isolated by mutanolysin/lysozyme digestion of *L. lactis* cells expressing SdrE; Lane 4, protoplast fraction of *L. lactis* cells expressing SdrC; Lane 5, protoplast fraction of *L. lactis* cells expressing SdrD; Lane 6, protoplast fraction of *L. lactis* cells expressing SdrE.

8.2.4 Interactions Between ClfA, ClfB, SdrC, SdrD and SdrE and Bovine Kappa-Elastin Peptides

S. aureus binds to soluble and immobilised elastin. It has been shown previously that EbpS is responsible for *S. aureus* binding to soluble elastin. However, it has recently been shown that EbpS does not mediate adherence to immobilised elastin (Roche, 2001). Therefore, an additional elastin-binding protein that can mediate adherence to immobilised elastin must exist in *S. aureus*. Adherence to elastin cannot be measured using the usual crystal violet assay (described in section 2.19.1), due to the fact that crystal violet binds to elastin. Instead, an alternative approach in analysing bacterial cell adherence to immobilised proteins is employed. This is a fluorometric assay in which cells are labeled with a fluorescent nucleic acid-binding probe (SYTO 13) and bacterial adherence measured in a luminescence spectrometer.

L. lactis cells expressing ClfA, ClfB, SdrC, SdrD or SdrE and the *L. lactis* wild type cells were grown to stationary phase and prepared as described in section 8.2.1. A 96-well ELISA plate was coated overnight under UV light with concentrations of κ-elastin ranging from 50 μg/well to 3 μg/well, as described in section 2.19.2. Following blocking of the plates with 5% (w/v) BSA, 100 μl of cells were added to each well and their ability to adhere to immobilised elastin tested, as described in section 2.19.2. Results showed that *L. lactis* cells expressing ClfB adhered to elastin in a dose-dependent manner (Figure 8.2). *L. lactis* cells that expressed ClfA, SdrC, SdrD or SdrE did not adhere. These data indicate that ClfB can bind to immobilised κ-elastin.

However, demonstrating this phenotype in *S. aureus* has not been so straightforward and several inconsistencies appear to exist. Although ClfB bound to elastin when expressed on the surface of *L. lactis* and also in its recombinant form (E. Walsh, unpublished), these data were not reinforced by binding studies with *S. aureus* mutants. A *S. aureus* strain P1 *ebpS* mutant still adheres to immobilised elastin (Roche, 2001). If ClfB was the only factor responsible for adherence to elastin in *S. aureus*, then a *clfB* mutant should not adhere. However, adherence to elastin was still observed in both a *clfB* and a *clfBebpS* mutant of *S. aureus* strain P1 (F. McAleese, unpublished). It appeared that FnBPA and FnBPB were the major determinants of binding to κ-elastin (F.

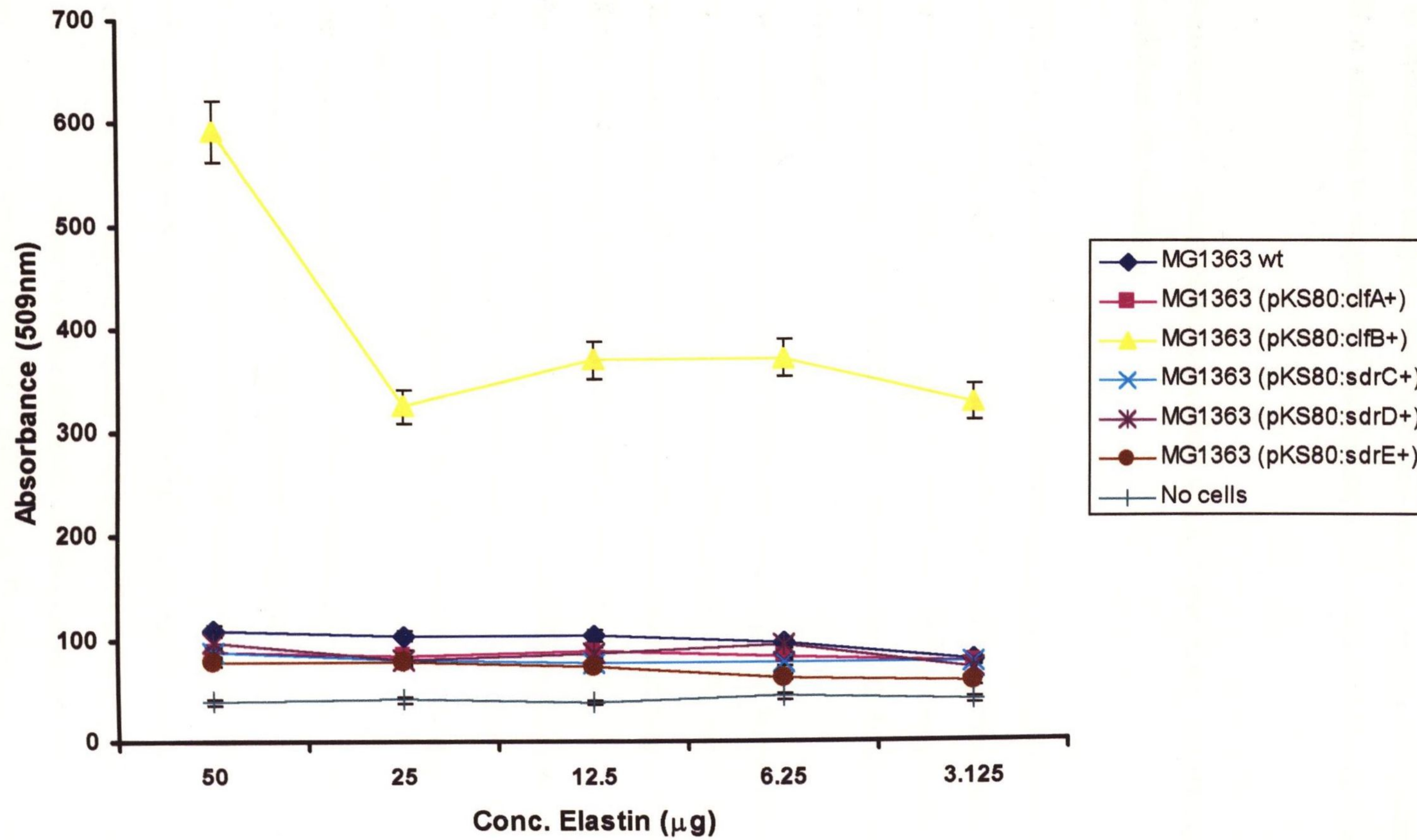


Figure 8.2 Adherence of *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD or SdrE to immobilised elastin. Elastin was coated on a 96-well plate at concentrations ranging from 50 μg- 3 μg/well.

Roche, unpublished). Furthermore, no reduction in elastin binding was observed in either of these mutant strains. These data suggest that while ClfB may be responsible for adhering to immobilised elastin, another protein is also present which can mediate adherence to immobilised elastin. Further experiments are required to characterise the role of ClfB in adhering to immobilised elastin and to show that this interaction occurs in *S. aureus*.

8.2.5 Adherence of *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD and SdrE to immobilised fibronectin.

In *S. aureus*, adherence to immobilised fibronectin is mediated by the fibronectin-binding proteins FnBPA and FnBPB. However, the possibility that other *S. aureus* proteins can interact with fibronectin cannot be ruled out. In this experiment, the ability of *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD or SdrE to bind to immobilised fibronectin was tested.

A 96-well ELISA plate was coated overnight with 20 μg/ml human fibronectin, as described in section 2.19.1. *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD or SdrE and the *L. lactis* wild type cells were grown to stationary phase and prepared as described in section 8.2.1. Following blocking of the plates with 2% BSA, 100 μl of cells were added to the wells and their ability to adhere to immobilised fibronectin tested, as described in section 2.19.1. Results indicated that the *L. lactis* cells expressing SdrC adhered to immobilised fibronectin (Figure 8.3). *L. lactis* cells expressing ClfA, ClfB, SdrD or SdrE did not adhere.

As the apparent interaction between SdrC and fibronectin was weak, this binding phenotype was studied in more detail. The ability of the *L. lactis* cells expressing SdrC to adhere to a range of fibronectin concentrations was tested. 96-well ELISA plates were coated as before with fibronectin concentrations ranging from 20 μg/ml to 0.6 μg/ml. *L. lactis* cells expressing SdrC and the *L. lactis* wild type cells were grown and prepared as before and their ability to adhere to these different concentrations of fibronectin tested as described above. Results showed that SdrC adhered to fibronectin in a dose-dependent fashion when expressed on the surface of *L. lactis* (Figure 8.4).

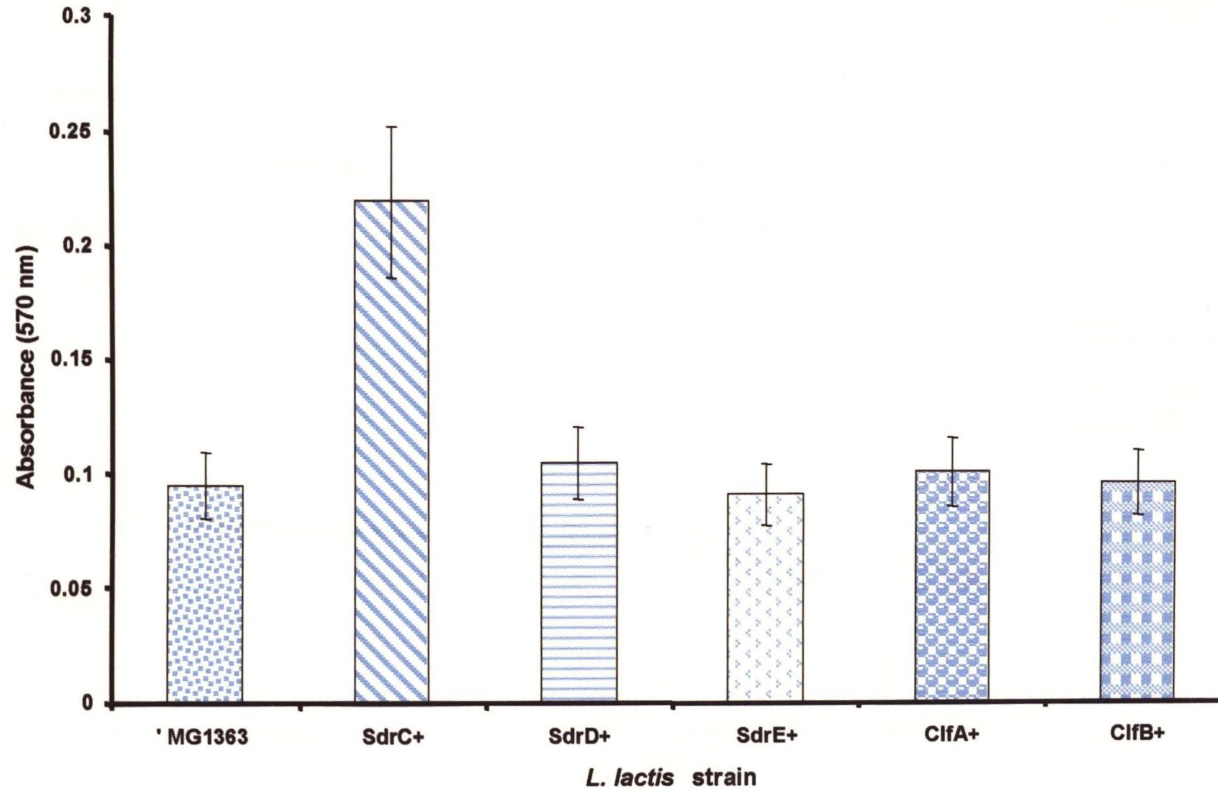


Figure 8.3 The interaction between *L. lactis* cells expressing CflA, ClfB, SdrC, SdrD and SdrE and immobilised human fibronectin. Plates were coated with 20 μg/ml fibronectin. This experiment was performed in duplicate.

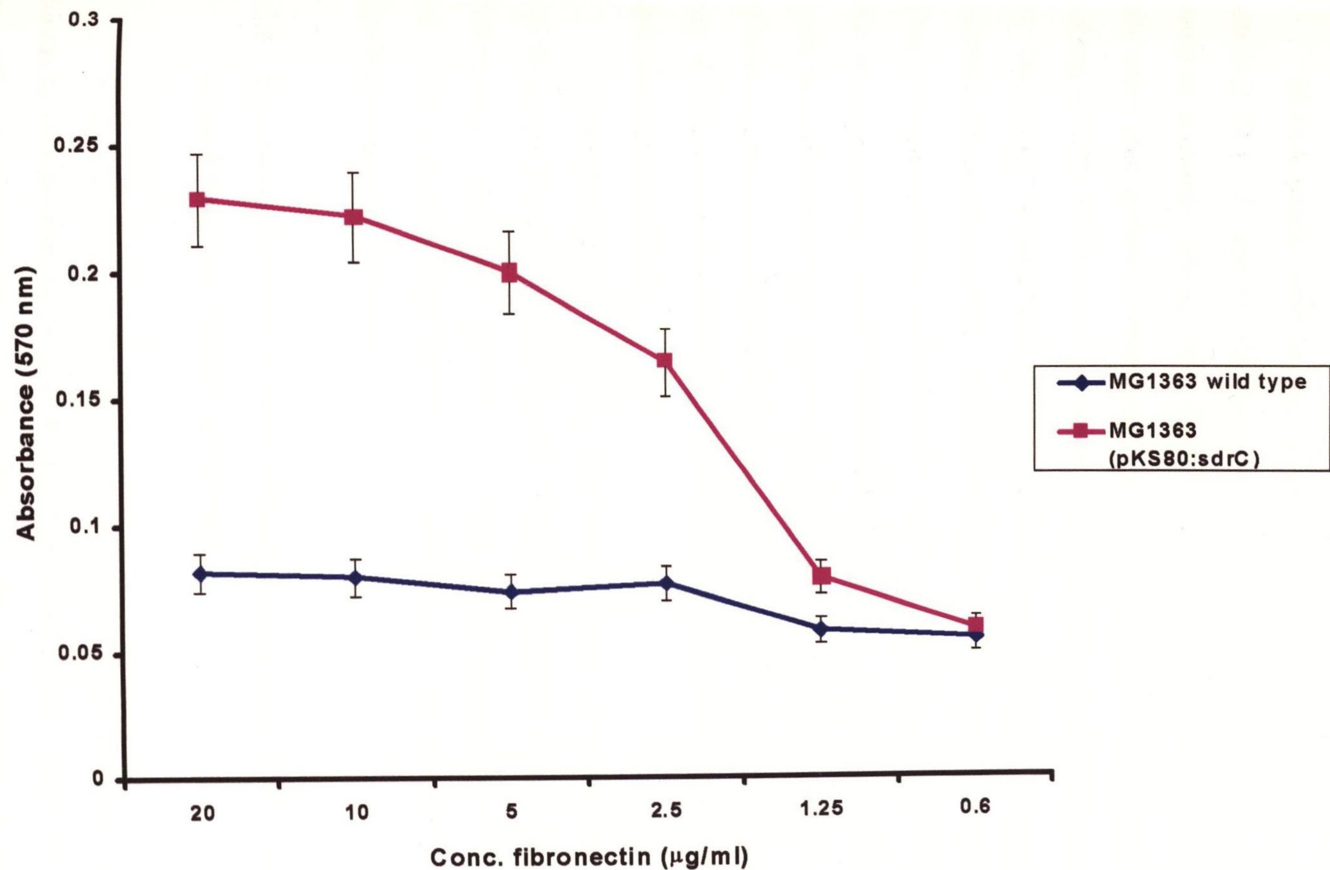


Figure 8.4 Dose-response adherence of *L. lactis* cells expressing SdrC to immobilised human fibronectin. 96-well ELISA plates were coated with different concentrations of immobilised fibronectin and the ability of *L. lactis* cells expressing SdrC to adhere tested. This experiment was performed in duplicate.

The ability of *S. aureus* Newman wild type cells and the Newman *sdrC* mutant to adhere to immobilised fibronectin was also tested. Because the *sdrC* mutation and the *fnbB* mutation (Greene *et al.*, 1995) were both marked with an erythromycin resistance determinant, these two mutations could not be combined and therefore, the fibronectin-binding capabilities of a *sdrC/fnbAB* mutation could not be assessed.

ELISA plates were coated overnight with 20 μg/ml fibronectin, as described in section 2.19.1. *S. aureus* Newman wild type cells and the Newman *sdrC* mutant were grown to stationary phase (16 hours) in 50 ml BHI broth, with shaking at 37°C. These cells were also grown to exponential phase (OD_{600nm} of 0.8) in 50 ml BHI broth, with shaking at 37°C. Cells were harvested, washed in PBS and resuspended to an OD_{600nm} of 1.0. The ability of these cells to adhere to immobilised fibronectin was then measured, as described in section 2.19.1. Results showed no difference in fibronectin binding with either the exponential or stationary phase cells (Figure 8.5). The fibronectin binding seen here was presumably due to the fibronectin binding proteins FnBPA and FnBPB. To assess the role of *sdrC* in *S. aureus*-mediated adherence to fibronectin, a novel *sdrC* allele replacement mutant would need to be constructed, containing either a silent mutation or a drug resistance marker other than Em^r (which marks the *fnbB* mutation) or Tet^r (which marks the *fnbA* mutation).

Although a role for SdrC in fibronectin binding was not shown in *S. aureus*, SdrC was shown to interact with fibronectin when expressed on the surface of *L. lactis*. This binding was weak when compared to the ligand binding capacity of proteins such as ClfA and ClfB. Further experiments are required in order to establish the biological significance of this interaction.

8.2.6 Interaction between *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD or SdrE immobilised von Willebrand factor.

S. aureus can interact with von Willebrand factor. Recently, this interaction was shown to be mediated by protein A (Hartlieb *et al.*, 2000). However, it is also possible that other *S. aureus* proteins can interact with von Willebrand factor. To test the possibility that ClfA, ClfB, SdrC, SdrD or SdrE might bind to von Willebrand factor, *L.*

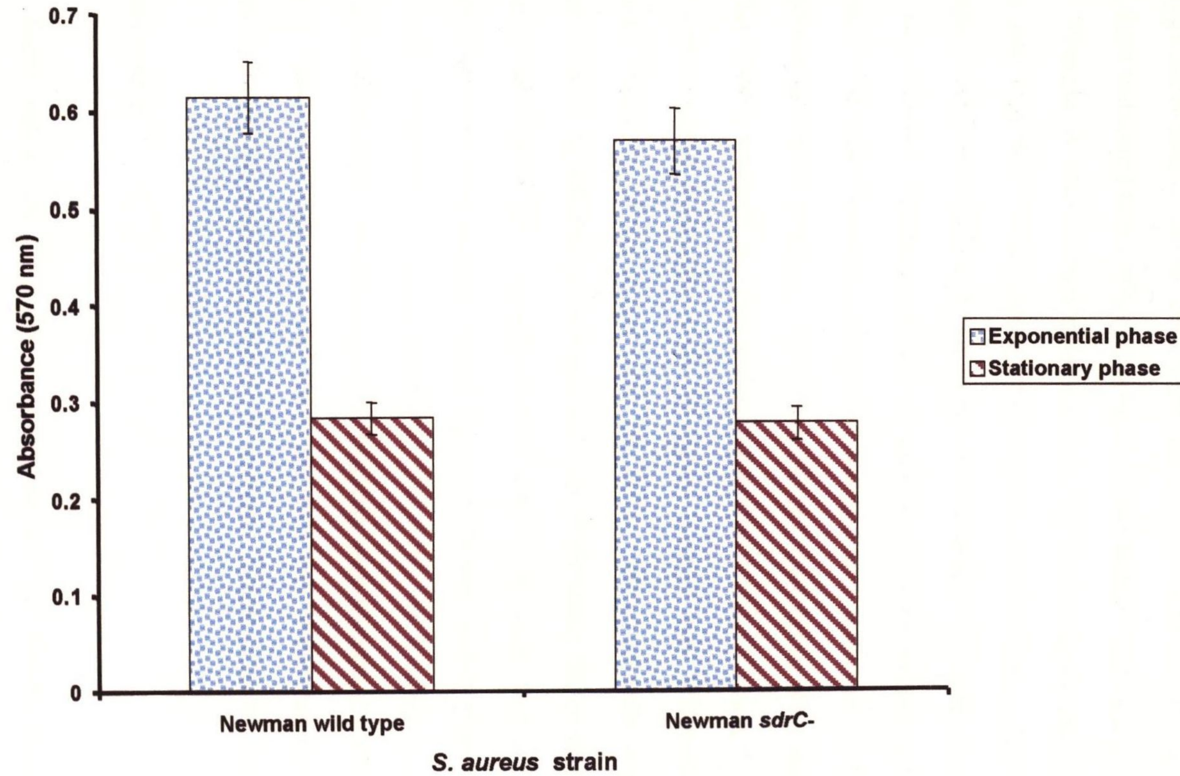


Figure 8.5 Interaction between *S. aureus* Newman wild type cells and the Newman *sdrC*- mutant and immobilised human fibronectin. Cells were grown to exponential and stationary phase and their ability to adhere to 20 μg/ml fibronectin tested. This experiment was performed in duplicate.

lactis cells expressing these proteins were grown to stationary phase and were prepared as described in section 8.2.1. 96-well ELISA plates were coated overnight with 20 μg/ml human von Willebrand factor as described in section 2.19.1. Following blocking of the plates with 2% BSA, 100 μl of cells were added to the wells and their ability to adhere to immobilised von Willebrand factor measured. Results showed that *L. lactis* cells expressing ClfA adhered to immobilised von Willebrand factor (Figure 8.6). *L. lactis* wild type cells and *L. lactis* cells expressing ClfB, SdrC, SdrD or SdrE did not adhere. These data indicated that ClfA could bind to von Willebrand factor.

Protein A was shown previously to be involved in the interaction between *S. aureus* and von Willebrand factor. To test the role of ClfA in this interaction, *S. aureus* Newman wild type cells and Newman cells containing mutations in the *spa* and *clfA* genes were tested for their ability to adhere to immobilised von Willebrand factor. Newman wild type, Newman *clfA*, Newman *spa* and Newman *clfAspa* cells were grown to exponential phase (OD_{600nm} of 0.8) in 50 ml BHI broth with shaking at 37°C. Cells were harvested, washed in PBS and resuspended to an OD_{600nm} of 1.0. 96-well ELISA plates were coated overnight with 20 μg/ml human von Willebrand factor, as before. Following blocking of the plates with 2% BSA, 100 μl of cells were added to the wells and their ability to adhere to immobilised von Willebrand factor tested as before. Results are shown in Figure 8.7. A reduction in von Willebrand factor binding was observed with the Newman *spa* mutant, which agrees with previously published data (Hartlieb *et al.*, 2000). However, a more dramatic reduction in binding was observed with the Newman *clfA* mutant. Furthermore, no binding was observed with the Newman *clfAspa* mutant. These data indicated that there are two von Willebrand factor-binding proteins expressed during exponential phase by *S. aureus*. These proteins are ClfA and protein A.

8.3 Discussion

L. lactis strain MG1363 does not appear to bind to any human matrix or plasma proteins. Therefore, expressing *S. aureus* cell wall-associated proteins in *L. lactis* provides an efficient mechanism for analysing their ligand-binding potential. This is usually difficult in *S. aureus* due to the problem of poor expression and functional

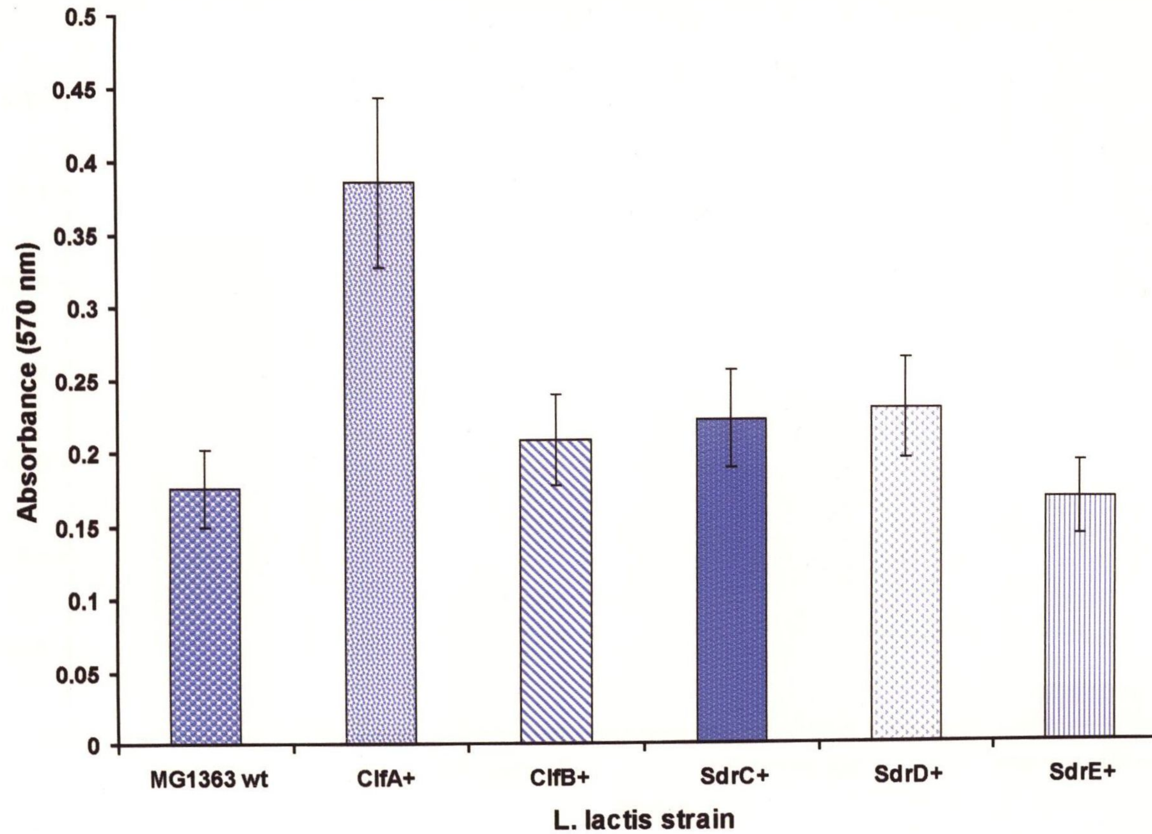


Figure 8.6 Adherence of *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD or SdrE to immobilised von Willebrand factor. 96-well ELISA plates were coated with 20 μg/ml human von Willebrand factor. This experiment was performed once.

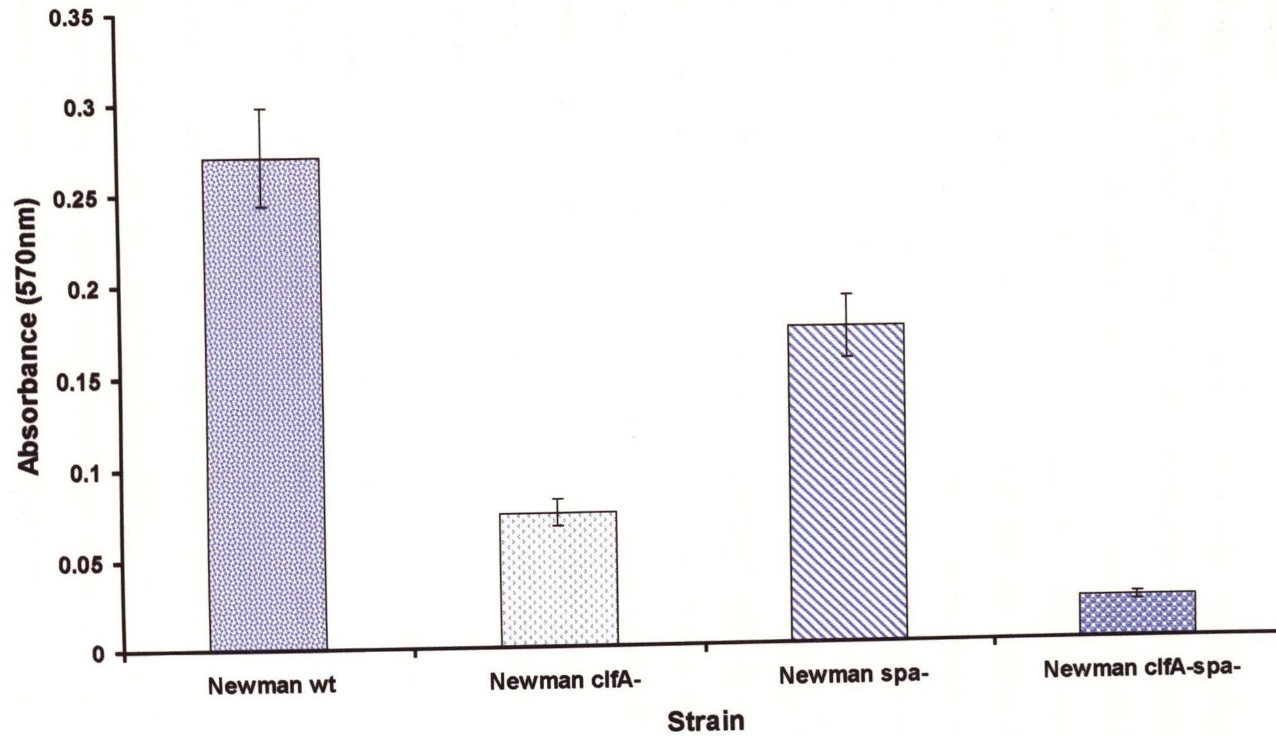


Figure 8.7 Adherence of *S. aureus* Newman wild type and *clfA*, *spa* or *clfAspa* cells to immobilised von Willebrand factor. 96-well ELISa plates were coated with 20 μg/ml human von Willebrand factor. This experiment was performed once.

redundancy. This chapter describes the interactions between *L. lactis*-expressed ClfA, ClfB, SdrC, SdrD and SdrE proteins and collagen, mucin, vitronectin, elastin, fibronectin and von Willebrand factor.

No interaction was observed between collagen and any of the Clf-Sdr proteins. This is not surprising as previous reports have indicated that no residual binding exists in a *S. aureus cna* mutant.

None of the Clf-Sdr proteins interacted with bovine mucin when expressed in *L. lactis*. To date, the *S. aureus* receptor for mucin remains uncharacterised. However, at least six novel LPXTG motif-containing *S. aureus* proteins have been identified through genome sequence analysis (M. Pallen, T. J. Foster & F. Roche, unpublished). These proteins contain several features which suggest that they are surface displayed and it is likely that at least one of these proteins is responsible for binding to mucin. This possibility will be tested in the future by similar heterologous expression of the candidate *S. aureus* protein in *L. lactis*.

Ligand affinity blotting of proteins isolated from SdrC, SdrD and SdrE-expressing *L. lactis* cells indicated that none these proteins interacted with vitronectin. These data agree with previous studies performed by Dr K. McCrea (Texas A&M, Houston) who showed that recombinant Sdr proteins did not interact with vitronectin. In addition, the ability of *L. lactis* cells expressing SdrC, SdrD and SdrE to bind to soluble I^{125}-labeled vitronectin was tested by Dr. K. McCrea. Results indicted that these strains did not bind to vitronectin either (K McCrea, unpublished). In addition, although a vitronectin-binding protein appeared to be present in the protoplast fraction of *L. lactis*, results found by Dr. K. McCrea indicated that *L. lactis* MG1363 wild type did not bind to soluble vitronectin (K. McCrea, unpublished data). To date, the *S. aureus* receptor for vitronectin remains uncharacterised.

The Clf-Sdr proteins were also tested for their ability to adhere to immobilised elastin. EbpS is known as the elastin-binding protein of *S. aureus* (Park *et al.*, 1996). However, a recent report has shown that while EbpS binds to soluble elastin, it does not mediate adherence to solid-phase or immobilised elastin (Roche, 2001). As *S. aureus* can adhere to immobilised elastin, another elastin-binding moiety must be present in *S. aureus*. Results presented in this chapter indicated that ClfB could mediate adherence to

immobilised elastin peptides in a dose-dependent fashion when expressed on the surface of *L. lactis*. None of the other Clf-Sdr proteins tested had this property.

However, experiments performed by F. McAleese and F. Roche (unpublished) indicated that another elastin-binding protein distinct from EbpS and ClfB is present in *S. aureus*. No role for ClfB in the adherence of *S. aureus* to elastin was shown in these experiments. These data do not agree with the results presented in this chapter, which show that ClfB mediated strong adherence of *L. lactis* cells to elastin. Further experiments are required to elucidate a role for ClfB in mediating the adherence of *S. aureus* cells to elastin. In *S. aureus*, ClfB is expressed at much lower levels than in *L.lactis* and is proteolytically cleaved to a truncated form during cell growth (McAleese *et al*., 2001). It is possible that the elastin-binding capacity of ClfB is far weaker in *S. aureus* than in *L. lactis* due to much lower expression levels in the native host and that this makes it difficult to detect the phenotype in *S. aureus*. Alternatively, the elastin-binding domain of ClfB may be present in the N-terminal domain N1 of region A, which is lost when the protein is expressed in *S. aureus*, but is still retained when ClfB is expressed in *L. lactis*. In addition, ClfB-mediated elastin-binding in *S. aureus* may be overshadowed by stronger adherence promoted by another protein. Further experiments are required to investigate these possibilities.

The interaction between SdrC and fibronectin is interesting. When SdrC was expressed in *L. lactis*, a weak dose-dependent interaction with fibronectin was detected. This was not observed with any of the other Clf-Sdr proteins tested. However, no difference in fibronectin-binding was seen with a *S. aureus sdrC* mutant when compared to the wild type cells. The adherence to fibronectin observed with the *S. aureus sdrC* mutant cells was most likely due to the fibronectin binding proteins, FnBPA and FnBPB. Both of these proteins are known for their ability to mediate adherence to fibronectin and until now were thought to be the only fibronectin-binding moieties expressed by *S. aureus*. However, a small amount of residual fibronectin-binding still occurs in a *S. aureus fnbAfnbB* double mutant, which could possibly be due to SdrC. In order to show a role for SdrC in the adherence of *S. aureus* to fibronectin, binding experiments should be performed in a *fnbAfnbB* and a *fnbAfnbBsdrC* mutant background. Unfortunately, this was not possible as both the *fnbB* and *sdrC* mutations were marked by an erythromycin

resistance determinant and therefore could not be combined by transduction. In order to perform these experiments, a novel allele replacement mutant in *sdrC* would need to be constructed.

The fact that only weak fibronectin-binding was observed with SdrC when expressed at very high levels in *L. lactis* suggests that SdrC probably only mediates weak fibronectin adherence in *S. aureus*. This hypothesis would agree with the fact that the residual fibronectin binding observed in a *S. aureus fnbAfnbB* double mutant is weak. This type of weak binding has been observed before. The A domain of FnBPA contains a region that binds to fibrinogen. However, this binding is weak and can only be demonstrated in *S. aureus* when the protein is over-expressed from a multi-copy plasmid (Wann *et al.*, 2000). Further experiments are required to investigate the possibility that a similar interaction occurs between SdrC and fibronectin.

All of the Clf-Sdr proteins were tested for their ability to adhere to immobilised von Willebrand factor (vWF). vWF is a large multimeric plasma protein that plays an important role in maintaining haemostasis. One of its main functions is to promote platelet adhesion at thrombotic surfaces and it is present in abundance at sub-endothelial sites of vascular damage. *S. aureus* can adhere to vWF, a property which probably contributes to the colonisation of damaged endothelial cells. Until recently, the *S. aureus* receptor responsible for binding to vWF factor was unidentified. However, a role for protein A in the binding of *S. aureus* cells to vWF has now been shown (Hartlieb *et al.*, 2000).

When ClfA was expressed in *L. lactis*, these cells could adhere to immobilised vWF. This was not observed with *L. lactis* cells expressing ClfB, SdrC, SdrD or SdrE. This observation suggested that perhaps protein A was not the only *S. aureus* protein that could interact with vWF and that ClfA was also involved. To test this observation further, *S. aureus clfA* and *spa* mutants were tested for their ability to mediate adherence to vWF. A significant reduction in vWF-binding was observed with the *clfA* mutant cells, suggesting that ClfA was important for the interaction between *S. aureus* and vWF. As was reported by Hartlieb *et al.* (2000), a reduction in vWF-binding was also seen with the *spa-* mutant cells. However, with a *clfAspa* double mutant, vWF-binding was completely eliminated, with only background levels detected. These data suggested that

in addition to protein A, ClfA was also responsible for the binding of *S. aureus* cells to vWF. Further experiments are required in order to characterise this novel interaction in more detail.

To summarise, expressing the Clf-Sdr proteins in *L. lactis* provided an excellent mechanism for studying their interactions with host ligands. In addition to overcoming the problem of redundancy in *S. aureus*, expressing the proteins in *L. lactis* also allows the detection of weak interactions that may be difficult to identify in *S. aureus* due to poor expression. The principle aim of these experiments was to identify novel host ligands for the *S. aureus* Sdr proteins. The role of these proteins was previously unknown and it was thought that they might mediate some of the *S. aureus*-host protein interactions for which no bacterial receptor had been identified. However, no interactions were observed between any of the Sdr proteins and vitronectin or mucin, and the *S. aureus* receptor for these proteins remains unidentified. Previous studies performed by Dr. K. McCrea indicated that the Sdr proteins did not bind to thrombospondin or laminin either. The *S. aureus* receptors for these proteins also remain unidentified.

However, novel interactions were identified for ClfA and vWF, ClfB and elastin, and SdrC and fibronectin. The binding of SdrC to fibronectin represents the only interaction that has been shown for any of the Sdr proteins and a host matrix or plasma protein. Each of these interactions need to be analysed in more detail in order to show their relevance to *S. aureus* and to determine their biological significance.

Chapter 9

Discussion

9.1 Discussion

Despite the fact that *S. aureus* is one of the most important human pathogens in the world today, the mechanisms by which it controls expression of virulence factors and interacts with the host are less well understood than for other bacteria such as *Escherichia coli*, *Salmonella typhimurium* or *Listeria monocytogenes*. However, in recent years the level of research that focuses on *S. aureus* has intensified and many groups are now attempting to identify how *S. aureus* interacts with the host to initiate infection. This will be helped enormously with the recent availability of the four different S. aureus genome sequences. Several genetic loci, which are important to the pathogenicity of *S. aureus* have now been identified. Among these, cell wall-associated proteins or MSCRAMMs have been shown to be of prime importance in establishing infections. To date, only about half of the known *S. aureus* MSCRAMMs have been characterised in detail. These include ClfA, ClfB, protein A, FnBPA, FnBPB, Cna and EbpS. Several of these proteins have been shown to be important virulence factors in animal models of infection. Characterising novel MSCRAMMs in terms of their expression patterns in *S. aureus* and their ability to interact with the host matrix is important in order to determine the role that they play in the infection process. Following the identification of the *S. aureus* proteins SdrC, SdrD and SdrE in 1998, this project was established in order to characterise their expression in *S. aureus* and to determine what host molecules the proteins interact with.

In order to study the expression and function of a novel MSCRAMM, isogenic mutants that are defective in that protein are required. One of the first aspects of this project was to generate mutations in the *sdrC*, *sdrD* and *sdrE* genes of the two classical *S. aureus* laboratory strains, Newman and 8325-4. However, because strain 8325-4 does not have a copy of the *sdrE* this meant that allele replacement mutants could not be moved between the two strains by transduction, as the *sdrE* gene would have either been lost or introduced to the recipient strain. Instead, an alternative approach to generating mutations in the *sdr* genes was employed. This approach used the temperature-sensitive shuttle plasmid, pG+host9, which integrates into the chromosome of *S. aureus* at the restrictive temperature and remains stably integrated at 37°C. An internal fragment from the unique A region of *sdrC* and *sdrD* was cloned into pG+host9 and these constructs

were integrated into the chromosome of *S. aureus* strains Newman and 8325-4, disrupting the *sdrC* and *sdrD* genes.

Attempts to generate a similar disruption mutant in *sdrE* were unsuccessful. It is not clear why this approach did not work, but it is possible that the area of the *S. aureus* chromosome that contains *sdrE* is resistant to homologous recombination. Instead, an allele replacement mutant in the *sdrE* gene was constructed by transduction. First, an allele replacement mutation was constructed in the *sdrC* and *sdrD* genes of *S. aureus* strain 8325-4 (which does not have a copy of the *sdrE* gene). This double mutation was then transduced into strain Newman, resulting in the loss of the *sdrE* gene and thus, the generation of a triple mutation in the *sdrC*, *sdrD* and *sdrE* genes. Using this strain for expression and functional studies is not of course ideal. However, in the absence of a single *sdrE* mutant it did provide a valuable tool for expression and functional studies.

These mutants were used to study Sdr protein expression in *S. aureus* strains Newman and 8325-4. Comparing the expression profiles of a particular protein with that of the corresponding mutant in Western immunoblotting allows its unambiguous identification. It was important to identify if the Sdr proteins were actually expressed by *S. aureus* and thus were not pseudogenes. In addition, it was important to show where in the growth cycle that the proteins were expressed, to characterise the level of expression and to determine the degree of proteolytic cleavage that occurred during cell growth. These factors had important implications for functional studies.

Some of the results from the expression studies in *S. aureus* were surprising. In strain Newman, SdrC was expressed throughout the growth cycle with no apparent proteolytic degradation. However, SdrD was only detected in cells from stationary phase with substantial proteolytic degradation. This was very unusual as no other *S. aureus* MSCRAMMs appear to be expressed exclusively in stationary phase. Because MSCRAMMs are anchored to peptidoglycan during its formation, it was not thought that surface proteins could become anchored to the cell wall after the cells had stopped dividing. It is possible that SdrD is present at low levels during exponential phase and only builds up to detectable levels as the cells enter stationary phase. In contrast, SdrE was present throughout the growth cycle in strain Newman and was proteolytically degraded as the cells entered stationary phase.

A different pattern of expression was observed for SdrC and SdrD in strain 8325-4. In strain 8325-4, SdrC was only detected as a proteolytically degraded form in cells from stationary phase. Again this result was surprising as (1) it is unusual for an MSCRAMM to be expressed exclusively in stationary phase and (2) this expression profile differed from that of SdrC in strain Newman (which was expressed throughout the growth cycle). SdrD was expressed throughout the growth cycle in strain 8325-4, with a progressive proteolytic degradation occurring as the cells entered stationary phase. This expression pattern also differed from that of SdrD in strain Newman (which was only detected on cells from stationary phase). Clearly, SdrC and SdrD are regulated by different factors in strains Newman and 8325-4. This may be due to the fact that strain 8325-4 produces a defective σ^B protein, which is known to interact with the global regulatory locus *sarA*. If the expression of the Sdr proteins is dependent on σ^B or SarA, then this could explain the difference in expression patterns seen between the two *S. aureus* strains. A more in-depth analysis of Sdr protein expression is required in order to answer the questions that have arisen from these experiments. However, for the purpose of this study, sufficient information has been gathered to show that the proteins are expressed and to provide the necessary information on expression patterns for future ligand-interaction studies.

It was clear from the expression studies in *S. aureus*, that the Sdr proteins were not expressed at high levels and that in some cases, substantial proteolytic degradation occurred when the cells were grown *in vitro*. In addition to these factors, the problem of functional redundancy that occurs in *S. aureus* meant that comparing the phenotypes of wild type and *sdr* mutant cells was not an ideal method for studying novel interactions. To overcome these problems, each of the Sdr proteins were expressed in the Gram-positive surrogate host *L. lactis*. In order to validate the system, three proteins of known function, ClfA and ClfB from *S. aureus* and SdrG from *S. epidermidis*, were also expressed in *L. lactis*. In their native host, each of these proteins can promote adherence to immobilised fibrinogen. The ability of these proteins to promote adherence of the *L. lactis* cells to immobilised fibrinogen was tested. In each case, *L. lactis* cells that expressed ClfA, ClfB or SdrG could adhere to immobilised fibrinogen in a dose-dependent and saturable fashion. In addition, each of the proteins that were expressed by *L. lactis* were of similar size to the native protein that was expressed in either *S. aureus* or

S. epidermidis. These data suggested that staphylococcal proteins were expressed in *L. lactis* in a form that was functional and non-degraded.

However, N-terminal sequencing of ClfA and SdrE proteins expressed by *L. lactis* showed that although SdrE was expressed in its full-length mature form, the ClfA molecule expressed by *L. lactis* appeared to be a truncated version of the full length form, with approximately 150 aa missing from the N-terminus. However, this 170 kDa ClfA protein expressed by *L. lactis* is the same size as the ClfA molecule detected on the surface of *S. aureus* Newman cells by Western immunoblotting. These data suggest that although the stpahylococcal proteins which are expressed in *L. lactis* may not be the full-length forms of the molecules, they are the same size as the proteins that are expressed in the native host.

Thus, *L. lactis* cells expressing ClfA, ClfB, SdrC, SdrD or SdrE were used to study interactions with various host molecules. In collaboration with a group in the RCSI, Dublin (D. Cox), a role for ClfA, ClfB and SdrE in activating human platelet aggregation was identified. Protein A was also shown to play an auxiliary role in the process. These interactions were initially shown through testing the ability of *L. lactis* cells expressing various *S. aureus* MSCRAMMs to induce platelet aggregation. Of the proteins tested, ClfA, ClfB and SdrE were the only MSCRAMMs that could independently activate platelet aggregation. Protein A was not capable of independently inducing platelet aggregation, but appeared to assist in SdrE-mediated activation of platelet aggregation. The ability of each of these proteins to activate platelet aggregation was also demonstrated in *S. aureus*.

L. lactis cells that expressed the Clf-Sdr proteins were also used in an attempt to identify novel interactions with various host matrix or plasma proteins. None of Clf-Sdr proteins appeared to interact with collagen, vitronectin or mucin. The *S. aureus* receptors for vitronectin and mucin remain unidentified. *L. lactis* cells expressing ClfA and ClfB adhered to fibrinogen, as was previously reported. However, despite similar structural organisation and some sequence identity, none of the Sdr proteins interacted with fibrinogen. Interestingly, although ClfA binds to fibrinogen, a novel interaction between this protein and human von Willebrand factor was identified, reinforcing the idea that MSCRAMMs are multifunctional. In addition, ClfB was shown to interact with both

fibrinogen and elastin when expressed on the surface of *L. lactis*. None of the Sdr proteins interacted with either von Willebrand factor or elastin. The only novel interaction between any of the Sdr proteins and a host matrix/plasma protein that was identified was the interaction between SdrC and fibronectin. When SdrC was expressed in *L. lactis*, it could promote a dose-dependent adherence to fibronectin. However, this interaction was rather weak and could not be demonstrated in *S. aureus*, as no decrease in fibronectin adherence was detected in a *S. aureus sdrC* mutant. This interaction needs to be studied in more detail in a *fnbAfnbB* negative background in order to establish its biological relevance.

These experiments have defined the necessary procedures that should be followed for the characterisation of a novel surface protein in *S.aureus*. Previous surface protein characterisations were based on identifying the protein(s) responsible for a known phenotype, such as fibrinogen, fibronectin or collagen binding. In these cases, transposon mutants defective in a particular phenotype were isolated and the gene and corresponding protein then identified. This is first example of how proteins of unknown function were characterised and has implications for future functional genomic studies. In principle, three steps in characterising a novel *S. aureus* surface protein should be followed. These include (1) the generation of a null mutation in the native host, (2) the generation of mono-specific polyclonal antibodies to the protein, which will allow the identification of the protein in the native host, showing temporal expression patterns and monitoring of proteolytic dgradation patterns. Mono-specific antibodies are also required for function-blocking studies when the ligand has been identified. (3) The gene that encodes the protein should be cloned in a surrogate Gram-positive host such as *L. lactis*. Mono-specific antibodies can be used to identify the clone that expresses the corresponding protein and to monitor expression and proteolytic degradation patterns. Expressing the protein in a surrogate host provides a powerful tool to investigate protein-ligand interactions.

To summarise, this study has shown that SdrC, SdrD and SdrE are expressed on the surface of *S. aureus* cells. The expression pattern revealed does not fit with the central dogma put forward by several groups working in the gene regulation field, which suggests that surface proteins are only expressed during exponential phase. Instead, the

Sdr proteins appear to be expressed either throughout the growth cycle (as is observed with proteins such as ClfA and EbpS), or during stationary phase. This idea that surface proteins are expressed in exponential phase is still accepted by many researchers. However, it is evidently not entirely correct, as many exceptions to the rule have now presented themselves. Regulation of surface protein expression in *S. aureus* is clearly not as simplistic as was originally proposed.

Identifying interactions between the Sdr proteins and the host has not been easy. It has been proposed that all *S. aureus* surface proteins interact with host matrix proteins, hence their term, "MSCRAMMs" (microbial surface components recognising adhesive matrix molecules). However, apart from the interaction identified between SdrC and fibronectin, SdrD and SdrE have not been shown to interact with any host matrix or plasma protein. It was originally suspected that one or more of the Sdr proteins were responsible for the adherence of *S. aureus* to either vitronectin, mucin, laminin or thrombospondin. However, data presented in this thesis indicates that this is not the case. It should be noted that because the interaction between SdrC and fibronectin is weak and only detected when the protein is over-expressed in *L. lactis*, it is unlikely that this is the main function of SdrC. It is possible that SdrC, SdrD and SdrE can interact with other host matrix/plasma proteins that have not been tested. Identifying this possibility requires further studies. Using *L. lactis* strains that express SdrC, SdrD and SdrE is probably the most efficient way to study these interactions in the future.

In addition, this study has reinforced the idea that *S. aureus* MSCRAMMs can interact with host factors other than matrix/plasma proteins. Protein A acts as a "true MSCRAMM" in the sense that it promotes adherence to von Willebrand factor. However, it also binds to other host factors such as IgG molecules and platelets. This study has shown that ClfA, ClfB and SdrE also interact with platelets to promote the activation of platelet aggregation. Therefore, although the term MSCRAMM is correct, it appears that it may not encompass all of the functions that *S. aureus* surface proteins are capable of.

This study has provided a strong contribution to the understanding of Sdr protein function and importantly, has generated excellent tools such as isogenic mutants and

heterologous expression systems that will be of great assistance in future host ligand interaction studies.

In order to gain a better understanding of how the Sdr proteins contribute to the pathogenicty of *S. aureus*, several further experiments are required. When attempting to characterize the function of a surface associated protein its native host, detailed knowledge of its display on the surface of the cell is required. These studies have shown that the Sdr proteins are expressed at different stages in the growth cycle of *S. aureus* and that all of the proteins appear to be processed by endogenous proteases. Transcript analysis of the *sdr* genes in the future will contribute to a greater understanding of Sdr protein expression patterns. In addition, studying the effect of staphylococcal proteases on Sdr protein expression will provide an insight into how and why these proteins are cleaved by endogenous proteases. This process is likely to have implications in the pathogenicity of *S. aureus* and may be involved in immunoevasion and/or detachment of the bacterium from specific infection sites. Future studies on Sdr proteins are also likely to focus on ligand interactions. Using phage display would probably be the most efficient method for identifying host molecules that interact with the Sdr proteins. Understanding these interactions has important implications for the development of future staphylococcal vaccines and/or novel therapies.

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