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Molecular analysis of surface proteins of staphylococci

A thesis submitted for the degree of Doctor of Philosophy

By

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April 2004

Declaration

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

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Summary

S. lugdunensis is an important human pathogen that causes 44% of coagulase negative staphylococcal native valve endocarditis as well as infections such as osteomyelitis and soft tissue abscesses. *S. lugdunensis* expresses a fibrinogen affinity factor the gene product of the 2426 bp *fbl* gene which encodes a protein of 881 amino acids.

In this study the *fbl* gene was detected in eight strains by PCR and Southern blotting. PCR amplification of DNA flanking the repeat region suggested that there are two distinct populations of *fbl* gene. This supports the notion that *S. lugdunensis* is a highly clonal species.

The Fbl protein is 58% identical to the fibrinogen-binding clumping factor A of *S. aureus* strain Newman (ClfA). The residues in ClfA predicted to bind fibrinogen are conserved in Fbl. *S. lugdunensis* strains were shown to adhere to an immobilised fibrinogen substrate in a dose-dependent and saturable manner.

In order to examine Fbl in isolation from other *S. lugdunensis* proteins, the *fbl* gene was cloned into the *L. lactis* pKS80 expression system. Like *L. lactis* ClfA$^+$, *L. lactis* Fbl$^+$ bound to fibrinogen in a dose-dependent manner. Whole cell dot blotting using the polyclonal anti-Fbl region A antibodies showed that there is 64-fold more Fbl expressed on the surface of *L. lactis* Fbl$^+$ cells than *S. lugdunensis* cells. This is reflected in the failure of *S. lugdunensis* to form clumps in soluble fibrinogen whereas *L. lactis* Fbl$^+$ has a high clumping titre. *L. lactis* Fbl$^+$ also rapidly activated platelet aggregation via Fbl, whereas *S. lugdunensis* had a significantly longer time to aggregation. Fbl-mediated adherence to fibrinogen was inhibited by increasing concentrations of Ca^{2+}, similar to ClfA. Fbl-mediated adherence of cells to immobilised fibrinogen was also inhibited by increasing concentrations of recombinant A domain of ClfA, which binds to the γ-chain of fibrinogen. Adherence was not inhibited by recombinant A domain of ClfB, a second clumping factor of *S. aureus*, which binds to the α-chain of fibrinogen. *L. lactis* Fbl$^+$ was shown to adhere in a dose-dependent manner to the recombinant γ-chain of fibrinogen.

ClfA is covalently anchored to the cell wall peptidoglycan of *S. aureus* and *L. lactis* by the enzyme sortase. Western immunoblotting of *S. lugdunensis* and *L. lactis*

Fbl$^+$ identified a protein of ~200kDa that was retained by the protoplast envelope and apparently not covalently anchored to the cell wall.

The A domain of Fbl (residues 40-534) was expressed as a soluble recombinant protein in the culture supernatant of *L. lactis*. Purified rFbl$_{40\text{-}534}$ bound to immobilised fibrinogen in a dose-dependent manner. rFbl$_{40\text{-}534}$ also inhibited the binding of *L. lactis* Fbl$^+$ and *L. lactis* ClfA$^+$ to fibrinogen. This confirms that the ligand binding activity of Fbl is located in the A domain between residues 40-534.

A collection of antibodies were tested for their ability to inhibit Fbl- and ClfA-mediated binding of cells to immobilised fibrinogen and to displace bound cells from an immobilised fibrinogen substrate. These included rabbit polyclonal anti-ClfA antibodies, rabbit polyclonal anti-Fbl antibodies, human immunoglobulin enriched for reactivity against ClfA of *S. aureus* and SdrG of *S. epidermidis*, and ten monoclonal antibodies (mAbs) generated against rClfA$_{40\text{-}559}$. All the polyclonal antibodies had inhibitory and displacing activity against both Fbl- and ClfA-expressing cells.

Seven of the 10 monoclonal antibodies inhibited ClfA-mediated binding to fibrinogen but none of the mAbs inhibited Fbl-mediated binding to fibrinogen. In ELISA tests all of the mAbs bound to rClfA$_{40\text{-}559}$ whereas none bound to rFbl$_{40\text{-}534}$. The mAbs also bound to rClfA$_{220\text{-}559}$ cloned from different *S. aureus* strains. This showed that the binding epitopes of the mAbs were conserved in naturally varying rClfA$_{220\text{-}559}$ but not in Fbl. In order to identify the binding epitopes of the mAbs, residues located on the surface of the rClfA$_{220\text{-}559}$ molecule were subjected to alanine scanning mutagenesis. Residues present in the epitopes mAbs 12-9 and 35-039 were identified.

The SdrH protein of *S. epidermidis* was also investigated. The *sdrH* gene is present in all *S. epidermidis* strains tested and homologous genes are present in *S. aureus*, *S. caprae* and *S. capitis*. In this study the *sdrH* gene was cloned into *L. lactis* in order to analyse its function as a possible ligand-binding surface protein. No ligand for SdrH was found by screening *L. lactis* SdrH$^+$ for binding to a variety of extracellular matrix and serum proteins in ELISA-type assays. Like Fbl, SdrH was found to be a membrane-anchored protein. Whole cell dot immunoblotting experiments using anti-SdrH region A and anti-SdrH region C antibodies showed that the A domain of SdrH is exposed on the surface of cells but the C domain appears to buried within the cell wall. Expression of SdrH was tested under different growth conditions. Growth of *S. epidermidis* in the presence of glucose and sucrose and on the surface of agar plates as sessile cultures increased the expression of SdrH.

Publications

Jin, T., Bokarewa, M., Foster, T.J., Mitchell, J., Higgins, J. and Tarkowski A. (2004). *Staphylococcus aureus* Resists Human Defensins by Production of Staphylokinase, a Novel Bacterial Evasion Mechanism. J Immunol. **172**(2): 1169-76.

Mitchell, J. and Foster T.J. Fbl is a fibrinogen binding protein of *Staphylococcus lugdunensis*. Manuscript in preparation.

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Key to abbreviations	
Single letter amino acid code	
A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine
Bases	
A	adenine
T	thymine
C	cytosine
G	guanine
Antibiotics	
Amp	Ampicillin
Cm	Chloramphenicol
Erm	Erythromycin
Kan	Kanamycin

Key to abbreviations contd.	
aa	amino acid
agr	accessory gene regulator
bp	base pair(s)
BSA	bovine serum albumin
CFU	colony forming units
ClfA	Clumping factor A
ClfB	Clumping factor B
Cna	collagen adhesin
CP	capsular polysaccharide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DIG	digoxygenin
dNTP	deoxy nucleoside triphosphate
Ebps	Elastin binding protein
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ET	exfoliative toxin
Fbl	Fibrinogen binding protein of *S. lugdunensis*
Fc	crystallisable fragment of IgG
FnBP	Fibronectin binding protein
hr	hour
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-β-D-thio-galactoside
kb	kilobase pair
kDa	kilodalton
LB	Luria broth
MAP	MHC class II analogous protein

Key to abbreviations contd.	
nt	nucleotide(s)
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocytes
PVL	Panton Valentine Leukocidin
rpm	revolutions per minute
sar	staphylococcal accessory regulator
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	staphylococcal enterotoxin
sigB	sigma factor B
TBS	tris-buffered saline
TNF	tumor necrosis factor
Tris	trishydroxymethylaminomethane
Ts	temperature sensitive
TSA	Trypticase soy agar
TSB	Trypticase soy broth
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin 1
v/v	volume per volume
w/v	weight per volume
wt	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Introduction

1.1 Biology of the staphylococci

Staphylococci are defined as non-motile gram-positive cocci (0.5-1.5μm in diameter) that characteristically divide in more than one plane to form irregular grape-like clusters (Greek: *staphyle* = bunch of grapes, *kokkus* = berry). They have a low G+C content of 30-39%. DNA sequence and hybridisation data indicates that the closest relatives of staphylococci are listeria, planococci and aerobic spore forming bacteria. To date there have been at least 32 species of staphylococci described, the majority of which have been isolated from non-human origins (Table 1.1).

Most strains of staphylococci are catalase positive and oxidase negative and are extremely halotolerant being able to withstand NaCl concentrations of up to 3.5M (Wilkinson, 1997). Staphylococci are ubiquitous commensals of the body including the mouth and throat and are commonly associated with the skin, skin glands and mucous membranes of warm-blooded animals. Certain species of staphylococci exhibit characteristic ecological niche preferences. For example *Staphylococcus aureus* is predominantly found colonizing the anterior nares whereas *S. lugdunensis* is usually isolated from the perineum and *S. epidermidis* is found widely distributed on human skin.

1.1.1 Classification and identification

S. aureus has the ability to clot blood plasma and this characteristic has been used to divide the staphylococci into two major groups, coagulase positive and coagulase negative (Phonimdaeng *et al.*, 1990). The isolation of coagulase-negative staphylococci from clinical specimens is not usually difficult, since staphylococci grow readily on commonly used media under a broad range of growth conditions. Initial identification is straightforward using conventional as well as automated and semi automated methods. The thermonuclease reaction is particularly useful for rapidly differentiating *S. aureus* (positive) from other staphylococcal species (negative) and is more accurate than tests based on coagulase production. Demonstration of resistance to novobiocin by disc diffusion testing is adequate for presumptive differentiation of *S. saprophyticus*. For most clinical isolates, the differentiation of *S. aureus*, *S. epidermidis*, and *S. saprophyticus* is sufficient. However, some other staphylococcal species, *S. haemolyticus*, *S. lugdunensis*, and *S. schleiferi* have been isolated more often from serious human infections

especially native valve endocarditis. Therefore, it may be useful in certain situations to be able to identify a clinical isolate to the species level because the repeated isolation of the same strain supports its role as an etiologic agent.

1.1.2 Coagulase negative staphylococci

Until 1975, coagulase-negative staphylococci (CoNS) were grouped together as *Staphylococcus albus* or *S. epidermidis* and distinguished from *S. aureus* by their inability to clot blood plasma and therefore presumed to be non-pathogenic. Yet today CoNS are the most commonly reported pathogens isolated from bloodstream infections in intensive care unit patients and surpass *S. aureus* as a cause of device-related nosocomial infections (NNIS, 1999). Nevertheless, recognition of infection is hampered by the difficulty in distinguishing the infecting strain from the normal flora. CoNS have long been dismissed as culture contaminants as they are commonly seen among the normal flora of human skin and mucous membranes. Treatment of these infections can prove difficult since many strains carry multiple antibiotic resistances and resistance is emerging to glycopeptide antibiotics, the last remaining antibiotic to which staphylococci were uniformly sensitive.

Thirty nine CoNS species have been published yet only half are seen in specimens of human origin. They can be divided into two groups differing in their resistance to or susceptibility to novobiocin. Those that are indigenous to human beings include the novobiocin-susceptible species *S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis, S. schleiferi*, as well as the novobiocin-resistant species *S. saprophyticus* and *S. xylosus* (Diekema *et al.*, 2001; Lina *et al.*, 2000)

Clinical manifestations of infections due to most CoNS markedly differ from those of *S aureus* infections. Normally, the clinical picture is subtle and non-specific and the clinical course more subacute or even chronic without fulminant signs of infection. Coagulase-negative staphylococcal bacteraemia is rarely life-threatening, especially if treated promptly and adequately. However, frank sepsis syndrome and fatal outcome may occur, especially in immunocompromised patients and/or if one of the more virulent species, such as *S. lugdunensis*, is involved.

The importance of CoNS as nosocomial pathogens has prompted more interest in their detailed characterization. Research on CoNS has proceeded on several fronts, including

Table 1.1

Staphylococcal species	Host
Staphylococcus aureus	Primates, Domestic animals, Poultry, Hares, Rodents
Staphylococcus epidermidis	Human
Staphylococcus lugdunensis	Human
Staphylococcus warneri	Human
Staphylococcus haemolyticus	Primates
Staphylococcus pettenkoferi	Human
Staphylococcus cohnii	Primates, Tree shrews
Staphylococcus saprophyticus	Primates, Tree shrews, Rodents
Staphylococcus saccharolyticus	Humans
Staphylococcus xylosus	Rodents, Mammals, Birds
Staphylococcus simulans	Carnivores, Domestic artiodactyls, Humans
Staphylococcus hominis	Humans
Staphylococcus capitis	Primates
Staphylococcus caprae	Humans, Goats
Staphylococcus delphini	Dolphins
Staphylococcus pasteuri	Humans, Domestic artiodactyls, Horses
Staphylococcus auricularis	Primates
Staphylococcus kloosii	Rodents, Domestic artiodactyls
Staphylococcus equorum	Domestic horses, cattle
Staphylococcus arlettae	Poultry
Staphylococcus galinarum	Poultry
Staphylococcus carnosus	Domestic artiodactyls
Staphylococcus felis	Carnivores
Staphylococcus piscifermentans	Unknown
Staphylococcus intermedius	Carnivores, Domestic horses, Poultry
Staphylococcus schleiferi	Humans
Staphylococcus hyicus	Artiodactyls, Poultry
Staphylococcus chromogenes	Artiodactyls, Horses
Staphylococcus muscae	Flies
Staphylococcus sciuri	Artiodactyls, Cetaceans, Rodents, Marsupials, Poultry
Staphylococcus lentus	Artiodactyls, Perissodactyls, Cetaceans
Staphylococcus vitulus	Artiodactyls, Perissodactyls, Cetaceans
Staphylococcus caseolyticus	Artiodactyls, Cetaceans
Staphylococcus succinus	isolated from 25 million yr old amber
Staphylococcus nepalensis	isolated from Himalayan goats
Staphylococcus ovis	Sheep
Staphylococcus pulvereri	Human
Staphylococcus lutrae	Otter
Staphylococcus fleurettii	Goat

development of more accurate methods for identifying species, for distinguishing infecting from contaminating isolates, and for epidemiological typing of strains. Furthermore, various virulence factors involved in the pathogenesis of infections due to CoNS, especially of polymer-associated staphylococcal infection, have been isolated and characterized during the past decade.

Staphylococcus lugdunensis is a coagulase negative staphylococcus that was first described in 1988 by Freney and colleagues in Lyon, France (Latin: *Lugdunum* = Lyon). It is a commensal organism of human skin and an opportunistic pathogen that is likely to colonize the perineum. Since its first description, *S. lugdunensis* has been associated with a wide variety of infections and has been since recognized as an unusually virulent coagulase negative staphylococcus which, unlike other coagulase negative staphylococci, is frequently implicated in soft tissue infections and has the ability to cause native valve endocarditis (Patel *et al.*, 2000; Kragsbjerg *et al.*, 2000; Ling and Yeo, 2000; Elliot *et al.*, 2001).

A number of bacteriological characteristics enabled the identification of *S. lugdunensis*: the absence of coagulase and protein A, and the presence of a fibrinogen affinity factor and thermonuclease and biochemical characteristics including ornithine and arginine decarboxylases, carbohydrate acidification and novobiocin susceptibility. These properties differentiated *S. lugdunensis* as a separate species from other staphylococci. However, *S. lugdunensis* does not possess virulence markers such as toxins or haemagglutinin (Fleurette *et al.*, 1989). The few known virulence factors of *S. lugdunensis* include the production of extracellular slime or glycocalyx, which can aid in the evasion of the host immune system, the production of three SLUSH toxins which act synergistically with β-hemolysin to produce complete hemolysis and the production of lipases and esterases (Donvito *et al.*, 1997a, b; Lambe *et al.*, 1990).

1.2 Virulence factors: Surface proteins

Infections caused by *S. aureus* vary from minor wound infections to severe sepsis and their ability to cause infection is due to the large range virulence factors it produces in order to colonize and survive within the host. Initial attachment of staphylococci to host tissues is considered the first crucial step in the disease process. Staphylococci produce a family of adhesins termed MSCRAMM (Microbial Surface Component Recognizing Adhesive Matrix Molecules) proteins that mediate adherence to extracellular matrix (ECM) proteins. The extracellular matrix is

A large number of these proteins are covalently linked to the cell wall by a process termed sorting, whereas others remain membrane anchored or non-covalently linked to the cell wall. Some surface proteins can also mediate binding of the bacteria to host factors other than those that comprise the extracellular matrix including protein A which binds to immunoglobulin in serum (Sjoquist *et al.*, 1972) and SasG which attaches to a lipid moiety in nasal epithelial cells (Huesca *et al.*, 2002; Roche *et al.*, 2003) and HarA which binds to haptoglobin and haptoglobin-haemoglobin complexes in blood (Dryla *et al.*, 2003).

1.2.1 Sorting

The bacterial cell wall is a heteropolymer containing glycan and peptide components (Strominger and Ghuysen, 1967). The glycan chains consist of a repeating disaccharide, *N*-acetylmuramyl-(β1–4)-*N*-acetylglucosamine (MurNAc-GlcNAc) (Ghuysen and Strominger, 1963). The lactyl of *N*-acetylmuramyl is amide linked to the amino of L-alanyl within the wall peptide (NH_2-L-Ala-D-iGln-L-Lys-D-Ala-D-Ala-COOH) (Tipper *et al.*, 1965; Tipper *et al.*, 1967; Tipper, 1968). Wall peptides of neighboring peptidoglycan strands can be cross-linked by a transpeptidation mechanism during which the terminal D-alanine is cleaved, and the liberated carboxyl of D-alanyl at position four is amide linked to the free amino of the cell wall cross-bridge. In staphylococci, the cross-bridge consists of five glycyl linked to the ∈-amino of the lysyl residue in the wall peptide [MurNAc-(L-Ala-D-iGln-L-Lys (NH2-Gly5)-D-Ala-D-Ala-COOH)-(β1–4)-GlcNAc] (Ghuysen, 1968; Tipper and Berman, 1969). The transpeptidation reaction is catalyzed by penicillin binding proteins and can be inhibited by β-lactam antibiotics (Navarre and Schneewind, 1994).

The pentaglycyl cross-bridge is synthesized via modification of the lipid II precursor (undecaprenylpyrophosphate-MurNAc(-L-Ala-D-iGln-L-Lys-D-Ala-D-Ala)-(β1–4) GlcNAc) (Matsuhashi *et al.*, 1965; Matsuhashi *et al.*, 1967). Three glycyl tRNA species are dedicated to this biosynthetic pathway. These tRNAs are thought to serve as substrate in a sequence of reactions that successively add glycine either directly to the ∈-amino of lysyl or to the amino of another glycyl (Roberts et al., 1974). It seems likely that the *femA*, *femB*, and *femX* genes specify enzymatic activities that catalyze these reactions (Kopp *et al.*, 1996).

The cell wall anchoring of surface proteins in *S. aureus* requires both an amino-terminal signal (leader) peptide and a carboxy-terminal cell wall sorting signal (Schneewind *et al.*, 1992).

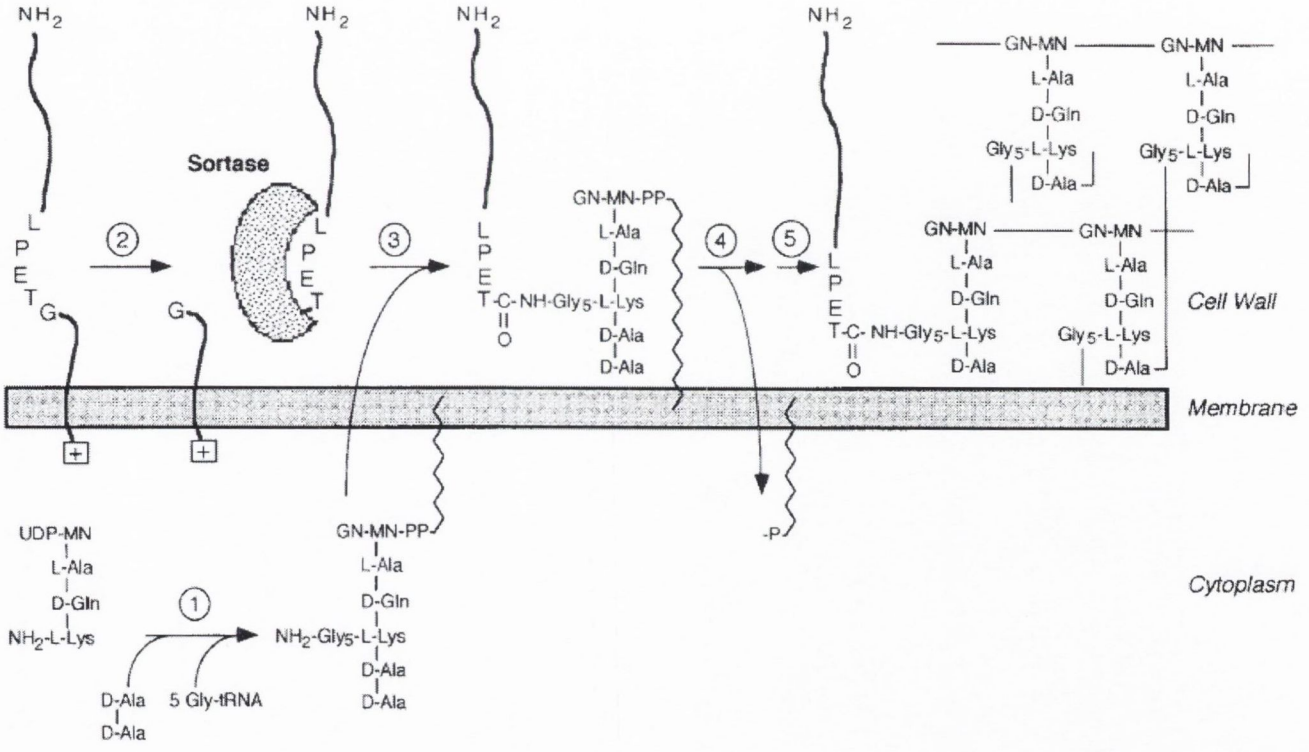


Figure 1.1 Sorting of surface proteins to the staphylococcal cell wall. Peptidoglycan precursor molecules are synthesized in the bacterial cytoplasm (1) and translocated across the membrane. Surface proteins are cleaved (2) between the threonine (T) and the glycine (G) of the LPXTG motif subsequently linked to a peptidoglycan precursor molecule (3). The intermediate surface protein linked to peptidoglycan precursor is then incorporated into the cell wall by a transglycosylase reaction (4). The mature anchored polypeptide chains are linked to the pentaglycine cross-bridge in the cell wall, which is tethered to the ∈-amino side chain of an unsubstituted cell wall tetrapeptide. A carboxypeptidase (5) may cleave the D-Ala-D-Ala bond of pentapeptide structures to yield the final branched anchor peptide in the staphylococcal cell wall. From Ton-That *et al.* (1997).

Table 1.2 Staphylococcal proteins containing serine-aspartate repeat regions.

Sdr Protein	Function	Strain	Reference
ClfA	Fibrinogen binding protein	*S. aureus*	McDevitt *et al.*, 1994
ClfB	Fibrinogen binding protein	*S. aureus*	Perkins *et al.*, 2001
SdrC	Unknown	*S. aureus*	Josefsson *et al.*, 1998
SdrD	Unknown	*S. aureus*	Josefsson *et al.*, 1998
SdrE	Unknown	*S. aureus*	Josefsson *et al.*, 1998
Bbp	Bone sialoprotein binding protein	*S. aureus*	Tung *et al.*, 2000
SdrF	Unknown	*S. epidermidis*	McCrea *et al.*, 2000
SdrG	Fibrinogen binding protein	*S. epidermidis*	McCrea *et al.*, 2000
SdrH	Unknown	*S. epidermidis*	McCrea *et al.*, 2000
Aap	Binding to squamous epithelia	*S. epidermidis*	Accession number: AJ249487, Roche *et al.*, 2003
SdrI	Fibronectin and fibrinogen binding protein	*S. saprophyticus*	Accession number: AF402316
SdrY	Fibronectin binding protein	*S. caprae*	Accession number: AY048593
SdrZ	Unknown	*S. caprae*	Accession number: AY048595
SdrX	Collagen IV binding protein	*S. capitis*	Accession number: AY510088
SdrZL	Unknown	*S. capitis*	Accession number: AY510087

Proteins are synthesized in the cytoplasm and then exported via a cleavable N-terminal signal peptide through the *sec* pathway (Schneewind *et al.*, 1993). Positively charged residues at the C-terminus of the protein allow it to be retained by the cell with the hydrophobic residues spanning the membrane (Fig. 1.1). The 35-residue sorting signal harbors an LP*X*TG sequence motif that is conserved within the sorting signals of more than 100 surface proteins of Gram-positive bacteria (Fischetti *et al.*, 1990; Schneewind *et al.*, 1993; Mazmanian *et al*, 1999). It serves as the recognition sequence for the proteolytic cleavage between its threonine and glycine residues by sortase (Navarre and Schneewind, 1994). Sortase links the carboxyl group of the threonine to the amino group of the pentaglycine cross-bridge of peptidoglycan precursor thus anchoring the protein to the cell wall peptidoglycan (Fig. 1.1). Sortase mutant strains of *S. aureus* fail to anchor protein A and ClfA to the cell wall and are less virulent in animal models of infection (Mazmanian *et al.*, 2000).

1.2.2 Surface proteins

Surface proteins have been found in many pathogenic gram-positive bacterial species. These structurally related proteins typically contain ~40 amino acid long N-terminal signal sequence(S), and C-terminal features that are required for sorting the proteins to the cell wall including a proline-rich wall-spanning region (W), the wall anchoring LPXTG motif, a hydrophobic transmembrane region (M), and a positively charged cytoplasmic tail (C) (Fig. 1.3). In addition to these C-terminal features, the members of the Sdr family of proteins, including the fibrinogen binding proteins ClfA and ClfB of *S. aureus* and SdrG of *S. epidermidis* contain a Ser-Asp repeat region (R region) just outside the cell wall-anchoring region (Table 1.2). This region allows the ligand binding domain to be functionally expressed by acting as an extended stalk that projects it through the cell wall peptidoglycan and away from the surface of the bacterial cell (Hartford *et al.*, 1997). The ligand binding activities of most have been localized to the N-terminal A regions that are approximately 500 amino acids long (Davis *et al.*, 2001; McDevitt *et al.* 1995). Fig. 1.4 illustrates the domain organisation of the SD-repeat proteins from *S. aureus* and *S. epidermidis*. The functions of many of these proteins remain to be elucidated while several have been thoroughly characterized and implicated as staphylococcal virulence factors as is discussed below.

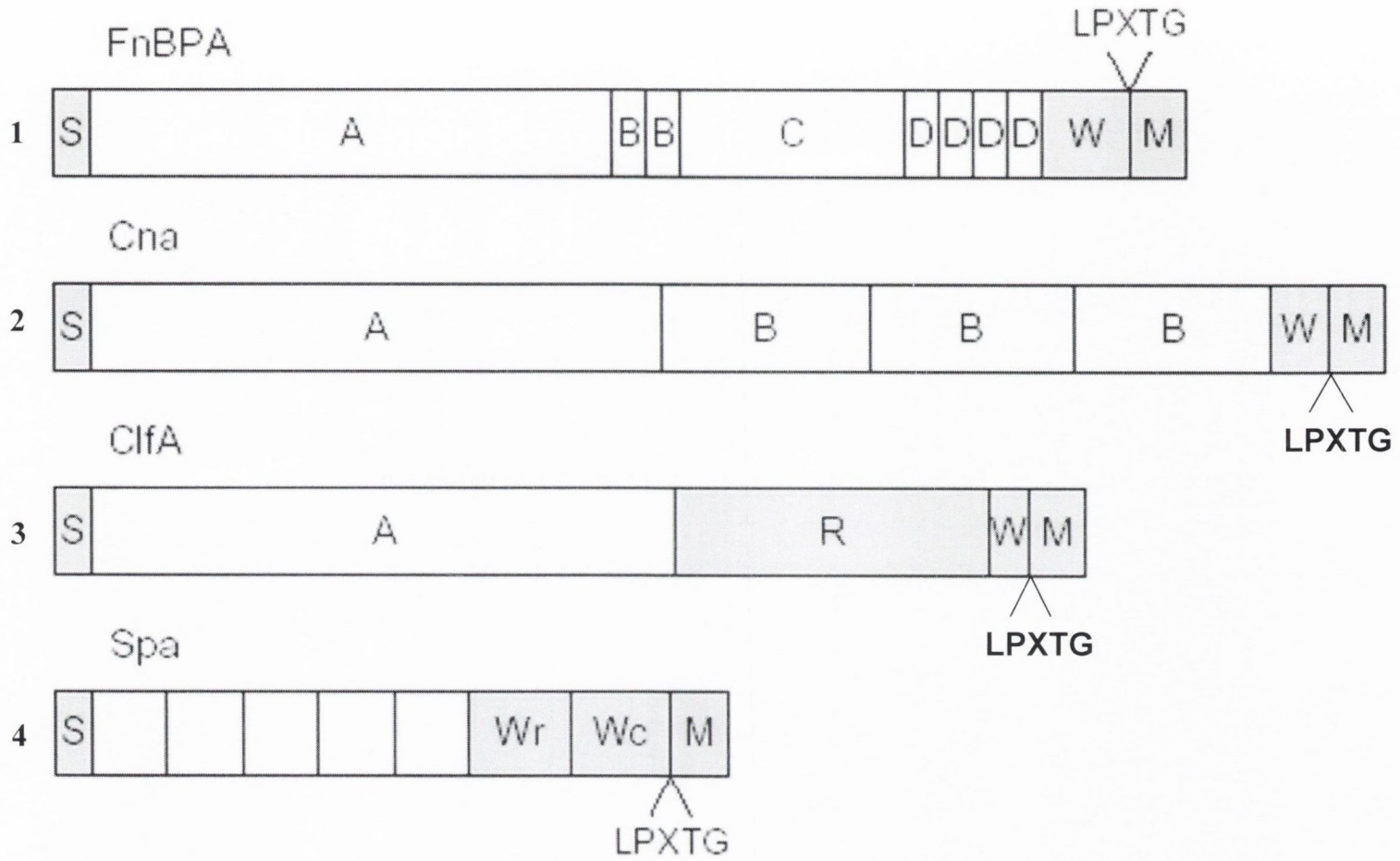


Figure 1.2 Structural organization of MSCRAMM (microbial surface components recognizing adhesive matrix molecules) proteins (1) FnBPA, (2) Cna, (3) ClfA and (4) Spa of Staphylococcus aureus. S. Signal sequence; R. Ser–Asp dipeptide repeats; W Wall-spanning region; M Membrane-spanning region and positively charged residues. Wr is composed of an octapeptide repeat, and Wc is a non-repeated region. The positions of the LPXTG motif and the A-, B-, C- and D-domains are indicated. From Foster and Hook (1998).

1.2.3 Protein A

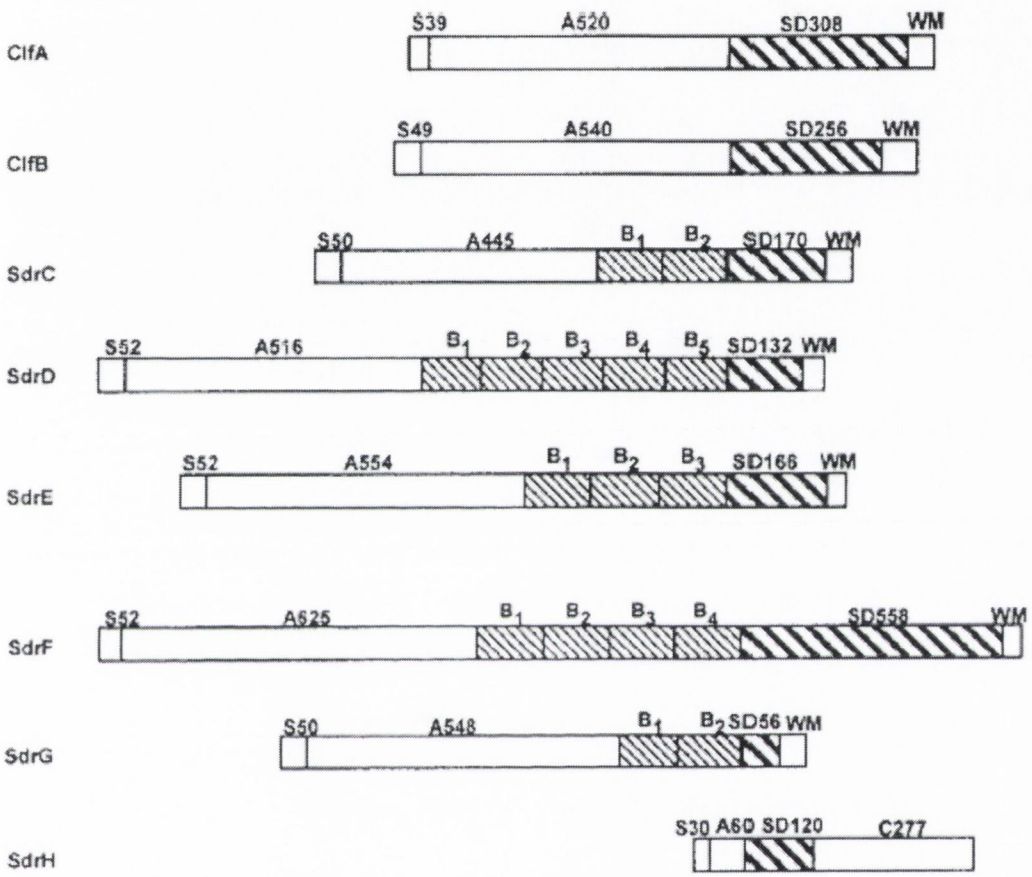
Protein A binds to the Fc region of mammalian IgG and was the first surface associated protein of *S. aureus* to be characterized (Söjdahl *et al.*, 1977; Moks *et al.*, 1986). Protein A is also a member of the MSCRAMM family of surface proteins as it has been recently shown to bind von Willebrand factor (Hartleib *et al.*, 2000). Protein A contains five homologous repeat regions each of which bind to the Fc region of mammalian IgG (Fig. 1.2). Co-crystallization of the B subdomain and IgG allowed identification by X-ray crystallography of 11 amino acid residues that are involved in binding to 9 residues in the IgG molecule (Starovasnik *et al.*, 1996). Protein A is a virulence factor (Patel et al., 1986; Palmqvist *et al.*, 2002). It binds to platelets (Herrmann *et al.*, 1993; Nguyen *et al.*, 2000) and may play a role in their activation, a phenomenon important in the development of infective endocarditis.

1.2.4 Fibronectin binding proteins

Fibronectin is a high molecular weight dimeric glycoprotein found in blood and body fluids, on many cell surfaces and in connective tissue matrices (Yamada and Olden, 1978). Fibronectin interacts with cell surfaces by binding to integrins through its RGD motif and is involved in the regulation of cell adhesion, cell substratum adhesiveness and cell motility (Mosesson and Amrani, 1980; Yamada and Olden, 1978).

The cell wall-attached fibronectin-binding proteins A and B (FnBPA and FnBPB) confer a tight association of the *S. aureus* with fibronectin (Fn) (Flock *et al.*, 1987; Jonsson *et al.*, 1991). *Staphylococcus aureus* targets host fibronectin in its adhesion to and invasion of host cells (Sinha *et al.*, 1999; Fowler *et al.*, 2000). Fibronectin-binding proteins (FnBPs), anchored in the bacterial cell wall, have multiple fibronectin-binding repeats in an unfolded region of the protein. The interaction of FnBPs with fibronectin is mediated by direct protein-protein interactions between multiple fibronectin-binding domains of the bacterial proteins connecting as extended antiparallel β-strands to the fibronectin type-1 modules in the amino-terminal domain of Fn (Schwarz-Linek *et al.*, 2003). Thus the unfolded repeated binding module forms a tandem array of anti-parallel β-strands when it complexes with the repeated array of F1 modules (Fig. 1.5). The binding site in the amino-terminal domain ($^{1-5}$F1) of fibronectin contains five sequential fibronectin type-1 (F1) modules. It was revealed by NMR that the structure of the

Figure 1.3 Schematic representation of the Sdr proteins of *S. aureus* and *S. epidermidis* showing the relative size of their signal sequences (S), A regions (A), B-repeat regions (B), SD-repeat regions (SD), C region (C) (SdrH only), and wall/membrane spanning regions (WM). From McCrea *et al.* (2001).



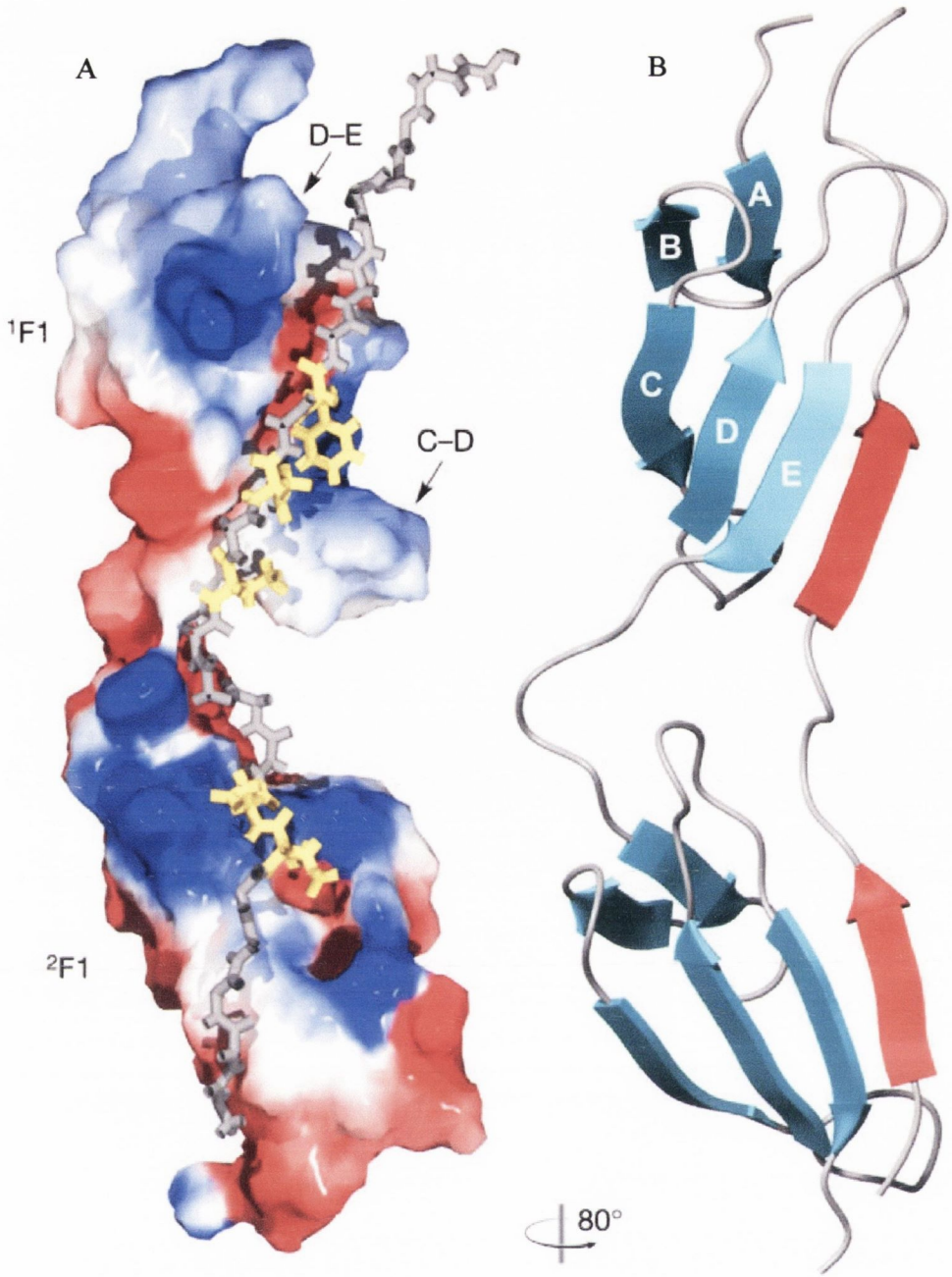


Figure 1.4 Molecular surface and ribbon diagram of the structure of the ^{1}F1^{2}F1 module pair from human Fibronectin complexed with B3 from *S. dysgalactiae*. **A**. Surface potential of ^{1}F1^{2}F1 with bound B3 (grey) and side chains of hydrophobic and acidic B3 residues (yellow). Negatively and positively charged regions of the ^{1}F1^{2}F1 surface are shown in red and blue, respectively. **B**. Ribbon diagram of the lowest-energy structure showing β-strands of the F1 modules (cyan) and the fourth strand formed by B3 (red). The difference in orientation between the two views is indicated.

revealed by NMR that the structure of the related streptococcal FnBP peptide (B3) forms a tandem β-zipper in complex with the module pair $^{1}F1^{2}F1$. Sequence analyses of larger regions of FnBPs from *S. aureus* reveal a repeating pattern of F1-binding motifs that match the pattern of F1 modules in $^{1-5}F1$ of Fn. The role of the FnBPA and FnBPB proteins as virulence factors, in bacterial invasion of host cells and in fibrinogen binding is discussed below.

1.2.5 Collagen binding proteins

Collagen is the main component of the extracellular matrix of most tissues. *S. aureus* can express a collagen-binding MSCRAMM, called CNA. CNA is necessary and sufficient for *S. aureus* to attach to cartilage *in vitro*, showing that CNA can act as an adhesin (Switalski *et al.*, 1993). CNA was also shown to be a virulence factor in experimental septic arthritis where CNA^{+} strains showed substantially increased virulence compared with the isogenic CNA^{-} strains as demonstrated by macroscopic clinical evaluation and histopathological analysis of the joints (Patti *et al.*, 1994). No viable *S. aureus* cells were recovered from the joints of mice injected with CNA^{-} strains, although significant numbers of *S. aureus* cells were isolated from joints of mice injected with CNA^{+} strains. Vaccination with a recombinant fragment of CNA protected mice from septic death induced by intravenously administered *S. aureus* (Nilsson *et al.*, 1998).

CNA consists of an N-terminal signal peptide, a non-repetitive A region, one to four repeated units (B-region), followed by a cell wall anchor region, a transmembrane segment, and a short positively charged cytoplasmic tail. The collagen binding activity of CNA is located within the A region of CNA between residues 30–531. The minimum binding domain was further localized to a 19-kDa fragment, $CNA_{151-318}$ (Patti *et al.*, 1993; Symersky *et al.*, 1997). $CNA_{151-318}$ forms a β-barrel structure composed of two antiparallel β-sheets and two short α-helices (Symersky *et al.*, 1997). β-Strands A, B, part of D, E, and H form β-sheet I, and strands C, part of D, F, G, I, and J form β-sheet II. β-Sheet I contains a surface trench into which a triple helical collagen molecule fits as shown by computer docking experiments using theoretical collagen probes of [(Gly-Pro- Pro) 4]3 or [(Gly-Pro-Hyp) 4]3. Site-directed mutagenesis of some residues in the putative binding trench of $CNA_{151-318}$ abolished collagen binding (Y175K, R189A, F191A, N193K, and Y233A) or caused reduced binding affinity (N223K and N278K) (Symersky *et al.*, 1997; Patti *et al.*, 1995), indicating that the trench constitutes the binding site.

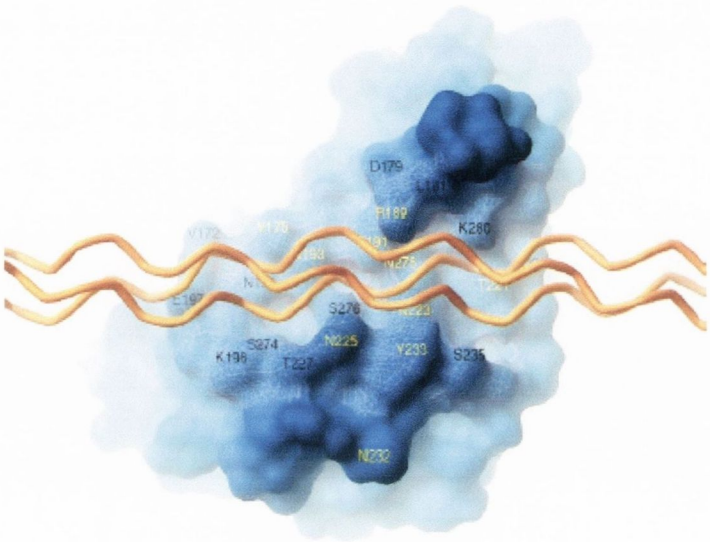


Figure 1.5 View of the molecular surface of the $Cna_{151-318}$ looking into the trench on the β-sheet. Residues within 6Å from the docked collagen are highlighted. The orange lines represent the collagen triple helix. From Foster and Hook (1998).

1.2.6 Fibrinogen binding proteins

Fibrinogen is a 340 kDa serum glycoprotein that undergoes polymerization upon vascular injury to prevent blood loss (Fig. 1.5). Staphylococci express several different proteins that bind to this host serum protein. The cell wall anchored proteins ClfA, ClfB, SdrG, FnBPA, and FnBPB all recognize fibrinogen. *S. aureus* also secretes several fibrinogen binding proteins including Map/Eap (Jonsson *et al.*, 1995; Palma *et al.*, 1999), Efb (Palma *et al.*, 2001), and Emp (Hussain *et al.*, 2001). However the amino acid sequence relatedness, similar modular design, and common binding domain organization of the cell wall anchored fibrinogen binding proteins suggest that they have arisen from a common ancestor. ClfA, FnBPA, and FnBPB bind to the extreme C-terminal residues of the γ-chain of fibrinogen (Wann *et al.*, 2000; McDevitt *et al.*, 1994; McDevitt *et al.*, 1997). ClfB binds in the centre of the α-chain and SdrG binds in close proximity to the thrombin cleavage site of the B β-chain of fibrinogen (Davis *et al.*, 2001).

Comparative structural analysis of the minimum binding regions of the apoprotein form of the ligand binding A domains of ClfA, ClfB and SdrG reveals that all three proteins adopt a similar structure (Fig. 1.6) comprised of two similarly folded domains termed a D E variant IgG fold (Fig. 1.7) (Deivanayagam *et al.*, 2002; Ponnuraj *et al.*, 2003). It is also proposed from amino acid sequence comparisons between all these proteins that the FnBPA and FnBPB proteins must adopt similar 3-D structures. The topology of the DEv-IgG fold is very similar to that of the C-type IgG fold, but displays variation between the D and E strands on one side. The only one of these proteins to have been co-crystallized with its ligand in situ is SdrG. Residues 273–597 constitute the minimum binding region of the SdrG ligand binding domain and this molecule was co-crystallized with a synthetic peptide analogous to its binding site in the B β-chain of fibrinogen (Ponnuraj *et al.*, 2003). This co-crystal revealed that SdrG bound to fibrinogen by a dock, lock, and latch model whereby the peptide bound in the hydrophobic cleft between the two similarly folded N2 and N3 domains. This appeared to be followed by a ligand binding-induced redirection of the C-terminal end of the N3 domain of SdrG which induced an insertion of this redirected C-terminal extension between strands E and D in the preceding N2 domain (Fig. 1.6). This molecular rearrangement represents a unique intramolecular β-sheet complementation. All of the other cell-wall sorted fibrinogen binding proteins contain a cleft in one of the IgG-like folds that could

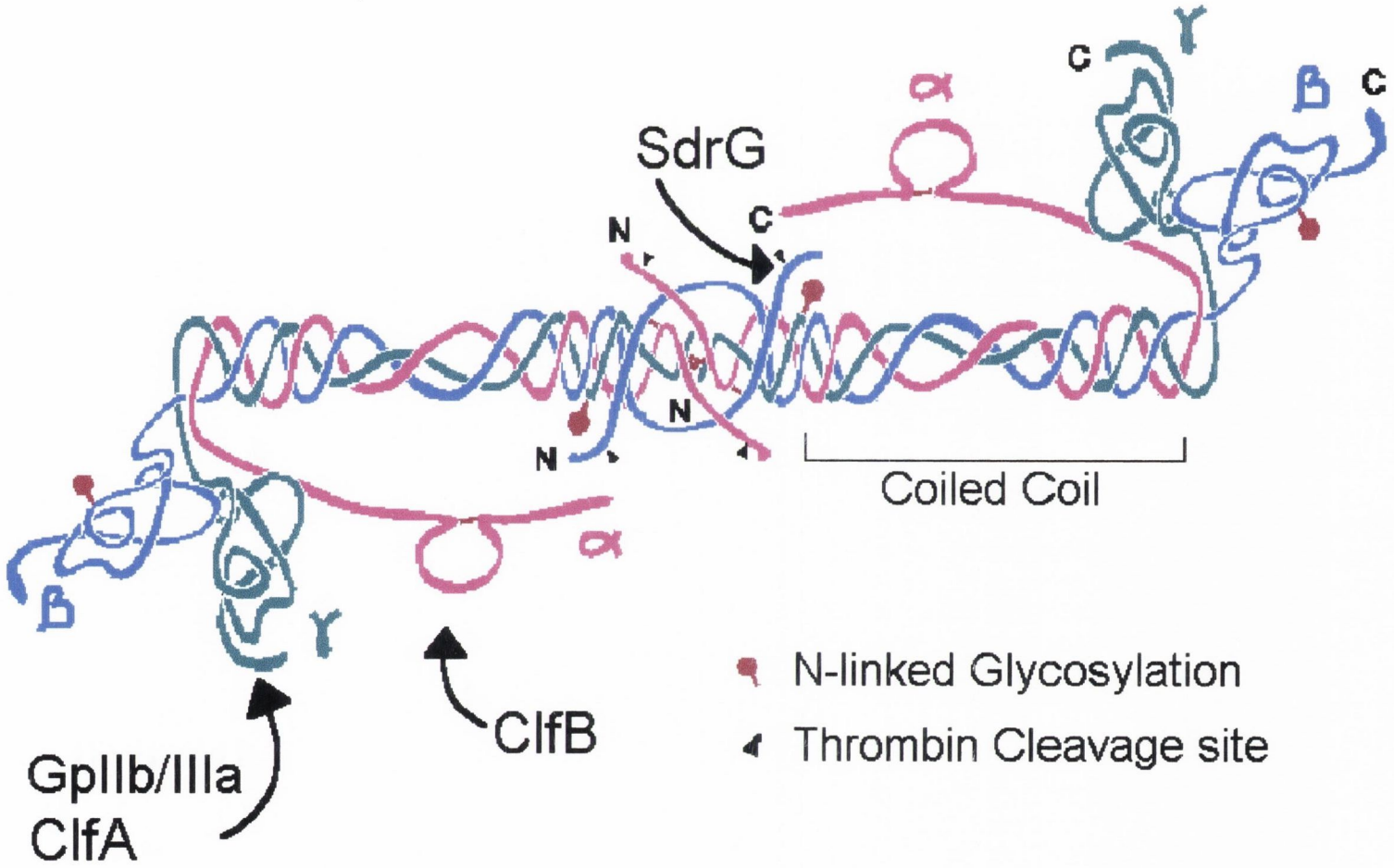


Figure 1.6 Diagrammatic representation of the structure of human fibrinogen. The positions of the binding sites of ClfA, ClfB, SdrG and the platelet glycoprotein GpIIb/IIIa are indicated by curved arrows. The thrombin cleavage sites are indicated by black triangles and sites of N-linked glycosylation are indicated with a red tag. From http://biology.ucsd.edu/faculty/doolittle.html.

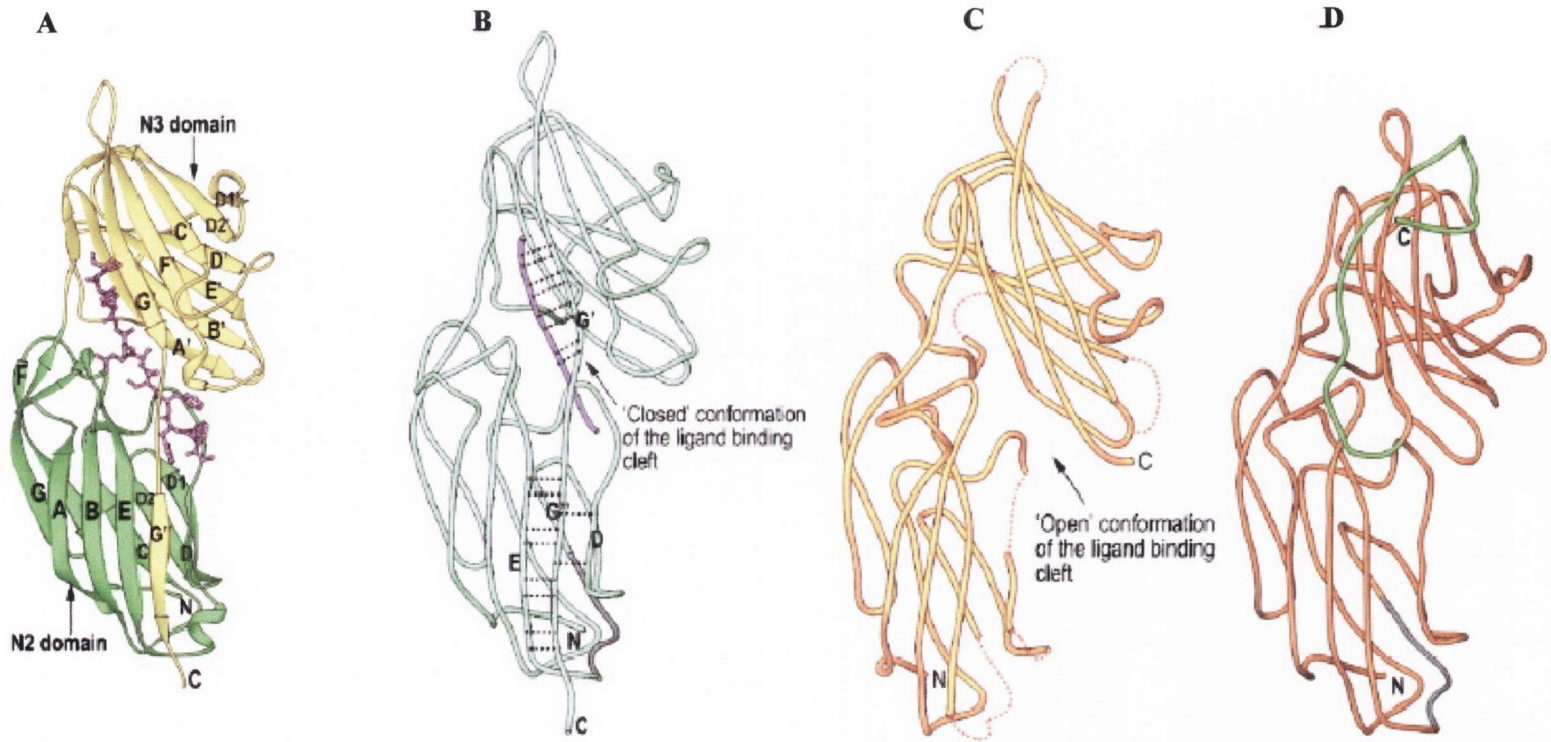


Figure 1.7 Backbone Representation and Comparison of the apo-rSdrG$_{273-597}$, rSdrG$_{273-597}$-Peptide Complex and apo-rClfA$_{221-559}$ Structures. (A) Ribbon representation of rSdrG$_{276-597}$-Fg (β6-20) peptide analog complex. The peptide is shown in the "ball and stick" model. (B) Representation of the "closed" rSdrG$_{273-597}$-peptide complex (light green and purple respectively) and (C) the "open" apo- rSdrG$_{273-597}$ conformation. (D) The apo structure of rClfA$_{221-559}$ (light brown). The C terminus of rClfA$_{221-559}$ (green) loops back and folds into the N3 domain, partially blocking the proposed ligand binding cleft. The conserved TYTFTDYVD motif is highlighted in gray (A and C). From Ponnuraj *et al.* (2003).

domain (Fig. 1.7). This molecular rearrangement represents a unique intramolecular β-sheet complementation. All of the other cell-wall sorted fibrinogen binding proteins contain a cleft in one of the IgG-like folds that could accommodate a latching strand and a "GSGSGDG" motif that could occupy the latching cleft. Therefore the dock, lock, and latch model has been proposed to apply not only to the interaction of these proteins with fibrinogen but to a general interaction of staphylococcal ligand binding proteins, related to SdrG and ClfA in domain structure organisation, and their various host ligands. Further discussion of the dock, lock, and latch model occurs in chapters 4 and 6.

1.2.7 Other surface proteins

The completion of the genome sequences of several strains of *S. aureus* and *S. epidermidis* has allowed extensive analysis of staphylococcal genes and their potential roles as virulence factors. Several studies have used comparative genomics to identify possible functions for staphylococcal genes due to their homology to genes of known function. The genomes have been extensively screened for the presence of genes using programs such as Artemis, which enable translation of a nucleotide sequence in all 6 translational reading frames and the prediction of open reading frames. Preliminary searches screening for the presence of a secretory signal sequence and a sortase substrate motif (LPXTG) followed by a hydrophobic stretch and a positively charged C-terminus within a single open reading frame led to the identification of 21 genes that could potentially encode proteins sorted to the cell wall (Mazmanian *et al.*, 2001; Roche *et al.*, 2003). These included *spa, cna, clfA*, *clfB*, *fnbA* and *fnbB* and also genes of unknown function *sdrC, sdrD, sdrE* and the *sasA-J* genes. Genes bearing homology to the *sas* genes are also present in the *S. epidermidis* genome sequences as are three members of the SD-repeat protein family *sdrF, sdrG and sdrH.* The function for many of these genes remains to be investigated but SdrE mediates platelet activation and its homologue Bbp has been shown to bind to bone sialoprotein (O'Brien *et al.*, 2002; Tung *et al.*, 2000).

It must also be observed that not all surface proteins are covalently sorted to the cell wall. The elastin binding protein (Ebps) is a transmembrane protein that binds tropoelastin monomers and soluble elastin peptides (Downer *et al.*, 2002) and Map/Eap is a multiple ligand binding protein that associates non-covalently with the cell wall of *S. aureus* (Jonsson *et al.*, 1995; Palma *et al.*, 1999).

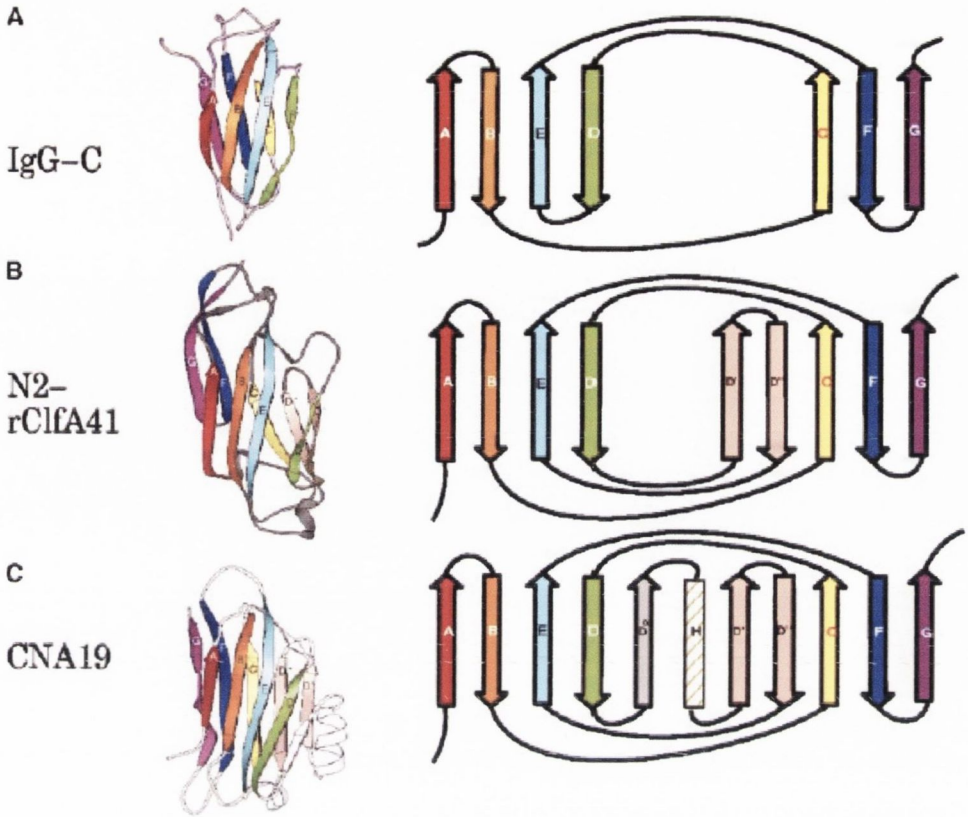


Figure 1.8 Topology of the IgG and MSCRAMM domains. The corresponding topology diagram of the structure is shown to the right. A. IgG-C domain. B. $rClfA_{377-559}$. C. CNA19 domain ($CNA_{169-318}$). From Deivanayagam *et al.* (2002).

1.2.8 Colonization

Staphylococcus aureus is an important cause of sepsis in community and hospital settings. The primary habitat of *S. aureus* is the anterior nares of humans. Nasal carriage is a major risk factor for the development of disease. Eradication of carriage by topical antibiotics reduces sepsis rates in high-risk individuals (Peacock *et al.*, 2001; Peacock *et al.*, 2003). Understanding the mechanisms by which *S. aureus* adheres to nasal epithelial cells *in vivo* may lead to alternative methods of decolonization that do not rely on sustained antimicrobial susceptibility. Colonization of the squamous epithelial cells that line this niche is postulated to be mediated in part by the direct interaction of *S. aureus* cells expressing ClfB with the squamous epithelial cells (O'Brien *et al.*, 2002). ClfB mediates adherence of *S. aureus* to cytokeratin 10, which is exposed on the surface of nasal squamous epithelia, through its ligand binding A domain. However ClfB is not the only factor that mediates the interaction of staphylococci with squamous epithelia as SasG and Pls of *S. aureus,* and Aap of *S. epidermidis* also mediate adherence to nasal epithelial cells through the N-terminal region of their ligand binding A-domains (Roche *et al.*, 2003). However the precise nature of this interaction has yet to be fully characterized.

1.2.9 Biofilm

Genetic analysis delineated two stages in biofilm formation by S. epidermidis (Heilmann *et al.*, 1997; Heilmann and Gotz, 1998). Firstly adhesion to the plastic surface mediated by surface exposure of the Atl protein. Following initial adherence to a foreign body surface, staphylococci multiply and accumulate as multilayered cell clusters. This biofilm accumulation involves intercellular adhesion. Purification and structural analysis showed that the polysaccharide intracellular adhesin (PIA) of *S. epidermidis* consists of two forms of the polysaccharide, a major polysaccharide I (>80%) and a minor polysaccharide II (<20%) (Mack *et al.*, 1996). Polysaccharide I is a linear β-1, 6-linked glucosaminoglycan mainly composed of at least 130 2-deoxy-2-amino-D-glucopyranosyl residues of which 80–85% are N-acetylated. Polysaccharide II is structurally related to polysaccharide I, with a lower content of non-N-

acetylated glucosaminyl residues and small amounts of phosphate and ester-linked succinyl residues.

The *icaABC* genes mediate cell clustering and PIA synthesis in *S. epidermidis*, along with an additional open reading frame *icaD* located between *icaA* and *icaB* and overlapping both genes (Gerke *et al.*, 1998). The *icaA* and *icaD* genes encode the proposed N-acetylglucosaminyltransferase activity. IcaAD in the presence of the IcaC protein catalyzes the synthesis of long-chain oligomers that react with PIA-specific antisera. A PIA-negative mutant of *S. epidermidis* is significantly less virulent than the isogenic wild-type strain in a mouse model of subcutaneous foreign body infection and in a rat model of central-venous catheter-associated infection (Rupp *et al.*, 1999a, b).

It has been shown that the synthesis of a similar if not identical polysaccharide from *S aureus* is mediated by a homologous *ica* gene cluster (Cramton *et al.*, 1999).

The 140 kDa extracellular protein AAP (accumulation-associated protein) is also associated with accumulative growth in certain *S. epidermidis* strains on polymer surfaces (Hussain *et al.*, 1997). It is proposed that AAP has a role in the anchoring of PIA to the cell surface because an AAP deficient strain produces PIA that is only loosely attached to the cell surface and antibodies specific for AAP inhibited accumulation by up to 98%.

The DtlA and Bap proteins have also been identified as contributors to staphylococcal biofilm formation. The *dtlABCD* operon is responsible for D-alanine esterification of teichoic acids. This reduces the negative charge on the surface of *S. aureus* cells that aids primary attachment to polystyrene or glass surfaces (Gross *et al.*, 2001). The Bap protein interferes with initial bacterial attachment of other surface proteins to host receptors and with cell internalization (Cucarella *et al.*, 2002), In contrast, staphylococcal isolates carrying the *bap* gene are strong biofilm producers, indicating a strong correlation between the presence of the protein and biofilm formation ability on abiotic surfaces (Cucarella *et al.*, 2001). It should be noted however that Bap is found in only 5% of bovine strains and not in *S. aureus* strains that infect humans.

1.2.10 Extracellular toxins

S. aureus elaborates a large number of toxins that are implicated in different staphylococcal disease phenotypes. Alpha-toxin (encoded by the *hla* gene) is a cytolytic pore-forming toxin that lyses mammalian erythrocytes and is an important virulence factor in

Table 1.3 Extracellular proteins of *S. aureus*

Secreted protein	Effect	Reference
α-toxin	Lyses mammalian erythrocytes, pore forming toxin	Bhakdi and Tranum-Jensen, 1991
β-toxin	Sphingomyelinase	Molby, 1983
δ-toxin	Peptide, inserts into mammalian membranes and lyses cells	Kreger *et al.*, 1971
γ-toxin	2-component synergohymenotropic toxin	Cooney *et al.*, 1993
PV-leukocidin	2-component synergohymenotropic toxin specific for leukocytes	Boussaud *et al.*, 2003
Enterotoxins	Superantigen, food poisoning	Bohach *et al.*, 1990
TSST-1	Superantigen, toxic shock	Schlievert *et al.*, 1981
Exfoliative toxins	Hydrolyses desmoglein 1	Amagai *et al.*, 2000
Lipase (Geh)	Hydrolyses glycerol esters, binds to collagen	Simons *et al.*,1996
Hyaluronidase	Hydrolyses hyaluronic acid, spread through tissue	Mims *et al.*, 1995
V8 protease	Serine protease, tissue damage	Potempa *et al.*, 1991
Staphopain A and B	Elastase, implicated in lung tissue damage	Potempa *et al.*, 1991
Metalloprotease	Tissue damage	Potempa *et al.*, 1991
Staphylokinase	Plasminogen activator. Binds to and activates defensins	Stephens and Vaheri, 1993 Jin *et al.*, 2004
Coagulase	Activates prothrombin, causes clotting of fibrinogen	Boden and Flock, 1989
CHIPS	Inhibits chemotaxis of neutrophils	Veldkamp *et al.*, 2000

staphylococcal infection (Bhakdi and Tranum-Jensen, 1991; Hildebrand *et al.*, 1991; Foster *et al.*, 1990). Beta-toxin (*hlb*) is an Mg^{2+}-dependent sphingomyelinase that causes invaginations of selected regions of the host cell membrane. Its toxicity depends on the sphingomyelin content of mammalian erythrocytes (Molby, 1983; Arbuthnott, 1982). Delta-toxin (hld) is a small heat stable 26 amino acid residue peptide that is thought to insert into a variety of membranes including erythrocytes, human cells and bacterial membranes (Kreger *et al.*, 1971; Mellor *et al.*, 1988). The epidermolytic toxins ETA and ETB are responsible for staphylococcal scalded skin syndrome characterized by the formation of blisters and epidermal splitting (Bailey *et al.*, 1995; Gemmell *et al.*, 1995). This blistering of the superficial epidermis is caused by hydrolysis of a single peptide bond, Glu381-Gly382, located between extracellular domains 3 and 4 of desmosomal cell adhesion molecule, desmoglein 1 (Amagai *et al.*, 2000; Amagai *et al.*, 2002; Yamaguchi *et al.*, 2002; Hanakawa *et al.*, 2002; Hanakawa *et al.* 2003). The staphylococcal enterotoxins and toxic-shock syndrome toxin-1 are members of a family of pyogenic toxins which behave as superantigens whose function is described below in section 1.2.12.3 (Ulrich *et al.*, 1995; Bohach *et al.*, 1990; Marrack and Kappler, 1990). Staphylococcal enterotoxins also cause staphylococcal food poisoning.

S. aureus also secretes a number of extracellular proteins that are likely to be associated with virulence. Table 1.3 summarizes these proteins and their roles in *S. aureus* pathogenesis.

1.2.11 Evasion of the host immune system

A notable feature of disease-causing staphylococci is their ability to subvert and modulate the host immune system in order to further the infectious process and survive within the hostile milieu of the host. Mechanisms of immune escape are used by many infectious agents affecting humans. The nature of these strategies vary significantly and can be classified as follows: (i) the generation of physical barriers that protect bacteria against immune attack (Kharazmi, 1991; Martin-Lopez *et al.*, 2002); (ii) the secretion of immuno-regulatory cytokine analogs or the induction of host cytokines that potentiate non-protective immunity (Suzuki *et al.*, 1995; Engele *et al.*, 2002; de Diego *et al.*, 1997), (iii) antigenic variation, which can prevent the generation of a protective immune response (Donelson *et al.*, 1998; Kyes *et al.*, 2001), (iv) antigenic mimicry, which can result in either immune modulation (e.g., MHC I analogs) or sequestration of the pathogen from the immune system (Wurzner 1999; Farrell *et al*, 1997), (v)

production, which can destroy different cell types, including immune cells (Lowy, 1998; Kharazmi, 1991), and (VI) direct modulation of host cell functions, which can result in the induction or inhibition of apoptosis (Boulton and Grey-Owen, 2002; Monack *et al.*, 1997).

1.2.11.1 Capsular polysaccharide

Capsular polysaccharides are produced by more than 90% of *S. aureus* strains. Eleven capsular serotypes of *S. aureus* have been identified. However, the majority of clinical isolates are either micro-encapsulated types 5 or 8 (Arbeit *et al.*, 1984). The production of capsular polysaccharide is encoded by the genes of the cap locus. There are 16 open reading frames within the locus. 12 of the 16 genes bear significant identity between the cap5 and cap8 loci (Sau *et al.*, 1997). The non-identical genes give rise to the serotype specificity by determining the exact structure of the repeating sugar monomer units that comprise the polysaccharide (Wann *et al.*, 1999; Kneidinger *et al.*, 2003). CP5 is composed of a trisaccharide repeat unit of: [→ 4)-3-O-Ac-β-D-Man*p*-NAcA-(1→ 4)-α-L-Fuc*p*-NAc-(1→ 3)-β-D-Fuc*p*-NAc-(1→]. This structure differs from that of CP8 only in the position of the O-acetyl group and in two of the three glycosidic linkages between the sugar residues (Fournier *et al.*, 1984, Moreau *et al.*, 1990).

Using isogenic capsule negative mutants it was shown that *S. aureus* capsule is anti-phagocytic and that specific anti-capsule antibodies in addition to complement are required for efficient opsonisation of micro-encapsulated *S. aureus* strains (Karakawa *et al.* 1988; Xu *et al.* 1992; Nilsson *et al.*, 1997). More recent studies have determined that type 5 capsular polysaccharide can inhibit the binding of opsonic complement C3 fragments to *S. aureus* (Cunnion *et al.*, 2001). This effect is enhanced by the ability of type 5 capsule to mask C3 fragments on bacterial cell surface from interacting with complement receptor 1 on neutrophils resulting in a markedly decreased level of phagocytosis (Cunnion *et al.*, 2003).

1.2.11.2 Masking cell surface by binding host proteins

The use of host protein to mask the surface of a bacterial cell such that the host immune system does not recognize it as foreign is not a novel phenomenon. *S. aureus* possesses the ability to mask themselves from recognition by the host immune system by virtue of their ability to bind to

to bind to serum proteins and thus coat themselves in host protein (Massey *et al.*, 2002). Protein A binds to the Fc portion of immunoglobulins. This is likely to coat the surface of bacterial cells with IgG and prevent opsonophagocytosis through the neutrophil Fc receptor. It is also possible that coating of bacterial cells with fibrinogen is antiphagocytic as isogenic ClfA negative strains are attenuated in a murine model of infection (Joseffson *et al.*, 2002).

Proteolysis of fibrinogen by thrombin during the formation of fibrin clots releases the chemotactic fragment fibrinopeptide B, which attracts leucocytes and fibroblasts to the site of injury (Kay *et al.*, 1974; Richardson *et al.*, 1976; Senior *et al.*, 1986; Skogen *et al.*, 1988). The binding of SdrG, the fibrinogen binding protein of *S. epidermidis*, to the B β chain of fibrinogen prevents the thrombin mediated release of chemotactic fibrinopeptide B (Davis *et al.*, 2001). This could reduce the influx of neutrophils to the site of infection as well as masking bacterial cells from recognition by neutrophils.

1.2.11.3 Resistance to antimicrobial peptides

Polymorphonuclear leukocytes (neutrophils) are the first leukocytes to appear at a site of infection. These cells elaborate several antimicrobial activities that participate in the killing of ingested microbes. In addition to the oxygen-dependent respiratory burst compounds, neutrophils produce bacteriolytic enzymes and cationic antimicrobial peptides (CAMPs). The antimicrobial activity of CAMPs involves insertion of the peptide into the bacterial cytoplasmic membrane. As the peptides accumulate in the membrane they form pores which result in leakage of bacterial cell contents and breakdown of membrane potential (Lehrer and Ganz, 1999). Peptides with similar properties and activity have been found in several vertebrates and invertebrates as well as in plants (Hoffmann *et al.*, 1999) and bacteria (Jack *et al.*, 1998) (Fig. 1.9). They include molecules with β-sheet structure such as porcine protegrins (Kokryakov *et al.*, 1993), α-helical peptides such as the amphibian magainins (Bechinger, 1997), and the bacterial lantibiotics bearing thioether bridges (Jack *et al.*, 1998). Human antimicrobial peptides are classified into three types; defensins, cathelicidins and histatins. LL37 is the only peptide associated with the cathelicidin family and is produced by neutrophils and epithelial cells (Larrick *et al.*, 1995). Histatins are histidine rich peptides found in human saliva (Gussman *et al.*, 2001).

Defensin peptides are cysteine-rich cationic peptides that are divided into two groups, (α and β) according to their structure and the cells that produce them. These peptides constitute a

shield against microbial infections on skin, on epithelia of the respiratory, gastrointestinal, and genitourinary tracts (ß-defensins), and are found in large amounts in the granules of phagocytes and intestinal Paneth cells (α-defensins) (Lehrer and Ganz, 1999). Although defensins account for 50% of the neutrophil granule proteins, they fail to kill *S. aureus* effectively. It is myeloperoxidase that mediates the bactericidal effect against staphylococci in neutrophils. Consequently, patients with inherited oxidative burst deficiency (chronic granulomatous disease) are particularly susceptible to *S. aureus* infections (Liese *et al.*, 1996). When keratinocytes from human skin come into contact with bacterial pathogens, they upregulate the expression of the defensin hBD-2 gene (Harder *et al.*, 1997; Midorikawa *et al.*, 2003) and there is evidence that airway epithelial cells respond in a similar manner (Hiratsuka *et al.*, 1998). Diminished defensin activity caused by increased salt concentration in airway fluids is thought to contribute to the susceptibility of cystic fibrosis patients to life-threatening *S. aureus* and *Pseudomonas aeruginosa* infections (Goldman *et al.*, 1997). Both *S. aureus* and the coagulase-negative *Staphylococcus xylosus* show high level innate tolerance to cationic antimicrobial peptides and lantibiotics (Harder *et al.*, 1997). The molecular basis of staphylococcal resistance to antimicrobial peptides involves several different mechanisms discussed below.

1.2.11.3.1 D-Alanine modification of teichoic acids

Teichoic acids are major components of the cell wall of staphylococci. They are polymers of glycerol-phosphate or ribitol-phosphate to which are attached glycosyl and D-alanyl ester residues. Wall teichoic acid is covalently linked to peptidoglycan, whereas lipoteichoic acid is attached to glycolipid intercalated in the cytoplasmic membrane. Together with peptidoglycan, these polymers make up a polyanionic matrix that function in cation homeostasis, the trafficking of ions, nutrients, proteins, and antibiotics, the regulation of autolysins, and in the presentation of envelope proteins. The esterification of teichoic acid with D-alanyl esters modulates the net anionic charge of the bacterial cell surface, determining the cationic binding capacity, and the display of cations in the cell wall.

The enzymes responsible for the D-alanylation of *S. aureus* teichoic acid are encoded by the *dlt* operon. The *dltABCD* genes of *S. aureus*, *S. xylosus*, *L. casei,* and *B. subtilis* are similar in sequence and organization. Studies in *L. casei* have demonstrated that DltA is a D-alanine-D-alanyl carrier protein ligase (Dcl), which activates D-alanine by hydrolysis of ATP and transfers

it to the phosphopantetheine cofactor of a specific D-alanine carrier protein (Dcp), which is encoded by *dltC* (Debabov *et al.*, 1996; Heaton and Neuhaus, 1992). The hydrophobic DltB is indispensable for D-alanine incorporation into teichoic acids and may be involved in the transfer of activated D-alanine across the cytoplasmic membrane (Perego *et al.*, 1995). The essential role of DltD and the presence of a putative N-terminal signal peptide suggest an involvement in the transfer of D-alanine from the membrane carrier to teichoic acids.

Targeted mutagenesis of the *dlt* operon has provided a greater understanding of how D-alanyl esterification of teichoic acids aids in the virulence of *S. aureus* and impedes host-mediated responses to infection. Inactivation of the *dlt* operon confers sensitivity to a wide host range of cationic antimicrobial peptides by reducing the overall negative charge of the cell surface (Peschel *et al.*, 1999). As the mode of action of these cationic peptides critically depends on their interaction with the negatively charged bacterial surface a reduction in negative charge repels the peptides from the bacterial surface and prevents them from inserting into the bacterial membrane. This effect also impacts on the susceptibility of *S. aureus* to vancomycin and other glycopeptide antibiotics which also appear to be repelled from the cell surface by D-alanylation of teichoic acids (Peschel *et al.*, 2000).

Furthermore, group IIA phospholipase A (2) binding to bacterial cells depends on the cationic properties of the enzyme that promote binding to the cell wall peptidoglycan. Consequently its antibacterial properties are also attenuated by D-alanylation of teichoic acids (Koprivnjak *et al.*, 2002). Further studies have since revealed that *S. aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are attenuated in a mouse model of sepsis and septic arthritis and a mouse tissue cage infection model (Collins *et al.*, 2002; Kristian *et al.*, 2003). Hence, modulations of the net charge in the bacterial cell envelope aid in reducing the accumulation of cationic antimicrobial peptides.

1.2.11.3.2 L-lysinylation of phosphatidyl glycerol

Transposon mutagenesis of *S. aureus* yielded a second genetic locus that contributed to resistance to human defensins and evasion of neutrophil killing. Peschel *et al.* (2001) showed that the novel virulence factor MprF modified membrane phosphatidyl glycerol with L-lysine. This produces lysyl-phosphatidylglycerol which, unusually, bears a net positive charge whereas other *S. aureus* phospholipids are anionic. The increased sensitivity of the mutant was restricted

to extended cationic peptides. The small, circular gramicidin S and the linear neutral gramicidin D from *Bacillus brevis* were almost equally active against wild-type and mutant strains. The resistance phenotype appeared to be mediated through modulation of surface anionic charges, as L-lysinylation reduces the overall anionic charge of the bacterial membrane. Kristian *et al.* (2003) subsequently confirmed that MprF only contributed to resistance to oxygen independent killing of *S. aureus*. Myeloperoxidase activity against both mutant and wild type strains was unaffected. Inactivation of *mprF* also increased the susceptibility of *S. aureus* to the glycopeptide antibiotic vancomycin (Ruzin *et al.*, 2003). Thus reduction of the overall anionic charge of the bacterial cell surface is a strategy employed by *S. aureus* to circumvent a wide range of antimicrobial compounds.

1.2.11.3.3 QacA efflux pump

QacA belongs to the major facilitator superfamily of efflux pumps which includes the EmrB protein from *E. coli* and the Bmr protein from *B. subtilis*. These proteins are energy dependent transporters that require a proton motive force. These proteins were originally shown to confer resistance to organic dyes and disinfectants (Mitchell *et al.*, 1998, Mitchell *et al.*, 1999) but have more recently been implicated in bacterial protection against cationic antimicrobial peptides. *S. aureus* strains differ in their susceptibility to the rabbit thrombocidin tPMP1 (Dhawan *et al.*, 1997). Reduced susceptibility is associated both with increased membrane fluidity (Bayer *et al.*, 2000) and with QacA (Kupferwasser *et al.*, 1999). However, questions remain about the mechanism of resistance as QacA-mediated tPMP1 resistance appears to be independent of the efflux properties of this pump, relying only on the presence of the QacA protein (Fig. 1.10). Interestingly, homologues of QacA are found in other staphylococcal species (Paulsen *et al.*, 1996).

1.2.11.3.4 Staphylokinase

Staphylokinase is a 136 residue secreted protein encoded by a *S. aureus* prophage-borne gene. Staphylokinase facilitates activation of plasminogen, the precursor of the fibrinolytic protease plasmin by forming a 1:1 stoichiometric complex with plasmin(ogen) that converts other plasminogen molecules to plasmin, a potent enzyme that degrades proteins of the

plasminogen molecules to plasmin, a potent enzyme that degrades proteins of the extracellular matrix. Staphylokinase structurally resembles other plasminogen activators, containing a plasminogen-binding site and a serine protease domain (Sakharov *et al.*, 1996). However, staphylokinase is not an enzyme and surprisingly has also been found to abrogate the bactericidal effects of α-defensins by binding to and neutralizing their antibacterial properties. This effect was found to be independent of the proteinase activity of staphylokinase. It is proposed that there are several defensin binding sites within staphylokinase, one of which lies in close proximity to the plasminogen binding site as defensin binding prevents plasminogen activation (Jin *et al.*, 2004).

1.2.12 Invasion of and survival inside host cells

Until recently *S. aureus* was considered to be an extracellular pathogen that did not invade host cells. However, an increasing body of evidence suggests that it may have the capacity to function as an intracellular pathogen. Clinical features of *S. aureus* infections that support this theory include its ability to cause endovascular infections, the frequency at which both localized and systemic infections metastasize and the frequent occurrence of disease relapses despite adequate antimicrobial chemotherapy (Lowy, 1998). *In vitro* studies have shown that professional and non-professional phagocytes internalize staphylococci (Yao 1995; Hudson *et al.*, 1995). The bacteria appear to be internalized by the zipper mechanism of invasion whereby the bacteria induce actin rearrangement, are taken up in membrane bound vacuoles and transported into the cell (Finlay and Cossart, 1997). *S. aureus* cells have also been observed to escape from the membrane bound vacuoles into the host cell cytoplasm where they induce apoptosis (Bayles *et al.*, 1998).

Invasion of eukaryotic cells by *S. aureus in vitro* is predominantly mediated by the fibronectin-binding proteins FnBPA and FnBPB (Sinha *et al.*, 1999; Dziewanowska *et al.*, 1999) although Map/Eap has also been implicated (Haggar et al., 2003). Eukaryotic cells also possess specific surface receptors that bind to fibronectin. The integrin $\alpha_5\beta_1$ serves as a fibronectin receptor on many cell types and recognizes a short peptide motif, the Arg-Gly-Asp (RGD) sequence, found within one of the type III repeats of Fn. FnBP associates with the N terminus of fibronectin, independently of the RGD sequence (Schwarz-Linek *et al.*, 2003; Massey *et al.*, 2001). Therefore simultaneous association of both *S. aureus* and human cells with a fibronectin molecule is possible, with fibronectin acting as a molecular bridge linking FnBP-expressing *S. aureus* with

aureus with integrin $\alpha_5\beta_1$ on the surface of human cells (Joh *et al.*, 1999). This interaction tightly anchors *S. aureus* to its eukaryotic host cell and promotes internalization of the bacterial cells by human epithelial and endothelial cells (Sinha *et al.*, 1999; Dziewanowska *et al.*, 1999; Jett and Gilmore, 2002).

Integrin binding to proteins of the extracellular matrix triggers cytoskeletal rearrangements (Schwarz and Ginsberg, 2002). Furthermore, Agerer *et al.* (2003) showed that indirect engagement of $\alpha_5\beta_1$ integrin by FnBPs induces actin-dependent uptake of *S. aureus* by its human host cell, a process that is critically dependent upon the Src family of protein-tyrosine kinases. However, the role of the *S. aureus* FnBP-Fibronectin-integrin interaction for the infection process *in vivo* is not yet understood. Agerer and colleagues postulated that the indirect attachment of the microorganisms to host cell integrins could promote internalization of the microbe *in vivo* as a means of sheltering the bacteria from host defense mechanisms or indeed from antimicrobial compounds that do not penetrate host cells. Indeed, in 163 clinical isolates of *S. aureus* either one or both FnBP genes were detected and presence of both FnBPs seemed to be correlated with invasive types of disease when compared with strains derived from nasal carriers (Peacock *et al.*, 2000).

Whether the survival of *S. aureus* cells inside non-professional phagocytes contributes to the infectious process remains to be fully investigated. Gresham *et al.* (2000) showed that survival of *S. aureus* inside neutrophils aids in the persistence of *S. aureus* infections *in vivo* and *in vitro*. The intracellular survival of the bacterial cells appeared to be mediated through uptake of some cells within macropinosomes that resemble the outcome of membrane ruffling as is observed with *Salmonella*, rather than uptake by the zipper mechanism which was associated with efficient destruction of the pathogen. This clearly demonstrated that *S. aureus* has the ability to invade and survive within the very cell that is responsible for its destruction, a trait reminiscent of other intracellular pathogens.

1.2.13 Immunomodulation by *S. aureus*

Reinfection of humans with *S. aureus* is one of the hallmarks of diseases caused by this pathogen. One possible reason for recurring infections is that leukocytes from patients with chronic or recurrent infections have impaired chemotactic, phagocytic, and bactericidal functions (Monteil *et al.*, 1987; Verbrugh *et al.*, 1983; Valmin *et al.*, 1982). *S. aureus* uses various

survival strategies that allow it to persist in mammalian hosts and recurrent infections point to mechanisms that obstruct the development of protective immunity. Elucidation of the function of these molecules and their mechanisms of action may be used to design strategies to prevent or treat disease.

1.2.13.1 MHC class II analog protein

Map (MHC class II analog protein) protein, also referred to as extracellular adherence protein (EAP) and p70, is a secreted protein that binds to extracellular matrix components, including fibronectin, fibrinogen, vitronectin, bone sialoprotein, and thrombospondin (Chhatwal *et al.*, 1987; Hudson *et al.*, 1999; Herrmann *et al.*, 1991). However, isogenic Map-deficient *S. aureus* mutants are not impaired in their adhesion to these extracellular matrix proteins. Other adhesins mediate these interactions and Map may have an alternative function (Kreikemeyer *et al.*, 2002; Chavakis *et al.*, 2002; Haggar *et al.*, 2003; Harraghy *et al.*, 2003). Map contains six repeated domains of 110 amino acids. Each repeat contains a 30 amino acid subdomain with similarity to a sequence in the peptide-binding groove of the MHC class II β chain (Jonsson *et al.*, 1995). The similarity between Map and the MHC class II molecules suggested that Map secretion may affect T cell function. Previous studies demonstrated that Map can induce immunoglobulin synthesis and the proliferation of blood mononuclear cells in addition to shifting T cell responses in a Th2 direction (Jahreis *et al.*, 2000; Chavakis *et al*, 2002; Jonsson *et al.* 1995).

The effects of Map on T cell responses may play a critical role in the survival of *S. aureus* as the induction of Th1 responses during the course of staphylococcal infections has been associated with bacterial clearance in mice (Guillen *et al.*, 2002). The role of T cells in protection against staphylococcal infections in humans is less well defined. Infection by *S. aureus* does not usually result in protective immunity and individuals can be subjected to persistent and repeated staphylococcal infections (Chang *et al.*, 2000; Hartstein *et al.*, 1992). Cellular immunity is critical in the clearance of systemic *S. aureus* infections and in preventing reinfection with the same or similar pathogens (Easmon and Glynn, 1975; Ficker *et al.*, 1989; Verbrugh *et al.*, 1983; Valmin *et al.*, 1982). Map may serve as a mechanism to facilitate the survival and persistence of *S. aureus* in the host due to its ability to inhibit or shift the T cell response in a Th2 direction (Lee *et al.*, 2002). Map is also an anti-inflammatory agent which

Map is also an anti-inflammatory agent which interferes with the innate host defense systems by preventing neutrophil recruitment, mainly by interacting with ICAM-1 on endothelial cells (Chavakis *et al.* 2000). Map is a virulence factor that interferes with acquired immunity in a *S. aureus* chronic murine infection model (Bremmell *et al.* 1991) and may play a role in the persistence and survival of *S. aureus* in the host by altering T cell function *in vivo* (Lee *et al.*, 2002).

1.2.13.2 CHIPS

Directed migration of neutrophils to a focus of infection is an important step in the eradication of invading bacteria by phagocytosis and intracellular killing. Staphylococci produce a number of proteins involved in phagocyte evasion, among them a 15 kD chemotaxis inhibitory protein (CHIPS) that binds to and attenuates the activity of the neutrophil receptors for complement and formylated peptides (W. Van Wamel *et al.*, 2001). Upon treatment of neutrophils with CHIPS, specific and total downregulation of the C5a and fMLP receptors is observed. Neutrophil chemotaxis towards fMLP and C5a is inhibited without interfering with chemotaxis to IL-8 (Veldkamp et al., 2000). This function is proposed to protect *S. aureus* from neutrophil-mediated killing (Van Wamel *et al.*, 2001). The gene encoding CHIPS (*chp*) has been shown to reside on a functional lysogenic phage that also carries the staphylokinase (*sak*) and enterotoxin A (*sea*) genes (Van Wamel *et al.*, 2001).

Staphylococci also produce the phage-encoded Panton-Valentine leukocidin (PVL), a cytotoxin with direct activity against human phagocytes (Kaneko et al., 1997). Thus, by inhibiting phagocytosis (CHIPS) and by directly attacking phagocytes (PVL), two different phage gene products counteract phagocyte-mediated destruction of their staphylococcal hosts.

1.2.13.3 Superantigens

The staphylococcal enterotoxins are a family of superantigens produced by *Staphylococcus aureus*. These protein toxins bind to major histocompatibility complex (MHC) class II molecules on antigen presenting cells and, with less affinity, to the T-cell antigen receptors. They cross-link the MHC class II to the T-cell receptor resulting in massive T-cell proliferation and cytokine

release that triggers acute pathological effects (Fraser, 1989; Marrack and Kappier, 1990; Ulrich *et al.*, 1995). Superantigens are associated with several serious diseases, including bacterial arthritis, and lethal toxic shock syndrome (Fraser *et al.*, 2000; Michie and Cohen, 1998). The main component of the intoxication process depends on the ability of superantigens to activate a large number of T cells, causing a massive release of inflammatory cytokines (Fraser *et al.*, 2000, Ulrich *et al.*, 1995). This inflammatory response does not serve to fight staphylococcal infection but rather overwhelms the host immune system leading to toxic shock, multiple organ system failure and death. Localized release of superantigens in minor infections is likely to reduce the specific immune response in the synthesis of appropriate antibodies.

1.3 Regulation of gene expression

1.3.1 Two component regulatory systems

The two-component regulatory systems that are known to be involved in expression of the *S. aureus* virulon include *agr* (Recsei *et al.*, 1986), *sae* (Giraudo *et al.*, 1994; 1999) and *srr* (Yarwood *et al.*, 2001; Throup *et al.* 2001).

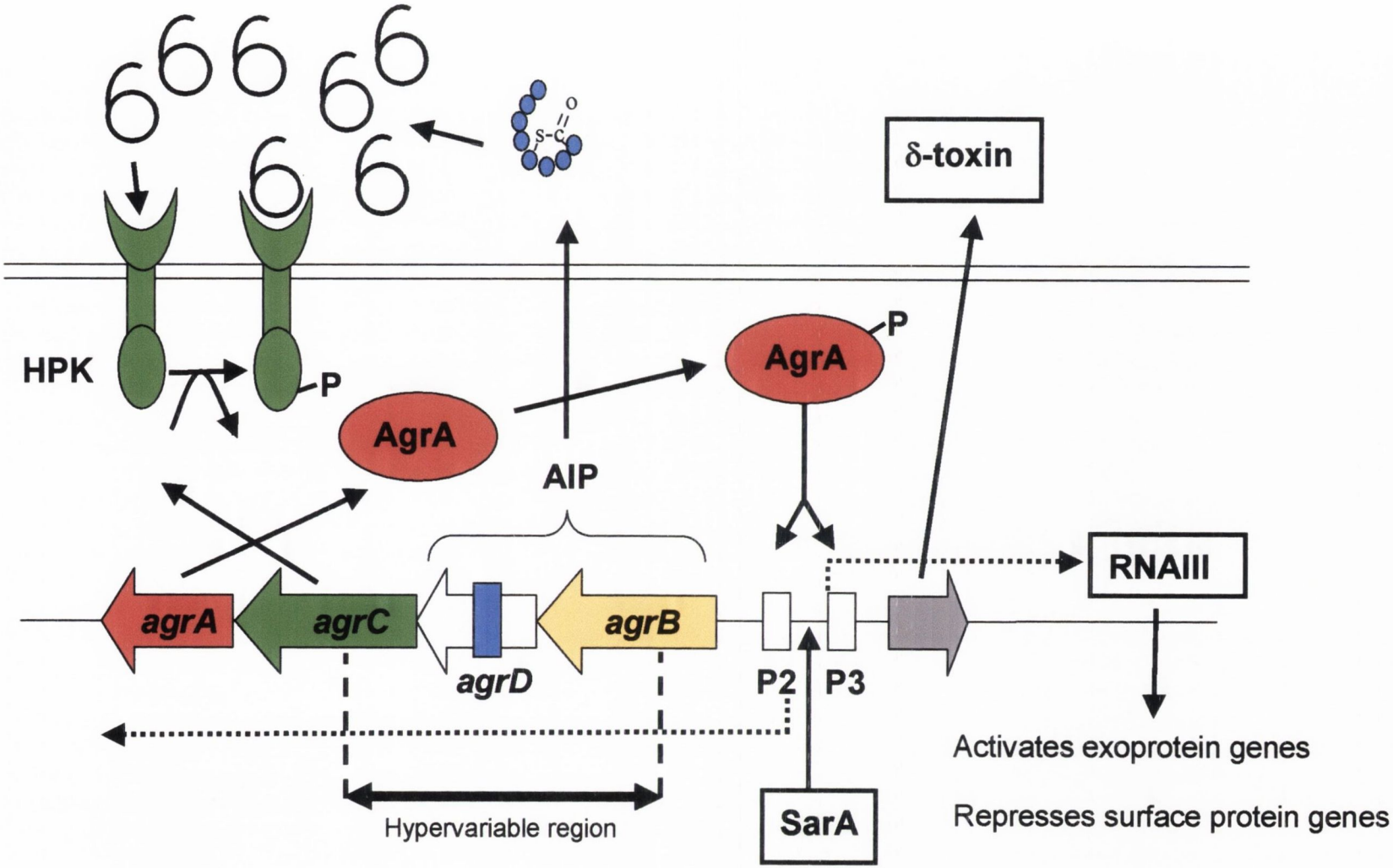
The *agr* system consists of 3 kb locus containing divergent transcription units, driven by promoters P2 and P3 (Fig. 1.9). The P2 operon encodes a two-component system and its autoinducing ligand (Novick *et al.*, 1995). The primary function of the operon is to activate the two *agr* promoters. The P3 transcript, RNA III, rather than the response regulator, AgrA, is the intracellular effector of target gene regulation (Janzon and Arvidson, 1990; Novick *et al.*, 1993). As *agr* is autoinduced by an extracellular ligand, it represents a sensor of population density. Because the activating ligand is encoded within the operon, the circuit is doubly autocatalytic, resulting in a very rapid burst of activity once the autoinduction threshold has been reached. The expression of this system entails a tremendous metabolic burden, resulting in frequent spontaneous *agr* mutants in the laboratory (Bjorklind and Arvidson, 1980; Somerville *et al.*, 2002b), especially in strains that lack σ^B, which modulates the *agr* regulon. The *agr*-activating ligand is a post-translationally modified peptide (AIP), seven to nine amino acid residues in length, which is processed from a propeptide encoded by *agrD* (Ji *et al.*, 1995). The AIP binds to the N-terminal transmembrane domain of the *agr* signal receptor, *agrC* (Ji *et al.*, 1995; Lina *et al.*, 1998; Lyon *et*

Agr is conserved throughout the staphylococci with variations in the *B–D–C* region. These variations have resulted in at least four *agr* specificity groups in *S. aureus* and one or more in each of 15 other staphylococcal species examined (Ji *et al.*, 1997; Otto *et al.*, 1999; Jarraud *et al.*, 2000; Dufour *et al.*, 2002). The groups are defined by the mutual inhibition by their peptides of the *agr* response in heterologous pairings, resulting in bacterial interference in which the *agr* regulon, rather than growth, is blocked (Ji *et al.*, 1997). The ability of an AIP to activate its cognate receptor is highly sequence specific; a single amino acid substitution can change group specificity. The N-terminal one-third of AgrB and the C-terminal (cytoplasmic) histidine protein kinase domain of AgrC are highly conserved, whereas the intervening sequences are highly divergent, constituting the hypervariable region. The divergent regions determine group specificity and must therefore have co-evolved. Functional variants within the *agr* locus are designed for cross-group and, presumably, cross-species interference rather than co-operative communication, so that they serve to isolate populations and may represent a major determinant of strain and species divergence. In keeping with this idea, it is predicted that the *agr* groups in *S. aureus* correlate with specific biotypes. For example most menstrual TSS strains belong to *agr* group III (Ji *et al.*, 1997), as do all the 16 strains recently found to cause leukocidin-induced necrotizing pneumonia (Gillet *et al.*, 2002). Most of the recently encountered VISA strains belong to *agr* group II (Sakoulas *et al.*, 2002) while most exfoliatin-producing strains belong to *agr* group IV (Jarraud *et al.*, 2000; McDowell *et al.*, 2001).

The *agrD*-encoded propeptide is N- and C-terminally processed to form a unique thiolactone ring between the conserved central cysteine and the peptide's C-terminal carboxyl (Ji *et al.*, 1997). The cyclic thiolactone is essential for AIP activity and is the hallmark of these peptides. The AIP of *S. intermedius* is the only exception where a serine replaces the cysteine (Dufour *et al.*, 2002). These strains produce an active nonapeptide AIP containing a cyclic lactone (Kalkum *et al.*, 2003). The propeptide is processed and secreted by AgrB, a polytopic transmembrane protein (Ji *et al.*, 1997; Saenz *et al.*, 2000; Zhang *et al.*, 2002). However, other proteins may be involved in AIP processing.

The group specificity of AgrB is less stringent than that of the AIP–receptor interaction. AgrB-I and AgrB-III each process AgrD-I and AgrD-III with equal efficiency, but neither will process AgrD-II or AgrD-*sl* (from *S. lugdunensis*) and vice versa (Ji *et al.*, 1997). The *agrD* sequence has been determined for nearly 30 different strains, including representatives of some 16 different staphylococcal species (Ji *et al.*, 1997; van Wamel *et al.*, 1998; Otto *et al.*, 1999;

Figure 1.9 Schematic representation of the *agr* regulon in *S. aureus*. AgrC. Membrane bound histidine protein kinase. AgrA. Response regulator. AgrD encodes autoinducing peptide (AIP). AgrB. Membrane bound protein that is responsible for processing the AIP. RNAIII. Effector molecule of the *agr* response.


δ-toxin
HPK
P
AgrA
AgrA
P
AIP
RNAIII
agrA
agrC
agrD
agrB
P2
P3
Activates exoprotein genes
Represses surface protein genes
Hypervariable region
SarA

from representative strains of *S. lugdunensis*, *S. warneri* and *S. epidermidis* have been sequenced and/or synthesized *in vitro* (Jarraud *et al.*, 2000; Lyon *et al.*, 2002a). AIPs I and IV are octapeptides, AIPs-II and *Si*-I are nonapeptides (Ji *et al.*, 1997; Kalkum *et al.*, 2002) and AIPs-III and *Sl*-I and II are heptapeptides. The AIPs form a coherent group with generally conserved structural features, including a strong gradient of increasing hydrophobicity from N- to C-termini, culminating in two bulky hydrophobic residues, limited to FLVY, plus an occasional M. The cyclical structure is required, whether the peptide is acting as an *agr* activator or as an inhibitor (Mayville *et al.*, 1999; Lyon *et al.*, 2000; McDowell *et al.*, 2001). Replacement of the thiolactone by a lactone or lactam bond eliminates the activation but not the cross-group inhibition function of the peptide, although these variant peptides are not self-inhibitors. Removal of the tail region of AIPs I or II converted either peptide into a universal inhibitor of *S. aureus agr* function (Lyon *et al.*, 2002a, b). The interaction between activating and inhibiting peptides at the receptor is strictly competitive (Lyon *et al.*, 2002b).

AgrC was inferred by sequence analysis to be a histidine protein kinase. It was shown by *phoA* fusions to have a polytopic transmembrane N-terminal domain (Lina *et al.*, 1998) and by pull-down studies to be the only cellular protein capable of binding the AIP (Ji *et al.*, 1995), thus confirming that AgrC is the receptor of the *agr* two-component system. Group specificity resides in the N-terminal transmembrane domain of AgrC (Lyon *et al.*, 2002a) whereby a single amino acid that differs between AIP I and IV (aspartate versus tyrosine at position 5) makes a specific contact in this region of the receptor.

AgrA has features of a response regulator (Nixon *et al.*, 1986) and is required for activation of the two *agr* promoters, P2 and P3, completing the autoinduction circuit (Novick *et al.*, 1993; 1995). However, binding of AgrA to the *agr* promoters or any other DNA has not been demonstrated (Morfeldt *et al.*, 1996a). Nevertheless, it seems likely that either AgrA or AgrA-P binds in this region.

The intergenic 120 bp region between *agr* promoters P2 and P3 must contain the site of activation. There is a 17 bp inverted repeat (IR) in this region that has been proposed to be a bidirectional regulatory protein binding site (Bayer *et al.*, 1996). There are also four heptanucleotide repeats, centered at -45 with respect to P2 and at -66 with respect to P3, of which the two middle repeats overlap with the IR. These repeats are conserved in *S. epidermidis*, *S. simulans* and *S. warneri*, in which activation of the P3 promoter by the *agr* two-component system

epidermidis, *S. simulans* and *S. warneri*, in which activation of the P3 promoter by the *agr* two-component system is also conserved (Benito *et al.*, 1998; Tegmark *et al.*, 1998). Only the two P3-proximal repeats are required for P3 activation, suggesting that the two *agr* promoters may be differentially regulated. Elimination of three nucleotides between the -10 and -35 elements of the promoter causes constitutive expression of the promoter (Morfeldt *et al.*, 1996a).

RNA III, the P3 transcript, is the intracellular effector of the *agr* regulon (Novick *et al.*, 1993). It is highly abundant and has a long half-life of ~15 min (Janzon and Arvidson, 1990). It has a complex secondary structure (Benito *et al.*, 2000), which is well conserved among several staphylococcal species (Benito *et al.*, 1998; Tegmark *et al.*, 1998), resulting in interspecific cross-reactivity of the molecule. RNA III has been shown to upregulate the transcription of most of the extracellular protein genes and to downregulate the transcription of several surface protein genes (Novick *et al.*, 1993; Saravia-Otten *et al.*, 1997).

Structure–function analysis of the RNA III molecule has revealed that the transcription termination loop is necessary and sufficient for repression of transcription of the protein A gene (Benito *et al.*, 2000). RNA III could also bind to individual transcription factors, causing allosteric modifications that affect their ability to bind target sequences (Arvidson and Tegmark, 2001). It is also possible that RNA III affects the stability of the target gene transcripts. RNA III also acts at the level of translation for two gene products, α-hemolysin (Novick *et al.*, 1993; Morfeldt *et al.*, 1995) and protein A. The 5' region of RNA III is complementary to the *hla* leader sequence which folds into an untranslatable configuration unless prevented from doing so by RNA III. It is likely that translation of the *hld* reading frame within RNA III is required for this interaction with the *hla* leader. The 3' end of RNA III is complementary to the translation initiation site of *spa* mRNA and blocks its translation. It is unknown whether translation of other *agr*-regulated proteins is affected by RNA III.

1.3.2 Transcriptional regulators

There is no consensus sequence that represents the regulatory target for *agr* among its target genes. Therefore RNA III must act via a multiplicity of intermediary proteins (Novick *et al.*, 1993). Studying the effects of blocking protein synthesis on target gene transcription by incubating an *agr*-null strain with erythromycin at an inhibitory concentration, and analyzing *hla* and *spa* transcripts tested this possibility (Novick *et al.*, 1995). Inhibition by erythromycin was

accompanied by a dramatic increase in *hla* transcription and an equally dramatic decrease in *spa*, mimicking the effects of RNA III and leading to the suggestion that *hla* expression is controlled by a labile protein repressor and *spa* by a labile activator. The hypothesis of intermediary proteins was subsequently confirmed by the isolation of mutations with phenotypes similar to the effects of erythromycin. In the absence of RNA III, *hla* is upregulated by mutation in *rot* or *sarT* (McNamara *et al.*, 2000; Schmidt *et al.*, 2001), and *spa* is downregulated by mutations in *sarS* (Tegmark *et al.*, 2000) or *rot* (Said-Salim *et al.*, 2003). SarS, SarT and Rot are SarA homologues that are members of a family of winged helix-turn-helix transcription factors. This family includes bacterial regulators of efflux pumps (Alekshun *et al.*, 2001; Godsey *et al.*, 2001), phage maturation proteins (de Beer *et al.*, 2002), the arginine repressor family (Holtham *et al.*, 1999) and response-regulator proteins (Martinez-Hackert and Stock, 1997).

SarA and its homologues (Tegmark *et al.*, 2000; Manna and Cheung, 2001) affect the transcription of a wide variety of genes (Dunman *et al.*, 2001). The DNA-binding segments of these proteins are closely conserved, all containing the motif KXRXXXDER. The first of these factors to be discovered was SarA (Cheung *et al.*, 1996), a 14.7 kDa DNA-binding protein, distantly related to VirF of *Shigella flexneri*. SarA binds as a dimer, and at least three of its homologues, SarS, SarU and SarY, appear to be the result of duplications and therefore are intrinsically dimeric. There is a high degree of structural and sequence similarity among the members of this family (Cheung and Zhang, 2002). The possibility of heterodimeric combinations has been suggested (Tegmark *et al.*, 2000).

The *sarA* locus is transcribed from three promoters, *sar*P1, *sar*P2 and *sar*P3 of which the last is a σ^B promoter. The three promoters are active at different times during the *in vitro* growth cycle (Manna *et al.*, 1998; Blevins *et al.*, 1999, 2002). There are two short reading frames upstream of the *sarA* coding sequence that appear to have a stimulatory effect on *sarA* function (Cheung *et al.*, 1997). *SarA* transcription from the *sar*P2 promoter is inhibited by another homologue, SarR (Manna and Cheung, 2001).

SarA binds as a dimer to AT-rich sequences distributed widely in the AT-rich staphylococcal genome, including the 5' regions of several genes. However there is no consensus SarA binding site (Wisell, 2000). Rechtin *et al* (1999) have described multiple SarA binding sites in the intergenic region between *agr* promoters P2 and P3, suggesting the possibility of cooperativity, whereas Cheung and coworkers have identified only one site (Morfeldt *et al.*, 1996a; Chien and Cheung, 1998).

SarA has been reported by Cheung *et al* (1997) to upregulate the transcription of several genes, including the two *agr* operons and *fnbA*, and to downregulate several others, especially including *ssp* (Chan and Foster, 1998b; Chien *et al.*, 1999) and *cna* (Blevins *et al.*, 1999). SarA is required for *saeRS* and *arlRS* activation, possibly via its effects on *agr* transcription, and downregulates the transcription of several exoprotein genes independently of its effects on RNA III transcription, including *ssp*, *aur*, *scp*, *cna* and *spa*. This effect is consistent with SarA binding in the 5' regions of these genes, deletions of which result in an increase in expression. Activation by SarA of *fnbA*, *tst* and *hla* has also been reported (Cheung *et al.*, 1999; Wolz *et al.*, 2000). However the reduced fibronectin-binding activity observed with *sarA* mutants may not be at the level of transcription. It is likely to be a consequence of the increased proteolytic activity seen with these mutants (Blevins *et al.*, 2002). *In vitro* analyses have revealed only inhibition by SarA (Chakrabarti and Misra, 2000), consistent with the suggestion by Arvidson and Tegmark (2001) that SarA is not ordinarily a transcriptional activator, and with the conclusion that SarA upregulates *hla* by downregulating the *hla* repressor, SarT (Schmidt *et al.*, 2001).

1.3.3 Alternative sigma factors

In bacteria, transcription initiation is the most important step for the regulation of gene expression. The single RNA polymerase core enzyme requires an additional subunit designated as the 'sigma factor' to recognize promoter sequences and to initiate transcription (Wösten, 1998). Most bacteria synthesize several sigma factors, each of which recognizes different consensus promoter sequences. This heterogeneity of the sigma factor is the primal transcriptional regulatory mechanism, i.e. a differential usage of distinct sigma factors allows bacteria to express different sets of genes, the regulation of which can be conditional or constitutive. The sigma factors are grouped into two families, the σ^{70} and the σ^{54} families. These two families share little sequence identity. The majority of the sigma factors belong to σ^{70} family and are divided into twelve subfamilies. Unlike *E. coli* and *B. subtilis*, which have 6 and 17 σ^{70}-family sigma factors, respectively, only two σ^{70}-family sigma factors have been identified in *S. aureus*. The primary sigma factor in all bacteria is σ^{A} (Deora and Misra 1996) while σ^{B} is a homologue of σ^{B} of *B. subtilis* (Wu *et al.* 1996; Kullik and Giachino 1997).

1.3.3.1 Sigma factor B

Expression of σ^B is activated by environmental stress and energy depletion (reduced ATP/ADP ratio) as well as by environmental stimuli such as ethanol (Chan and Foster, 1998a) and salicylic acid (Bayer *et al.*, 2000). Its activity is regulated by a complex post-translational pathway consisting of *rsbU*, *rsbV* and *rsbW* (Scott *et al.*, 1999). σ^B is usually bound by RsbW, an antisigma factor that phosphorylates RsbV, an anti-antisigma factor. Under conditions of environmental stress, RsbV~P is dephosphorylated by either of two phosphatases, RsbU or RsbP, and then binds RsbW, releasing and activating σ^B. σ^B recognizes a unique promoter ($GTTT(N_{14-17})GGGTAT$), which has been identified in 23 different *S. aureus* genes (Gertz *et al.*, 2000), including one of the three *sarA* promoters and one of the three *sarS* promoters, as well as genes encoding transport functions and others involved in generating $NADH_2$.

In *S. aureus*, σ^B feeds into the global regulatory network governing the expression of accessory genes. It acts mainly through other regulatory genes and transcription factors. However σ^B can also act directly on genes that have σ^B-dependent promoters. Thus, it has reciprocal activities, upregulating some exoprotein genes at a very early stage of growth, such as *coa* and *fnbB* (Nicholas *et al.*, 1999), and downregulating others at the end of the exponential phase *in vitro*. As many of the latter are involved in virulence, σ^B seems to be antagonistic to *agr*. A mutation of *sigB* had little or no effect on the virulence of one clinical strain (Nicholas *et al.*, 1999) although it greatly decreased the biofilm-forming ability of another (Rachid *et al.*, 2000).

S. aureus strain 8325 and related strains do not produce the characteristic *S. aureus* golden pigment, a σ^B-dependent function, owing to a natural deletion in *rsbU*. However, these strains are not totally deficient in σ^B function, as pigment synthesis can be induced by subinhibitory clindamycin. It is suggested that this could involve σ^B activation via RsbP. A small fraction of *S. aureus* clinical isolates are non-pigmented and overproduce various exoproteins. These are likely to be σ^B defective. This supports the idea that σ^B may not be required for pathogenesis. Derivatives of 8325 strains in which *rsbU* has been repaired (Bischoff *et al.*, 2001; Horsburgh *et al.*, 2002) show important differences including a reduction in the lag phase of growth, an increase in overall growth yield and in starvation survival (Horsburgh *et al.*, 2002), as well as in the expression of both regulatory and exoprotein genes. In summary, σ^B is

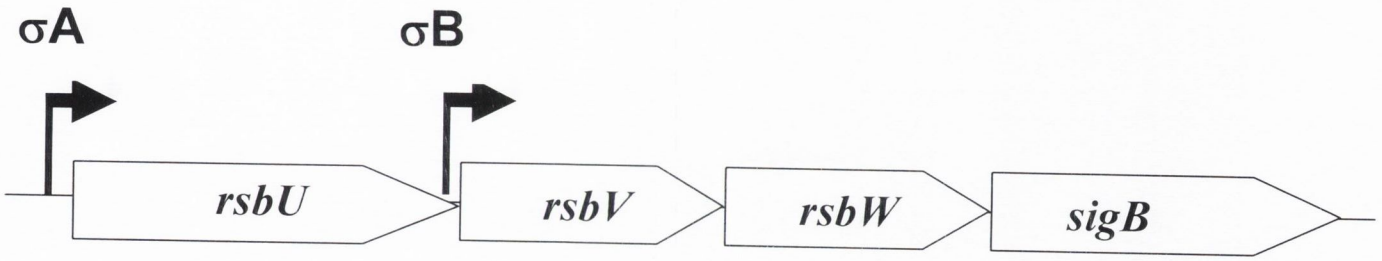


Figure 1.10 The *S. aureus sigB* operon. The positions of the σ^A and σ^B promoters are indicated.

involved in the signaling pathways that regulate virulence and other accessory gene functions in staphylococci. However, its precise role in the overall regulatory network remains to be determined.

1.4 Treatment of staphylococcal infections

In order to treat staphylococcal infections successfully intracellular targets vital to the survival of the bacterium *in vivo* must be identified. Antimicrobial chemotherapy targets bacterial metabolism and biosynthesis leading to either lysis or suspension of bacterial growth which aids the host immune system to clear infections. Vaccination primes the immune system to specifically recognize surface components or secreted toxins of bacterial pathogens so that infections can be quickly and efficiently cleared. Passive immunization aids in the clearance of bacteria specifically recognised by the antibodies, by neutrophil opsonophagocytosis. However virulence factors and drug targets have to be first identified and a number of methods have been utilized to this end. The elucidation of these gene products should provide new targets for antimicrobial drug development. Most if not all chemical classes used in antimicrobial chemotherapy were identified by empirical screening methods. First for example, by testing the essentiality of the gene for survival *in vitro* (or *in vivo*), high throughput screening of chemical libraries and structure based drug design methods that identify and improve inhibitors. Several novel techniques have been devised to identify bacterial proteins that are potential vaccine candidates or drug targets. The application of some of these to staphylococci is revised below.

1.4.1 Identification of genes that are expressed or that are required for *in vivo* growth

Several methods have been employed to identify genes that are essential for the survival of staphylococci *in vivo*. The technologies discussed below have confirmed the contribution of genes of known function to survival within the host by assessing their

essentiality of genes of unknown function prompted investigation into their function and role as potential antimicrobial targets.

In vivo expression technology (IVET) identifies bacterial genes that are induced when a pathogen infects its host. Among induced genes are those that encode virulence factors and products specifically required for the infection process. The system is based on complementation of an attenuating auxotrophic mutation by gene fusion. In *Salmonella typhimurium*, the system was successfully used to identify a number of genes that were induced in a mouse infection model, and when subsequently mutated, conferred a virulence defect. The IVET system has several applications in the area of vaccine and antimicrobial drug development. The IVET system in *S. aureus* facilitated the isolation of mutations in genes involved in virulence and identified biosynthetic, catabolic, and regulatory genes that are required for growth of microbes in animal tissues (Lowe *et al.*, 1998). IVET does not determine whether a gene is required for *in vivo* growth.

Signature tagged mutagenesis (STM) allows a large number of insertion mutants to be screened for loss of virulence in a single animal (Hensel *et al.*, 1995). STM allowed the identification of genes such as ClpX and ClpP in stress resistance, whose mutation by insertion with Tn*917* led to a virulence-attenuated phenotype in a mouse model of infection (Mei *et al.*, 1997; Frees *et al.*, 2003). Differential fluorescence induction analysis allowed the identification of promoters that were preferentially induced under infection simulating growth conditions (Schneider *et al.*, 2002). Genes were identified that represented possible *in vivo* targets for both antimicrobial therapy and vaccination. It was encouraging to note that genes encoding known virulence factors such as *clfA* and the beta hemolysin gene were identified by this method. Ji and colleagues (2001) successfully employed an inducible antisense RNA strategy in order to identify essential genes. Several of the genes that were identified in each of these studies were unknown or had poorly defined biological function indicating a potential for further investigation of novel targets.

1.4.2 Screening convalescent patients' sera

The screening of convalescent patients' sera for antibodies against staphylococcal proteins has proved a valuable tool for assessing whether a particular gene is expressed *in vivo* during the infectious process. Several studies have utilized the screening of recombinant

proteins (Roche *et al.*, 2003, McCrea *et al.*, 2000), staphylococcal protein fragment expression libraries (Etz *et al.*, 2002, Weichhart *et al.*, 2003) or 2D-PAGE analysis of staphylococcal lysates (Vytvytska *et al.*, 2002) in order to identify staphylococcal proteins expressed during infections. The ClfA protein and the SdrH-like protein of *S. aureus* have been identified by these technologies. Comparative analysis of convalescent patients' sera and healthy donor sera can also determine which of the proteins are preferentially expressed during an infection. Those that are secreted or surface expressed are potential virulence factors and vaccine candidates. These comparative screens have identified a number of gene products that are preferentially expressed during infections e.g. SdrD, IsaA, protein A, lipase, FnbpA and FnbpB (Etz *et al.*, 2002). The function of several of these proteins is known. However several genes of unknown function have been identified and further investigation is warranted into their function and possible roles as potential vaccine candidates. To date, antibodies against one protein of unknown function, SA1781, have been shown to mediate opsonophagcytosis of *S. aureus* by macrophages (Etz *et al.*, 2002). Screening for *in vivo*-expressed antigens remains a valid method for the identification of potential vaccine candidates and supports the case for the use of antigens of unknown function in protection studies using animal models.

1.4.3 Microarray transcription analysis

Using a whole genome microarray has provided investigators with a means of taking a snapshot view of the effects of different growth conditions and regulatory mutations on the transcriptional profile of the entire staphylococcal genome. Microarray analysis has been used to investigate the effects of the antibiotics vancomycin, erythromycin and ramoplanin on the transcriptome (Mongodin *et al.*, 2003, Volokhov *et al.*, 2003, Fan *et al.*, 2002) and to identify the specific effects of mutants of the two-component signal transduction system Agr and the global regulators SarA and Rot (Kuroda *et al.*, 2003; Dunman *et al.*, 2001; Said-Salim *et al.*, 2003). Transcriptional profiling is important in identifying the coordinated expression of networks of genes and identifying regulatory cascades by accurately quantifying genes that are over- or under- expressed. It can also help evaluate the contribution of regulatory elements and growth conditions on the expression of genes of unknown function whose impact on the pathogenesis of staphylococci has yet to be determined. For example, the expression of *sdrH* has been shown by

microarray analysis to be significantly upregulated when *S. epidermidis* is treated with ramoplanin (Fan *et al.*, 2002).

Microarray technology has also been used to identify the genes in *ex vivo* human leukocytes that are upregulated in response to stimulation with *S. aureus* (Feezor *et al.*, 2003). Microarray transcriptional analysis of genes expressed *in vivo* can provide a more complete picture of the host-pathogen interplay and aid in evaluation of *in vivo*-expressed genes as antimicrobial and vaccine targets and characterization of the host response to disease. It is difficult to obtain sufficient bacteria from a diseased host to adequately analyse the *in vivo* transcriptome but growth of bacteria in conditions that simulate the hostile host environment will provide an alternative.

1.4.4 Vaccines

The resistance of staphylococci to methicillin and other antibiotics, and emerging resistance to vancomycin have led to a search for alternative strategies to effectively combat infections. Active immunization by vaccination with bacterial surface components and therapeutic treatment by passive transfer of immunoglobulin have yielded promising results. Several of these approaches have reached clinical trial status (Shinefield *et al.*, 2002; Vernachio *et al.*, 2003) and may prove to be powerful tools in the fight against MRSA.

Several criteria are important in choosing an effective vaccine candidate antigen. These include (I) distribution among a high percentage of strains, (II) evidence that the antigen is expressed *in vivo* during infections, (III) that immunization with the candidate antigen provides a high titer of specific opsonic antibodies that protect against staphylococcal infection. The work carried out by InterCell in Austria clearly demonstrates that comparative screening of convalescent and normal patient sera for antibodies against staphylococcal antigens provides a means to identify immunogenic proteins of known and unknown function that could act as potential vaccine candidate antigens (Etz *et al.*, 2002, Vytvytska *et al.*, 2002, Weichhart *et al.* 2003). This method also highlights immunogenic antigens that are recognised as non-self. This helps to avoid choosing antigens as vaccine targets that could lead to autoimmunity.

1.4.4.1 Active immunization

A live-attenuated vaccine is probably not a viable option for prevention of staphylococcal infection. Vaccination depends upon successful stimulation of immunological memory and as discussed in section 1.3 *S. aureus* can express factors involved in immune evasion and immunomodulation that inhibit the induction of immunological memory. Many factors contribute to disease and pathogenesis. Clearance of staphylococci from a diseased host relies mainly on opsonophagocytosis of bacteria by polymorphonuclear leukocytes. A more reliable method would be to develop a subunit vaccine comprised of vaccine candidate antigen(s) that elicit a high titer of specific opsonic antibodies that mediate clearance of bacteria from the host.

1.4.4.1.1 Bacterial extracts

Due to the low immunogenicity of capsular polysaccharides and the lack of in-depth knowledge of which staphylococcal antigens mediate immunity against staphylococcal disease in cattle, vaccines for bovine mastitis are composed of whole bacterial cell lysates and bacterial supernatants (O'Brien *et al.*, 2001). To date, one bovine vaccine, MASTIVAC, has been brought to veterinary challenge and field trials and has had success at protecting cattle against challenge *S. aureus* (Leitner *et al.*, 2003b, c). The MASTIVAC preparation also elicited a broad spectrum of antibodies that protected against *S. aureus* challenge in mouse models of infection (Leitner *et al.*, 2003a). The regulations governing human vaccines are far more stringent and require exact specifications of the composition of the vaccine administered something that cannot be defined with bacterial lysates.

1.4.4.1.2 Capsular polysaccharide as a subunit vaccine

Capsular polysaccharides type 5 and type 8 occur on 85% of strains that infect humans. Antibodies to capsular polysaccharides can protect against *S. aureus* challenge (Fattom *et al.*, 1996; Fattom *et al.*, 1998) but polysaccharides are poorly immunogenic and vaccination with the polysaccharides does not yield long lasting immunity. The capsular polysaccharides type 5 and type 8 were conjugated to *Pseudomonas aeruginosa* exotoxin A toxoid which improved antigenicity. The conjugate vaccine has been developed as an anti-staphylococcal vaccine

STAPHVAX. Concerns exist that a vaccine that depends upon capsular serotypes specificity will contribute to serotype redistribution. Immunization with capsular polysaccharide generates opsonic antibodies against both backbone and O-acetyl side chain sugar moieties that would potentially extend the efficacy of the vaccine beyond specified serotypes (Fattom *et al*, 1998). Clinical trials of STAPHVAX have been successful in generating specific antibodies to capsular polysaccharides in ~75% of patients tested (Shinefield *et al.*, 2002) however no significant difference in the incidence of infection between the vaccinated and non-vaccinated groups was observed. Therefore the clinical trial is being repeated and a second generation of vaccine is being developed that also include capsular polysaccharide from strain 336 which elaborates a capsule serotype representing a further 10% of strains that infect humans, resulting in 95% coverage of clinically observed *S. aureus* strains. A significant improvement to the vaccine would involve conjugation of all three capsular polysaccharide types to a staphylococcal toxoid thus protecting against infection and potentially neutralizing a toxin that would be secreted by a majority of *S. aureus* strains that infect humans.

1.4.4.1.3 Surface proteins and toxoids as vaccines

Adherence of staphylococci to the extracellular matrix is vital to the initiation of *S. aureus* infections. Consequently, proteins which play a role in adherence to host proteins provide a target for antibodies. Surface proteins are also important in preventing opsonophagocytosis and in metastatic spread of infection by mediating tissue specificity. Antibodies against MSCRAMM proteins exhibit at least two biological properties. Antibodies prevent microbial adherence (McDevitt *et al.*, 1995; Mohamed *et al.*, 1999; Rennermalm *et al.*, 2001), as well as recolonization of host tissues or biomaterials. Also, the increased level of anti-MSCRAMM antibodies bound to the bacterial surface facilitates rapid clearance of the organism by promoting opsonophagocytosis (Rozalska *et al.*, 1993; Nilsson *et al.*, 1998). Several surface proteins have been investigated as potential vaccine candidate antigens. The collagen binding protein provided protection against *S. aureus* strains expressing the *cna* gene in the septic arthritis model (Nilsson *et al.*, 1998). Unfortunately, as *cna* is present in only 40-60% of *S. aureus* strains it does not represent a viable

FnBPA in complex with fibronectin, creating ligand induced binding site (LIBS) epitopes (Casolini *et al.*, 1998). Therefore the antibodies never block fibronectin binding greater than 50%. The A domain of FnBPA may represent a better vaccine target.

The *clfA* gene is present in 100% of invasive isolates and 98% of carriage isolates of *S. aureus* (Peacock *et al.*, 2002). ClfA is known to be expressed during infection (Etz *et al.*, 2002; Weichhart *et al.*, 2003). Isogenic *clfA* mutants are attenuated in several different animal models of *S. aureus* infection (Moreillon *et al.*, 1995; Que *et al.*, 2000; Stutzman *et al.*, 2001). Accordingly, it represents a good vaccine candidate antigen. Immunization with ClfA can protect against lethal intravenous challenge with *S. aureus* in mice (Brouillette *et al.*, 2002) and that the severity of disease was markedly reduced in a mouse model of septic arthritis (Josefsson *et al.*, 2001).

Toxins also represent potential vaccine candidate antigens. They are expressed by a wide variety of strains and contribute to the severity of many staphylococcal diseases. However, the toxins cannot be injected into the host without modification as they would damage the host either by their superantigenicity (enterotoxins and TSST-1) or cytotoxicity (alpha toxin). Strategies to circumvent these problems include the generation of recombinant mutant forms of the toxins that retain immunogenic epitopes whilst abrogating their toxicity or superantigenicity (Bavart *et al.*, 1996; Ulrich *et al*, 1995). The α-toxin toxoid protected against lethal challenge with *S. aureus* (Nilsson *et al.*, 1999; Ulrich *et al.*, 1998). However toxoids will only ever be used as components of a multivalent vaccine. Alone they cannot prevent infection, only neutralize some of the more potent toxic effects.

1.4.4.1.4 DNA vaccines

DNA vaccines consist of non-replicating plasmid vectors derived from bacteria that contain heterologous genes inserted under the control of a eukaryotic promoter allowing expression of the protein in mammalian cells (Davis, 1997). Vaccination involves the intra-muscular injection of the bacterial plasmid encoding the antigen. To date two anti-staphylococcal DNA vaccination strategies have been used. The first approach involved immunization with a plasmid that contained the *clfA* gene under the control of the CMV promoter (Brouillette *et al.*, 2002). Mice immunized in this way generated a strong and specific antibody response to ClfA. Preincubation of *S. aureus* cells with these antibodies inhibited

adherence to fibrinogen up to 92%. However this form of vaccination did not protect mice against intraperitoneal challenge with *S. aureus*. The second approach involved vaccination with a plasmid bearing the *mecA* gene which encodes the PBP2a protein responsible for methicillin resistance in MRSA (Senna *et al.*, 2003). This approach appeared to provide protection against a sub-lethal dose of MRSA injected intraperitoneally. Therefore DNA vaccination is a viable approach towards protecting against *S. aureus* infections. However improvements to the immunizing vector such as tissue specific promoters and immunizing in conjunction with recombinant antigen may help direct a more protective immune response.

1.4.4.2 Passive immunization

Passive immunization is the administration of exogenously produced polyclonal or monoclonal antibodies in order to prevent infections in exposed individuals, to inactivate bacterial toxins or to remedy hypogammaglobulinaemia in immunocompromised patients. One rationale for the use of intravenous immunoglobulin (IVIG) is to replace a broad spectrum of missing antibodies in an immunocompromised host (Sacher, 2001) thereby providing protection against common pathogens. IVIG has the potential to be used prophylactically post-exposure. The successful use of polyclonal antibodies to prevent viral infections has been demonstrated (Sawyer, 2000). Specific hyperimmune immune globulins against hepatitis B (Perillo, 2000) or cytomegalovirus (Snydman, 2001) for the prevention of infection in high-risk or exposed patients have been used effectively for a number of years.

During bacterial infections the administered antibodies can directly affect bacterial adherence, neutralize toxins and superantigens, facilitate opsonisation and complement activation, and modulate cytokine production. Several strategies have been undertaken to combat staphylococcal infections by passive immunoprophylaxis, each targeting different cellular components. Higuchi *et al.* (1994) used monthly injections of IVIG to treat patients predisposed to recurrent episodes of toxic shock. Fischer et al. (1994) showed the potential benefit of IVIG in a neonatal animal model of *S. epidermidis* infection. Clinical trials are currently in progress to evaluate the safety and efficacy of donor-selected IVIG in the prevention of staphylococcal infections in very low birth-weight neonates (Bloom *et al.*, 2003).

The *clfA* gene is present in 100% of invasive strains tested and is expressed during infection (Peacock *et al.*, 2002, Etz *et al.*, 2002, Weichhart *et al.*, 2003). Therefore ClfA

represents a viable target for passive immunoprophylaxis. Joseffson *et al.* showed that both human and rat antibody preparations containing high titers of anti-ClfA-specific IgG could be used prophylactically to decrease mortality in a murine model of *S. aureus* sepsis (2001). Inhibitex Inc. have developed a hyperimmunoglobulin, SA-IGIV, derived from plasma donors with high titers of anti-ClfA IgG, for the treatment of staphylococcal infections in very low birthweight infants (Vernachio *et al.*, 2003). This IgG preparation recognised the *S. aureus* cell surface, specifically inhibited *S. aureus* adherence to immobilised fibrinogen, acted as an efficient opsonin in an *in vitro* phagocytosis assay and finally, when used in conjunction with vancomycin was therapeutically effective in a rabbit model of catheter-induced infective endocarditis caused by MRSA. A phase II clinical trial of SA-IVIG is currently underway to evaluate its safety and efficacy.

In addition to the SA-IVIG product, Inhibitex have developed a monoclonal antibody product called Aurexis. This monoclonal antibody was raised against residues 200-559 of the ligand binding A domain of ClfA. It inhibits ClfA-mediated adherence of bacteria to fibrinogen. Consequently, when this antibody was tested in *S. aureus* animal models of infection by passive transfer it efficiently protected against intravenous challenge (Hall *et al.*, 2003) and significantly enhanced the therapeutic efficacy of vancomycin in a model of already-established infectious endocarditis (Domanski *et al.*, submitted).

The neutralization of staphylococcal toxins plays a role in the treatment of infections. Several studies have addressed this issue by using anti-toxin antibodies to passively treat animal models of infection (Gauduchon *et al.*, 2004). Passive transfer of immunoglobulin Y antibody generated in chickens against whole enterotoxin B toxin suppressed cytokine responses and was protective in mice. Furthermore rhesus monkeys treated with the IgY specific for SEB up to 4 h after challenge survived lethal SEB aerosol exposure (LeClaire *et al.*, 2002). Antibodies against non-toxic recombinant mutant toxins that retain their antigenicity have also been shown to confer protection. Hu *et al.* (2003) showed that passive transfer of antibodies specific for a non-toxic mutant TSST-1 toxin provided protection against *S. aureus* induced septic death in a mouse model of infection. Protection was also observed in mouse models of infection by antibodies generated against a non-toxic mutant alpha toxin and an enterotoxin A molecule devoid of superantigenicity (Menzies *et al.*, 1996 Nilsson *et al.*, 1999).

McKenney *at al.* (1999) used rabbit antibodies generated against poly-1-6 β-D-N-succinylglucosamine, the product of the *ica* locus in *S. aureus*, to protect mice against lethal

intravenous challenge with *S. aureus*. This polysaccharide adhesin is serologically distinct from the capsular polysaccharide type 5 or 8. In contrast, antibodies generated against type 5 capsule conjugated to Pseudomonas exotoxin A toxoid provided protection against endocarditis, bacteraemia and renal abscess formation in rats challenged intraperitoneally with *S. aureus* (Lee, 1996). Previous studies showed that passive transfer of antibodies against type 5 capsular polysaccharide alone did not provide sufficient protection against staphylococcal disease (Nemeth and Lee, 1996; Fattom *et al.*, 1996). Therefore pseudomonas exotoxin A was used as a conjugate adjuvant. Treatment with a mAb BSYX-A110, generated against lipoteichoic acid of staphylococci was shown to improve the outcome of neonatal rats intraperitoneally injected with a lethal dose of *S. epidermidis* (Weisman *et al.*, 2001). A clinical trial of BSYX-A110 is currently underway to evaluate its safety and efficacy (Weisman *et al.*, 2003).

Flooding an infected host with highly specific antibodies aids in efficient opsonophagocytosis (Domanski *et al.*, 2004) and aids in complement deposition and the development of protective immunity. Antibodies have also been observed to penetrate biofilms (Maira-Litran *et al.*, 2002) and can detach bound cells from their substrate (Visai *et al.*, 2000) resulting in the formation of planktonic rather than sessile, biofilm bound bacteria which would then be rendered more susceptible to both antibiotics and the host immune system. Therefore, the evidence presented above clearly reinforces the rationale for using protective antibody preparations in both prophylactic and therapeutic applications with regard to existing staphylococcal infection and individuals at risk of developing *S. aureus* infections.

1.5 Aims and rationale of this study

The initial aim of this study was to characterize the function of the *fbl* gene and its gene product Fbl. The prevalence of the *fbl* gene in clinically relevant strains of *Staphylococcus lugdunensis* was analysed by Southern hybridisation and PCR amplification of the gene from genomic DNA. Once the presence of the *fbl* gene was verified the ability of Fbl expression to confer a fibrinogen binding phenotype to *S. lugdunensis* was verified by analyzing the binding of whole cells to immobilised and soluble fibrinogen. The genetics of *S. lugdunensis* are not well defined and it was not possible to make a *fbl* knockout mutant. However the expression of Fbl on the surface of a bacterium was analysed in isolation by expressing the *fbl* gene from the heterologous expression system *L. lactis* MG1363 pKS80. This allowed examination of the

function of Fbl in comparison to that of its closely related homologue, the clumping factor of *S. aureus* (ClfA) in order to discover whether it exhibited similar fibrinogen binding characteristics, the ability to activate platelet aggregation and the effects of divalent cation on its ligand binding activity.

Analysis of the Fbl protein was furthered by expression and purification of the ligand binding A domain of Fbl from a *L. lactis* expression system. This recombinant molecule, $rFbl_{40-534}$, was used in order to raise antibodies against Fbl and to accurately quantify the kinetics of the interaction of $rFbl_{40-534}$ with fibrinogen. Fbl antibodies allowed analysis of the topology of Fbl in both *S. lugdunensis* and *L. lactis* by digestion of cell walls with murolytic enzymes and analysis of cell wall-associated and protoplast associated proteins by SDS-PAGE and Western immunoblotting and by whole cell dot immunoblotting.

The ability of both anti-Fbl and anti-ClfA antibodies to both inhibit the binding of Fbl- and ClfA-expressing bacteria to fibrinogen and to displace bound bacteria from a fibrinogen substrate was then compared and contrasted. Rabbit polyclonal antibodies raised against either Fbl or ClfA, human antibodies enriched for reactivity against ClfA and murine monoclonal antibodies raised against $rClfA_{40-559}$ were all analysed in this study. As a result of this study it was decided to attempt to identify the epitopes to which a selection of the murine monoclonal antibodies bound. In order to find the residues responsible for forming the non-continuous conformational epitope residues 40-559 of the A domain of ClfA from several strains of *S. aureus* were cloned and expressed as recombinant molecules. The ability of mAb 12-9 to bind to these recombinant molecules was analysed and when this revealed that naturally variant ClfA molecules had identical binding profiles, a structure-based strategy of site directed mutagenesis was employed. Several site-directed mutants of $rClfA_{40-559}$ were analysed for their ability to bind to the murine monoclonal antibodies and used to try to define an epitope to which mAb 12-9 binds.

Finally the SdrH protein of *Staphylococcus epidermidis* was analysed in order to reveal its role as a possible virulence factor. The *sdrH* gene was cloned into the surrogate expression host *L. lactis* MG1363 and its expression in *L. lactis* and its natural host *S. epidermidis*, was analysed by digestion of cell walls with murolytic enzymes and analysis of cell wall-associated and protoplast associated proteins by SDS-PAGE and Western immunoblotting and by whole cell dot immunoblotting. *L. lactis* $SdrH^+$ was also used as a tool for identification of a putative ligand or function for SdrH. Blast analysis of the amino acid sequence of SdrH revealed a distant homologue

distant homologue in *Ruminococcus flavifasciens*, *xyn*A, a gene encoding a xylanase. Therefore it was decided to test the effects of growth of *S. epidermidis* in different sugars on the expression of SdrH.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

Escherichia coli, *Staphylococcus aureus*, *Staphylococcus lugdunensis*, *Staphylococcus epidermidis* and *Lactococcus lactis* strains are listed in Table 2.1. *E. coli* was routinely grown on Luria (L) agar or broth at 37°C. *S. aureus* was grown on Trypticase Soy agar (TSA, Oxoid) or broth (TSB) at 37°C unless otherwise stated. For expression studies and adherence assays *S. aureus* strains were grown in 50ml volumes in 250ml flasks in an orbital shaker at 200rpm at 37°C. *S. epidermidis* and *S. lugdunensis* strains were grown on TSA or TSB at 37°C. For expression studies and adherence assays *S. epidermidis* cultures were grown in 50ml volumes in 250ml flasks either in an orbital shaker at 200rpm or statically at 37°C. *L. lactis* cultures were grown statically on M17 (Difco) agar or broth incorporating 0.5% glucose at 30°C. Stationary phase cultures were grown for approximately 16 hours. Exponential phase cultures were inoculated 1:100 from overnight starter cultures. Cells were washed in phosphate buffered saline 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 medium where appropriate: ampicillin (Amp), 100 μg/ml; erythromycin (Em), 10 μg/ml or 5 μg/ml; tetracycline (Tet), 2 μg/ml; chloramphenicol (Cm), 10 μg/ml; Kanamycin (Kan), 100 μg/ml. Antibiotics were purchased from Sigma Chemical Co.

2.2 Plasmids

All plasmids and derivatives are listed in Table 2.2.

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. lugdunensis, 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 sphaeroplast buffer used to resuspend the bacterial pellet. The mixture was incubated at 37°C for 40 min until bacterial lysis had occurred. The remainder of the procedure was carried out according the manufacturer's instructions. Plasmid DNA was isolated from *E. coli* strains using the Promega Wizard SV Plus Minipreps DNA purifications system, as recommended by the manufacturer. The same kit was used to isolate DNA from *S. aureus* and *L. lactis*, with the following modifications. 200μg 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 mins prior to proceeding with the DNA isolation.

DNA hybridization was carried out according to the method of Southern (1975). Approximately 5 μg of genomic DNA was digested for 16 hours with 20U enzyme in a total volume of 70 μl. Digested DNA fragments were separated for 16hrs 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, 10min) denatured (1.5 M NaCl, 0.5 M NaOH, 45 min) and neutralized (1.5 M NaCl, 0.15 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 prehybridized in a standard prehybridization solution (5 x SSC, 0.1% N-laurylsarcosine, 0.02% SDS, 1 x Blocking reagent (Roche)) for 2 hours at 68°C and then hybridized for 16 hours with the probe (0.5 μg DIG-labeled PCR product/ml prehybridization solution) at 68°C. Following hybridisation, the membrane was washed twice at room temperature with 2 x SSC, 0.2% 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, pH 7.5, Tween 20 (0.3% v/v). Blocking was performed for 1 hour in

1 x blocking reagent (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 100mM Tris, pH 9.5, 100mM NaCl and then incubated with the chemiluminescent substrate Disodium 3-(4-metho xyspiro {1,2-dioxetane-3,2-(5-chloro)tricyclo [3.3.1.1^{3},7]decan}-4-yl)phenyl phosphate (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 was performed at Lark Technologies

2.5 Polymerase chain reaction (PCR)

All primers are listed in Table 2.3. Where possible, plasmid DNA (10 ng) was used as a template, otherwise 100ng of chromosomal DNA was used. Reaction mixtures (100 µl) contained 100pM each of the relevant forward and reverse primers, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, template DNA and 1.25 U *Taq* DNA polymerase in a standard *Taq* reaction buffer (Promega). Amplification was performed in a Hybaid Omnigene thermocycler with denaturation for 1 min at 94°C, annealing for 1 min at a temperature corresponding to the melting temperatures of the relevant primers and extension at 72°C for a length of time dependent on the size of the fragment to be amplified (1 min/kb). This cycle was repeated 30 times followed by a final extension of 10 min. DIG-labeled probes were constructed by PCR as described above using 10 µl DIG-dUTPs (Roche) in place of dNTPs and plasmid DNA or a gel purified PCR-product as the template.

DNA fragments to be used for cloning were amplified using the high fidelity polymerase *Pfu* (Promega), according to the manufacturer's instructions. Reaction mixtures (100 µl) contained 100pM forward and reverse primers, 2.5 mM dNTPs, 1.5 mM $MgSO_4$, template DNA and 5 U of *Pfu* polymerase in a standard Promega *pfu* reaction buffer. Amplification was carried out with an initial denaturation step at 94°C for 1 min before addition of the enzyme. This was followed by 30 cycles of denaturation

for 30 sec, annealing for 1 min at a temperature dependent on the primers and elongation at 72°C for 2 min/kb product. This was followed by incubation at 72°C for 10 min. In all cases, PCR products were purified either directly or from an agarose gel using the High Pure™ PCR purification kit (Roche).

2.6 Transduction, transformation and conjugation

Plasmids were transformed into *E. coli* strain XL-1 Blue or TOPP3 made competent by $CaCl_2$ treatment (Sambrook *et al.*, 1989) or electroporated into *S. aureus* strain RN4220 (Augustin and Gotz, 1990; Foster, 1998) or *L. lactis* MG1363 (Wells *et al.*, 1993). *E. coli* transformants were screened for the presence of chimeric plasmids using the rapid colony screening procedure developed by Le Gouill and Dery (1991). *S. aureus* and *L. lactis* transformants were screened by a colony PCR screening procedure. Briefly, the colony to be screened was emulsified in 25 µl lysis buffer (50mM NaOH, 0.25% SDS) and boiled for 15 mins. 200 µl of nuclease free H_20 was added to this and 5 µl of the final solution was used as a template in a PCR reaction.

Plasmid pROJ6448 and derivatives were transferred from *S. aureus* RN4220 carrying pGO1 and pC221 to *S. epidermidis* 9142 by conjugational mobilization. Stationary-phase cultures of *S. aureus* RN4220 (pGO1, pC221, pROJ6448) and *S. epidermidis* 9142 grown in BHI broth were lawned on the surface of BHI agar and grown for 16 h at 30 °C. The lawns were emulsified in PBS and resuspended at an OD_{600} of 1·0. Portions of 1 ml of each were mixed, centrifuged and resuspended in 100 µl PBS and pipetted onto a 2 cm^2 Protran nitrocellulose membrane (Schleicher & Schuell) on the surface of a PY (Peptone-Yeast extract) agar plate and incubated for 48 h at 30 °C. Cells were harvested in PBS and spread onto PY agar containing 5 µg norfloxacin ml^{-1} and 10 µg erythromycin ml^{-1} and incubated for 3–4 d at 30 °C. Transconjugants were screened for those that were sensitive to 50 µg kanamycin ml^{-1}.

2.7 Cloning and expression of Fbl and SdrH in *L. lactis*

The *fbl* genes of *S. lugdunensis* strain 1003 and 1004 and the *sdrH* gene from *S. epidermidis* strain 9491 were amplified using Pfu polymerase from the putative translational initiation codon to the TAA stop codon and cloned into the *L. lactis* expression vector pKS80 as previously described (Wells *et al.* 1993). The vector provides a lactococcal promoter, ribosome binding sites and a translational coupling mechanism to ensure efficient initiation of translation of the cloned gene. The primers used are illustrated in Table. 2.3. Expression of the target gene is achieved by cloning into the *Bcl*I site TGATCA. Translation is optimized by fusing the target open reading frame ATG codon to the ATG codon of the expression cassette. This overlaps the stop codon TGA of the upstream open reading frame.

2.8 Isolation of cell wall associated proteins from *S. lugdunensis, S. epidermidis* and *S. aureus*

Cell wall associated proteins were isolated from *S. aureus*, *S. lugdunensis* and *S. epidermidis* strains by the following method. Cells were grown to the required growth phase and then harvested by centrifugation. Cells were washed twice in PBS and adjusted to an OD_{600nm} of 40 in PBS. Cells were centrifuged and resuspended in 1 ml 30% (w/v) raffinose (Sigma), in 20mM Tris (pH 8.0), 10 mM $MgCl_2$. The following components were then added; 400 µg recombinant lysostaphin, 75 µl Complete® mini protease inhibitor cocktail (Roche) and the reactions incubated in a 37°C water bath for 10 minutes. Protoplasts were formed and cell wall-associated proteins were released into the supernatant. Finally samples were centrifuged at 6,000 rpm for 10 min to pellet the protoplasts. The supernatant containing solublised cell wall-associated proteins, was removed for further analysis.

2.9 Isolation of cell wall proteins from *L. lactis*

Heterologously expressed proteins were isolated from the cell wall of *L. lactis* in a manner similar to that described for staphylococci in section 2.8, with the following modifications. Cells were grown for 16 hours in 50ml volumes of M17 broth containing

0.5% glucose and 5 μg/ml Erm at 30°C statically. Cells were harvested by centrifugation and washed twice with PBS and adjusted to an OD_{600nm} of 40 in 1 ml 30% 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, 5mM EDTA, 500U mutanolysin (Sigma) and 200μg lysozyme (Sigma). The remainder of the procedure was carried out as described in section 2.8.

2.10 SDS-PAGE and Western immunoblotting

Protein samples were analysed by SDS-PAGE and Western immunoblotting. Samples were boiled for 10 min in 2x final sample buffer (10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 3% (w/v) bromophenol blue in 62.5 mM Tris-HCl, pH 6.8), and 20 μl aliquots were separated by SDS-PAGE (Laemmli, 1970), using 4.5% stacking and either 7.5%, 10% or 12.5% separating acrylamide gels, where appropriate. Prestained molecular weight markers from New England Biolabs were separated alongside the protein samples which included protein bands in the range of 175 kDa, 83 kDa, 62 kDa, 47.5 kDa, 32.5 kDa, 25 kDa, 16.5 kDa and 6.5 kDa. Use of these markers allowed accurate determination of the size of analysed proteins. Following separation, proteins were either stained with Coomassie blue or transferred for 1 hour at 18 V using a Semi-Dry transfer cell (BioRad) onto PVDF membrane (Roche). After protein transfer membranes were incubated overnight at 4°C in 10% Marvel (w/v) in TS buffer to block any non-specific interactions. Primary antibodies were then suitably diluted (usually 1:1000) in TS buffer containing 10% Marvel, and incubated with the membrane for 1.5 hours. Unbound antibody was removed by washing the membrane three times for 10 min in TS buffer. Protein A coupled to horseradish peroxidase (Sigma), diluted 1:500 in TS buffer containing 10% Marvel or goat anti-rabbit IgG coupled to horseradish peroxidase (HRP) (DAPI), diluted 1:1000 into 10% Marvel TS buffer, was used to detect bound primary antibody by incubating with the membrane for 1 hour. Excess protein A coupled to horseradish peroxidase or goat anti-rabbit IgG coupled to horseradish peroxidase was removed by washing three times for 10 min in TS buffer. The blot was then visualized

by developing in the dark using the chemiluminescent substrate "Lumiglo" (New England Biolabs), and finally exposed to X-Omat autoradiographic film (Kodak).

2.11 Whole cell dot immunoblotting

Bacterial cells to be tested were grown to stationary phase in TSB with aeration. Cells were harvested by centrifugation, washed in PBS, and resuspended at an $A_{600\ nm}$ = 2. Ten microliters from this suspension were dotted onto a nitrocellulose membrane (Protran) and dried for 30 min. The membrane was blocked with 10% (w/v) skim milk (Marvel) in PBS for 1 h. Primary antibodies were then suitably diluted (usually 1:1000) in TS buffer containing 10% Marvel, and incubated with the membrane for 1.5 hours. Unbound antibody was removed by washing the membrane three times for 10 min in TS buffer. Unbound Protein A coupled to horseradish peroxidase (Sigma), diluted 1:500 in TS buffer containing 10% Marvel or goat anti-rabbit IgG coupled to horseradish peroxidase (HRP) (DAPI), diluted 1:1000 into 10% Marvel TS buffer, was used to detect bound primary antibody by incubating with the membrane for 1 hour. Excess protein A coupled to horseradish peroxidase or goat anti-rabbit IgG coupled to horseradish peroxidase was removed by washing three times for 10 min in TS buffer. The blot was then visualized by developing in the dark using the chemiluminescent substrate "Lumiglo" (New England Biolabs), and finally exposed to X-Omat autoradiographic film (Kodak).

2.12 Adherence assays

Adherence of staphylococcal and lactococcal cells to immobilised fibrinogen or other serum and matrix proteins was performed as described by Wolz *et al.* (1996), with modifications made in this laboratory. 96-well flat bottomed microtitre plates (Sarstedt) were coated with 100µl of a solution of host extracellular matrix or serum protein diluted to a specific concentration in either PBS or coating solution (0.02% sodium carbonate buffer, pH 9.6) and incubated overnight at 4°C. Proteins used were fibrinogen (Calbiochem), collagen (Sigma), laminin (sigma), keratin (Sigma) and mucin

(Calbiochem). Human serum was obtained from donors. Control wells contained PBS or coating solution only. Bovine serum albumin (BSA; 2 mg/ml) was added and the plates incubated at 37°C for 1 hour. The plates were then washed three times with PBS. Cells to be tested were washed in PBS and adjusted to an OD600nm of 1 and 100µl of this suspension was added to the plates and incubated with gentle agitation for 1.5 hours at room temperature. The plates were subsequently washed three times with PBS and bound cells were fixed with 100µl formaldehyde per well (25% w/v) for 30 min. The plates were washed again three times with PBS and then stained with 100µl crystal violet per well (0.5% v/v) for 1 min. Absorbance was measured at 570nm in an ELISA plate reader (Labsystems Multiskan Plus). For increased sensitivity in certain cases, crystal violet was solublised by addition of 10% acetic acid (50µl/well) and the plates read at 570nm in an ELISA plate reader. Wells without fibrinogen and blocked with BSA were used as controls. This background value was subtracted from the values obtained for sample wells. Error bars are representative of the standard error values for triplicate samples on the same plate.

2.13 Inhibition of bacterial adherence to immobilised fibrinogen with antibodies

The ability of antibodies to inhibit the adherence of *S. lugdunensis, S. aureus* and *L. lactis* strains expressing either Fbl or ClfA on their cell surface to immobilised fibrinogen was performed as described previously (Perkins *et al.*, 2001) with the following modifications. Microtitre plates were coated with 0.5 µg/ml of the protein in PBS and incubated overnight at 4 °C. Bovine serum albumin (2% w/v in PBS) was added, and the plates were incubated for 1 h at 37 °C. 100 µl of a bacterial cell suspension (1×10^8 colony-forming units/ml) was subsequently incubated with doubling dilutions of an antibody solution and incubated with gentle agitation for 1hr at room temperature. This cell suspension was then added to the plate and incubated for a further 1 hour at room temperature with gentle agitation. Plates were subsequently washed three times with PBS, and bound cells were fixed with formaldehyde (25% v/v) for 30 min and then stained with crystal violet (0.5% v/v) for 1 min. Absorbance was measured at 570 nm in an enzyme-linked immunosorbent assay plate reader (Labsystems Multiskan

Plus). Wells without fibrinogen and blocked with BSA were used as controls. This background value was subtracted from the values obtained for sample wells. Error bars are representative of the standard error values for triplicate samples on the same plate.

2.14 Inhibition of bacterial adhering to immobilised fibrinogen with recombinant protein

The ability of recombinant Fbl region A and recombinant ClfA region A to inhibit the adherence of *L. lactis* strains to immobilised fibrinogen was performed as is described previously (Perkins *et al.*, 2001) with the following modifications: Microtitre plates were coated with 0.5 μg/ml of the protein in PBS and incubated overnight at 4 °C. Bovine serum albumin 2% w/v in PBS was added, and the plates were incubated for 1 h at 37 °C. 100μl of recombinant protein was then added to the wells in doubling dilutions and incubated with gentle agitation for 1hr at room temperature. After this 100 μl of a bacterial cell suspension (1×10^8 colony-forming units/ml) was added and the plates were incubated with gentle agitation at room temperature for 1.5hr. Plates were subsequently washed three times with PBS, and bound cells were fixed with formaldehyde (25% v/v) for 30 min and then stained with crystal violet (0.5% v/v) for 1 min. Absorbance was measured at 570 nm in an enzyme-linked immunosorbent assay plate reader (Labsystems Multiskan Plus). Wells without fibrinogen and blocked with BSA were used as controls. This background value was subtracted from the values obtained for sample wells. Error bars are representative of the standard error values for triplicate samples on the same plate.

2.15 Displacement of bacterial adherence to immobilised fibrinogen with antibodies

The ability of antibodies to displace bound *S. lugdunensis, S. aureus* and *L. lactis* strains expressing either Fbl or ClfA on their cell surface from an immobilised fibrinogen substrate was analysed. Briefly, microtitre plates were coated with 0.5 μg/ml of the protein in PBS and incubated overnight at 4 °C. Bovine serum albumin 2% w/v in PBS was added, and the plates were incubated for 1 h at 37°C. 100 μl of a bacterial cell

suspension (1×10^8 colony-forming units/ml) was added to the plates and incubated with gentle agitation for 1hr at room temperature. Plates were then washed three times with PBS and doubling dilutions of an antibody solution were then added to wells in the plate and incubated for a further 1 hour at room temperature with gentle agitation. Plates were subsequently washed three times with PBS, and bound cells were fixed with formaldehyde (25% v/v) for 30 min and then stained with crystal violet (0.5% v/v) for 1 min. Absorbance was measured at 570 nm in an enzyme-linked immunosorbent assay plate reader (Labsystems Multiskan Plus). Wells without fibrinogen and blocked with BSA were used as controls. This background value was subtracted from the values obtained for sample wells. Error bars are representative of the standard error values for triplicate samples on the same plate.

2.16 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 two-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, whereby the clumping titre was determined to be the lowest dilution of fibrinogen at which cell clumping still occurred.

2.17 Expression and purification of His_6-tagged proteins by immobilised metal chelate affinity chromatography

Recombinant proteins of $ClfA_{40-559}$, $ClfA_{220-559}$ and SdrH were purified by immobilised metal chelate affinity chromatography. The DNA sequences encoding the specific amino acid ranges of these proteins were amplified by PCR and cloned into the expression vector pQE30 which contains an IPTG-inducible promoter allowing for the control of target gene expression. In *E. coli* strains XL-1 Blue and TOPP3 the pQE30 target gene promoter is repressed by the $LacI^Q$ repressor which prevents transcription of

the target gene. IPTG binds to $LacI^{Q}$ leading to derepression of the promoter and consequent induction of target gene expression. Target genes were expressed as recombinant proteins with an N-terminal hexahistidine affinity tag.

E. coli TOPP3 strains containing the pQE30 plasmid and the target gene of choice were grown to on OD600nm of 0.4-0.6 and then induced for 4 hours at 37°C by addition of 1 mM IPTG (Biosynth). The cells were then harvested by centrifugation and lysed in a French pressure cell. The lysates were centrifuged at 18,000 rpm for 15 min and the supernatant filtered to remove cell debris. A 5 ml HiTrap™ Chelating HP column was charged with 150 mM Ni^{2+} and equilibrated with binding buffer (0.5 M NaCl, 20 mM Tris-HCl, pH7.9). The supernatant was then applied to the column at a constant flow rate of 1 ml/min and washed with binding buffer until the absorbance at 280nm of the eluate was <0.001. Bound protein was eluted with a continuous linear gradient of imidazole (5-100mM; total volume of 100ml) in 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9). Eluted fractions were monitored by absorbance at 280nm and peak fractions were analysed by SDS-PAGE and Western Immunoblotting. Protein fractions that contained the recombinant protein were pooled and dialyzed against 50 mM NaCl, 20 mM Tris-HCl (pH 7.4) and then applied to a 5ml HiTrap Q Sepharose column (Amersham Pharmacia Biotech). Bound protein was eluted with a continuous linear gradient of NaCl (50-500 mM; total volume 100 ml) in 20 mM Tris and 2 mM EDTA (pH 7.9). Peak fractions were analyzed by SDS-PAGE, pooled and concentrated.

2.18 Expression and purification of GST-Tagged proteins by affinity chromatography

The DNA encoding SdrH region A was cloned into the IPTG-inducible expression vector pGEX by Dr. Orla Hartford. Proteins cloned into pGEX are expressed as fusion proteins with the 26 kDa glutathione S-transferase (GST). The GST gene contains an ATG and ribosome-binding site, and is under control of the IPTG inducible *tac* promoter. The resultant fusion protein was purified as follows. TOPP3 containing the pGEX:SdrH region A plasmid were grown to on OD600nm of 0.4-0.6 and then induced for 4 hours at 37°C by addition of 1 mM IPTG (Biosynth). The cells were then

harvested by centrifugation and lysed in a French pressure cell. The lysate was centrifuged at 18,000 rpm for 15 min and the supernatant filtered to remove cell debris.

Subsequently 20 ml glutathione-Sepharose 4B resin was poured into a 2.5 x 8-cm column. The column was then washed with 5 to 10 bed volumes PBS at a flow rate of 1.5 ml/min to remove the ethanol storage solution. The cleared cell lysate was then loaded onto the pre-equilibrated glutathione column and then washed with 10 column volumes of PBS. Bound protein was eluted by passing 5 bed volumes of glutathione buffer (50 mM Tris-HCl, 10 mM reduced glutathione (Sigma), pH 8.0) through the column at a constant flow rate of 1 ml/min and 1 ml fractions were collected. Fractions were analysed by SDS-PAGE and those containing the GST-fusion protein were pooled and concentrated.

2.19 Cloning and expression of rFbl$_{40\text{-}534}$ in *L. lactis*

In order to construct a *L. lactis* vector that would express recombinant Fbl region A a reverse primer was designed encoding the C-terminal amino acids of Fbl region A, and a forward primer was designed encoding the stop codon of *fbl* and adjoining flanking sequence encoding the terminator sequence of plasmid pKS80 (Table 3). These primers amplified a 7Kb fragment from the pKS80Fbl^{+} plasmid which was purified using the High Pure PCR Product Purification Kit from Roche Diagnostics GmbH. The product was cut using *Sac*I enzyme overnight at 37°C, repurified and then ligated and transformed into *L. lactis* MG1363. Clones positive for rFbl region A were identified by using supernatants of each transformant in a dot-immunoblot probing the blot with anti-ClfA region A antibodies.

2.20 Purification of rFbl$_{40\text{-}534}$ from *L. lactis* supernatants

L. lactis pKS80:*fbl*A was inoculated into GM17 supplemented with 10μg/ml erythromycin and grown overnight at 30°C statically. The following day a 1/100 dilution of this overnight culture was made into 1L amounts of GM17 (M17 broth supplemented with 0.5% glucose) supplemented with 10μg/ml erythromycin and grown overnight at

30°C statically. The overnight cultures were then pelleted by centrifugation at 7000 rpm at 4°C and 445g ammonium sulphate per litre was added to the supernatant and mixed well. The precipitated protein that rose to the surface of the treated supernatant was removed and resuspended in 50ml redistilled H_20. The protein solution was then dialysed overnight into 5L 10mM Tris-HCl 0.9% NaCl pH 7.4 at 4°C. This solution was concentrated 10-fold in a stirred cell ultrafiltration chamber (Amicon, Beverly, MA) equipped with a 50,000 molecular-mass cut-off membrane. The resulting solution was first separated by size-fractionation through sephadex G-50 and eluted fractions were monitored by absorbance at 280 nm. Peak fractions were then applied to a 5 ml HiTrap Q Sepharose column (Amersham Pharmacia Biotech). Bound protein was eluted with a continuous linear gradient of NaCl (50-500 mM; total volume 100 ml) in 20 mM Tris and 2 mM EDTA (pH 7.9). Peak fractions were analyzed by SDS-PAGE, pooled and concentrated.

2.21 N-terminal sequencing of *L. lactis* expressed $rFbl_{40-534}$

N-terminal sequencing of $rFbl_{40-534}$ was performed by Dr. Mike Weldon at the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge.

2.22 Generation of rabbit polyclonal antibodies to recombinant proteins

Antibodies to the A region of ClfA (residues 40-559) were kindly provided by Ms. Judy Higgins. Antibodies to $rFbl_{40-534}$ and rSdrH were raised in young New Zealand White rabbits (2 kg) whose pre-immune sera showed no reaction with *S. lugdunensis* ($rFbl_{40-534}$) or *S. epidermidis* (rSdrH) wall-associated antigens in Western blots. The protein (25 μg) in 10mM Tris-HCl 0.9% NaCl pH 7.4 was emulsified with an equal volume of Freund's complete adjuvant (500 μl) and injected subcutaneously. Three subsequent injections given at 2 week intervals contained Freund's incomplete adjuvant. The rabbits were bled, serum-recovered, and the immunoglobulin fraction was purified by the method of Owen (1985).

2.23 Affinity-purification of polyclonal antibodies to SdrH region A.

5 mg of SdrH region A was dialysed overnight in a coating buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) at 4°C. 1g of CNBr-activated Sepharose (Amersham Pharmacia Biotech) was washed ten times in ice-cold 1 mM HCl. The recombinant protein was incubated overnight at 4°C with washed CNBr-activated sepharose in coating buffer (final volume of 10 ml) with gentle shaking. The resulting gel was allowed to settle on ice for 1 hour. Excess liquid was removed and the gel was gently resuspended in blocking buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl) and then left at room temperature for 3 hours to block any remaining active sites. The gel was then washed six times alternating between blocking buffer and wash buffer (0.1 M CH_3CO_2Na, 0.5 M NaCl, pH 3.9) and left at room temperature overnight in blocking buffer. The gel was then placed in a XK-16 column (Pharmacia) and equilibrated with blocking buffer. Antiserum was applied to the column at a flow rate of 1 ml/min and washed with blocking buffer. Bound antibody was eluted by passing through 30ml of elution buffer (50 mM glycine, 0.5 M NaCl, pH 2.7) at a flow rate of 1 ml/min. Eluate fractions were measured at an absorbance of 280nm and protein containing fractions were dialysed overnight at 4°C in preserve buffer (PBS, 15 mM NaN_3) and concentrated using the Amicon ultrafiltration device (YM-3).

2.24 Enzyme linked immunosorbent assays

The ability of recombinant proteins to bind to immobilized fibrinogen was analysed using an enzyme-linked immunosorbent assay. Microtitre plates (Sarstedt) were coated with fibrinogen (10 μg/ml in PBS) overnight at 4°C. The plates were washed three times with PBS 0.5% Tween 20 and blocked with 2% bovine serum albumin for 2 h at 37 °C. After an additional three washes with PBS 0.5% Tween 20, recombinant protein was added and incubated at 37 °C for 2 h. The wells were washed again and incubated with

anti-ClfA region A antiserum diluted 1:2,000 in PBS, at 37 °C for 1 h. After further washing, horseradish peroxidase-labeled goat anti-rabbit IgG (Sigma) was added at a 1:1,000 dilution. Following incubation at 37 °C for 1 h and washing with PBS, 100 µl of chromogenic substrate (580 µg/ml tetramethylbenzidine and 0.0001% H_2O_2 in 0.1 M sodium acetate buffer (pH 5.2)) was added per well and developed for 10 min in the dark. The reaction was stopped by the addition of 2 M H_2SO_4 (50 µl/well). Plates were read at 450 nm.

2.25 Inhibition of rFbl$_{40-534}$ or rClfA$_{40-559}$ binding to whole fibrinogen using fibrinogen γ-chain peptides

Microtitre plate wells (Immulon 4, Dynatech Laboratories Inc.) were coated with 1 µg of Fg in TBS, pH 7.5, for 18 h at 4 °C. Plates were washed three times with TBST, and remaining protein binding sites were blocked with 5% (w/v) bovine serum albumin (BSA) in TBS for 2 h at room temperature. The wells were then washed three times with TBST. The recombinant proteins were pre-incubated for 1.5 h at room temperature in TBS, 0.1% (w/v) BSA with a synthetic peptide composed of the 17 C-terminal residues of the γ-chain of Fg (GEGQQHHLGGAKQAGDV) or one of the mutant gamma chain peptides that contain an alanine or serine substitution at each of the 17 amino acids as illustrated in Table 2.4. The reactions were then added to the fibrinogen-coated wells and the plates were incubated for 1 h at room temperature. The plates were again washed with TBST, and bound protein was detected by incubation with a 1:1000 dilution of rabbit polyclonal anti-ClfA region A antibody in TBST, 0.1% (w/v) BSA for 1 h at room temperature. After three further washes with TBST, a 1:2,000 dilution of goat anti-rabbit AP-conjugated polyclonal Abs (Bio-Rad) in TBST, 0.1% (w/v) BSA was added to the wells and the plates were incubated for 1 h at room temperature. Finally, bound alkaline phosphatase-conjugated antibodies were detected by the addition of *p*-nitrophenyl phosphate (Sigma) in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.0, at room temperature for 30-60 min. Plates were read at 405 nm using an ELISA plate reader (Thermomax microplate reader, Molecular Devices).

2.26 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*, *S. lugdunensis* and *L. lactis* cells were washed once in PBS and adjusted to an OD600nm of 1.6. Aggregations were measured in a platelet aggregometer at 37°C. The aggregometer was first blanked using PPP. 450µl PRP was added to a siliconised 1 ml tube and placed into the aggregometer. 50 µl of the bacterial cell suspension was then added and the reaction allowed to proceed. If aggregation had not occurred by 25 min, then the result was considered negative.

2.27 Mutagenesis of *sdrH* by allele replacement

Plasmid pROJ6448 *sdrH::tet* was constructed by Orla Hartford. DNA fragments corresponding to 197 bp upstream of the start codon up to 270 bp downstream of the start codon and bases 631-1310 downstream of the start codon were amplified by PCR and ligated either side of the *tet* gene and cloned into pROJ6448 (Fig. 8.1). This plasmid was electroporated into *S. aureus* RN4220 pGO1 pC221 and then transferred to *S. epidermidis* 9142 by conjugational mobilization as described in section 2.6. Standard temperature shift experiments (Foster, 1998) were performed whereby *S. epidermidis* carrying pROJ6448*sdrH::tet* was grown in TSB containing erythromycin (100 µg ml^{-1}) at 28 °C and cultures were subsequently diluted and spread on TSA containing erythromycin and incubated at 45 °C. Tetracycline resistant colonies were tested by Southern blotting.

2.28 Assay of biofilm formation on polystyrene and glass.

The method of Heilmann *et al.* (1996) was modified in order to quantitate the biofilm-forming capacity of *S. lugdunensis* and *S. epidermidis* strains. Bacterial cells were grown overnight in TSB at 37°C. The culture was diluted 1:200 in TSB-supplemented with 0.5% glucose, and 200µl of this cell suspension was used to inoculate sterile, 96-well polystyrene microtitre plates (Sarstedt). After cultivation for 24 h at 37°C, the wells were gently washed twice with 200 ml of sterile phosphate-buffered saline (PBS). The plates were air dried, and the remaining surface-adsorbed cells of the individual wells were stained with 100 µl crystal violet per well (0.5% v/v) for 1 min. Absorbance was measured with a ELISA plate reader (Labsystems Multiskan Plus) at 570 nm. A well to which sterile TSB lacking cells was added served as a control. The value for this well was subtracted from the experimental readings. Each assay was performed in triplicate.

Biofilm formation on a glass surface was determined by the presence or absence of a ring of biofilm at the air-liquid interface on the inner surface of glass test tubes after 5ml of TSB or TSB supplemented with 0.5% glucose was grown at 37°C for 16 hours with shaking.

Table 2.1. Bacterial strains

Strain	Relevant Genotype	Relevant Properties	Source/ Reference
E. coli			
XL-1 Blue		Propagation of plasmids	Stratagene
TOPP3		Protease deficient strain. Used for the expression of recombinant proteins	Stratagene
L. lactis			
MG1363		Plasmid free derived strain of NCDO 712	Gasson, 1983
S. epidermidis			
9142		Biofilm former	Mack *et al.*, 1994
9491		SdrH and SdrF prototype strain	ATCC strain
K28		SdrG prototype strain	McCrea *et al.*, 2000
HB		Osteomyelitis strain	Nilsson *et al.*, 1998
1457		Biofilm former	Mack *et al.*, 1994
RP62a		Slime/Biofilm former	Christensen *et al.*, 1982
Tu3298		Transformable strain	Augustin and Gotz, 1990
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 *clfA clfB*	*clfB*::Tet^r	Tet^r	Ní Eidhin *et al.*, 1998
8325-4		NCTC 8325 cured of prophages	Novick, 1967
RN4220		Restriction-deficient derivative of 8325-4	Kreiswirth *et al.*, 1983
P1		Rabbit virulent strain	Sherertz *et al.*, 1993

Table 2.1 Contd.

Strain	*Relevant Genotype*	*Relevant Properties*	*Source/ Reference*
COL		MRSA isolate	Wilkinson *et al.*, 1978
EMRSA-16		MRSA isolate	Cox *et al.*, 1995
EMRSA-3		MRSA isolate	Enright *et al.*, 2000
MSSA			Enright *et al.*, 2000
S. lugdunensis			
963	*fbl*$^+$		F. Vandenesch
964	*fbl*$^+$		F. Vandenesch
1003	*fbl*$^+$		F. Vandenesch
1004	*fbl*$^+$		F. Vandenesch
1005	*fbl*$^+$		F. Vandenesch
1006	*fbl*$^+$		F. Vandenesch
1007	*fbl*$^+$		F. Vandenesch
1008	*fbl*$^+$		F. Vandenesch

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
pUC18	High copy number *E. coli* plasmid. Used for general cloning	Amp^r	
pQE30	Expression vector, recombinant proteins fused to N-terminal hexahistidine tag.	Amp^r	Qiagen Inc.
pGEX	Expression vector, recombinant proteins fused to N-terminal Glutathione-S-Transferase.	Amp^r	Amersham Pharmacia Biotech
pCU1	Shuttle vector derived from pC194 and pUC19, maintain high copy number in *E. coli* and *S. aureus*	Amp^r in *E. coli* Cm^r in *S. aureus*	Augustin *et al.*, 1992.
pKS80	*L. lactis* plasmid with a high level expression system for heterologous surface proteins	Em^r	Hartford *et al.*, 2001.
pKS80:*fbl*$_{1003}$	pKS80 containing the full length *fbl* gene cloned from *S. lugdunensis* strain 1003 in frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	This work
pKS80:*fbl*$_{1004}$	pKS80 containing the full length *fbl* gene cloned from *S. lugdunensis* strain 1004 in frame with the ATG start codon located within the BclI site of the vector	Em^r	This work

Table 2.2 Contd.

pKS80:*clfA*	pKS80 containing the full length *clfA* gene cloned from *S. aureus* strain Newman in frame with the ATG start codon located within the BclI site of the vector	Em^r	O'Brien *et al.*, 2002
pKS80:*clfB*	pKS80 containing the full length *clfB* gene cloned from *S. aureus* strain Newman in frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	O'Brien *et al.*, 2002
pKS80:*sdrH*	pKS80 containing the full length sdrH gene cloned from *S. epidermidis* strain 9491 in frame with the ATG start codon located within the *Bcl*I site of the vector	Em^r	This work
pKS80:*fblA*	pKS80:*fbl*1003 deletion mutant modified by inverse PCR lacking bases –2624 of the *fbl* gene	Em^r	This work
pCF40	pQE30 containing the full length A region from *clfA* cloned from *S. aureus* Newman	Amp^r	O'Connell *et al.*, 1998
pCF41	pQE30 containing a fragment of the A region of *clfA* encoding residues 220-559 cloned from *S. aureus* Newman	Amp^r	O'Connell *et al.*, 1998
pCF41COL	pQE30 containing a fragment of the A region of *clfA* encoding residues 220-559 cloned from *S. aureus* COL	Amp^r	This work

Table 2.2 Contd.

pCF41P1	pQE30 containing a fragment of the A region of *clfA* encoding residues 220-559 cloned from *S. aureus* P1	Amp^r	This work
pCF41MSSA	pQE30 containing a fragment of the A region of *clfA* encoding residues 220-559 cloned from *S. aureus* MSSA	Amp^r	This work
pCF41EMRSA-16	pQE30 containing a fragment of the A region of *clfA* encoding residues 220-559 cloned from *S. aureus* EMRSA-16	Amp^r	This work
pCF41EMRSA-3	pQE30 containing a fragment of the A region of *clfA* encoding residues 220-559 cloned from *S. aureus* EMRSA-3	Amp^r	This work
pCF41206	pQE30 containing a fragment of the A region of *clfA* encoding residues 220-559 cloned from *S. aureus* 206	Amp^r	This work
pCF41670	pQE30 containing a fragment of the A region of clfA encoding residues 220-559 cloned from *S. aureus* 670	Amp^r	This work
pClfA-(221-559) (K417A)	A derivative of pCF41 where residue K_{417} has been changed to A by site directed mutagenesis	Amp^r	This work
pClfA-(221-559) (N406A)	A derivative of pCF41 where residue N_{406} has been changed to A by site directed mutagenesis	Amp^r	This work

Table 2.2 Contd.

pClfA-(221-559) (T413A)	A derivative of pCF41 where residue T_{413} has been changed to A by site directed mutagenesis	Amp^r	This work
pClfA-(221-559) (G404A)	A derivative of pCF41 where residue G_{404} has been changed to A by site directed mutagenesis	Amp^r	This work
pClfA-(221-559) (S535A)	A derivative of pCF41 where residue S_{535} has been changed to A by site directed mutagenesis	Amp^r	This work
pClfA-(221-559) (K473A)	A derivative of pCF41 where residue K_{473} has been changed to A by site directed mutagenesis	Amp^r	This work
pClfA-(221-559) (I495A)	A derivative of pCF41 where residue I_{495} has been changed to A by site directed mutagenesis	Amp^r	This work
pClfA-(221-559) (F539A)	A derivative of pCF41 where residue F_{539} has been changed to A by site directed mutagenesis	Amp^r	This work
pClfA-(221-559) (I517A)	A derivative of pCF41 where residue I_{517} has been changed to A by site directed mutagenesis	Amp^r	K. McFarland
pClfA-(221-559) (R519A)	A derivative of pCF41 where residue R_{519} has been changed to A by site directed mutagenesis	Amp^r	K. McFarland
pClfA-(221-559) (Y376A)	A derivative of pCF41 where residue Y_{376} has been changed to A by site directed mutagenesis	Amp^r	K. McFarland

Table 2.2 Contd.

pSdrHC	pQE30 containing the full length C region from *sdrH* cloned from *S. epidermidis* 9142	Amp^r	This work
pSdrHA	pGEX containing the full length A region from *sdrH* cloned from *S. epidermidis* 9142	Amp^r	O. Hartford

Table 2.3. Primers

Primer Name	Sequence (5' to 3')	5' Restriction Site or codon change
SdrHF	GGCGGATCCGAAAGTTTAACATTAAACATTCA	*Bam*HI
SdrHR	GGCGGATCCTTACTTTTTTCTTCTAAAGATATA	*Bam*HI
SdrHCF		*Bam*HI
SdrHCR	GGCGGATCCTTACTTTTTTCTTCTAAAGATATA	*Bam*HI
FblF	GGAAGATCTTAACAAAGAAAGAAAAGTTTTCAA	*Bgl*II
FblR	GCAAGATCTTTATTTATCTTCTTTTTTTGAACG	*Bgl*II
FblAF	TCCGAGCTCTAACAAAGCCCGAAAGGAAG	*Sac*I
FblAR	TCCGAGCTCCCCGGTTGATCCGGTAT	*Sac*I
Clf41F	CGCGGATCCGTAGTGCAGATGCACCGGTAGC	*Bam*HI
Clf41R	CCCAAGCTTCTCTGGAATTGGTTCAATTTCACCAG	*Hind*III
G404AF	GTCAATCCAAGTGCAGATAACGTTATTG	G404A
G404AR	CAATAACGTTATCTGCACTTGGATTGAC	G404A
N406AF	CCAAGTGGAGATGCAGTTATTGCGCCG	N406A
N406AR	CGGCGCAATAACTGCATCTCCACTTGG	N406A
T413AF	TGCGCCGGTTTTAGCAGGTAATTTAAAA	T413A
T413AR	TTTTAAATTACCTGCTAAAACCGGCGCA	T413A
K417AF	ACAGGTAATTTAGCACCAAATACGGATAGT	K417A
K417AR	ACTATCCGTATTTGGTGCTAAATTACCTGT	K417A
F539AF	AACGAAGTAGCAGCAAATAACGGATCAGG	F539A
F539AR	CCTGATCCGTTATTTGCTGCTACTTCGTT	F539A
I517AF	ATAACTCGAATATAGCTTGGCGCTCTATG	I517A
I517AR	CATAGAGCGCCAAGCTATATTCGAGTTAT	I517A
R519AF	GAATATAATTTGGGCCTCTATGTCATGGG	R519A
R519AR	CCCATGACATAGAGGCCCAAATTATATTC	R519A
S535AF	AACGGATCAGGTGCAGGTGACGGTATC	S535A
S535AR	GATACCGTCACCTGCACCTGATCCGTT	S535A
K473AF	TCCAAATCAATATGCAGTAGAGTTTAATACG	K473A
K473AR	CGTATTAAACTCTACTGCATATTGATTTGGA	K473A
I495AF	GTTAATGGTCATGCAGATCCGAATAGCAAA	I495A
I495AR	TTTGCTATTCGGATCTGCATGACCATTAAC	I495A
Y376AF	GAAAAATATGGTAAGTTTGCTAACTTATCTAT	Y376A
Y376AR	ATAGATAAGTTAGCAAACTTACCATATTTTTC	Y376A

Table 2.4 Sequence of the synthetic γ-chain peptides

Peptide name	Sequence	Source
Wild type	GEGQQHHLGGAKQAGDV	S. Gurusiddappa
P1	AEGQQHHLGGAKQAGDV	S. Gurusiddappa
P2	GAGQQHHLGGAKQAGDV	S. Gurusiddappa
P3	GEAQQHHLGGAKQAGDV	S. Gurusiddappa
P4	GEGAQHHLGGAKQAGDV	S. Gurusiddappa
P5	GEGQAHHLGGAKQAGDV	S. Gurusiddappa
P6	GEGQQAHLGGAKQAGDV	S. Gurusiddappa
P7	GEGQQHALGGAKQAGDV	S. Gurusiddappa
P8	GEGQQHHAGGAKQAGDV	S. Gurusiddappa
P9	GEGQQHHLAGAKQAGDV	S. Gurusiddappa
P10	GEGQQHHLGAAKQAGDV	S. Gurusiddappa
P11	GEGQQHHLGGSKQAGDV	S. Gurusiddappa
P12	GEGQQHHLGGAAQAGDV	S. Gurusiddappa
P13	GEGQQHHLGGAKAAGDV	S. Gurusiddappa
P14	GEGQQHHLGGAKQSGDV	S. Gurusiddappa
P15	GEGQQHHLGGAKQAADV	S. Gurusiddappa
P16	GEGQQHHLGGAKQAGAV	S. Gurusiddappa
P17	GEGQQHHLGGAKQAGDA	S. Gurusiddappa

Table 2.5 Antibodies used in this study

Antibody	Cognate antigen	Source
Anti-His_6 mAb	Hexahistidine peptide	P. Speziale
Anti-SdrH region A	$SdrH_{31-91}$	O. Hartford
Anti-SdrH region C	$SdrH_{210-487}$	This study
Anti-ClfA region A	$ClfA_{40-559}$	J. Higgins
Anti-Fbl region A	Fbl_{40-534}	This study
Veronate	ClfA, SdrG	P. Domanski, Inhibitex
mAb 12-9	ClfA N3	P. Domanski, Inhibitex
mAb 13-1	ClfA N2	P. Domanski, Inhibitex
mAb 13-2	ClfA N3	P. Domanski, Inhibitex
mAb 15-6	ClfA N2	P. Domanski, Inhibitex
mAb 33-104	ClfA N3	P. Domanski, Inhibitex
mAb 35-006	ClfA N3	P. Domanski, Inhibitex
mAb 35-039	ClfA N3	P. Domanski, Inhibitex
mAb 35-041	ClfA N3	P. Domanski, Inhibitex
mAb 35-052	ClfA N2	P. Domanski, Inhibitex
mAb 35-220	ClfA N3	P. Domanski, Inhibitex

Chapter 3

The fibrinogen binding protein Fbl of *Staphylococcus lugdunensis*

3.1 Introduction

Several species of coagulase negative staphylococci (CoNS) are commensals of human skin. They are opportunistic pathogens, most frequently associated with infections of indwelling medical devices. *Staphylococcus lugdunensis*, however, is an unusually virulent CoNS that was first described by Freney *et al.* (1988). It can cause a wide range of invasive infections usually associated with *Staphylococcus aureus* such as osteomyelitis, peritonitis, soft tissue abscesses and infective endocarditis (Bellamy and Barkham, 2002). Indeed, *S. lugdunensis* accounts for 18% of infective endocarditis and 44% of native valve endocarditis caused by CoNS (Patel *et al.*, 2000). *S. lugdunensis* endocarditis is more aggressive than that caused by other CoNS and often resembles that caused by *S. aureus*. *S. lugdunensis* endocarditis is characterised by a high fatality rate even with antimicrobial treatment (Kragsbjerg *et al.*, 2000).

S. lugdunensis expresses relatively few putative virulence factors compared to *S. aureus*. Known factors include the SLUSH synergistic toxins, a haemolysin, extracellular enzymes, a glycocalyx and a fibrinogen binding protein (Donvito *et al.*, 1997, Leung *et al.*, 1998). A genetic system for *S. lugdunensis* has not been established. The species is highly clonal as indicated by genotyping studies. van der Mee-Marquet *et al.* (2003) found two PFGE genotypes associated with a collection of clinical and commensal *S. lugdunensis* isolates. Dufour *et al.* (2002) reported that there are two *agr* types associated with their collection of *S. lugdunensis* clinical isolates.

Recently the sequence of a gene encoding a fibrinogen binding protein of *S. lugdunensis* (*fbl*) was reported (Accession number: AF404823). The *fbl* gene is 2646bp in length and is predicted to encode a protein of 881 residues. This gene bears significant organisational and sequence similarity to the clumping factor A of *S. aureus* (ClfA) (Nilsson *et al.*, 2001). ClfA is an important virulence factor of *S. aureus* that contributes to the pathogenesis of experimental septic arthritis and endocarditis (Josefsson *et al.*, 2001, Moreillon *et al.*, 1995). It is also a potential protective antigen for active and passive immunization (Josefsson *et al.*, 2001; Vernachio *et al.*, 2003). ClfA binds to the extreme C-terminus of the γ-chain of fibrinogen at the same site as the platelet integrin GPIIb/IIIa (McDevitt *et al.*, 1995; McDevitt *et al.*, 1997; Medved *et al.*, 1997). Structural analysis of ClfA and the related proteins SdrG and ClfB

revealed that the ligand binding A domain is composed of three subdomains called N1, N2 and N3 (Davis *et al.*, 2001; Perkins *et al.*, 2001; Ponnuraj *et al.*, 2003; Deivanayagam *et al.*, 2002). The structure of N2N3 of ClfA has been solved. Each subdomain is independently folded and comprises 9 β-sheets that form a novel IgG type fold (Deivanayagam *et al.*, 2002). The structure of N1 was not determined. The γ-chain peptide binding site was predicted to occur in a hydrophobic groove located at the junction of N2 and N3. Substitution mutants of several residues predicted to contact the peptide ligand caused defects in fibrinogen binding (Hartford *et al.*, 2001; Deivanayagam *et al.*, 2002).

The purpose of this study was to examine the expression and fibrinogen binding activity of Fbl on the surface of bacterial cells. Fbl expression was analysed in both its native host, *S. lugdunensis* and also in the surrogate expression host *Lactococcus lactis*. The ability of Fbl to promote adherence to fibrinogen and the purified recombinant γ-chain of fibrinogen was compared with ClfA expressed by *L. lactis*. The ability of Ca^{2+} ions to interrupt Fbl-mediated binding of cells to fibrinogen was also analysed. Finally the ability of bacteria expressing Fbl on their cell surface to initiate activation of platelet aggregation was investigated and compared to that of ClfA expressing bacteria.

3.3 Results

3.3.1 DNA sequence analysis of the *fbl* gene and amino acid sequence analysis of the Fbl protein of *S. lugdunensis*.

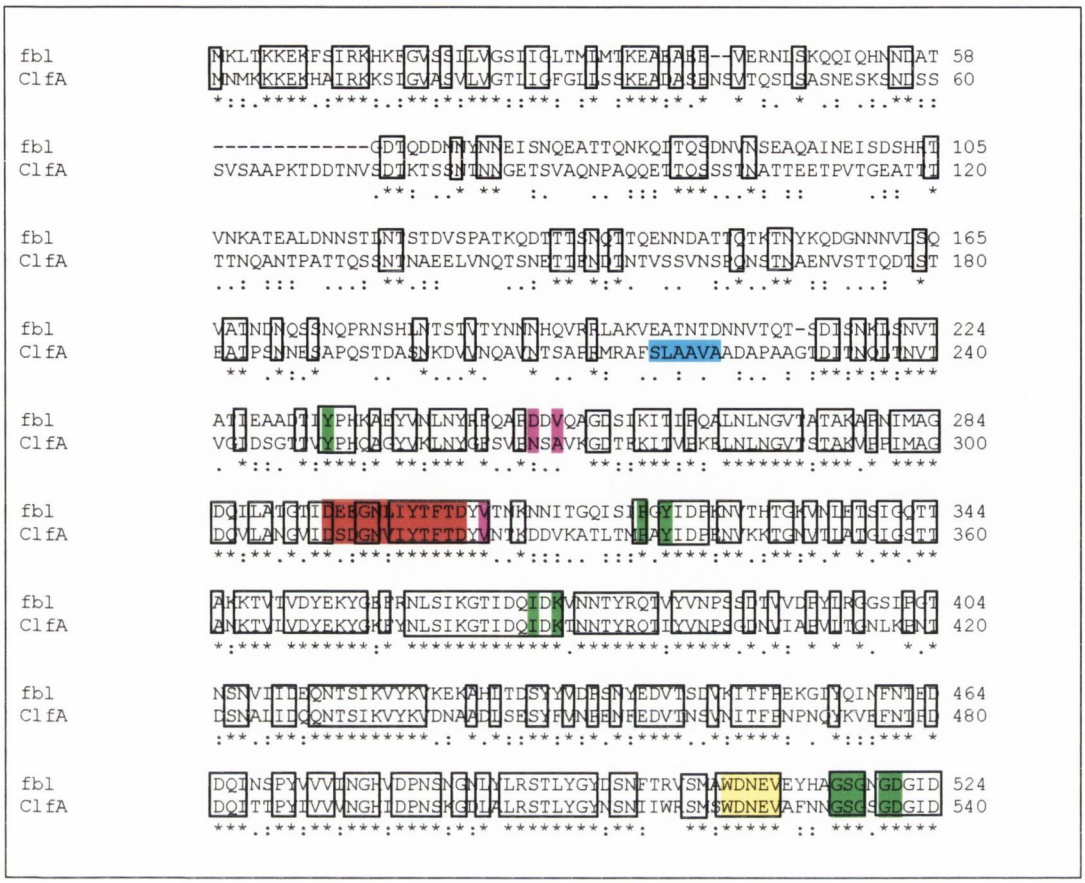
The *fbl* gene of *S. lugdunensis* encodes a protein of 881 amino acids. The amino acid sequence of the Fbl protein was analysed by several web-based sequence analysis tools. This revealed that the entire amino acid sequence of Fbl is 58% identical to that of ClfA of *S. aureus* strain Newman. Following a 40 residue signal sequence, the A domain of Fbl differs markedly from that of ClfA over the subdomain N1 of ClfA with only 19% identical residues (Figure 3.1 A). In contrast, subdomains N2 and N3 of Fbl are 60% identical to ClfA. Subdomain N1 of ClfA is separated from N2 by the metalloprotease cleavage site SLAAVA (McAleese *et al.*, 2001). This motif is absent in Fbl. The ligand binding A domain of ClfA is linked by a DS dipeptide repeat region R to the C-terminal wall-anchoring LPDTG motif, a hydrophobic membrane-spanning domain and a positively charged tail. The ClfA R region is encoded by an 18bp DNA repeat GAY TCN GAY TCN GAY AGY (Hartford *et al.*, 1997). In Fbl region R comprises DSDSDA repeats encoded by GAY TCN GAY AGY GAY GCR (where R=a purine, Y=a pyrimidine and N is any base).

The first 174 amino acids of the A domain of Fbl (region N1) were significantly divergent in sequence from the corresponding first 219 amino acids of ClfA (19% identical). In contrast the domain of ClfA that contains the minimum fibrinogen-binding site (221-559) has approximately 60% identity to the equivalent region of Fbl. Comparative analysis by using a Kyte-Doolittle hydropathy plot revealed that the hydropathy profiles of both Fbl and ClfA are very similar except for the N1 regions. The hydropathy index of Fbl N1 indicates that the region is more hydrophilic than region N1 of ClfA and there is a high probability of N1 of Fbl region A forming coiled-coils but not the N1 of ClfA as indicated by the Predict Protein server at http://maple.bioc.columbia.edu/pp/.

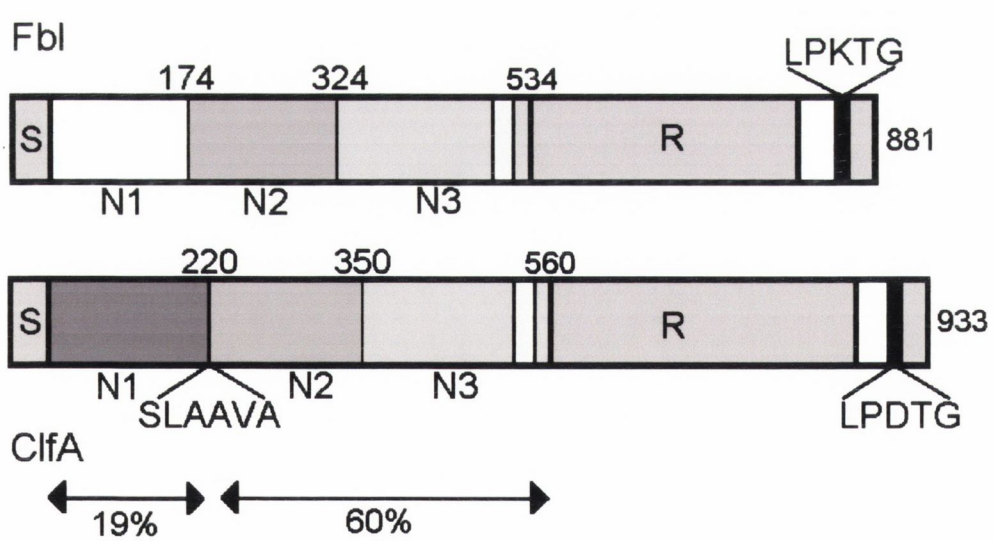
There are cell wall and hydrophobic membrane spanning regions at the C-terminus of each protein, which are bisected by the cell wall sorting motif LPXTG. The LPKTG motif of Fbl is 37 amino acids before the C terminus of the protein and the LPDTG motif of ClfA is 38

Figure 3.1 A. ClustalW alignment of the amino acid sequences of the A domains of Fbl and ClfA. The WDNEV motif is indicated in yellow and other residues important in binding, as predicted by the crystal structure and brute-force docking between the 3D protein and peptide structures using SoftDock are indicated in green. The metalloprotease cleavage site is indicated in blue. The residues that were predicted to form a putative EF-hand motif are highlighted in red (O'Connell *et al.*, 1998). The residues that co-ordinate with divalent cations in the crystal structure of $rClfA_{220-559}$ (Deivanayagam *et al.*, 2002) are highlighted in magenta. Identical residues are boxed. **B.** Schematic representation of the domain structure of Fbl and ClfA indicating the percentage identity between the N1 regions and the N23 regions of the A domains of each protein. Fbl does not contain a SLAAVA metalloprotease cleavage motif bisecting the N2 and N3 regions. The repeated R region of Fbl differs from that of ClfA as it is comprised of a repeating SDSDSA hexapeptide motif rather than SD repeats in ClfA.

A.


fbl
ClfA

B.


Fbl
174
324
534
LPKTG
S
R
881
N1
N2
N3
220
350
560
S
R
933
N1
SLAAVA
N2
N3
LPDTG
ClfA
19%
60%

amino acids before the C terminus. Both N3 of Fbl and N3 of ClfA contain the putative ligand binding motif $_{523}$WDNEV$_{527}$ (Fig. 3.1 B). Mutations of E_{526} and V_{527} abrogate binding of ClfA to fibrinogen (Hartford *et al.*, 2001). The residues predicted to line the ligand-binding pocket of ClfA and to contact the γ-chain peptide (P_{336}, Y_{338}, Y_{256}, K_{389}, I_{387}) are also conserved in Fbl (Deivanayagam *et al.*, 2002). The residues that correspond to ClfA$_{550\text{-}559}$ are notably missing from the amino acid sequence of Fbl. However these residues are not predicted to affect binding of ClfA to fibrinogen as the minimum binding region was shown to reside within residues 220-550 (McDevitt *et al.*, 1995).

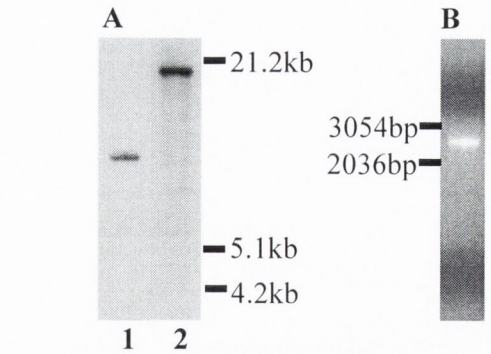
3.3.2 Detection of the *fbl* gene by Southern blotting and PCR

Southern hybridisation analysis was performed with genomic DNA from eight *S. lugdunensis* strains. A fragment of the *clfA* gene of *S. aureus* Newman was amplified by PCR from bases 1400 to 3001 (Table 2.3). This fragment encodes the conserved part of the A domain, region R and the wall spanning region. When the *S. lugdunensis* genomic DNA was cleaved with *Hind*III a single hybridising band of 9kb was detected. When it was cleaved with *Bam*HI one fragment of approximately 20kb was detected (Fig. 3.2 A).

PCR amplification of the entire *fbl* gene using primers that recognized bases 1 to 2646 (Table 2.3) yielded a fragment of approximately 2.7kb in all strains (Fig. 3.2 B). This suggests that the *fbl* gene is present in each strain and that there is little if any diversity in the locus. PCR amplification of the eight strains was also carried out using primers that recognized unique DNA sequence immediately 5' and 3' to the sequence encoding the DSDSDA-repeat region (Table 2.2). This PCR experiment yielded one of two distinct products of either 900kb or 810kb for each strain (Fig. 3.2 C). This indicates that only two size variants of Fbl region R occur among the limited strain collection tested.

3.3.3 *S. lugdunensis* adheres to immobilised fibrinogen

The eight *S. lugdunensis* strains were tested for their ability to adhere to immobilised human fibrinogen. Strains 1003 and 1004 adhered strongly in a dose-dependent and saturable



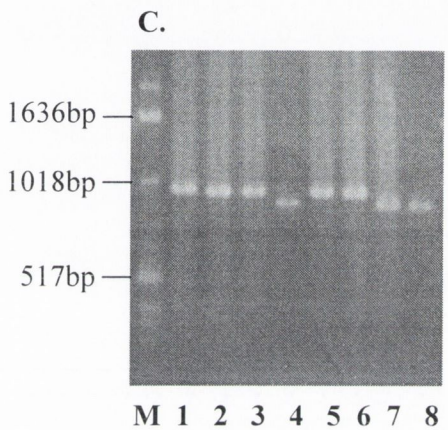


Figure 3.2 **A.** Southern hybridisation of genomic DNA from a representative *S. lugdunensis* strain probed using DIG labeled DNA corresponding to the *clf*A gene from base pair 1400-3001. This encodes the ClfA protein from the SD-repeat region to the C-terminal residue. Lane 1: *S. lugdunensis* strain 1003 genomic DNA digested with *Hind*III. Lane 2: *S. lugdunensis* strain 1003 genomic DNA digested with *Bam*HI. Seven other strains tested gave similar results. **B.** The entire *fbl* gene (2721bp) amplified using *S. lugdunensis* strain 1003 genomic DNA as a template. **C.** PCR analysis of the DNA flanking the repeated R region of *fbl*. Lane M= Molecular weight marker. Lane 1, *S. lugdunensis* strain 1003, lane 2, *S. lugdunensis* strain 1004, lane 3, *S. lugdunensis* strain 1005, lane 4, *S. lugdunensis* strain 1006, lane 5, *S. lugdunensis* strain 1007, lane 6, *S. lugdunensis* strain 1008, lane 7, *S. lugdunensis* strain 963, lane 8, *S. lugdunensis* strain 964. All DNA was separated on 1% agarose gels.

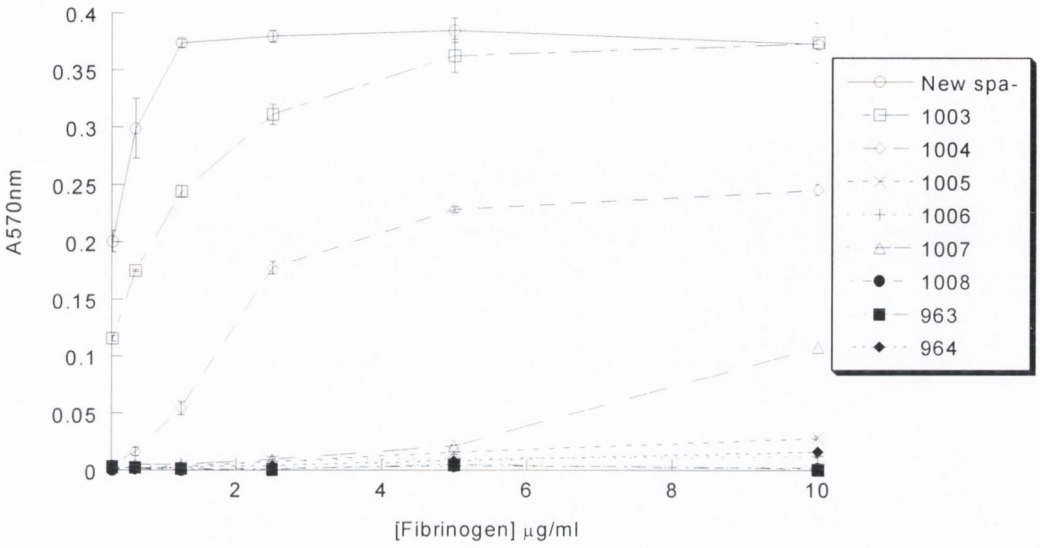


Figure 3.3 Adherence of *S. lugdunensis* strains to immobilised fibrinogen. 96 well ELISA plates were coated with ten serial dilutions of fibrinogen ranging from 10µg/ml - 0.01µg/ml. *S. lugdunensis* bacteria grown to stationary phase were collected by centrifugation, washed and resuspended to an OD_{600nm}=1. *S. aureus* strain Newman was used as a control. The other samples are strains of *S. lugdunensis*. Cells were stained with crystal violet and washed with PBS. Cells that bound to fibrinogen coated microtitre plate wells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells. This experiment was carried out three times with similar results.

fashion although strain 1004 adhered less avidly than 1003 (Fig. 3.3). Strain 1007 adhered very weakly. The other five strains did not adhere detectably under the conditions tested. This seemed to correspond to expression of glycocalyx because non-adherent strains formed biofilm when grown in glass test tubes and planktonic cells were difficult to resuspend after centrifugation.

3.3.4 Cloning of Fbl into the surrogate expression host *Lactococcus lactis*

In order to determine unambiguously that Fbl is a fibrinogen binding adhesin similar to ClfA of *S. aureus*, the entire coding sequence of the *fbl* genes from *S. lugdunensis* strains 1003 and 1004 were amplified by PCR and cloned into the *L. lactis* expression vector pKS80 (Fig. 3.4). Primers FblF and FblR (Table 2.3) amplified precisely from the ATG codon at the 5' end of the gene to the stop codon at the 3' end of the gene. *Bgl*II sites were incorporated into the 5' extensions of each primer. The *Bgl*II-cut PCR product was then ligated to pKS80 which was digested with *Bcl*I, forming a transcriptional fusion between the strong plasmid borne lactococcal bacteriophage C2 promoter, LPS2, which provides constitutive expression of a short open reading frame. Translation is optimized by fusing the target open reading frame ATG codon to the ATG codon of the expression cassette (Fig. 3.5). This overlaps the start codon TGA of the upstream open reading frame and translational coupling occurs. The resultant chimeric plasmids were transformed into *L. lactis* strain MG1363. Transformants were detected by whole cell dot-immunoblotting with anti-ClfA antibodies (Fig. 3.6). The fidelity of the chimaeric plasmid was checked by restriction analysis and the DNA fragment was sequenced. Two selected strains were studied further. Whole cells bound human fibrinogen. This was detected by HRP-conjugated rabbit-anti-human fibrinogen IgG antibodies (Fig. 3.7 A).

3.3.5 Detection of Fbl expression by SDS-PAGE and Western Immunoblotting of cell wall extracts of *S. lugdunensis*

Lysostaphin solublised cell wall extracts and stabilised protoplasts that were lysed by boiling in final sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004%

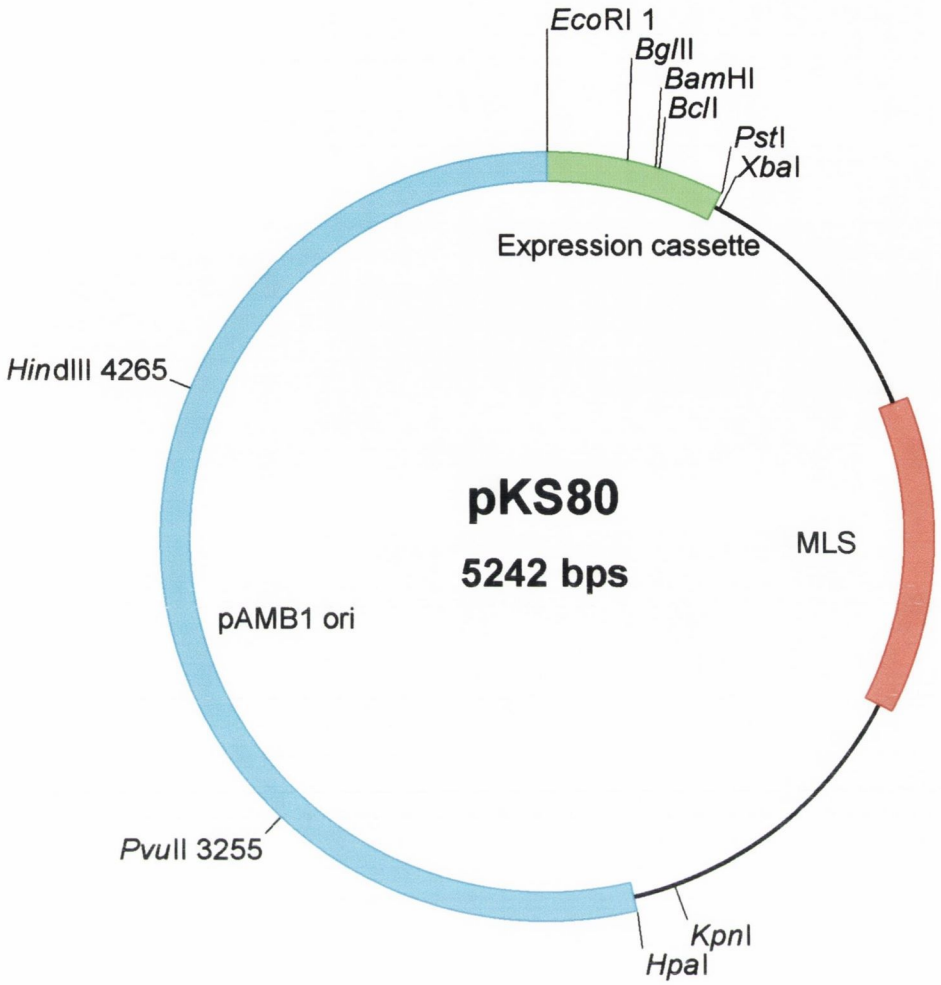


Figure 3.4 Map of pKS80. Positions of the plasmid replication origin of pAMß1, the macrolide, lincosamide and streptogramin B (MLS) marker and the expression cassette are indicated.

Figure 3.5 The pKS80 expression cassette. The lactococcal bacteriophage C2 promoter LPS2 provides constitutive expression of a short open reading frame. Expression of the target gene is achieved by cloning into the *Bcl*I site TGATCA within the striped box. The grey box indicates the position of the Shine–Delgarno (SD) sequence of the second translation initiation signal used by the fused orf. Translation is optimized by fusing the target open reading frame ATG codon to the ATG codon of the expression cassette. This overlaps the stop codon TGA of the upstream open reading frame. The ribosome-binding site of the upstream orf is indicated by the upstream red box. Curved lines indicate the translation products.

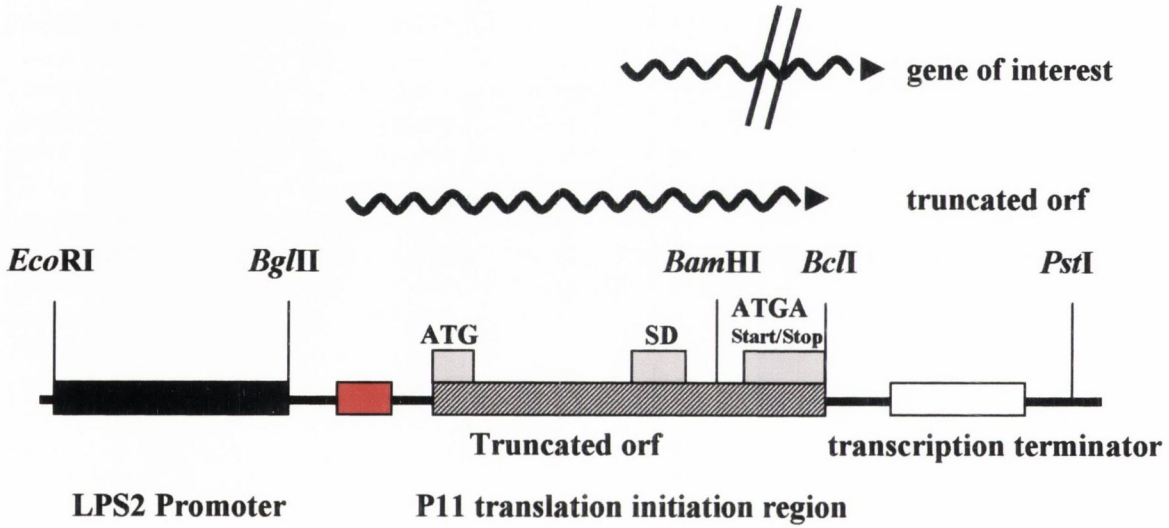
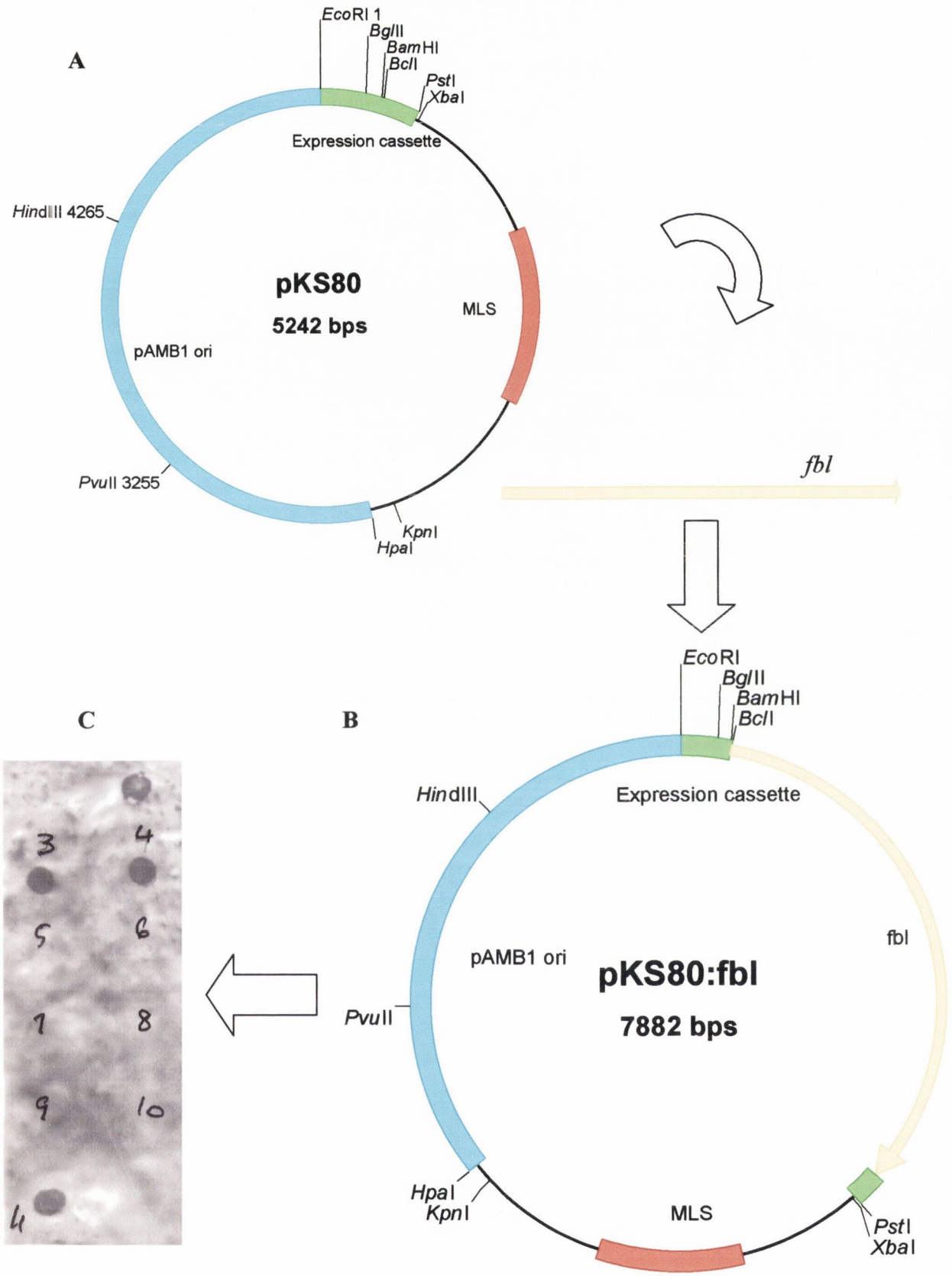


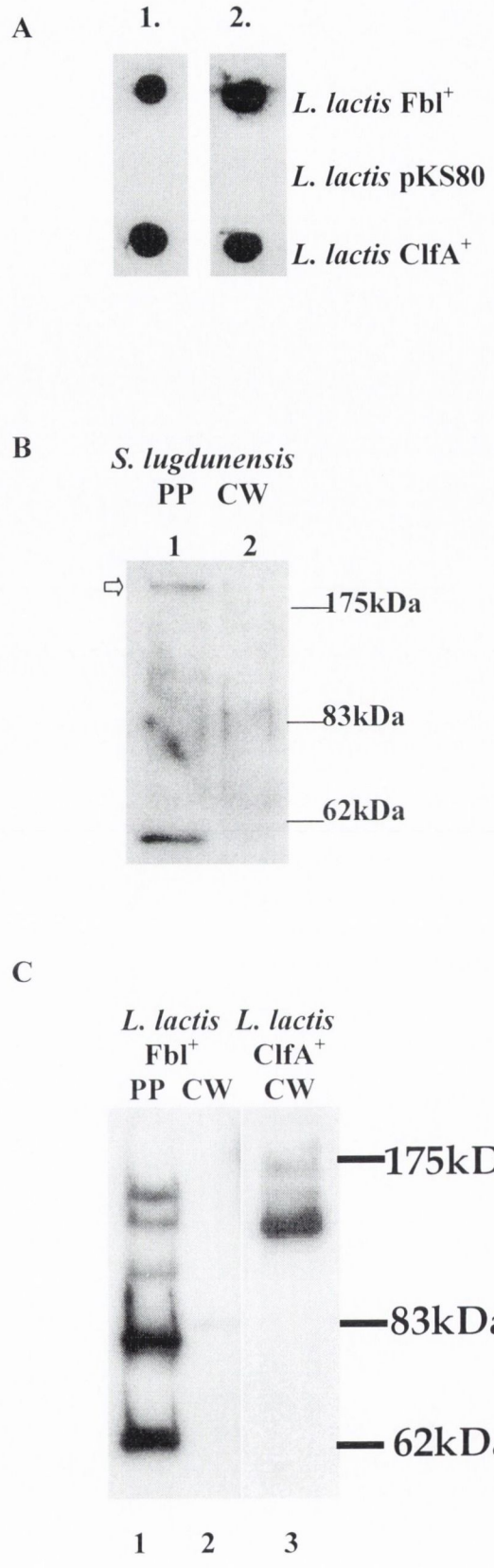
Figure 3.6 Cloning of the *fbl* gene into the *L. lactis* expression system pKS80. **A.** Map of pKS80. Positions of the plasmid replication origin of pAMß1, the macrolide, lincosamide and streptogramin B (MLS) resistance marker and the expression cassette are indicated. **B.** The entire *fbl* gene was amplified by PCR, digested using *Bgl*II and ligated to pKS80 digested using *Bcl*I. **C.** Whole cell dot blot of putative *L. lactis* Fbl^+ cells probed with polyclonal rabbit anti-ClfA region A antibodies showing that clones 2, 3, 4 and 11 are positive for Fbl expression.


A
EcoRI 1
BglII
BamHI
BclI
PstI
XbaI
Expression cassette
HindIII 4265
pKS80
5242 bps
MLS
pAMB1 ori
PvuII 3255
KpnI
HpaI
fbl
EcoRI
BglII
BamHI
BclI
B
HindIII
Expression cassette
fbl
pAMB1 ori
pKS80:fbl
7882 bps
PvuII
HpaI
KpnI
MLS
PstI
XbaI
C
3
4
5
6
7
8
9
10
4

bromophenol blue and 0.125 M Tris HCl pH 6.8), of *S. lugdunensis* were separated by SDS-PAGE through a 10% acrylamide gel. Cell wall associated proteins were solublised by lysostaphin in the presence of 30% raffinose. The stabilized protoplasts were separated by centrifugation. Each sample was boiled in final sample buffer containing SDS and β-mercaptoethanol. Cell wall associated proteins and proteins from the protoplasts were separated by SDS-PAGE. The separated proteins were then transferred to PVDF membranes and probed using anti-ClfA region A antibodies. An immunoreactive band with an apparent molecular weight ~200kDa was detected for each strain although only 964 is shown here (Fig. 3.7 B). This most likely corresponds to the full length Fbl protein. The majority of immunoreactive protein was associated with the protoplast fraction suggesting that Fbl was not efficiently sorted to the cell wall. Strain 964 was chosen because as it expressed the highest level of Fbl that was detectable by Western immunoblotting. Strain 964 also produced the highest level of biofilm of the strains tested. This is presumably why it failed to adhere to immobilised fibrinogen as production of a glycocalyx has been shown previously to mask cell surface proteins (Gillaspy *et al.*, 1998).

Western immunoblotting of *L. lactis* Fbl^+ cell wall proteins that were solublised using lysozyme and mutanolysin was performed to investigate whether Fbl is sorted to the cell wall. When the protoplasts were analysed, several strongly immunoreactive bands were detected which were absent in the MG1363 (pKS80) control. The largest corresponded in size to the single immunoreactive band in *S. lugdunensis* cell wall extracts. The smaller bands are most likely products of proteolytic degradation. This problem often confounds analysis of heterologously expressed proteins in *L. lactis* (O'Brien *et al.*, 2002; Poquet *et al.*, 2000). No immunoreactive protein was detected in the cell wall fraction. It can be concluded that Fbl is expressed on the surface of *L. lactis* but that it appears that sorting of the protein to the cell wall fraction is incomplete and much remains associated with the membrane and cytoplasmic fractions (Fig. 3.7 C). This contrasts with the ClfA control which is associated with the cell wall fraction in *S. aureus* and *L. lactis*. ClfA protein has been mostly degraded to a 130kDa form. The apparent molecular weight of >175kDa indicates that Fbl is migrating at twice its predicted size of 95kDa in SDS-PAGE gels. This is typical of ClfA and other serine-aspartate-rich proteins (Hartford *et al.*, 1997; McCrea *at al.*, 2000).

Figure 3.7 Expression of the Fbl protein in the heterologous expression host *L. lactis* MG1363. **A.** Whole cell dot blot of *L. lactis* Fbl^{+}_{1003} cells. 1. *L. lactis* cells probed with rabbit anti-ClfA region A antibodies and HRP-conjugated goat anti-rabbit IgG. 2. *L. lactis* cells probed with 10 μg/ml human fibrinogen and HRP-conjugated rabbit anti-human fibrinogen IgG antibodies. **B.** Lane 1. Protoplast fraction of *S. lugdunensis* 964. Lane 2. Lysostaphin-solublised cell wall fraction of *S. lugdunensis* 964. Lanes 1-2 probed with anti-ClfA region A antibodies. **C.** Lanes 1-3 probed with anti-ClfA region A antisera. 1. Protoplast fraction of *L. lactis* Fbl^{+}_{1003}. 2. Mutanolysin- and lysozyme-solublised cell wall fraction of *L. lactis* Fbl^{+}_{1003}. 3. Mutanolysin- and lysozyme-solublised cell wall fraction of *L. lactis* $ClfA^{+}$.


A
1.
2.
L. lactis Fbl+
L. lactis pKS80
L. lactis ClfA+
B
S. lugdunensis
PP CW
1 2
175kDa
83kDa
62kDa
C
L. lactis Fbl+
PP CW
L. lactis ClfA+
CW
175kDa
83kDa
62kDa
1 2 3

3.3.6 *L. lactis* cells expressing Fbl adhere to immobilised fibrinogen

L. lactis Fbl^{+}_{1003} was tested for its ability to adhere to different amounts of immobilised human fibrinogen (Fig. 3.8). This strain adhered in a dose dependent and saturable manner and bound to the same level as *L. lactis* $ClfA^{+}$. *L. lactis* Fbl^{+}_{1004} also adhered to immobilised fibrinogen in a similar fashion to *L. lactis* Fbl^{+}_{1003} (data not shown). This clearly indicates that Fbl is an adhesin that mediates strong adhesion to immobilised human fibrinogen. All subsequent experiments were performed with *L. lactis* Fbl^{+}_{1003}.

3.3.7 Effect of divalent cations on adherence of *L. lactis* Fbl^{+} to immobilised fibrinogen.

Alignment of the A-domain sequences of ClfA and Fbl indicated that the putative Ca^{2+} binding motif present in ClfA (O'Connell *et al.*, 1998) is conserved in the A domain of the protein (Fig. 3.1). Ca^{2+} has been shown previously to regulate the interaction of ClfA with fibrinogen. Therefore the effect of Ca^{2+} ions on the fibrinogen binding activity of Fbl in *L. lactis* Fbl^{+} was tested. The data in Fig. 3.9 clearly shows that Fbl-dependent adherence to immobilised fibrinogen was inhibited by Ca^{2+} ions in a concentration-dependent manner and to a greater extent than ClfA-dependent adherence. The IC_{50} of *L. lactis* Fbl^{+} is 1.3 fold greater than that of *L. lactis* $ClfA^{+}$.

3.3.8 Blocking of Fbl-promoted adherence of *L. lactis* Fbl^{+} to immobilised fibrinogen by recombinant ClfA region A.

In order to determine the part of the fibrinogen molecule recognized by Fbl, recombinant ClfA region A protein, $rClfA_{40\text{-}559}$ (residues 40-559) and recombinant ClfB region A protein, $rClfB_{197\text{-}542}$ (residues 197-542) were tested for their ability to block adherence of *L. lactis* Fbl^{+} and *L. lactis* $ClfA^{+}$ to immobilised fibrinogen (Fig. 3.10 A). $rClfA_{40\text{-}559}$ recognizes the extreme C-terminal residues of the γ-chain of fibrinogen whereas $rClfB_{197\text{-}542}$ recognizes the α-chain of

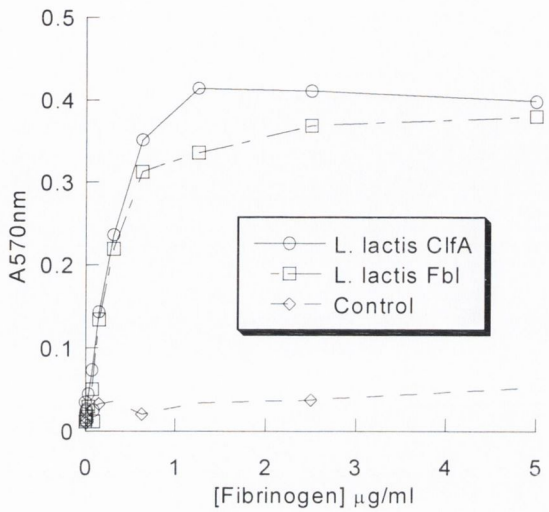


Figure 3.8 Adherence of *L. lactis* Fbl^{+}_{1003} and *L. lactis* $ClfA^{+}$ strains to immobilised fibrinogen. 96 well ELISA plates were coated with ten serial dilutions of fibrinogen ranging from 10µg/ml to 0.01µg/ml. *L. lactis* Fbl^{+} and *L. lactis* $ClfA^{+}$ bacteria grown to stationary phase were collected by centrifugation, washed and resuspended to an OD_{600nm}=1. *L. lactis* pKS80 was used as a control. Cells were stained with crystal violet and washed with PBS. Cells that bound to fibrinogen coated microtitre plate wells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells. This experiment was carried out three times with similar results.

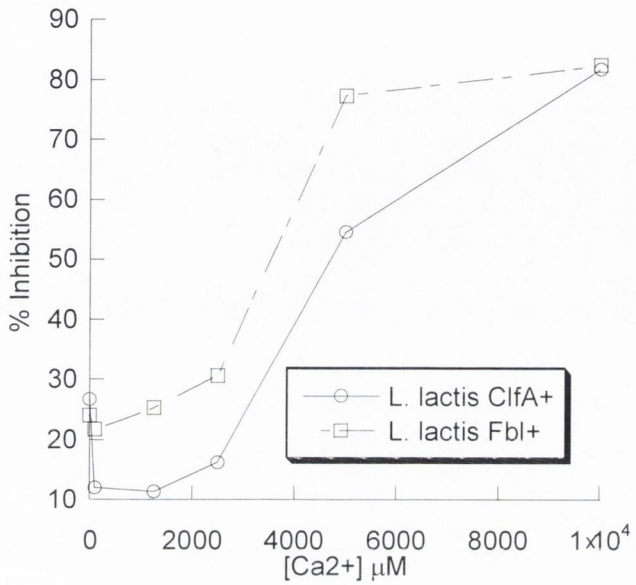


Figure 3.9 The effect of Ca^{2+} on the adherence of *L. lactis* $ClfA^{+}$ and *L. lactis* Fbl^{+} to immobilised fibrinogen. The fibrinogen binding activity of *L. lactis* $ClfA^{+}$ and *L. lactis* Fbl^{+} was tested over ten serial doubling dilutions of Ca^{2+} varying from 10mM to 19.5µM. Cells were stained with crystal violet and washed with PBS. Cells that bound to fibrinogen coated microtitre plate wells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages inhibition of binding of cells to fibrinogen compared to control (no Ca^{2+}). This experiment was repeated three times with similar results.

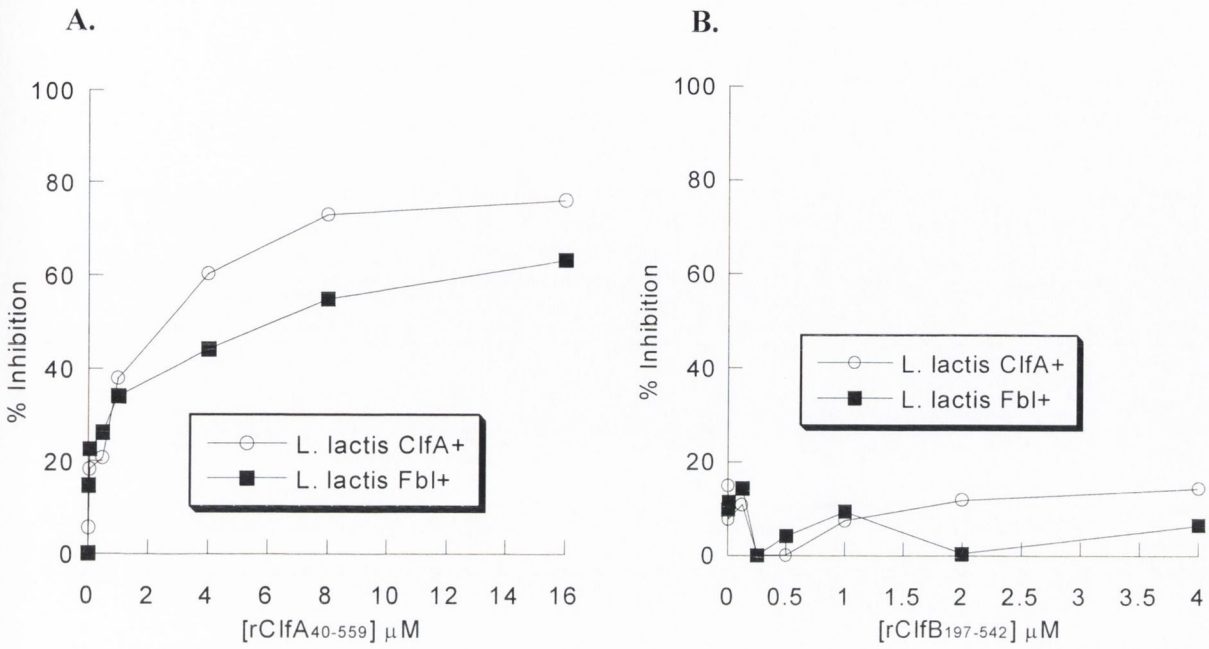


Figure 3.10 The effect of recombinant fibrinogen binding proteins on the adherence of *L. lactis* Fbl$^+$ to fibrinogen. **A.** The ability of *L. lactis* ClfA$^+$ and *L. lactis* Fbl$^+$ to adhere to microtitre wells coated with 100μl 0.5μg/ml fibrinogen was tested in the presence of concentrations of rClfA$_{40\text{-}559}$ varying from 16μM to 31.25nM. **B.** *L. lactis* ClfA$^+$ and *L. lactis* Fbl$^+$ fibrinogen binding activity was tested in the presence of varying concentrations of rClfB$_{197\text{-}542}$. Cells were stained with crystal violet and washed with PBS. Cells that bound to fibrinogen coated microtitre plate wells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages inhibition of binding of cells to fibrinogen compared to control (no rClfA$_{40\text{-}559}$ or no rClfB$_{197\text{-}524}$). This experiment was repeated three times with similar results.

fibrinogen (Deivanayagam *et al.*, 2002; Ni Eidhin *et al.*, 1998). The adherence of both *L. lactis* Fbl$^+$ and *L. lactis* ClfA$^+$ to fibrinogen was inhibited by rClfA$_{40\text{-}559}$ in a dose-dependent manner and was completely unaffected by rClfB$_{197\text{-}542}$ (Fig. 3.10 B). This indicates that Fbl binds to the same region in the gamma chain of fibrinogen as ClfA.

3.3.9 Binding of *L. lactis* Fbl$^+$ to the γ-chain of fibrinogen.

The fibrinogen binding protein ClfA binds to the γ-chain of fibrinogen. DNA encoding the γ-chain of fibrinogen had been cloned into the *E. coli* expression vector pQE30 and the protein was purified by Ni^{2+} chelate affinity chromatography. The adherence of *L. lactis* Fbl$^+$ to recombinant γ-chain of fibrinogen purified from *E. coli* was analysed in order to determine if Fbl also mediates binding to the γ-chain. Both *L. lactis* Fbl$^+$ and *L. lactis* ClfA$^+$ bound in a dose-dependent and saturable manner to immobilised γ-chain of fibrinogen in an ELISA-type assay, whereas *L. lactis* ClfB$^+$, which adheres to the α-chain of fibrinogen (Ni Eidhin *et al.*, 1998), and *L. lactis* pKS80 did not (Fig. 3.11). ClfA appeared to promote stronger binding to the purified gamma-chain of fibrinogen than Fbl. This agrees with data presented in Chapter 4 which indicates that the recombinant A domain of Fbl has a lower affinity for fibrinogen than recombinant ClfA A domain. Possible reasons for this are discussed in Chapter 4. However differing levels of protein expression and proteolysis could also affect the binding of *L. lactis* Fbl$^+$ and *L. lactis* ClfA$^+$ to immobilised fibrinogen.

3.3.10 Clumping of Fbl expressing bacteria in soluble fibrinogen

L. lactis Fbl$^+$ strains 1003 and 1004 were analysed for their ability to clump in soluble fibrinogen in comparison to *L. lactis* ClfA$^+$, *S. aureus* strain Newman and their parent *S. lugdunensis* strains 1003 and 1004 (Table 3.1). Neither of the *S. lugdunensis* strains exhibited an ability to clump in soluble fibrinogen. The *L. lactis* Fbl$^+$ had a similar high clumping titre as *L. lactis* ClfA$^+$. The inability of *S. lugdunensis* to clump in soluble fibrinogen is likely to be due to poor expression of Fbl. Thus, when expressed at high levels in *L. lactis* Fbl$^+$, Fbl is a clumping factor, like ClfA.

Table 3.1 Clumping titre of bacteria in soluble fibrinogen. Strains to be tested for clumping were grown to stationary phase, harvested by centrifugation and washed in PBS. A suspension of cells at an OD_{600nm}=6 in 20µl was added to 50µl of doubling dilutions of fibrinogen in microtitre wells. The highest dilution of fibrinogen that caused clumping after 5 minutes of gentle agitation was defined as the clumping titre.

Strain	Clumping Titre
S. aureus Newman *spa*$^-$	612
L. lactis ClfA$^+$	612
L. lactis Fbl^+_{1003}	306
L. lactis Fbl^+_{1004}	306
S. lugdunensis 1003	0
S. lugdunensis 1004	0

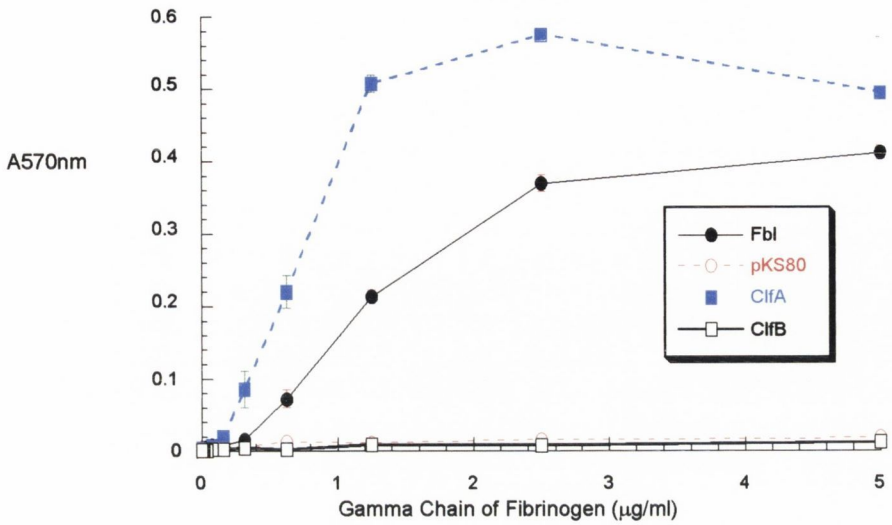


Figure 3.11 Adherence of *L. lactis* Fbl$^+$ and *L. lactis* ClfA$^+$ to immobilised recombinant γ-chain of fibrinogen. 96 well ELISA plates were coated with ten serial dilutions of recombinant γ-chain of fibrinogen ranging in concentration from 5μg/ml to 9.7ng/ml. *L. lactis* cells grown to stationary phase were collected by centrifugation, washed and resuspended to an OD_{600nm}=1. *L. lactis* pKS80 and *L. lactis* ClfB$^+$ were used as controls. Cells were stained with crystal violet and washed with PBS. Cells that bound to fibrinogen coated microtitre plate wells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells. This experiment was carried out three times with similar results.

3.3.11 Fbl expressing bacteria induce activation of platelet aggregation

ClfA is an important surface component of *S. aureus* and is a potent cause of platelet activation when expressed on the surface of *S. aureus* or *L. lactis* (O'Brien *et al.*, 2002). Platelet aggregation by staphylococci is thought to be a virulence trait associated with the ability to cause endovascular infections including endocarditis. The ability of *S. lugdunensis* strain 1003 cells to induce platelet aggregation in PRP was tested as it is a strong indicator of the ability of the bacteria to form platelet-bacteria thrombi which lead to infective endocarditis.

Cells grown to stationary phase were added to platelet rich plasma in order to analyse whether they could induce platelet aggregation. *S. lugdunensis* cells induced platelet aggregation with a lag time of 12.75 min with a monophasic response. *S. aureus* Newman *spa* induced platelet aggregation with a lag time of 0.75 min (Fig. 3.12) also with a monophasic response as had been observed previously (O'Brien *et al.*, 2002). Protein A binds to IgG which interferes with accurate determination of immunoreactive bands in Western immunoblots and has been observed to contribute to platelet aggregation (O'Brien *et al.*, 2002). A protein A mutant (*spa*) of *S. aureus* Newman was used throughout this work.

These results showed that *S. lugdunensis* can cause activation of platelet aggregation. *L. lactis* Fbl^+ cells grown to stationary phase were tested for their ability to activate platelet aggregation. These cells induced platelet aggregation with a lag time of 1.75 min as compared to a lag time of 1.5 min for *L. lactis* $ClfA^+$. *L. lactis* pKS80 cells were used as a control to show that *L. Lactis* cells containing the empty vector did not initiate platelet aggregation. This experiment shows that the ability of Fbl to cause activation of platelet aggregation is comparable to that of ClfA and that consequently Fbl is likely to be a major contributing factor to the ability of *S. lugdunensis* cells to cause endocarditis.

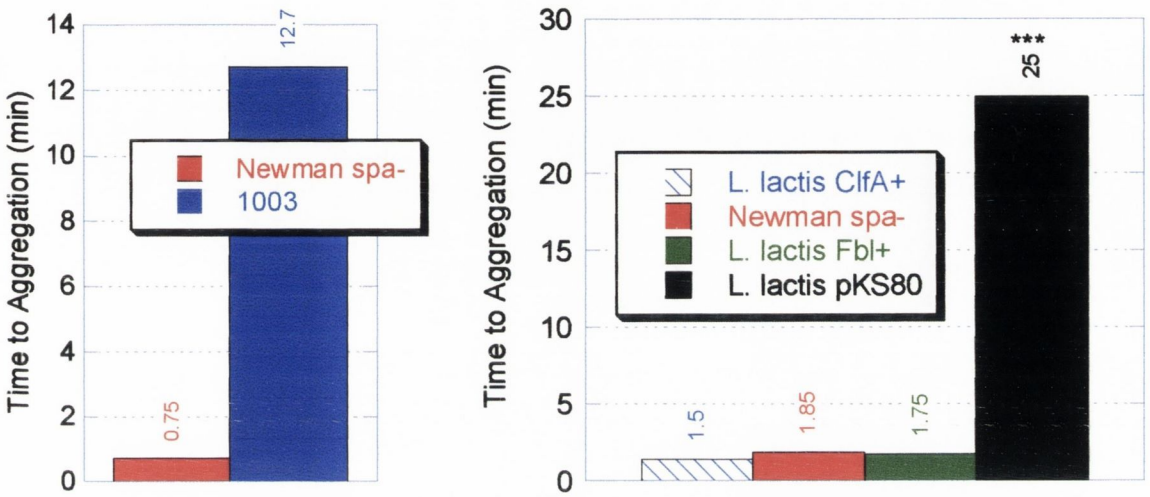


Figure 3.12 Fbl-mediated activation of platelet aggregation. **A.** Activation of platelet aggregation by *S. aureus* strain Newman *spa* and *S. lugdunensis*. *S. aureus* Newman *spa* and *S. lugdunensis* strain 1003 were grown to stationary phase and tested for their ability to activate platelet aggregation in PRP. Results are expressed as time taken to aggregation. **B.** Activation of platelet aggregation by *L. lactis* Fbl^+, *L. lactis* $ClfA^+$, *L. lactis* pKS80 and *S. aureus* Newman *spa*. *** indicates that aggregation did not occur after 25 min incubation. These experiments were carried out three times with similar results.

3.4 Discussion

The data presented here show that Fbl is a fibrinogen binding protein and a clumping factor. The *fbl* gene is present in all eight strains of *S. lugdunensis* tested. The locus is highly conserved throughout a range of clinically relevant strains as is indicated by PCR and Southern blotting analysis. Fbl appears to mediate binding of *S. lugdunensis* strains to fibrinogen. However the fibrinogen binding activity of some of the *S. lugdunensis* strains appears to be compromised by the presence of capsular polysaccharide on *in vitro* grown bacteria. However, it is unclear whether this level of capsule production is produced *in vivo*. The strains that did adhere to fibrinogen bound in a dose-dependent and saturable manner and paradoxically appeared to express much lower levels of Fbl protein than the strains that did not bind fibrinogen. Perhaps the higher levels of Fbl expression in non-binding strains is an attempt by the cells to compensate for the high levels of extracellular polysaccharide which may impede the fibrinogen binding activity. Masking of cell surface proteins by capsular polysaccharide has been previously observed in staphylococci (Gillaspy *et al.*, 1998).

Because a genetic system has not been established in *S. lugdunensis* it was not possible to generate a *fbl* null mutant of *S. lugdunensis* by either plasmid integration or allele replacement mutagenesis. The eight strains tested in this study proved to be non-transformable by electroporation. The known tight clonality of the *S. lugdunensis* species (van der Mee-Marquet *et al.*, 2003) was further verified by PCR amplification of the DNA encoding the R region of Fbl. This PCR analysis yielded two distinct sizes of DNA encoding the repeated R region that differ by approximately 100bp. When similar analysis was performed on the *clfA* gene, varying sizes of the DNA encoding the R region was observed, a feature that reflects the more diverse population structure of the *S. aureus* species as is seen in PFGE studies (McDevitt and Foster, 1995; van der Mee-Marquet *et al.*, 2003).

Fbl conferred fibrinogen-binding activity to *L. lactis*. Fbl, when expressed on the surface of *L. lactis*, behaved in a very similar manner to ClfA. It was subject to inhibition when incubated with increasing levels of Ca^{2+}. Recombinant ClfA region A inhibited the adherence of

L. lactis Fbl$^+$ to immobilised fibrinogen whereas recombinant ClfB region A did not. This indicated that Fbl interacts with the γ-chain of fibrinogen rather than the α-chain of fibrinogen, to which ClfB binds. *L. lactis* Fbl$^+$ was subsequently shown to adhere to the immobilised recombinant γ-chain of fibrinogen in a dose-dependent and saturable manner. Thus it can be concluded that Fbl binds to the same region of the γ-chain of fibrinogen as ClfA but whether both proteins bind to the same residues will be clarified by future experiments with the recombinant A domain of Fbl. This is the same region that the platelet integrin GPIIb/IIIa interacts with (residues 400-411). GPIIb/IIIa has been shown to have two distinct classes of Ca^{2+} binding sites; high affinity binding sites which promote ligand binding and low affinity binding sites which inhibit ligand binding (Hu *et al.*, 1996). A similar situation is thought to affect ClfA-fibrinogen binding whereby three Ca^{2+} ions are required for structural integrity of the ClfA molecule but a high concentration of calcium disrupts the interaction of ClfA and fibrinogen (Deivanayagam *et al.*, 2002; O'Connell *et al.*, 1998). It is likely given the high level of homology between Fbl and ClfA that this is the reason why an excess of Ca^{2+} ions can inhibit Fbl-mediated adherence of cells to fibrinogen.

S. lugdunensis cells did not clump in soluble fibrinogen. However upon over-expression of the Fbl protein in *L. lactis,* Fbl did cause clumping of the lactococcal cells. This suggests a dose-dependent effect for the clumping phenomenon whereby the level of expression of the fibrinogen binding protein is proportional to the clumping titre observed. It is also clear from Western Immunoblotting that there are significantly more Fbl molecules expressed on the surface of *L. lactis* Fbl$^+$ than *S. lugdunensis.* This effect also appears to impact on the ability of *S. lugdunensis* to cause platelet aggregation. We know that *S. aureus* Newman causes activation in seven minutes (exponential phase) and 1.5 minutes (stationary phase) and that there is approximately 7-fold more ClfA on the surface of stationary phase cells than exponential phase cells (O'Brien *et al.*, 2002). It is also clear from Western Immunoblotting experiments that there are significantly more Fbl molecules expressed on the surface of *L. lactis* Fbl$^+$ than *S. lugdunensis.* This might explain the shorter lag time of *L. lactis* Fbl$^+$ to initiate activation of platelet aggregation than *S. lugdunensis*. The ability of bacteria to initiate activation of platelet aggregation is a strong indicator of their involvement in the formation of vegetative platelet-bacteria thrombi that lead to infective endocarditis. It is, therefore, a strong possibility that Fbl is

the major contributing factor to the ability of *S. lugdunensis* to cause platelet aggregation and may shed some light on why *S. lugdunensis* infective endocarditis follows such an aggressive course that is reminiscent of *S. aureus* infective endocarditis.

The predicted molecular weight of full length Fbl is 95kDa. However, when protoplasts of *S. lugdunensis* and *L. lactis* Fbl$^+$ were analysed by SDS-PAGE and Western Immunoblotting the highest reacting band had an apparent molecular weight of 175kDa. This aberrant migration of approximately twice the predicted molecular weight is similar to that of other Clf/Sdr proteins and is attributed to the presence of the repeating serine-aspartate residues in region R (Hartford *et al.*, 1997). When Fbl was isolated from *L. lactis* Fbl$^+$ the protein appeared to have undergone proteolytic degradation. This is proposed to happen during cell wall digestion and does not appear to affect either the cell surface exposure of Fbl or its fibrinogen binding activity. A previous study concluded that the majority of molecules of heterologously expressed protein were intact and fully functional on the surface of *L. lactis* cells and that degradation only occurs during isolation of the proteins when they are exposed to membrane-associated proteases (O'Brien *et al*, 2002). This was verified by the whole cell dot blotting experiments in section 3.3.4 whereby Fbl protein bound fibrinogen on the surface of *L. lactis* cells. It is interesting to observe that while ClfA is covalently sorted to the cell wall peptidoglycan in *S. aureus* and *L. lactis*, Fbl is not as efficiently sorted. The sortase substrate motif LPKTG is present in Fbl. However other factors must impact on its ability to become anchored to the cell wall. It has been shown that mutagenesis of a consensus sequence SIRK-G/S in the signal sequence of some staphylococcal surface proteins can impede efficient sorting (Bae and Schneewind, 2003). Changes to the signal sequence of Fbl at three residues might provide an explanation as to why Fbl sorting is inefficient. It is unlikely that the position of the LPXTG motif affects Fbl sorting. The LPXTG motif of Fbl is only one amino acid closer to the hydrophobic membrane spanning domain and positively charged C-terminal residues than the LPXTG motif of ClfA.

Considering that Fbl exhibits many similar characteristics to ClfA it is reasonable to assume that it is one of the main virulence factors of *S. lugdunensis*. An effective anti-staphylococcal approach such as monoclonal or polyclonal antibody therapy and/or vaccination using the A domain of Fbl could provide a tool to fight *S. lugdunensis* infections much more

quickly and effectively than antibiotics while also potentially providing protection against *S. aureus* infections.

Chapter 4

Properties of the Recombinant A domain of Fbl

4.1. Introduction

ClfA and Fbl bind to human fibrinogen, a 340kDa serum glycoprotein that consists of two disulfide bonded subunits which in turn comprise three nonidentical polypeptide chains Aα, Bβ, and γ (Herrick *et al.*, 1999). Following cleavage by thrombin, the molecule undergoes polymerization to fibrin to prevent the loss of blood upon vascular injury. ClfA binds to residues 398-411 of the γ-chain of fibrinogen, which are the same residues that the platelet integrin GPIIb/IIIa binds to in order to initiate fibrinogen-dependent platelet aggregation (Kloczewiak *et al.*, 1984). The binding of ClfA to fibrinogen is inhibited by millimolar concentrations of Ca^{2+} and Mn^{2+} which are thought to interact with cation binding sites in the ligand binding A domain of ClfA (O'Connell *et al.*, 1998). It was originally postulated that the cations interacted with putative EF-hand and MIDAS motifs. However, 3D structural analysis discounted the interaction of the EF-hand motif with Ca^{2+} ions as it did not co-ordinate any of the three Ca^{2+} ions that co-crystallized with the recombinant protein (Deivanayagam *et al.*, 2002). However the interaction of the MIDAS motif has not been refuted and the nature of the cation binding sites awaits further analysis.

The minimum fibrinogen-binding site of ClfA resides between residues 220-550 in the A-domain (McDevitt *et al.*, 1995). This region contains two similarly folded sub-domains. The crystal structure of the minimum binding region of ClfA region A suggests that the ligand-binding site resides in a hydrophobic pocket formed between the two sub-domains (Deivanayagam *et al.*, 2002). Several residues in this region (Tyr256, Pro336, Tyr338 and Lys389) were proposed to contact the terminal AGDV residues of the γ-chain of fibrinogen as predicted by SoftDock, a computer generated brute force docking procedure (Jiang and Kim, 1991). When these residues were subjected to mutational substitution, the fibrinogen binding activity of the resultant proteins was markedly reduced or completely abrogated. Valine 527, which was previously shown to be essential for binding to fibrinogen, also resides within this pocket. Several other staphylococcal fibrinogen binding proteins are proposed to have a similar domain architecture, including ClfB, which binds to the α-chain of fibrinogen,

and the fibronectin binding proteins FnBPA and FnBPB, the A domains of which also bind to the fibrinogen γ-chain.

Unfortunately, it has not as yet proven possible to co-crystallise the fibrinogen gamma-chain peptide with the minimum binding domain of ClfA region A. This would give a greater understanding of how exactly the terminal residues of the gamma chain of fibrinogen interact with the residues of ClfA involved in fibrinogen binding. Co-crystallization would also aid in the elucidation of the mechanism of binding. Ponnuraj *et al.* (2003) successfully co-crystallized the minimum binding domain of SdrG, the fibrinogen binding protein of *S. epidermidis*, with a modified β-chain peptide. They solved the structure of SdrG bound to its ligand and compared it with the structure of the unbound apo-SdrG. This led to the proposal of a "Dock, Lock and Latch" model for binding whereby an "open" form of SdrG first binds to the fibrinogen β-chain peptide, a loop of SdrG then encloses the peptide within the binding groove and the peptide is locked into position (Fig. 1.7). It was proposed that this could be a universal mechanism for staphylococcal proteins binding to host ligands but whether this actually applies to proteins such as Fbl and ClfA remains unclear.

Expression of the Fbl protein on the cell surface confers a fibrinogen binding phenotype to bacteria as the data in Chapter 3 shows. In this chapter, in order to further dissect its fibrinogen binding activities, the A domain of Fbl was expressed as a recombinant protein and purified. This recombinant protein was used to generate antibodies to Fbl, and in ELISA-based assays to compare and contrast its affinity for fibrinogen with that of ClfA, the fibrinogen binding protein of *S. aureus*. The recombinant $Fbl_{40\text{-}534}$ molecule was also used in ELISA assays in order to determine whether Fbl binds to the γ-chain of fibrinogen and C-terminal γ-chain peptides. Peptides conjugated to Glutathione-S-Transferase for ease of purification were analysed for their ability to inhibit the interaction of recombinant $Fbl_{40\text{-}534}$ with fibrinogen. Antibodies generated against Fbl were also analysed for their ability to recognize Fbl and ClfA released from solublised cell walls and stabilized protoplasts of *S. lugdunensis*, *S. aureus* and *L. lactis* expressing either Fbl or ClfA.

4.2 Results

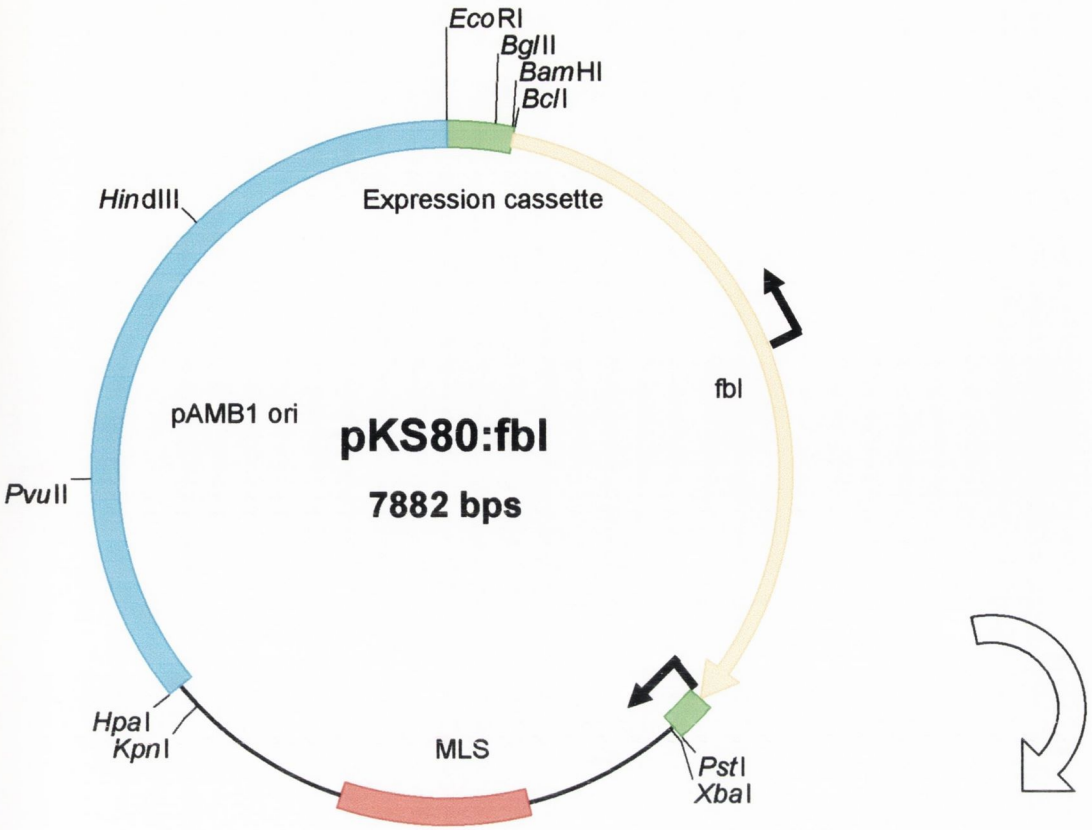
4.2.1. Expression and purification of recombinant Fbl region A (Fbl_{40-534})

Many attempts were made to clone the DNA encoding the A domain of Fbl into the expression vectors pQE30, and pGEX and cloning vectors pUC18 and pBLUESCRIPT in *E. coli*. Despite changing cloning sites and using host strains that supported the establishment of unstable chimeras, low frequencies of transformation were always obtained, and any putative recombinants had undergone rearrangements. An alternative strategy to obtain recombinant Fbl A domain involved inverse PCR of the pKS80:*fbl* plasmid to delete the DNA encoding the SD repeat region and the cell wall and membrane-spanning region of Fbl. These sequences were replaced with a stop codon which would generate a gene product 532 amino acids in length (Fig. 4.1). The ligated DNA was transformed into *L. lactis* MG1363. This resulted in a plasmid that expressed a truncated form of the Fbl protein stretching from the N-terminal methionine to the end of the A region. Fbl region A protein was secreted into the growth medium and purified by ammonium sulfate precipitation followed by ion exchange chromatography and separation by size fractionation. The recombinant protein had an apparent molecular weight of 70kDa as determined by SDS-PAGE with 10% acrylamide as opposed to its predicted molecular weight of 54.33kDa (Fig. 4.2). The sequence of the N-terminal 10 amino acids was determined to be EVERNLSKQQ showing that the gene product had been cleaved at the signal sequence cleavage site thus releasing the Fbl A domain into the supernatant. A subsequent experiment to generate a recombinant pKS80:*fblA* construct by inverse PCR that would secrete only the N23 domains of the A domain of Fbl was unsuccessful.

4.2.2. Generation of antibodies to $rFblA_{40-534}$

In order to generate antibodies that reacted with the Fbl protein, the purified A region of Fbl (residues 40-534) was used to immunise a New Zealand white rabbit as described in section 2.21. The titre of Fbl-specific antibodies was relatively high as a 1:5000 dilution of the antisera reacted with 1μg $rFblA_{40-534}$ in a Western immunoblot.

Figure 4.1 Schematic Diagram of pKS80:*fbl*A. The pKS80:*fbl* plasmid was amplified by inverse PCR to generate a PCR product that could be cut with *Sac*I and ligated to itself. The forward primer amplified pKS80:*fbl* from the stop codon after the *fbl* gene and the reverse primer amplified the pKS80:*fbl* DNA from the DNA encoding residue 534. The resultant recombinant plasmid when transformed into *L. lactis* MG1363 produced a truncated Fbl protein comprising amino acids 1 to 534. When secreted the processed molecule represented the entire A domain of Fbl comprising residues 40-534.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BamHI
BclI
HindIII
Expression cassette
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pKS80:fbl
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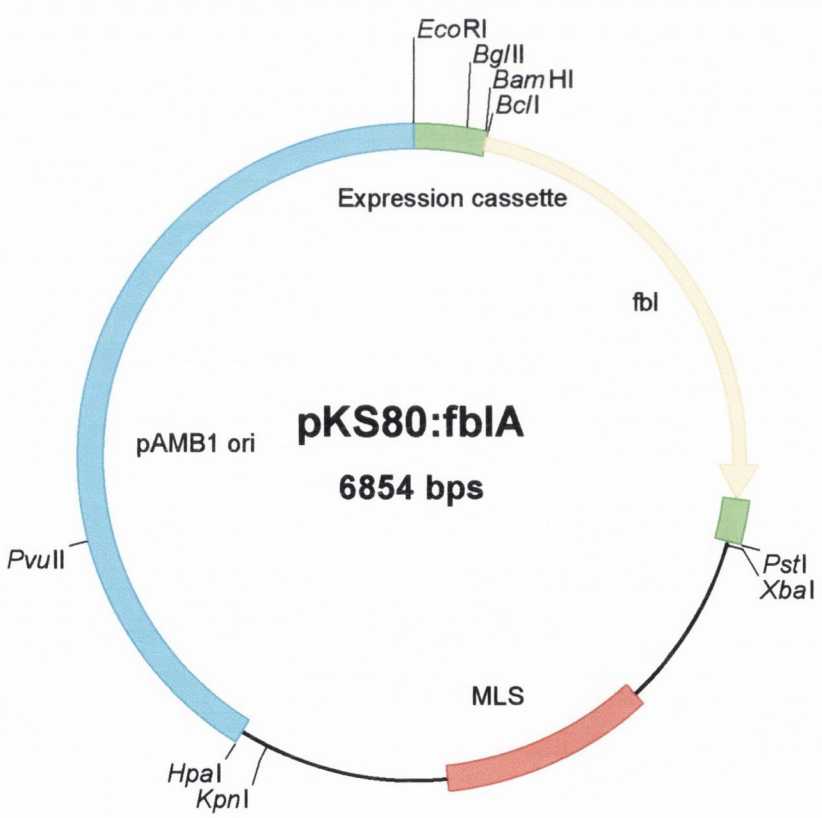
KpnI
MLS
PstI
XbaI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BglII
BamHI
BclI
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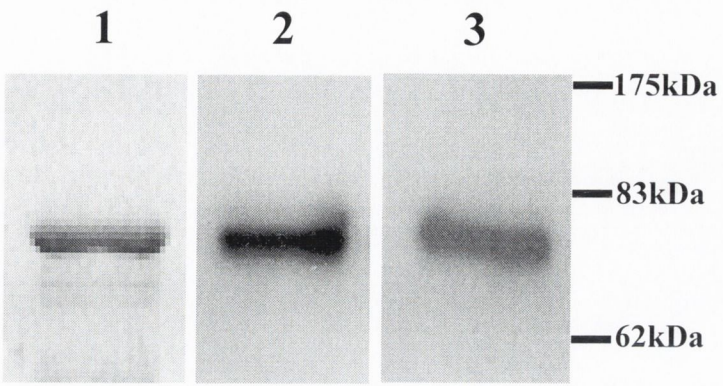
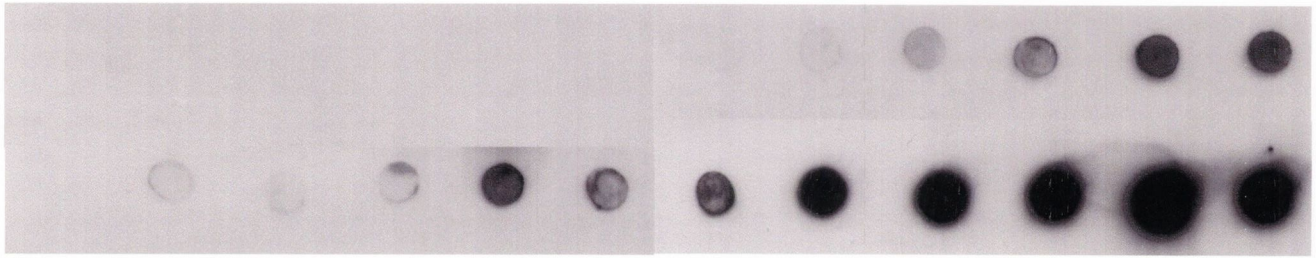


Figure 4.2 Recombinant Fbl_{40-534}. Lane 1. Coomassie stained SDS-PAGE gel of 20μg $rFbl_{40-534}$ purified from the supernatants of *L. lactis* pKS80:*fblA*. Lane 2. Western immunoblotting analysis of $rFbl_{40-534}$. $rFbl_{40-534}$ was separated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-Fbl region A antibodies generated against $rFbl_{40-534}$. Lane 3. Western immunoblotting analysis of $rFbl_{40-534}$ probing with polyclonal anti-ClfA region A antibodies generated against $rClfA_{40-559}$.

Figure 4.3 Comparative expression of Fbl on the surface of *S. lugdunensis* 1003 and *L. lactis* Fbl^{+}_{1003}. *S. lugdunensis* 1003 and *L. lactis* Fbl^{+}_{1003} cells at an OD600nm=2 were serially diluted two-fold and dotted onto nitrocellulose membrane. The membranes were probed with polyclonal anti-Fbl region A antibodies and visualised using horse radish peroxidase conjugated goat anti-rabbit IgG. This experiment was carried out twice with similar results.


S. lugdunensis 1003
L. lactis Fbl+1003
1/2048 1/1024 1/512 1/256 1/128 1/64 1/32 1/16 1/8 1/4 1/2 2

The antibodies did not need to be affinity purified as they did not show any significant cross reaction with cell wall associated proteins from either control *L. lactis* MG1363 pKS80 or a ClfA$^-$ mutant of *S. aureus*.

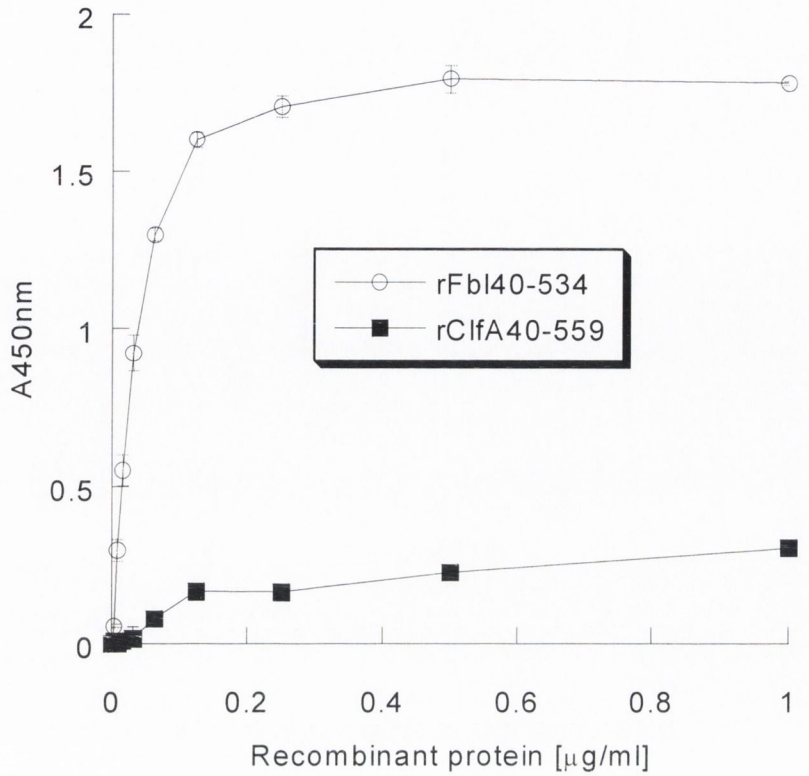
The generation of anti-Fbl region A antibodies also allowed estimation of the level of expression of Fbl on the surface of *L. lactis* Fbl$^+$ compared to *S. lugdunensis* strain 1003. Fig 4.3 shows that when serial two-fold dilutions of *L. lactis* Fbl$^+$ and *S. lugdunensis* cells were made and analysed by whole cell dot immunoblotting, *L. lactis* Fbl$^+$ expressed approximately 64-fold more Fbl protein on its surface than *S. lugdunensis*. This explains why, in the platelet aggregation study in Chapter 3, *L. lactis* Fbl$^+$ has a far shorter time to aggregation than *S. lugdunensis* and why *L. lactis* Fbl$^+$ has a high clumping titre whereas *S. lugdunensis* did not clump in soluble fibrinogen. There must be a threshold level of Fbl expression on the surface of bacterial cells for promoting clumping of cells in soluble fibrinogen and activation of platelet aggregation.

4.2.3. Affinity of antibodies for rFblA$_{40\text{-}534}$ and rClfA$_{40\text{-}559}$

Recombinant FblA$_{40\text{-}534}$ was analysed by SDS-PAGE and Western immunoblotting, probing with polyclonal rabbit anti-Fbl region A antibodies and polyclonal rabbit anti-ClfA region A antibodies. Figure 4.2 shows that both antibodies recognized FblA$_{40\text{-}534}$. The interaction between the antibodies and antigens was also analysed by ELISA. Figure 4.4 A indicates that the polyclonal rabbit antibodies generated against the A domain of Fbl have a 6-fold higher affinity for their cognate antigen rFbl$_{40\text{-}534}$ at saturation than the ClfA A domain rClfA$_{40\text{-}534}$. Similarly, rabbit polyclonal antibodies generated against rClfA showed a higher affinity for their cognate antigen rClfA40-559 than for rFbl40-534 (Fig. 4.4 B). However, anti-ClfA region A antibodies had only a 1.28-fold higher affinity for rClfA40-559 at saturation than rFbl40-534. Whether this difference in affinity for antigen affects the ability of different antibodies to inhibit the interaction of Fbl and ClfA with fibrinogen will be examined in Chapter 5.

Figure 4.4 Rabbit Anti-Fbl region A antibodies and Rabbit Anti-ClfA region A antibodies recognize $rFblA_{40\text{-}534}$ and $rClfA_{40\text{-}559}$. **A.** 96-well ELISA plates were coated with serial two-fold dilutions of either $rFbl_{40\text{-}534}$ or $rClfA_{40\text{-}559}$ ranging from 10μg/ml-150ng/ml. The plates were blocked with 2% BSA and then probed with 35μg/ml polyclonal rabbit anti-Fbl region A antibodies. **B.** 96-well ELISA plates coated with $rFbl_{40\text{-}534}$ or $rClfA_{40\text{-}559}$ were probed with 35μg/ml polyclonal rabbit anti-ClfA region A antibodies. Bound antibody was detected with goat anti-rabbit AP-conjugated antibodies followed by development with p-nitrophenyl phosphate substrate. Values represent the means of triplicate wells. This experiment was repeated three times with similar results.

A.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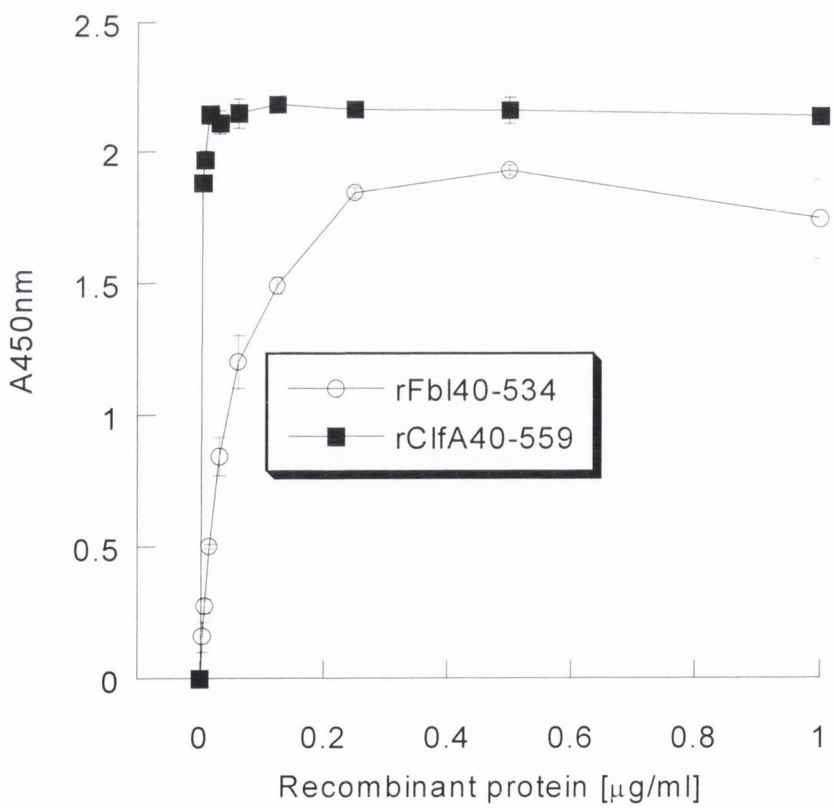
1.5
1
0.5
0
A450nm
rFbl40-534
rClfA40-559
0
0.2
0.4
0.6
0.8
1
Recombinant protein [μg/ml]

B.


2.5
2
1.5
1
0.5
0
A450nm
rFbl40-534
rClfA40-559
0
0.2
0.4
0.6
0.8
1
Recombinant protein [μg/ml]

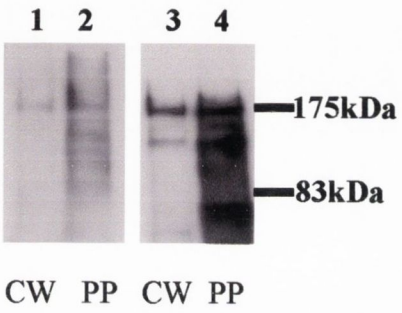
4.2.4. Rabbit antibodies raised against Fbl region A recognize ClfA expressed by of *S. aureus* and *L. lactis* ClfA$^+$

Cell surface-associated proteins were released from *S. aureus* Newman *spa* by lysostaphin digestion as described in section 2.8. The released proteins and the proteins from the stabilized protoplasts were then separated by SDS-PAGE on 10% acrylamide gels and analysed by Western immunoblotting, as described in section 2.10, using polyclonal rabbit anti-Fbl region A antisera. The cell wall protein fraction isolated from *S. aureus* yielded two immunoreactive proteins of 175kDa and 130kDa (Fig. 4.5 A lane 1 and 2). These are native and truncated forms of the ClfA protein that are also recognised by polyclonal anti-ClfA region A antibodies (O'Brien *et al.*, 2002). The anti Fbl-region A antibodies have been shown to be less efficient at recognizing ClfA than anti-ClfA region A antibodies in Fig. 4.4. This probably accounts for the weakness in labeling of ClfA by anti-Fbl antibodies (Fig. 4.5 A lane 3 and 4). It is evident from Fig. 4.5 that some ClfA is retained in the protoplast in stationary phase *S. aureus* Newman cells. This is contrary to studies performed on *S. aureus* 8325-4 where ClfA was shown to be completely sorted to the cell wall (Downer *et al.*, 2002). However, *S. aureus* strain Newman produces substantially more ClfA than *S. aureus* strain 8325-4.

Cell surface associated proteins were released from the stabilized *L. lactis* protoplasts carrying the pKS80:*clfA* plasmid and pKS80 with no insertion, by lysozyme and mutanolysin digestion as described in section 2.9. Proteins were separated by SDS-PAGE and analysed by Western immunoblotting, as described in section 2.10, using polyclonal rabbit anti-Fbl region A antisera. The proteins isolated from stabilized protoplasts of *L. lactis* ClfA$^+$ yielded several immunoreactive proteins, two of which correspond to the 175kDa and 130kDa bands that were recognized by anti-ClfA region A antibodies (Fig. 4.5 B lane 1 and 2). The anti-Fbl region A antibodies have insufficient affinity for ClfA to detect the smaller amount of cell wall located ClfA. This is likely due to the low affinity of the anti-Fbl region A antibodies for ClfA as demonstrated in Fig. 4.4. Therefore despite the presence of ClfA in the cell wall fraction of *L. lactis* it is not detected in a Western immunoblot by anti-Fbl region A antibodies. No immunoreactive bands were present in the cell wall solublised protein fraction or stabilized protoplast fraction isolated from *L. lactis* pKS80 (not shown).

Figure 4.5 Polyclonal anti-Fbl region A antibodies recognize Fbl in *S. lugdunensis*, ClfA in *S. aureus* and Fbl and ClfA expressed in the heterologous expression *L. lactis* MG1363. **A.** Lanes 1 and 3, Lysostaphin solublised cell wall fraction of *S. aureus* Newman *spa*, lanes 2 and 4, stabilised protoplasts of *S. aureus* Newman *spa*. **B.** Lanes 1 and 3, stabilised protoplast fraction of *L. lactis* ClfA$^+$, lanes 2 and 4, mutanolysin/lysozyme solublised cell wall fraction of *L. lactis* ClfA$^+$. **C.** Lanes 1 and 3, stabilised protoplasts of *S. lugdunensis* 964, lanes 2 and 4, lysostaphin solublised cell wall fraction of *S. lugdunensis* 964. **D.** Lanes 1 and 3, stabilised protoplast fraction of *L. lactis* Fbl$^+$, lanes 2+4, mutanolysin/lysozyme solublised cell wall fraction of *L. lactis* Fbl$^+$. Lanes 1 and 2 of each section probed with 1:1000 dilution of polyclonal anti-Fbl region A antisera and lanes 3 and 4 of each section probed with 1:1000 dilution of polyclonal anti-ClfA region A antisera.

A.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83kDa
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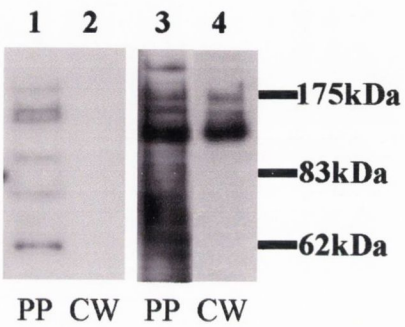
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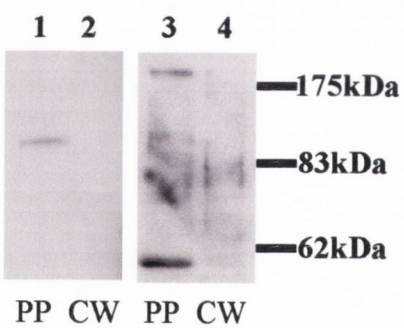
83kDa
62kDa
PP CW PP CW

C.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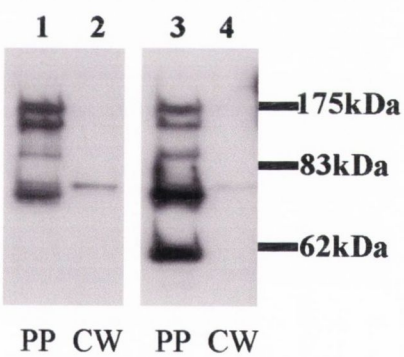
175kDa
83kDa
62kDa
PP CW PP CW

D.


1 2 3 4
175kDa
83kDa
62kDa
PP CW PP CW

4.2.5. Rabbit Anti-Fbl region A antibodies recognize Fbl expressed by of *S. lugdunensis* and *L. lactis* Fbl+

Cell surface associated proteins were released from the stabilized *S. lugdunensis* strain 964 protoplasts by lysostaphin digestion as described in section 2.8. The released proteins were then separated by SDS-PAGE and analysed by Western immunoblotting, as described in section 2.10, using polyclonal rabbit anti-Fbl region A antisera. The lysed protoplast fraction isolated from *S. lugdunensis* yielded one immunoreactive band at 200kDa when probed with anti-ClfA region A antibodies (Fig. 4.5 C lane 3) and there was one band at 90kDa present in the stabilized protoplast fraction when probed with anti-Fbl region A antibodies (Fig. 4.5 C lane 1). This is likely to be a breakdown product of Fbl.

The size of full length Fbl was verified by analysis of Fbl expressed in the *L. lactis* surrogate heterologous expression system. Cell surface associated proteins were released from the stabilized *L. lactis* protoplasts carrying the pKS80:*fbl* plasmid and pKS80 with no insertion, by lysozyme/mutanolysin digestion as described in section 2.9 for 10 minutes. These proteins were then separated by SDS-PAGE in 10% acrylamide gels and analysed by Western immunoblotting, as described in section 2.10, using polyclonal rabbit anti-Fbl region A antisera. The lysozyme/mutanolysin solublised cell wall protein fraction isolated from *L. lactis* carrying the pKS80:*fbl* plasmid yielded one immunoreactive protein of 70kDa (Fig. 4.5 D lane 2). This band was also observed when the same samples were probed using polyclonal anti-ClfA region A antisera indicating that both sets of antibodies appear to be recognizing similar epitopes in Fbl whereas the ability of the Fbl antibodies to recognize ClfA is significantly less efficient. This correlates with the data seen in Fig. 4.4. No immunoreactive bands were present in the protein fraction isolated from *L. lactis* pKS80 suggesting that the bands observed in lane are representative of the Fbl protein (data not shown). When *L. lactis* Fbl^+ cells were digested with lysozyme/mutanolysin for longer than 15 minutes a progressive proteolytic degradation of the Fbl protein was observed (data not shown), a phenomenon that has previously observed when *L. lactis* ClfA+ cells are subjected to lysozyme/mutanolysin digestion for longer than 15 mins (O'Brien *et al.*, 2002).

4.2.6. Binding of recombinant Fbl_{40-534} to fibrinogen

ELISA type binding assays are used to study the binding of recombinant MSCRAMMS to their ligands. Microtitre wells were coated with a fixed concentration of human fibrinogen and then incubated with varying concentrations of $rFbl_{40-534}$. Binding of increasing concentrations of $rFbl_{40-534}$ to immobilised fibrinogen was dose dependent and saturable indicating that the interaction is specific. Fbl has a lower affinity for immobilised fibrinogen than ClfA (apparent K_D of 1.5μM) as determined by direct binding in an ELISA assay (Fig. 4.6 A) whereas $ClfA_{40-559}$ bound to fibrinogen with an apparent K_D of 100nM (Fig. 4.6 B). This experiment also showed that the ligand binding region of Fbl is the A domain between residues 40 and 534.

4.2.7. Blocking of Fbl- and ClfA-promoted adherence of *L. lactis* to immobilised fibrinogen by recombinant Fbl_{40-534}

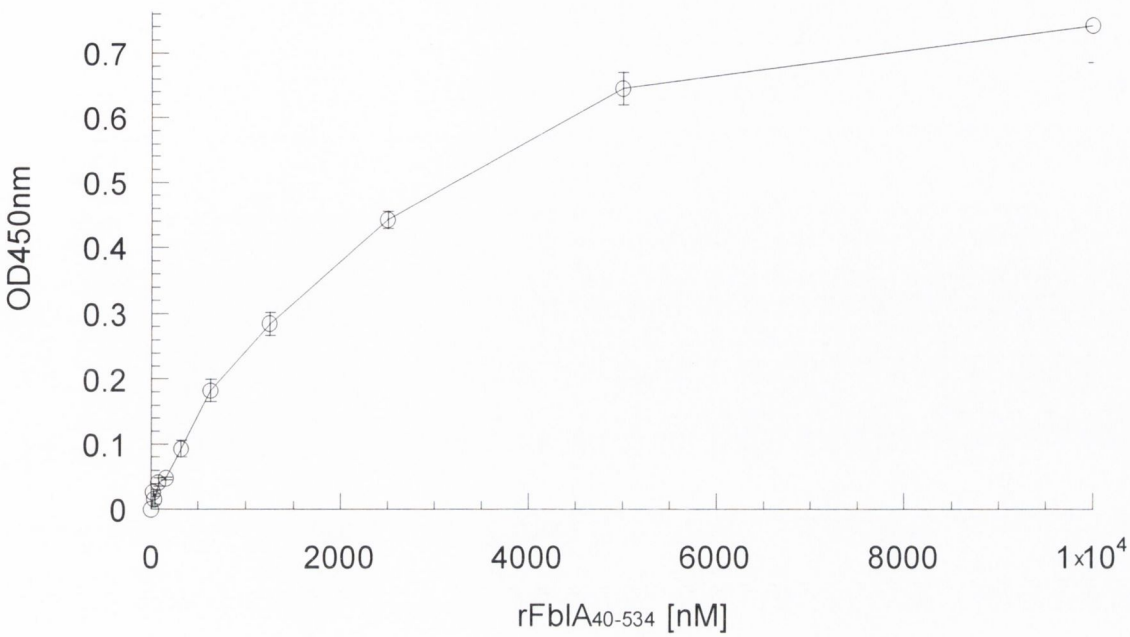
In order to determine the part of the fibrinogen molecule recognized by Fbl, the ability of $rFbl_{40-534}$ to cause inhibition of fibrinogen binding activity of *L. lactis* Fbl^+ and *L. lactis* $ClfA^+$ was determined. Adherence of both strains to immobilised fibrinogen was inhibited by increasing concentrations of $rFbl_{40-534}$ in a dose-dependent manner (Fig. 4.7). Thus rFbl can inhibit ClfA promoted adherence of *L. lactis* to immobilised fibrinogen. This suggests that Fbl and ClfA are competing for the same binding site in the γ-chain of fibrinogen.

4.2.8. Binding of $rFbl_{40-534}$ and $rClfA_{40-559}$ to the fibrinogen γ-chain peptide

In order to define the binding site of Fbl to a specific region within the γ-chain of fibrinogen two γ-chain peptides fused to the GST affinity purification tag were tested in an ELISA-type binding assay. The GST-tag facilitated purification of the peptides. The γ-chain-GST comprised the C-terminal 17 residues of the wild type γ-chain peptide sequence. p16-GST comprised the C-terminal 17 residues of the γ-chain peptide

Figure 4.6 Binding of recombinant Fbl_{40-534} and $ClfA_{40-559}$ to immobilised fibrinogen. Microtitre wells were coated with a solution of 10μg/ml human fibrinogen. Increasing concentrations of $rFbl_{40-534}$ (**A**) or $rClf_{40-559}$ (**B**) were incubated in the wells for 1 hr at room temperature. Bound protein was detected by polyclonal anti-ClfA region A antibody and goat anti-rabbit AP-conjugated antibodies followed by development with p-nitrophenyl phosphate substrate. Values represent the means of triplicate wells. This experiment was repeated three times with similar results.

A.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0.6
0.5
0.4
0.3
0.2
0.1
0
0
2000
4000
6000
8000
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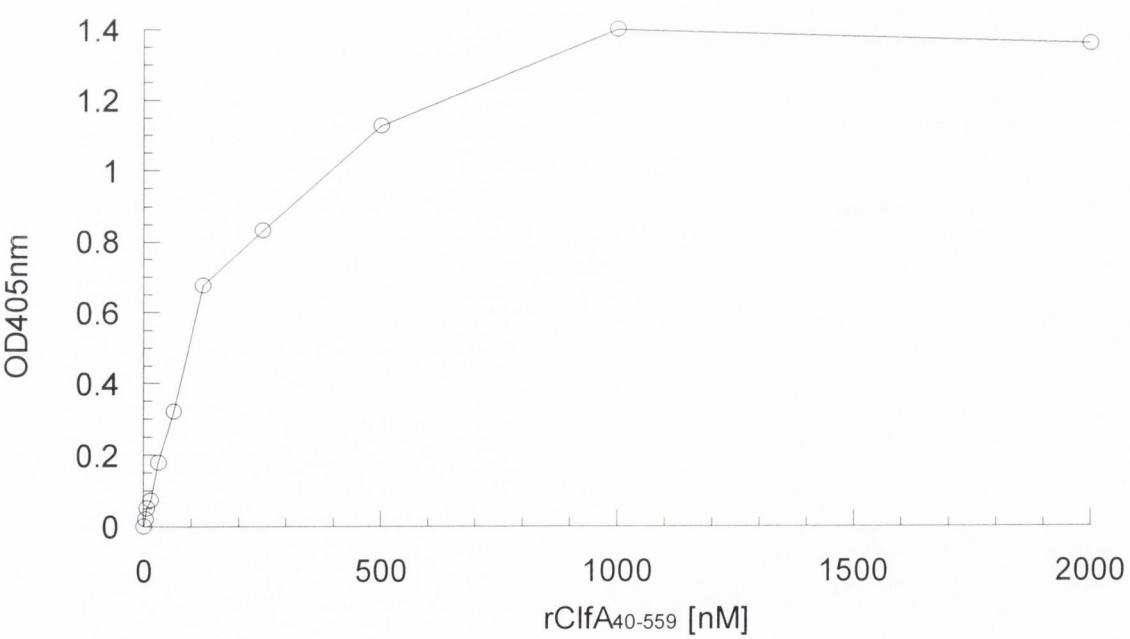
rFblA40-534 [nM]

B.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0.6
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0.2
0
0
500
1000
1500
2000
rClfA40-559 [nM]

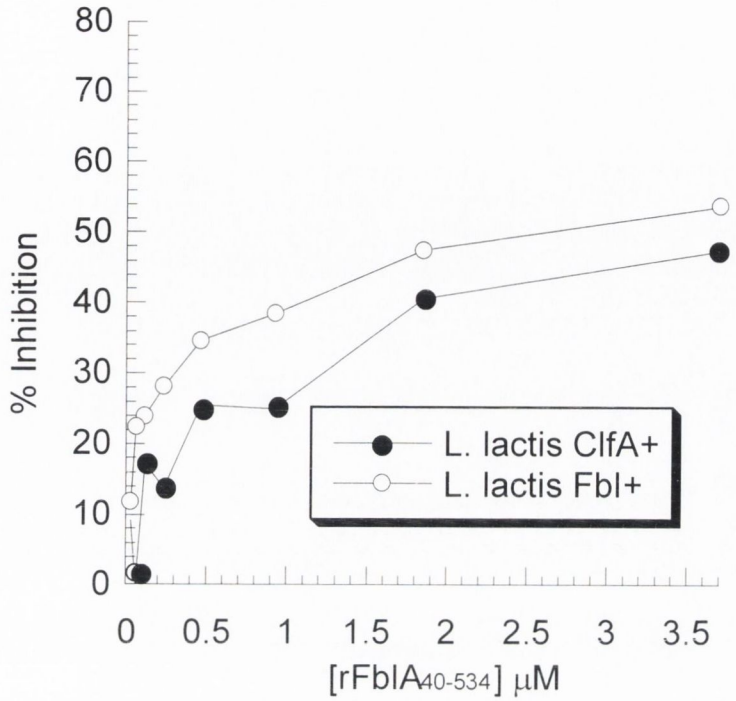
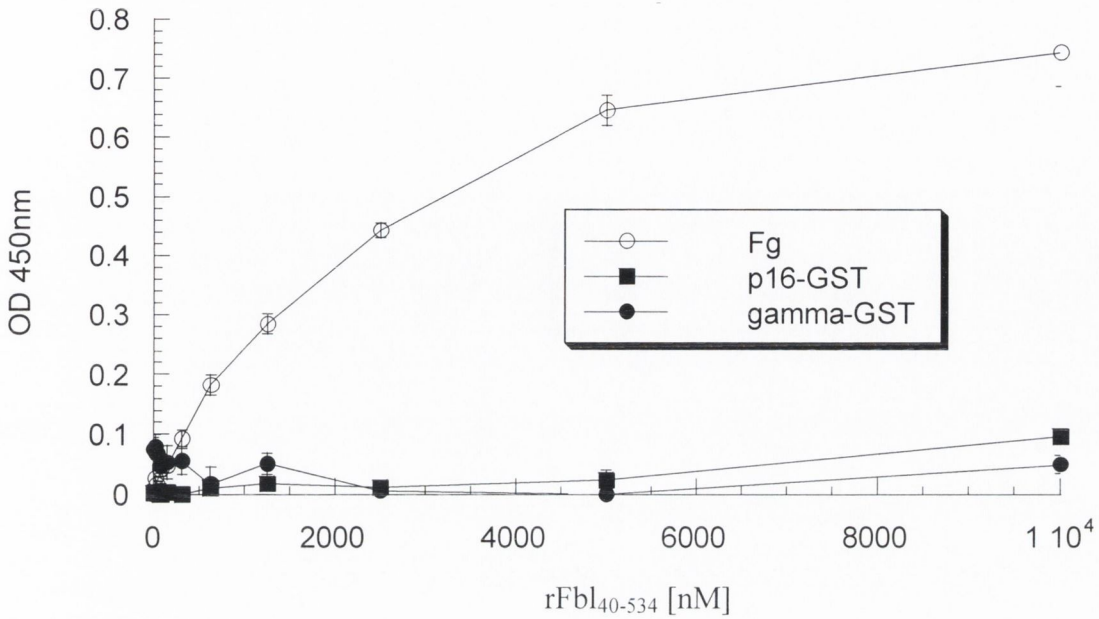


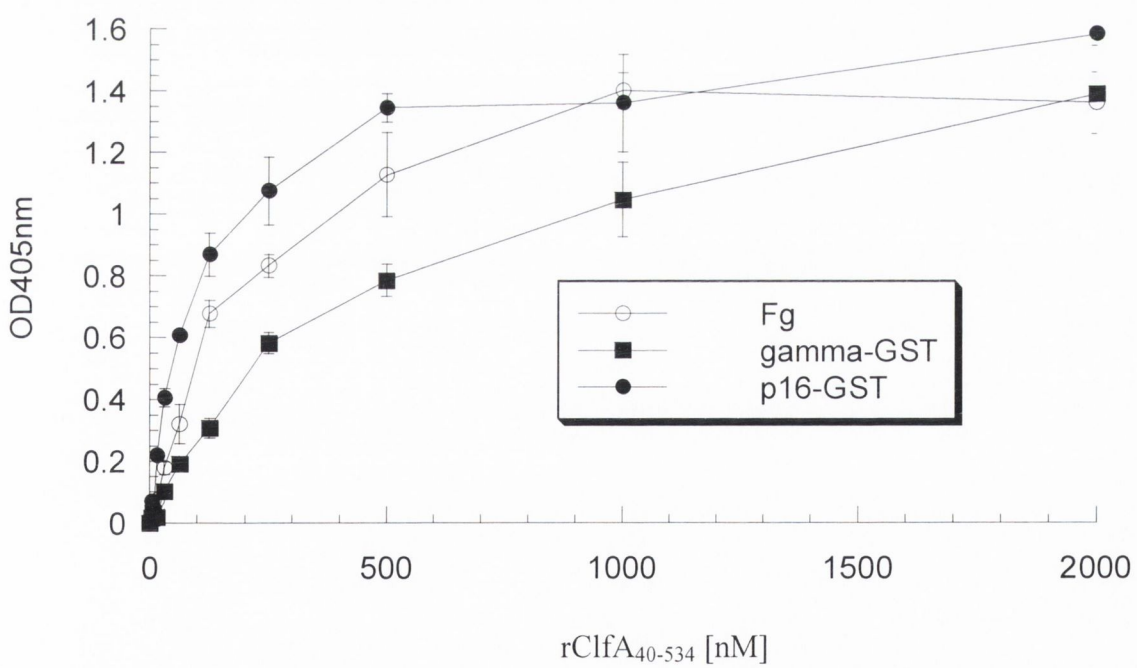
Figure 4.7 Inhibition of Fbl and ClfA mediated bacterial adherence to fibrinogen by recombinant $Fbl_{40\text{-}534}$. *L. lactis* $ClfA^+$ and *L. lactis* Fbl^+ fibrinogen binding activity was tested in the presence of varying concentrations of $rFbl_{40\text{-}534}$. Cells were stained with crystal violet and washed with PBS. Cells that bound to fibrinogen coated microtitre plate wells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages inhibition of binding of cells to fibrinogen compared to control (no $rFbl_{40\text{-}534}$). This experiment was repeated three times with similar results. Error bars do not expand outside the circles.

Figure 4.8 Comparative binding of recombinant Fbl_{40-534} and recombinant $ClfA_{40-559}$ to fibrinogen, a GST fusion to the gamma chain of fibrinogen and a GST fusion to the p16 gamma chain mutant peptide. Microtitre wells were coated with 1μg human fibrinogen, γ-GST or p16-GST. Increasing concentrations of $rFbl_{40-534}$ (**A**) or $rClf_{40-559}$ (**B**) were incubated in the wells for 1 hr at room temperature. Bound protein was detected by polyclonal anti-ClfA region A antibody and goat anti-rabbit IgG conjugated to alkaline phosphatase followed by development with p-nitrophenyl phosphate substrate. Values represent the means of triplicate wells. This experiment was repeated three times with similar results.

A.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 450nm
0
2000
4000
6000
8000
1 10^4
rFbl40-534 [nM]
Fg
p16-GST
gamma-GST

B.


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD405nm
0
500
1000
1500
2000
rClfA40-534 [nM]
Fg
gamma-GST
p16-GST

sequence with a D410A substitution. The p16-GST peptide has been shown to bind to ClfA with a greater affinity than the wild-type γ-chain-GST peptide (M. Hook, personal communication). Both peptide-GST fusions were coated onto 96-well microtitre plates. Fig. 4.8 B shows that the binding of increasing concentrations of $rClfA_{40-559}$ to immobilised fibrinogen, γ-chain-GST and p16-GST exhibited saturation kinetics. This demonstrated a specific interaction between ClfA and fibrinogen and localized the binding region to the C-terminal γ-chain peptide. Fbl, however, has a lower affinity for fibrinogen than ClfA and bound to the γ-chain-GST and p16-GST too weakly for the kinetics of the interaction to be determined.

The ability of synthetic γ-chain peptide and p-16 peptide to inhibit the interaction of $rFbl_{40-534}$ and $rClfA_{40-559}$ with fibrinogen was analysed (Fig. 4.9). The γ-chain peptide inhibited the interaction of $rClfA_{40-559}$ poorly, reaching a maximum of 25% inhibition. The p-16 peptide, however, inhibited the interaction of $rClfA_{40-559}$ and fibrinogen up to 75%. Interestingly, a different profile of inhibition was observed with $rFbl_{40-534}$. The γ-chain peptide inhibited the binding of $rFbl_{40-534}$ and fibrinogen to a maximum of 50%. However, increasing the concentration of inhibitor above 15μM decreased the observed inhibition. A similar phenomenon was observed for the p-16 peptide where a maximum inhibition of 70% was observed but the highest inhibitor concentration decreased inhibition.

4.2.9. Blocking of $rFbl_{40-534}$ and $rClfA_{40-559}$ binding to fibrinogen by γ-chain peptides

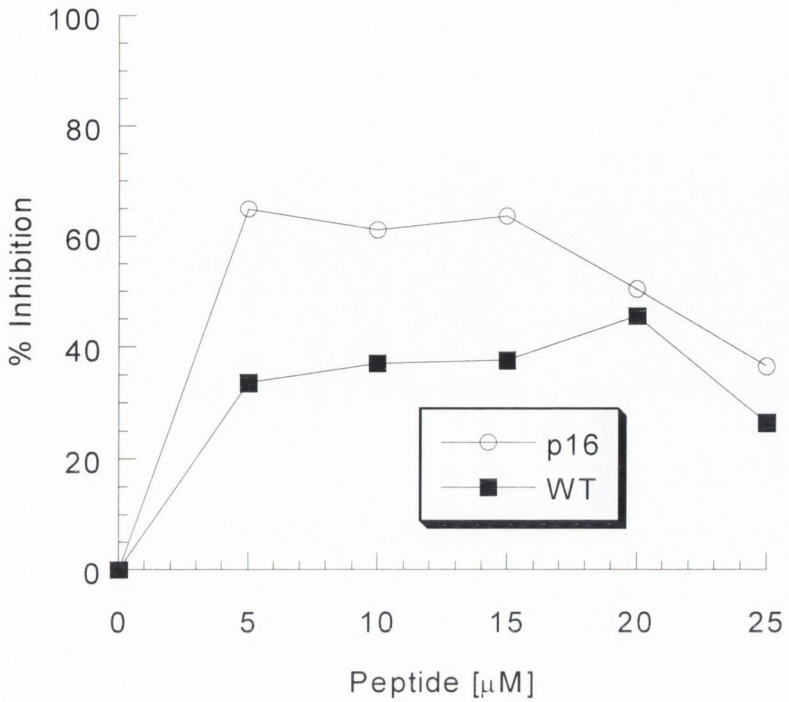
The interaction of $rFbl_{40-534}$ and $rClfA_{40-559}$ with fibrinogen was further analysed by testing the ability of a panel of synthetic γ-chain peptides representing a complete alanine scan of the terminal 17 residues of the γ-chain of fibrinogen to inhibit $rFbl_{40-534}$ and $rClfA_{40-559}$ adherence to fibrinogen. Table 4.1 shows the complete set of peptides used. Fig. 4.10 shows the peptides having the greatest inhibition of binding of $rFbl_{40-534}$ to fibrinogen were p7, p8 and p16. However only p7 and p8 caused a markedly greater inhibition of binding of $rFbl_{40-534}$ compared to $rClfA_{40-559}$. Conversely, p11, previously

Table 4.1 Sequence of the synthetic γ-chain peptides

Peptide name	Sequence
Wild type	GEGQQHHLGGAKQAGDV
P1	AEGQQHHLGGAKQAGDV
P2	GAGQQHHLGGAKQAGDV
P3	GEAQQHHLGGAKQAGDV
P4	GEGAQHHLGGAKQAGDV
P5	GEGQAHHLGGAKQAGDV
P6	GEGQQAHLGGAKQAGDV
P7	GEGQQHALGGAKQAGDV
P8	GEGQQHHAGGAKQAGDV
P9	GEGQQHHLAGAKQAGDV
P10	GEGQQHHLGAAKQAGDV
P11	GEGQQHHLGGSKQAGDV
P12	GEGQQHHLGGAAQAGDV
P13	GEGQQHHLGGAKAAGDV
P14	GEGQQHHLGGAKQSGDV
P15	GEGQQHHLGGAKQAADV
P16	GEGQQHHLGGAKQAGAV
P17	GEGQQHHLGGAKQAGDA

Figure 4.9 Inhibition of recombinant Fbl_{40-534} and recombinant Clf_{40-559} bindng to immobilized fibrinogen by synthetic C-terminal γ-chain peptides. Microtitre wells were coated with 10μg/ml human fibrinogen. $rFbl_{40-534}$ (**A**) or $rClf_{40-559}$ (**B**) were preincubated with increasing concentrations of a synthetic C-terminal γ-chain peptide ($_{395}$GEGQQHHLGGAKQAGDV$_{411}$) or with increasing concentrations of the p16 peptide which substitutes D410A, for 1 h at room temperature. After incubation in the wels for 1hr at room temperature, bound protein was detected with polyclonal anti-ClfA region A antibody and goat anti rabbit Alkaline Phosphatase-conjugated antibodies, followed by development with p-nitrophenyl phosphate. Values represent the means of triplicatewells and are expressed as the percentages inhibition of binding to fibrinogen compaed to control (no inhibitor peptide). This experiment was repeated three times with smilar results.

A.


100
80
60
40
20
0
% Inhibition
p16
WT
0
5
10
15
20
25
Peptide [μM]

B.

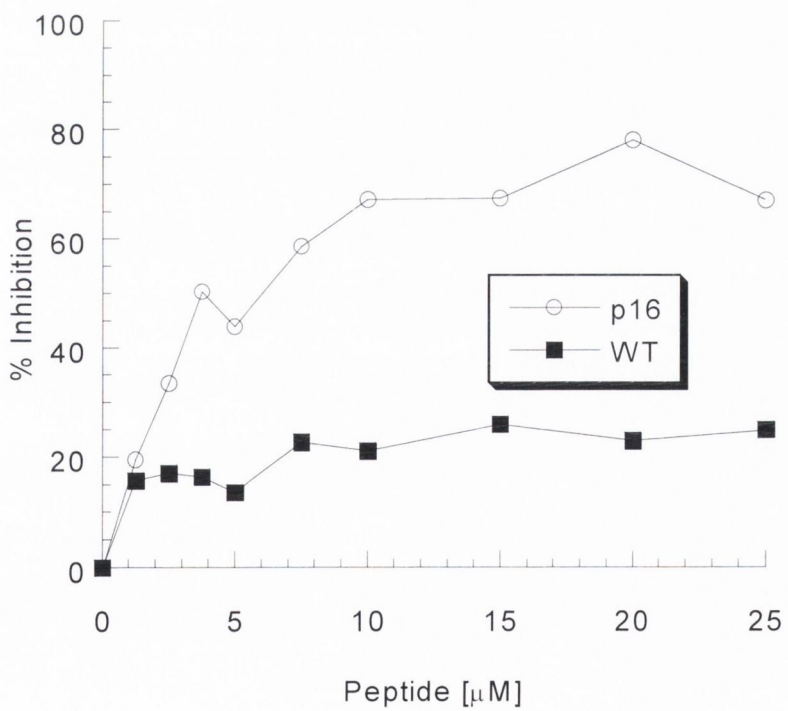
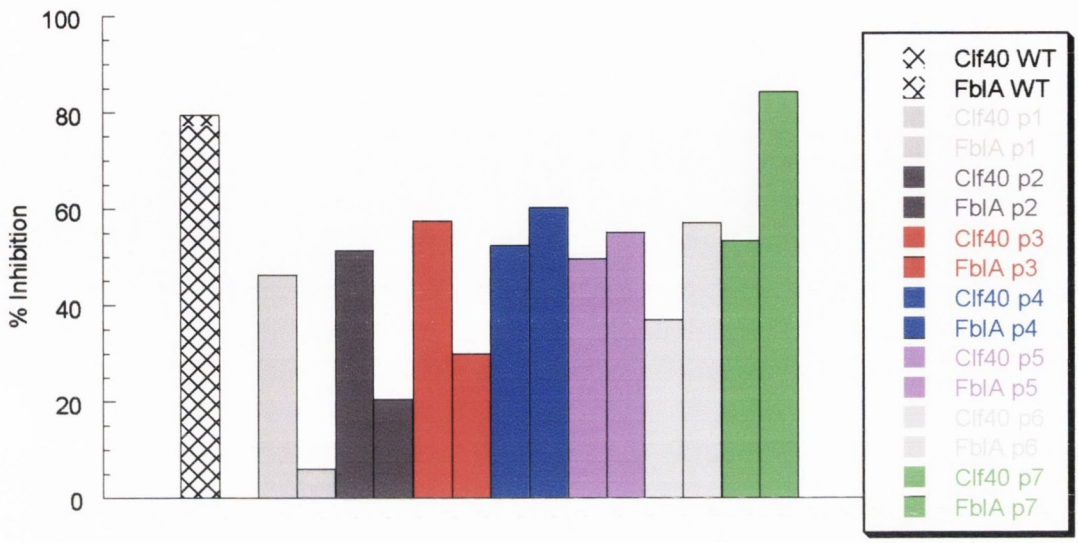

100
80
60
40
20
0
% Inhibition
p16
WT
0
5
10
15
20
25
Peptide [μM]

Figure 4.10 Inhibition of recombinant Fbl_{40-534} and recombinant Clf_{40-559} binding to immobilized fibrinogen by synthetic C-terminal γ-chain peptides representing an alanine scan of the terminal 17 amino acids of the γ-chain of fibrinogen. Microtitre wells were coated with 1μg human fibrinogen. $rFbl_{40-534}$ or $rClf_{40-559}$ were preincubated with 25μM of each of the synthetic C-terminal γ-chain peptides for 1 h at room temperature. After incubation in the wells for 1 h at room temperature, bound protein was detected polyclonal anti-ClfA region A antibody and goat anti-rabbit antibodies conjugated to alkaline phosphatase, followed by development with p-nitrophenyl phosphate. Values represent the means of triplicate wells and are expressed as the percentages inhibition of binding to fibrinogen compared to control (no inhibitor peptide). This experiment was repeated twice with similar results.


100
80
60
40
20
0
% Inhibition
Clf40 WT
FblA WT
Clf40 p1
FblA p1
Clf40 p2
FblA p2
Clf40 p3
FblA p3
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FblA p4
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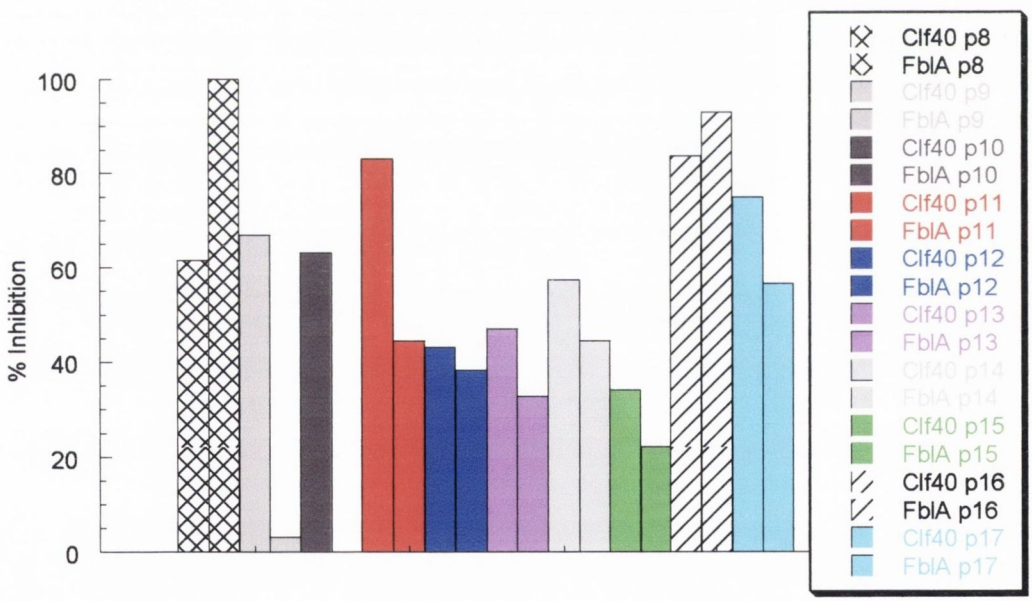
FblA p6
Clf40 p7
FblA p7


100
80
60
40
20
0
% Inhibition
Clf40 p8
FblA p8
Clf40 p9
FblA p9
Clf40 p10
FblA p10
Clf40 p11
FblA p11
Clf40 p12
FblA p12
Clf40 p13
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Clf40 p17
FblA p17

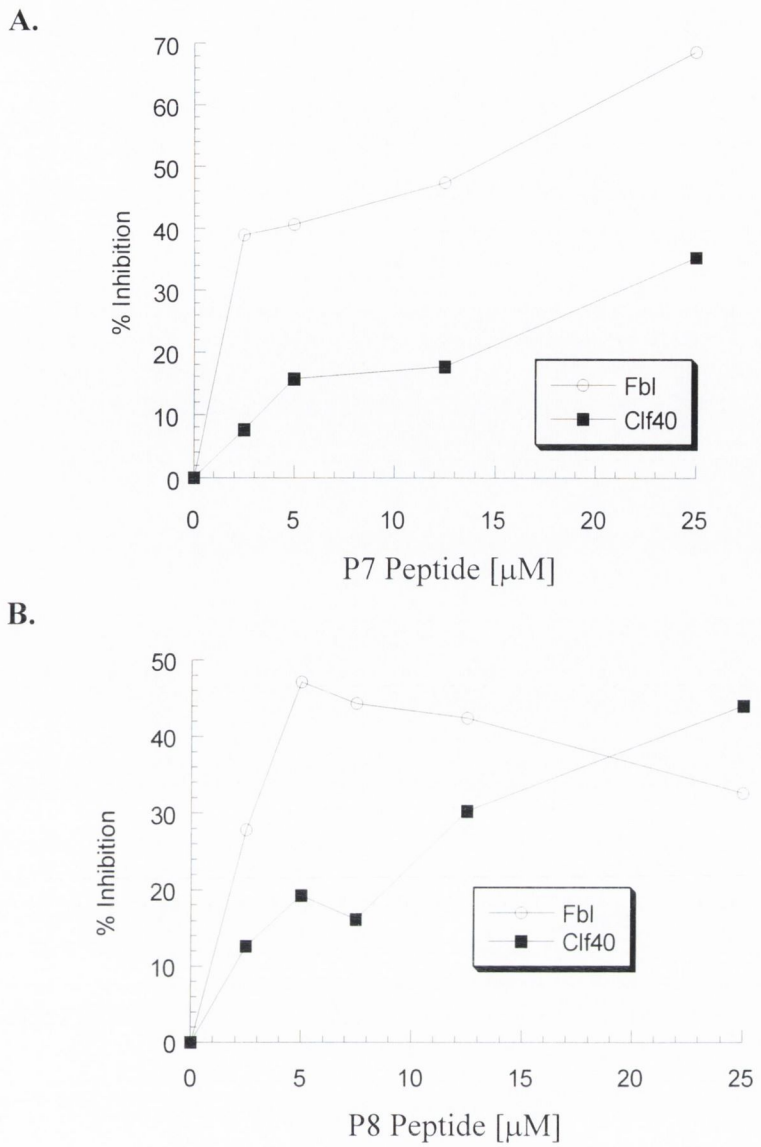


Figure 4.11 Inhibition of $rFbl_{40-534}$ and $rClfA_{40-559}$ binding to fibrinogen by synthetic γ-chain peptides p7 and p8. Microtitre wells were coated with 1μg human fibrinogen. $rFbl_{40-534}$ or $rClf_{40-559}$ were preincubated with doubling dilutions of either p7 (**A**) or p8 (**B**) C-terminal γ-chain peptides for 1 h at room temperature. After incubation in the wells for 1 h at room temperature, bound protein was detected polyclonal anti-ClfA region A antibody and goat anti rabbit AP-conjugated antibodies, followed by development with p-nitrophenyl phosphate. Values represent the means of triplicate wells and are expressed as the percentages inhibition of binding to fibrinogen compared to control (no inhibitor peptide). This experiment was repeated twice with similar results.

shown to cause a greater inhibition of binding of $rClfA_{40-559}$ to fibrinogen was less able to inhibit binding of $rFbl_{40-534}$.

A dose-response inhibition of binding of $rFbl_{40-534}$ to fibrinogen by p7 and p8 was carried out to determine whether the peptides inhibited binding of $rFbl_{40-534}$ to fibrinogen to a greater extent than $rClfA_{40-559}$ binding to fibrinogen. Both peptides must have a greater affinity for $rFbl_{40-534}$ than $rClfA_{40-559}$ because they both inhibited the interaction of $rFbl_{40-534}$ with fibrinogen to a greater extent than they inhibited $rClfA_{40-559}$. The p7 peptide appeared to inhibit $rFbl_{40-534}$ binding to a greater extent than $rClfA_{40-559}$ binding and caused up to 65% inhibition whereas the maximum inhibition of ClfA binding was 35% (Fig 4.11 A). The p8 peptide was less effective than the p7 peptide at inhibiting $rFbl_{40-534}$ binding to fibrinogen yet still had a greater affinity for $rFbl_{40-534}$ than $rClfA_{40-559}$ (Fig 4.11 B).

Some of the peptides exhibited none or minimal inhibitory effects upon the binding of $rFbl_{40-534}$ to fibrinogen. The wildtype, p1, p9, p10 and p11 peptides had a significantly lower inhibitory effect upon $rFbl_{40-534}$ binding than $rClfA_{40-559}$ binding to fibrinogen (Fig. 4.11).

4.3. Discussion

The cloning and expression of the *fbl* gene and purification of recombinant Fbl in *L. lactis* MG1363 represents a novel method for the generation of native recombinant proteins. It has many potential applications. *L. lactis* has been investigated as a potential live vaccine vector (Dieye *et al.*, 2003) and as a delivery mechanism for IL-10 as a treatment for inflammatory bowel disease (Steidler, 2002). The difficulty in cloning the DNA encoding the Fbl A encoding region was not anticipated when this project was begun and the failure of all the procedures tested to clone the Fbl A region remains a mystery. Previous publications cite unusual DNA conformations and stem-loop structures alongside modifications of DNA molecules as reasons for the unclonability of certain genes (Razin *et al.*, 2001). However, in the future, difficulties in cloning and expressing genes from a Gram positive background could be resolved by cloning in *L. lactis* vectors. The one drawback appeared to be that less protein was obtained using this expression system compared to *E. coli* expression systems. Nevertheless *L. lactis* could be developed to express larger amounts of proteins from stronger promoters. Affinity tags could be incorporated for more efficient purification of recombinant proteins.

Recombinant $Fbl_{40\text{-}534}$ was used to generate specific anti-Fbl region A polyclonal antibodies. Bands in the Western immunoblot of proteins solublised from the cell wall of *L. lactis* Fbl^+ are attributable to full length Fbl and its proteolytic degradation products because the antibody did not react with extracts of *L. lactis* pKS80. As an Fbl^- isogenic mutant of *S. lugdunensis* was not available, it must be assumed that the 200kDa band observed in Western immunoblots of *S. lugdunensis* proteins separated by SDS-PAGE is full length Fbl. Interestingly, the anti-Fbl and anti-ClfA antibodies recognized both ClfA and Fbl but reacted with different patterns of degradation products. The proteins have 60% identical residues and must have some epitopes that are not recognized by the heterologous antibody.

The majority of cell wall anchored proteins studied thus far (ClfA, ClfB, FnBPA, FnBPB) were intact and fully functional when expressed on the surface of *L. lactis* MG1363, but had undergone proteolytic degradation when the solubilized cell wall-associated and envelope proteins were analysed by SDS-PAGE and Western

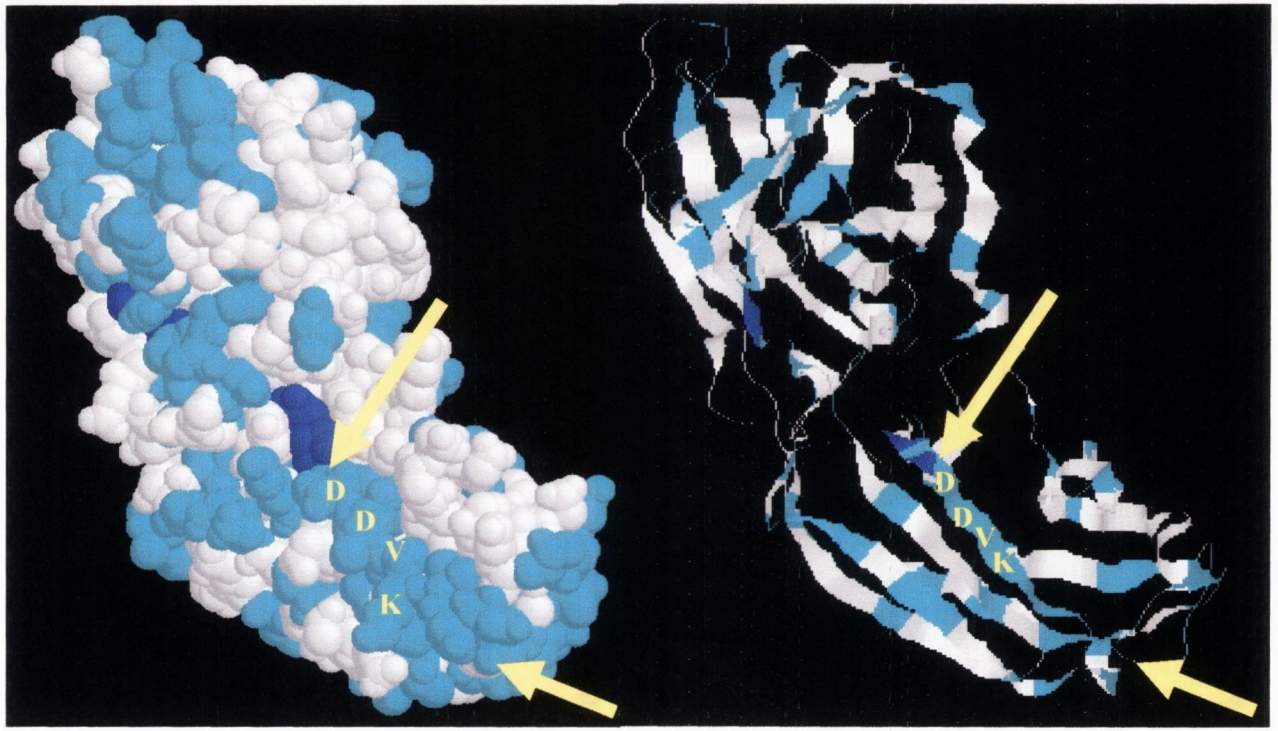
immunoblotting (O'Brien *et al.*, 2002). Degradation appeared to occur when proteins were isolated from the cell wall by digestion of cells with murolytic enzymes. This is proposed to expose the cells to the membrane located protease HtrA. HtrA is the only chromosomally encoded protease in *L. lactis* that is involved in degradation of heterologous extracellular or misfolded secreted proteins (Poquet *et al.*, 2000; Miyoshi *et al.*, 2002). MG1363 has been cured of plasmids that express extracellular proteases. Therefore a Δ*htrA* strain of MG1363 would represent an ideal expression host for heterologous expression of unstable staphylococcal surface proteins. Nevertheless it is clear that the majority of Fbl protein is intact and fully functional on the surface of *L. lactis* MG1363 pKS80:*fbl*. It is concluded that Fbl is necessary and sufficient for bacterial attachment to fibrinogen.

ClfA is regulated by the stationary phase sigma factor σ^B (Nicholas *et al.*, 1999). It has been shown to be translated and transcribed in stationary phase. It is possible that protein sorting is inefficient in cells that are not actively growing and synthesizing new peptidoglycan. This could provide an explanation as to why there is a substantial amount of ClfA protein retained by protoplasts after lysostaphin digestion of *S. aureus* Newman cells.

It is interesting that rabbit polyclonal anti-ClfA region A antibodies and rabbit polyclonal anti-Fbl antibodies exhibited such a striking difference in affinity for the two antigens. It should be noted that the Fbl antibodies were raised in a specific-pathogen-free rabbit whereas the ClfA antibodies were raised in a rabbit that was not kept in specific-pathogen-free conditions. It is possible that the ClfA-immunized rabbit was exposed to a wider range of antigens which enabled it to recognize Fbl with a comparable affinity to that of ClfA. The specific-pathogen-free rabbit would not have come into contact with staphylococcal antigens. This might explain the lower level of anti-Fbl region A antibodies as compared to ClfA. The reaction of the anti-Fbl region A antibodies with $rFbl_{40\text{-}534}$ and $rClfA_{40\text{-}559}$ may more closely represent the differences in immunogenicity between Fbl and ClfA indicated by the 40% difference in amino acid sequence. Epitopes that differ between the two proteins may be the immunodominant epitopes.

Figure 4.12 A RasMol generated space-filled diagram illustrating the amino acid sequence differences between Fbl and ClfA as determined by ClustalW mapped onto the $rClfA_{220-559}$ crystal structure. **A.** The dark blue residues represent the residues that, when mutated to alanine, abrogate binding of $rClfA_{220-559}$ fibrinogen. The cyan residues represent the variant residues between Fbl and ClfA. The yellow arrows indicate the residues lining the trough into which the proposed latching β-strand binds. **B.** A comparison of the residues, in Fbl and ClfA, that line the trough into which the proposed latching β-strand binds. The properties of each amino acid are indicated under each peptide sequence. P. Polar amino acid. 0. Uncharged amino acid. +. Positively charged amino acid. -. Negatively charged amino acid

A



B

$_{310}$NNITGQISI$_{319}$ Fbl

PP000P0P0

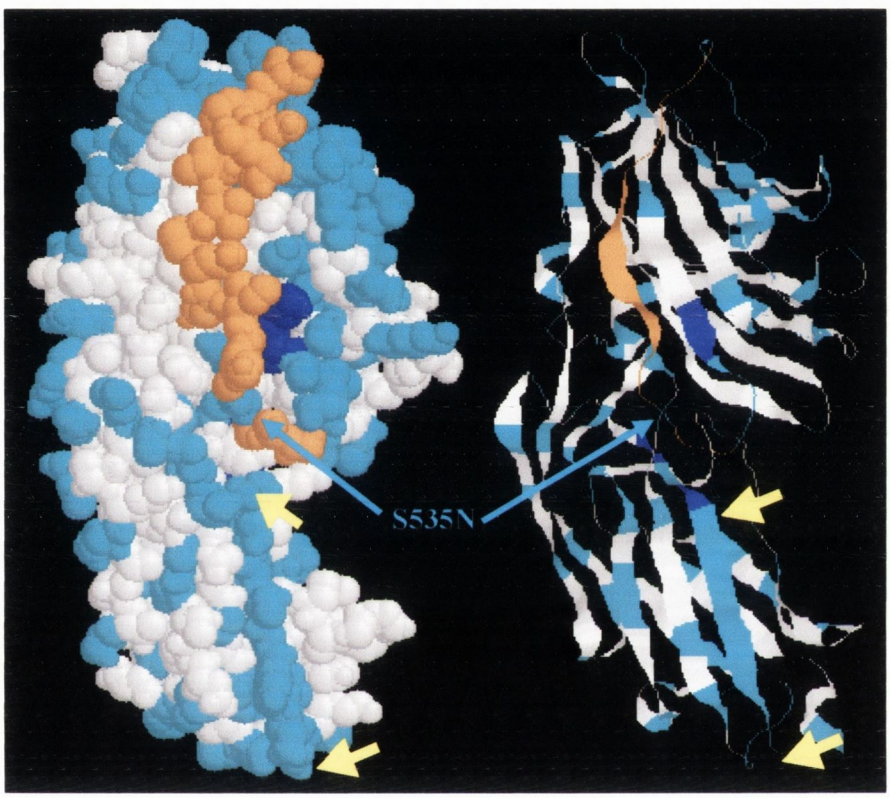
$_{326}$DDVKATLTM$_{335}$ ClfA

--0+0P0P0

The work presented in this chapter shows that the binding domain of Fbl is located in the A region between residues 40-534. Interestingly, Fbl has a lower affinity for the γ-chain of fibrinogen than ClfA. It is possible that this is due in part to the absence of residues equivalent to $ClfA_{550-559}$. Previous studies showed that $ClfA_{220-559}$ had a higher affinity for fibrinogen than $ClfA_{220-550}$ (McDevitt *et al.*, 1997). Nevertheless $ClfA_{220-550}$ still bound to fibrinogen in a dose-dependent and saturable manner. It is possible that residues 550-559 play a role in stabilizing the interaction between ClfA and fibrinogen while not actually contributing to the specific binding to fibrinogen. Also there might be subtle differences in the structure of the ligand binding trench. This could be the cause of the different inhibition profiles by the γ-chain variant peptides.

The residues in ClfA that have been shown to interact directly with the extreme C-terminal AGDV residues of the γ-chain peptide of fibrinogen are conserved in Fbl. The majority of amino acid residue differences between Fbl and ClfA occur away from the fibrinogen binding site and are proposed to contribute to antigenic rather than functional differences in the protein. This is discussed further in chapters 5 and 6. The unusual profile of inhibition of $rFbl_{40-534}$ binding to fibrinogen by the γ-chain peptide and the p-16 peptide, where the highest concentrations have lower inhibitory effect than the smaller ones, might be due to initial inhibition of fibrinogen binding due to the γ-chain peptide binding within the $rFbl_{40-534}$ binding site, followed by a subsequent conformational change that releases the peptide and allows $rFbl_{40-534}$ to bind to fibrinogen. Alternatively, the structurally altered $rFbl_{40-534}$ molecule could then bind elsewhere in fibrinogen. What is certain is that Fbl exhibits a different binding profile in its interaction with fibrinogen in comparison to that of ClfA. It has a lower affinity for intact human fibrinogen and even though it undoubtedly binds to the γ-chain it does not recognize exactly the same residues as ClfA. Preliminary isothermal titration calorimetry experiments were carried out to determine the kinetics of interaction of $rFbl_{40-534}$ with the p-16 γ-chain peptide in comparison to the interaction of $rClfA_{40-559}$ with the p-16 γ-chain peptide. These experiments suggested that Fbl interacted in a different manner with the γ-chain peptide than ClfA (data not shown). It is possible that the reason that Fbl did not adhere to immobilised γ-chain-GST constructs as well as

Figure 4.13 The latching peptide of ClfA. RasMol generated diagrams showing the latching peptide of $ClfA_{220-559}$ (orange) with respect to the groove formed in N2 of the molecule (indicated by yellow arrows). Cyan residues represent residues that change between ClfA and Fbl. The position of the S535N substitution between Fbl and ClfA that is located in the centre of the putative latching peptide is indicated.



ClfA is due to a conformational change caused by the GST moiety or inaccessibility of the residues that Fbl interacts with due to masking by the GST moiety. This could explain why the synthetic peptides in solution inhibited $rFbl_{40-534}$ binding to fibrinogen. The residues in these peptides to which $rFbl_{40-534}$ binds may be more accessible in the synthetic peptide. Clues to the residues involved in coordinating the interaction of Fbl and fibrinogen are revealed in Fig 4.10 and 4.11 whereby the p8 and p7 peptides appear to inhibit $rFbl_{40-534}$ binding to fibrinogen more strongly than $rClfA_{40-559}$ binding to fibrinogen. Further analysis of the kinetics of the interaction of these peptides with $rFbl_{40-534}$ by ELISA, fluorescence polarization and isothermal titration calorimetry experiments will confirm whether these observations identify residues involved in the interaction of Fbl with fibrinogen.

The "dock, lock and latch" model proposed by Ponnuraj *et al.* (2003) for the binding of the SdrG protein of *S. epidermidis* to the β-chain of fibrinogen indicated that changes in the β-chain peptide that added an additional phenylalanine between Gly9 and Phe10 contributed to the hydrophobic stacking interactions between the β-chain peptide and the $rSdrG_{273-597}$ molecule that stabilized the interaction of SdrG and its ligand even more than the native β-chain peptide. It is possible that the H401A substitution in the p7 γ-chain peptide and the L402A substitution in the p8 γ-chain peptide alter the interaction of $rFbl_{40-534}$ and the γ-chain peptide such that the affinity of Fbl for the mutant ligand is stronger than that of the wild type.

Despite 40% of amino acid residues changing between Fbl and ClfA the ligand binding activity of the proteins is similar. Both proteins bind to the γ-chain of fibrinogen and the residues proposed to contact the γ-chain peptide are conserved but ClfA has a higher affinity for fibrinogen. It is possible that the amino acid changes in the latching peptide trough affect the on and off rates of Fbl and fibrinogen. There are a greater proportion of charged residues in ClfA than Fbl in this region (Fig. 4.12). The residues that comprise the putative latching peptide in Fbl are conserved in Fbl apart from a S535N substitution (Fig. 4.13). Both serine and asparagine are uncharged hydrophobic residues and it is unlikely that this substitution would affect the function of the latching peptide. In order to test this theory it would be necessary to sequentially mutate the residues in the latching trough in ClfA to those present in the Fbl sequence in

order to determine whether this affects the kinetics of interaction of ClfA with fibrinogen. The critical region is $_{277}$DDVK$_{280}$ which contains the charged residues. These mutant proteins would have to be tested by techniques such as surface plasmon resonance and isothermal titration calorimetry in comparison to both wild type ClfA and Fbl. Any increase in off rate observed would confirm the theory that Fbl has a higher off rate when interacting with fibrinogen. Mutation of these trough residues would also determine whether the specificity for the variant γ-chain peptides is altered. Perhaps this is why Fbl has a markedly different profile of binding.

The experimentation in this chapter shows that the binding of Fbl to fibrinogen exhibits saturation kinetics that are lower than that of ClfA. Fbl also binds to a similar region of the γ-chain of fibrinogen as ClfA. However differences occur in the precise interaction between residues in the binding trough and the γ-chain, regardless of the conservation of all the residues previously shown to coordinate the interaction of ClfA with fibrinogen. Which residues are involved in coordinating the interaction of Fbl with fibrinogen will be elucidated by future experiments.

Chapter 5

Studies with antibodies that inhibit Fbl- and ClfA-mediated bacterial adherence to immobilised human fibrinogen

5.3. Introduction

In recent years the emergence of methicillin resistance in both hospital and community *S. aureus* and, more recently, vancomycin resistance has become of major concern. The ability of staphylococci to become resistant to new antibiotics occurs faster than the pharmaceutical and biotechnology industries can develop new drugs. As a result of financial inviability of antimicrobial development the majority of large pharmaceutical companies have moved away from developing lead compounds for antimicrobial chemotherapy (Projan, 2003). Several smaller biotechnology companies are developing alternative anti-staphylococcal therapies including vaccines and the use of anti-staphylococcal polyclonal and monoclonal antibodies in prophylaxis and therapy (Dunman and Nesin, 2003). Several candidate antigens have been investigated in the search for a vaccine that generates the production of the most effective protective antibodies. Proteins which are well conserved among the majority of isolates represent the most appropriate candidates. Polysaccharides vary among isolates and often fail to induce a long lasting immune response unless conjugated to a protein such as *Pseudomonas* exotoxin A (Fattom *et al.*, 1993; Fattom *et al.*, 1995).

ClfA has been shown to be a virulence factor in several animal models of *Staphylococcus aureus* infection including endocarditis, infection of implanted medical devices and septic arthritis (Moreillon *et al.*, 1995, Vaudaux *et al.*, 1995, Joseffson *et al.*, 2001). Furthermore the *clfA* gene is present in 100% of invasive isolates (Peacock *et al.*, 2002) and therefore represents a viable target for both active and passive immunization. A recent study by Weichart and colleagues (2003) identified ClfA by screening of whole-genome expression libraries as an important vaccine candidate antigen. Previous studies have shown that immunization with ClfA protected mice against *Staphylococcus aureus* sepsis and septic arthritis (Joseffson *et al.*, 2001).

The primary adaptive immune response of humans to any antigen is polyclonal. A wide range of antibodies that vary in both their antigen binding sites and their effector regions are generated against any single antigen. However it is difficult to obtain large

quantities of human antibodies that could be used as donor selected intravenous immunoglobulin (IVIG). Furthermore, polyclonal antibodies are a heterogeneous pool of antibodies that recognize many different antigens. Monoclonal antibodies overcome these difficulties. A monoclonal antibody is generated by immunizing a mouse with the appropriate antigen. The B-lymphocytes from the immunized mouse are then fused with myeloma cells to produce an immortal antibody-producing cell line (hybridoma) whose antibodies recognize a single epitope on the immunizing antigen. The major drawback of using murine monoclonal antibodies in humans is that the human immune system will recognize the murine antibodies as non-self. Antibodies recognizing mouse IgG will form immune complexes that could damage the kidneys. However murine monoclonal antibodies can be humanized. The humanization of monoclonal antibodies involves cloning the DNA encoding the antigen binding loops of a murine monoclonal antibody into a human immunoglobulin gene. Secondly those residues in the specificity determining regions of the heavy and light chains that are predicted to occur on the surface but are not involved in the binding site itself can be changed by site-directed mutagenesis, a process called "veneering". These procedures generate a monoclonal antibody that has an identical complementarity-determining region as the parent murine monoclonal antibody but the resultant recombinant molecule is also antigenically identical to human immunoglobulin, thus avoiding the threat of anaphylaxis when using monoclonal antibodies therapeutically in humans.

Inhibitex Inc is a clinical stage, Biopharmaceutical Company focused on developing and commercializing novel antibody-based products to prevent and treat staphylococcal infections. Their first product to gain approval for clinical trial development is Veronate®, a novel polyclonal antibody-based investigational drug being developed to prevent both *Staphylococcus aureus* and *Staphylococcus epidermidis* infections in very low birth weight infants (Vernachio *et al.*, 2003). A second antibody-based anti-*S. aureus* product that Inhibitex is developing is Aurexis®, a humanized monoclonal antibody generated against $rClfA_{220-559}$. Preclinical studies have demonstrated that when used prophylactically as monotherapy, or therapeutically in combination with antibiotics, Aurexis® can significantly reduce the incidence and

severity of *S. aureus* infections, including those caused by antibiotic-resistant *S. aureus* (Hall *et al.*, 2003, Domanski *et al.*, 2004).

The work presented in this chapter characterizes the ability of different antibodies, both polyclonal and monoclonal, to inhibit the interaction of staphylococcal fibrinogen binding proteins with their host ligand fibrinogen. The ability of these antibodies to detach bound cells from a fibrinogen substrate was also analysed. The antibodies used were rabbit polyclonal anti-$ClfA_{40-559}$, rabbit polyclonal anti-Fbl_{40-534}, Veronate, a pooled human IgG product enriched for reactivity against staphylococcal surface proteins (*S. aureus* ClfA and *S. epidermidis* SdrG), and a panel of ten murine monoclonal antibodies generated against $rClfA_{220-559}$. Among the murine monoclonal antibodies was monoclonal antibody 12-9 which was chosen by Inhibitex Inc to be humanized and developed as Aurexis® (Hall *et al.*, 2003). Both the inhibitory and displacing characteristics of these antibodies are likely to contribute to their overall ability to neutralize infections by functional inhibition, as well as their ability to promote the opsonisation of cells.

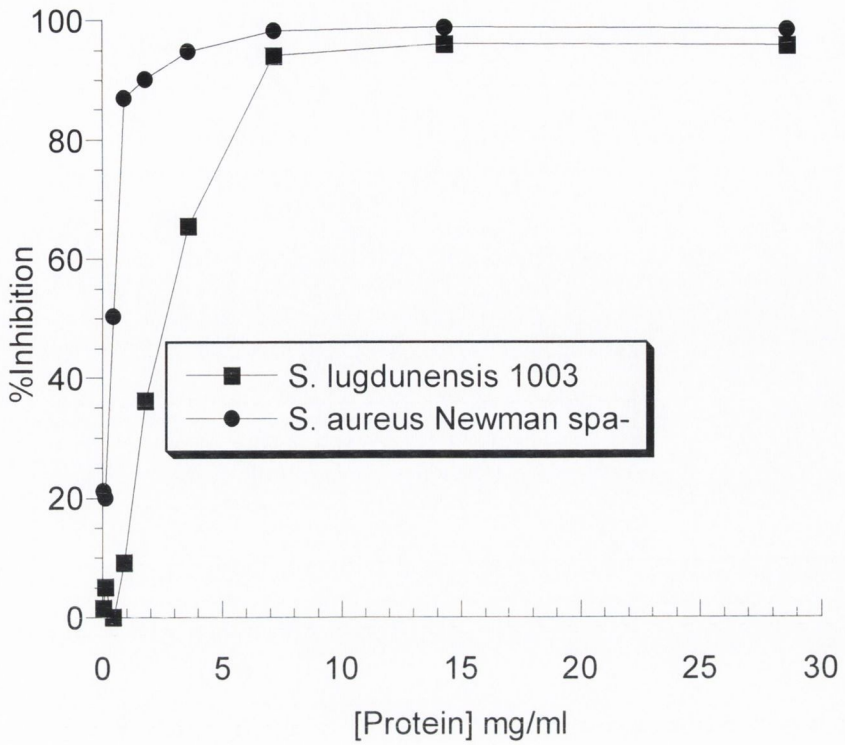
5.2. Results

5.2.1. Inhibition of bacterial adherence using polyclonal anti-ClfA region A antibodies

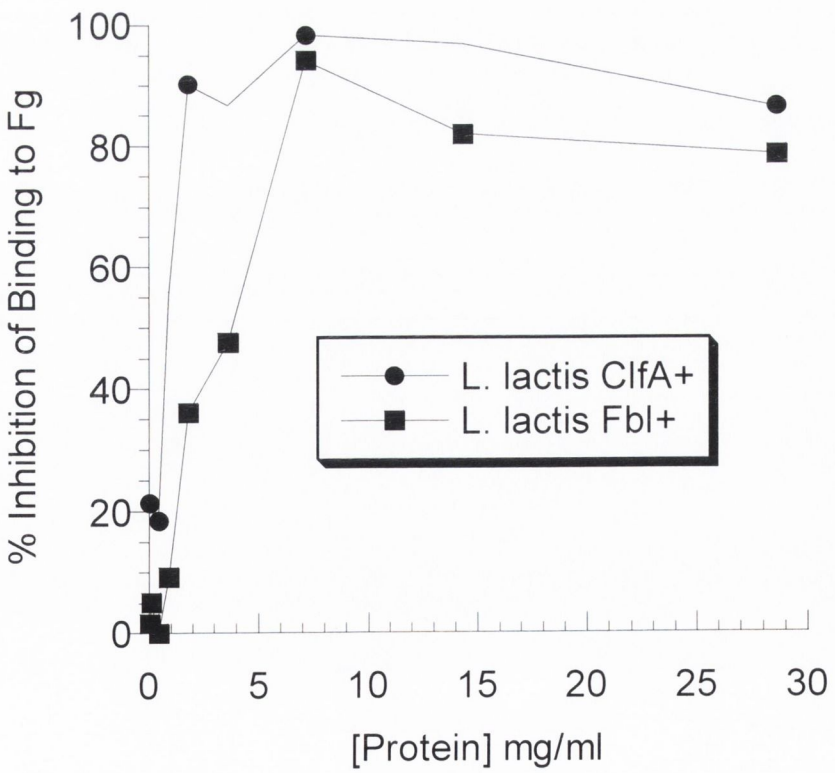
Polyclonal antibodies that were generated by immunizing rabbits with $ClfA_{40-559}$ were tested for their ability to inhibit the adherence of *S. aureus* strain Newman *spa* and *S. lugdunensis* strain 1003 cells to immobilised human fibrinogen. A protein A mutant of *S. aureus* Newman was used throughout. The *spa* mutation eliminates the IgG binding protein A. Growth to stationary phase eliminated the expression of the other fibrinogen binding proteins ClfB, FnbpA and FnbpB. Figure 5.1 clearly shows that treating the staphylococcal cells with increasing amounts of polyclonal antibodies inhibited the adherence of the cells to fibrinogen. In order to confirm the hypothesis that the inhibitory effect was attributable to the recognition of ClfA in *S. aureus* and Fbl, in *S. lugdunensis*, each protein was expressed in *L. lactis*. *L. lactis* has no indigenous fibrinogen binding protein and therefore any fibrinogen binding activity exhibited by the *L. lactis* $ClfA^+$ and *L. lactis* Fbl^+ strains is solely due to the expression of the heterologous fibrinogen binding protein on the surface of these cells. The interaction of both strains with fibrinogen was inhibited by rabbit polyclonal anti-ClfA region A antisera in a dose-dependent manner. Complete (100%) inhibition of *S. lugdunensis* binding was observed, indicating that the binding site of Fbl can be completely blocked by polyclonal rabbit anti-ClfA region A antibodies. However more anti-ClfA region A antibodies are required to block Fbl-mediated adherence to fibrinogen than ClfA-mediated adherence to fibrinogen. This suggests that there is a higher titre of anti-ClfA antibodies than antibodies that cross-react with Fbl. Goat anti-rabbit IgG added alone as a negative control caused no inhibition of adherence of either strain to immobilised fibrinogen.

Figure 5.1 The inhibitory effects of polyclonal rabbit anti-$ClfA_{40-559}$ antibodies on Fbl- and ClfA-mediated adherence to fibrinogen. *S. aureus* Newman spa, *S. lugdunensis* 1003 (1×10^8 colony-forming units/ml) (**A**) and *L. lactis* $ClfA^+$ and *L. lactis* Fbl^+ (1×10^8 colony-forming units/ml) (**B**) cells were preincubated with varying concentrations of polyclonal rabbit anti-$ClfA_{40-559}$ antibodies ranging from 28mg/ml to 109.375μg/ml. The ability of the cells to bind to immobilised fibrinogen was then tested. Cells that bound to fibrinogen coated microtitre plate wells were stained with crystal violet and washed with PBS. Adherent cells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages inhibition of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.

A


100
80
60
40
20
0
%Inhibition
S. lugdunensis 1003
S. aureus Newman spa-
0
5
10
15
20
25
30
[Protein] mg/ml

B


100
80
60
40
20
0
% Inhibition of Binding to Fg
L. lactis ClfA+
L. lactis Fbl+
0
5
10
15
20
25
30
[Protein] mg/ml

5.2.2. Displacement of attached bacteria polyclonal anti-ClfA region A antibodies

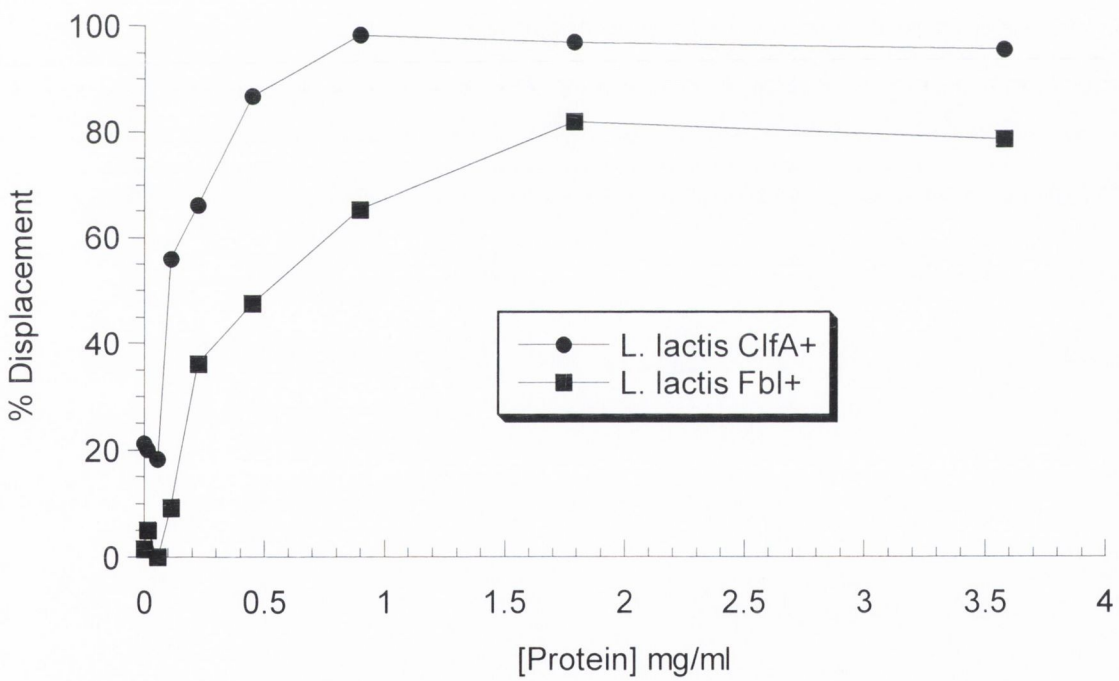
The preceding experiments confirmed that immunization with $ClfA_{40-559}$ resulted in the generation of antibodies that functionally blocked the interaction of bacterial cells expressing ClfA and Fbl on their surface with fibrinogen. Next antibodies were tested for the ability to detach bacterial cells adhering to an immobilised fibrinogen substrate. A series of experiments were then carried out whereby *L. lactis* Fbl^+ and *L. lactis* $ClfA^+$ cells were attached to a fibrinogen substrate, and were then treated with increasing concentrations of rabbit polyclonal anti-ClfA region A antibodies. Rabbit polyclonal anti-ClfA region A antisera contains antibodies that displaced bacteria expressing ClfA or Fbl from a fibrinogen substrate in a concentration dependent manner (Fig. 5.2).

5.2.3. Inhibition using polyclonal anti-Fbl region A antibodies

Polyclonal anti-ClfA region A antibodies specifically blocked the adherence of ClfA- and Fbl-expressing bacteria to immobilised fibrinogen. The ability of polyclonal rabbit antibodies generated against the A region of Fbl to inhibit the binding of *S. aureus* Newman *spa* and *S. lugdunensis* strain 1003 to immobilised fibrinogen was then tested. Figure 5.3 shows that polyclonal anti-Fbl region A antibodies blocked both ClfA-mediated adherence of *S. aureus* and Fbl-mediated adherence of *S. lugdunensis*. The polyclonal anti-Fbl region A antisera also inhibited the adherence of *L. lactis* Fbl^+ and *L. lactis* $ClfA^+$ cells to immobilised fibrinogen.

The antibodies blocked *S. lugdunensis* more efficiently than *L. lactis* Fbl^+. This is presumably because, there is 64-fold less protein expressed on the surface of *S. lugdunensis* than *L. lactis* Fbl^+ (section 4.2). A greater concentration of antibody was required to inhibit the larger number of Fbl molecules on the surface of *L. lactis*. The antibodies appeared to block *S. aureus* more avidly than *L. lactis* $ClfA^+$. There are a greater number of ClfA molecules on the surface of *L. lactis* $ClfA^+$ than *S. aureus* Newman (O'Brien *et al.*, 2002). Therefore the two proteins must share epitopes that

Figure 5.2 The ability of polyclonal rabbit anti-$ClfA_{40-559}$ antibodies to detach bound Fbl- and ClfA-expressing *L. lactis* cells from an immobilised fibrinogen substrate. Ten serial doubling dilutions of polyclonal rabbit anti-$ClfA_{40-559}$ antibodies ranging from 14mg/ml to 54.6μg/ml were added to microtitre wells containing *L. lactis* Fbl^+ and *L. lactis* $ClfA^+$ bound to an immobilised fibrinogen substrate. Cells that remained bound to fibrinogen coated microtitre plate wells after incubation with antibodies were stained with crystal violet and washed with PBS. Adherent cells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentage displacement of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.



allow functional blocking by this pool of anti-Fbl region A polyclonal antibodies. However their antigenic differences are reflected by the greater ability of the polyclonal anti-Fbl region A antibodies to inhibit Fbl-mediated adherence to fibrinogen than ClfA-mediated adherence. There must be a lower titre of antibodies that inhibit ClfA-mediated adherence to immobilised fibrinogen as anti-Fbl region A antibodies fail to inhibit *L. lactis* ClfA$^+$ more than 50%.

5.2.4. Displacement using polyclonal anti-Fbl region A antibodies

Section 5.2.3 demonstrated that ClfA and Fbl share antigenic epitopes that are responsible for functional blocking by antibody binding. The proteins share a high level of sequence identity. Therefore it is likely that both proteins bind to fibrinogen by the same mechanism. Characterization of the ability of polyclonal anti-Fbl region A antibodies to displace bound bacteria from a fibrinogen substrate was performed in order to test this hypothesis. Figure 5.4 shows that polyclonal anti-Fbl region A antibodies efficiently displaced Fbl-expressing bacteria from a fibrinogen substrate but they did not displace bound ClfA-expressing bacteria from fibrinogen as efficiently.

5.2.5. Inhibition and displacement using Veronate®

Rabbits immunized with recombinant ClfA$_{40\text{-}559}$ generated antibodies that both blocked the adherence of ClfA and Fbl to fibrinogen and displaced bound ClfA and Fbl from fibrinogen. Similarly antibodies to Fbl$_{40\text{-}534}$ blocked ClfA- and Fbl-promoted adherence to fibrinogen and stimulated the detachment of adherent bacteria. The human plasma mixture, Veronate®, was also tested for the presence of inhibitory antibodies against ClfA and Fbl, and for the presence of antibodies that would displace bound bacteria from a fibrinogen substrate. Encouragingly, Veronate® inhibited both ClfA- and Fbl-expressing bacteria from adhering to immobilised fibrinogen in a concentration-dependent manner (Fig. 5.5 A). Veronate® blocked *S. lugdunensis* more efficiently than *L. lactis* Fbl$^+$. This must be because there is less protein expressed on the surface of *S.*

Figure 5.3 The inhibitory effects of polyclonal rabbit anti-Fbl_{40-534} antibodies on Fbl- and ClfA-mediated adherence to fibrinogen. *S. lugdunensis* 1003, *S. aureus* Newman *spa*, *L. lactis* $ClfA^+$ and *L. lactis* Fbl^+ cells were preincubated with varying concentrations of polyclonal rabbit anti-Fbl_{40-534} antibodies ranging from 32.5mg/ml to 63.5μg/ml. The ability of the cells to bind to immobilised fibrinogen was then tested. Cells that bound to fibrinogen coated microtitre plate wells were stained with crystal violet and washed with PBS. Adherent cells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages inhibition of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.

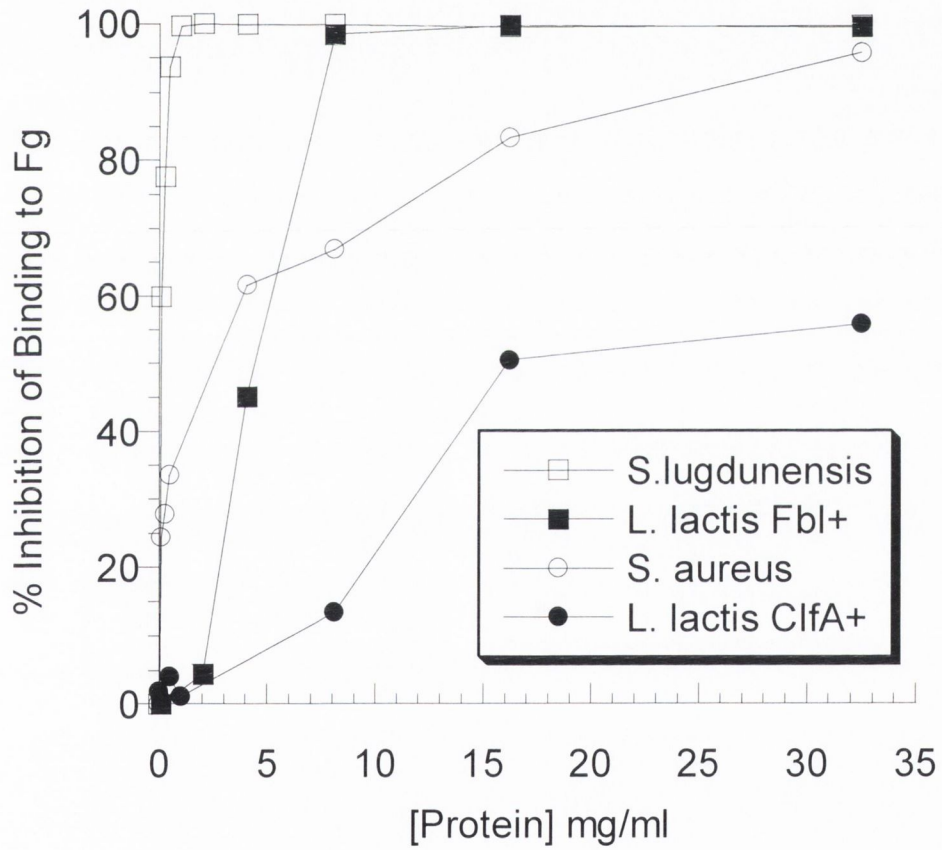


Figure 5.4 The ability of polyclonal rabbit anti-Fbl_{40-534} antibodies to detach adherent Fbl- and ClfA-expressing cells from an immobilised fibrinogen substrate. Varying concentrations of polyclonal rabbit anti-Fbl_{40-534} antibodies ranging from 32.5mg/ml to 63.5μg/ml were added to microtitre wells containing *L. lactis* Fbl^+ and *L. lactis* $ClfA^+$ bound to an immobilised fibrinogen substrate. Cells that remained bound to fibrinogen coated microtitre plate wells after incubation with antibodies were stained with crystal violet and washed with PBS. Adherent cells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentage displacement of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.

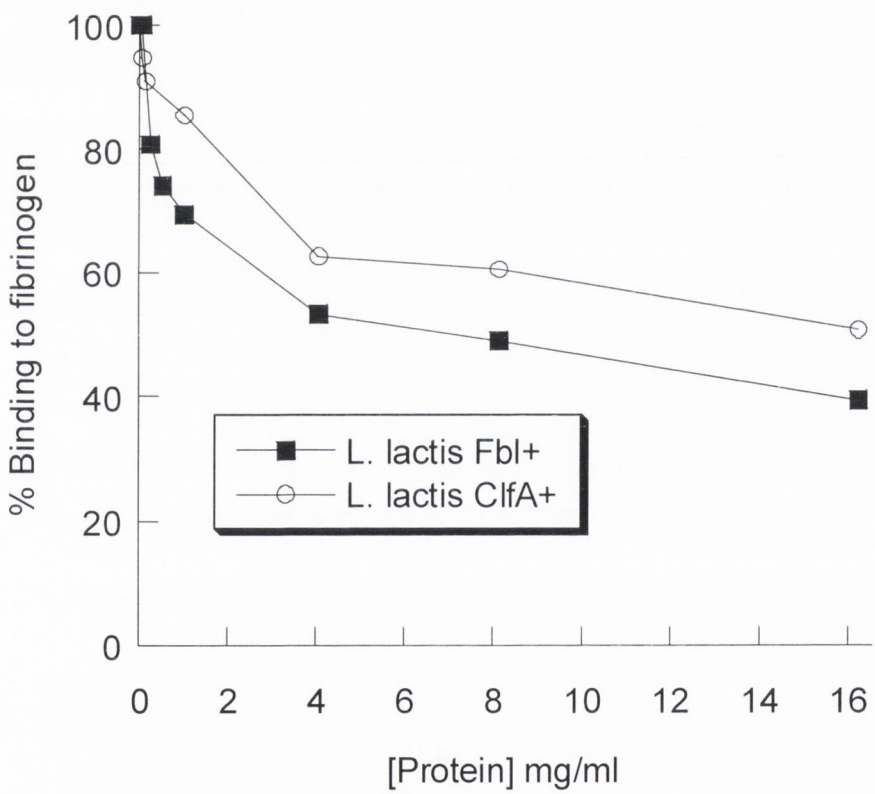
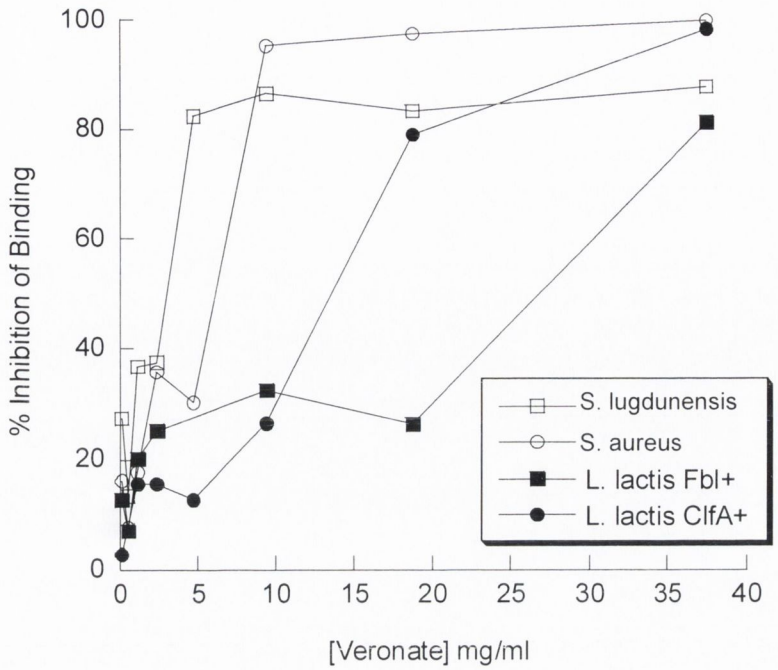
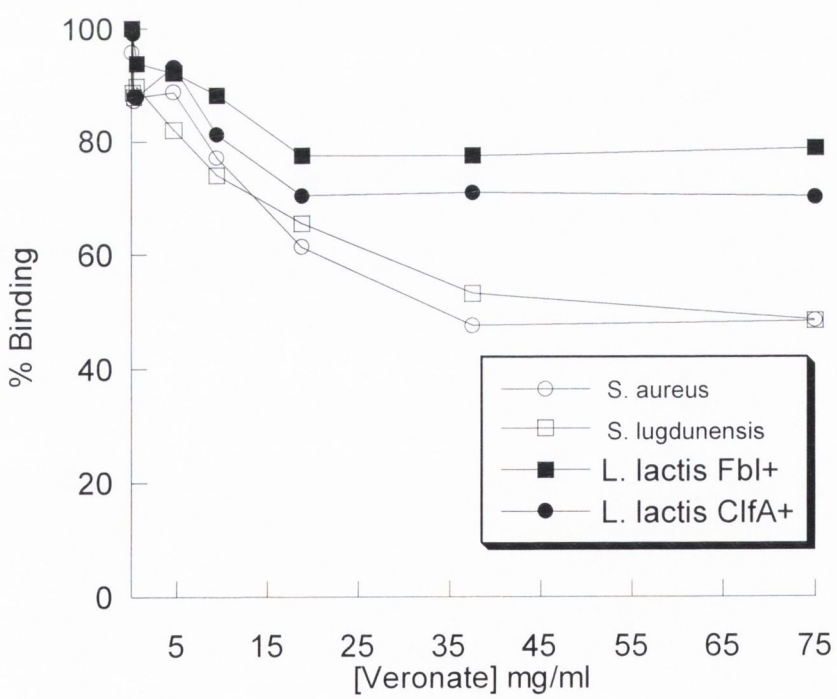


Figure 5.5 The inhibitory and displacing effects of Veronate® on Fbl- and ClfA-mediated adherence to immobilised human fibrinogen. **A.** Varying concentrations of Veronate® ranging from 37.5mg/ml to 73.2μg/ml were tested for their ability to inhibit the binding of *S. lugdunensis* 1003, *S. aureus* Newman *spa*, *L. lactis* Fbl$^+$ and *L. lactis* ClfA$^+$ to immobilised fibrinogen in a concentration-dependent manner. **B.** Varying concentrations of Veronate® ranging from 75mg/ml to 146.5μg/ml were tested for their ability to displace bound *S. lugdunensis* 1003, *S. aureus* Newman *spa*, *L. lactis* Fbl$^+$ and *L. lactis* ClfA$^+$ from an immobilised fibrinogen substrate. Cells that bound to fibrinogen coated microtitre plate wells were stained with crystal violet, washed with PBS and measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages of inhibition or displacement of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.

A.


100
80
60
40
20
0
% Inhibition of Binding
0
5
10
15
20
25
30
35
40
[Veronate] mg/ml
S. lugdunensis
S. aureus
L. lactis Fbl+
L. lactis ClfA+

B.


100
80
60
40
20
0
% Binding
5
15
25
35
45
55
65
75
[Veronate] mg/ml
S. aureus
S. lugdunensis
L. lactis Fbl+
L. lactis ClfA+

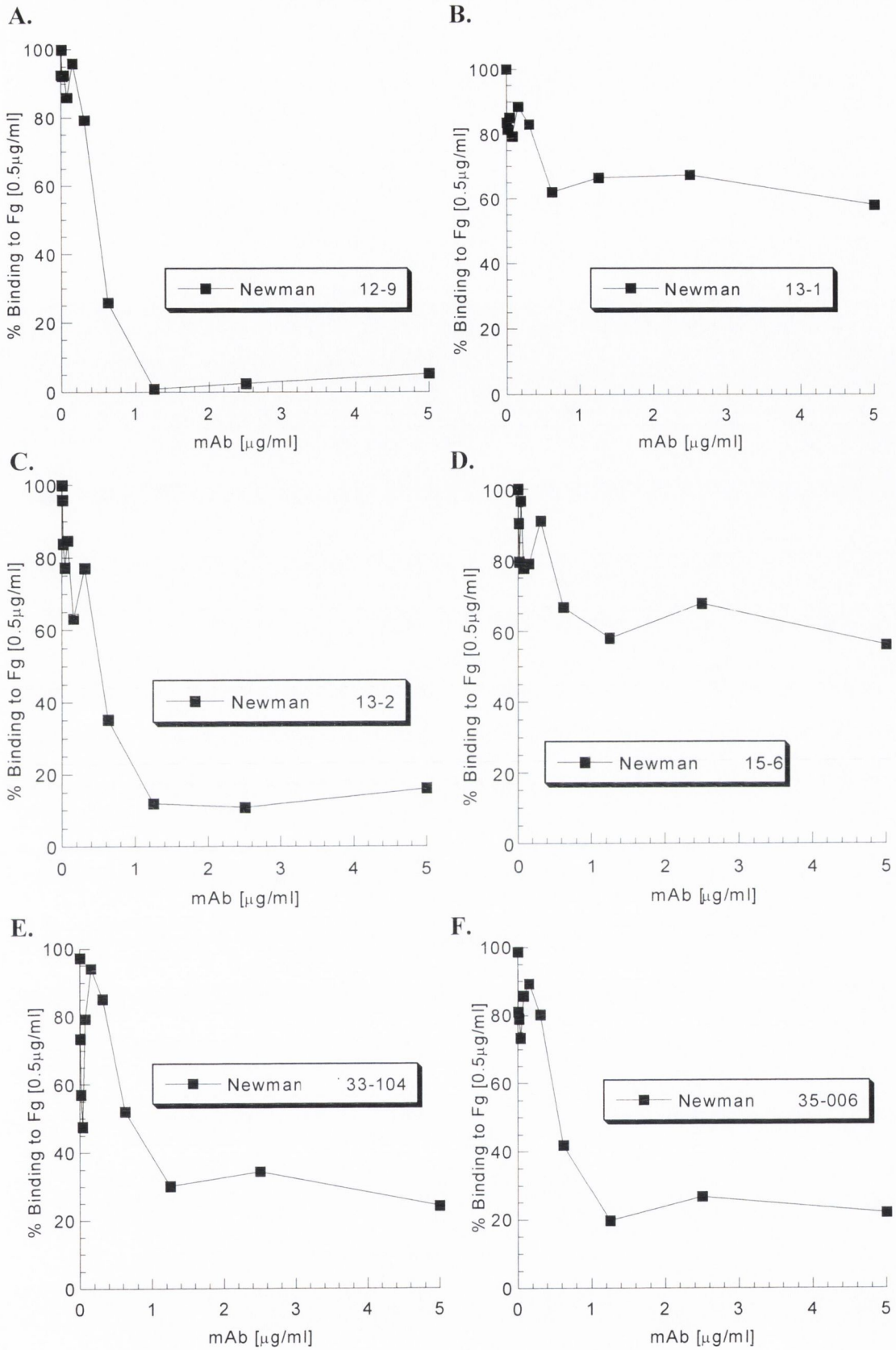
lugdunensis than *L. lactis* Fbl$^+$. Similarly Veronate® blocked *S. aureus* Newman adherence to immobilised fibrinogen more efficiently than *L. lactis* ClfA$^+$.

Veronate® was then tested for its ability to promote displacement of adherent bacteria. It displaced bound bacteria expressing Fbl and ClfA from a fibrinogen substrate in a concentration-dependent manner. Again displacement correlated with the number of molecules on the surface of *L. lactis* Fbl$^+$ and *L. lactis* ClfA$^+$. However, displacement did not exceed 50% for any strain. This must be due to a lower titre of antibodies in Veronate® that mediate displacement. In contrast there is a high titre of antibodies that inhibit ClfA- and Fbl-mediated adherence to immobilised fibrinogen. The differences in displacing ability of the antibody are not as marked as with anti-Fbl region A antibodies. This is probably due to the fact that Veronate is a pool of polyclonal IgG molecules presumably raised as a result of exposure to *S. aureus*. It may contain antibodies to bacterial components whose interaction with IgG might mask the interaction of Fbl- and ClfA-expressing bacteria to fibrinogen. Alternatively there could be a lower titre of Fbl- and ClfA-specific antibodies in Veronate®. Nevertheless, this data provides further evidence that the two proteins might share epitopes that allow blocking of adherence to fibrinogen. The result in this section also suggest that inhibition and displacement may be mediated by different antibodies and that Veronate® contains fewer of the displacing antibodies than the rabbit polyclonal anti-ClfA region A antibodies.

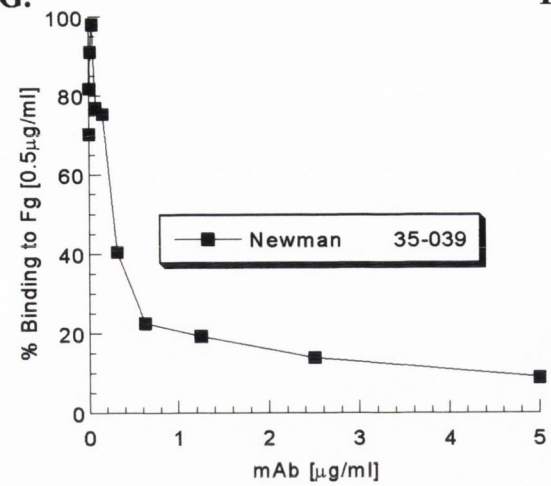
5.2.6. Inhibition of ClfA- and Fbl-mediated bacterial cell binding to fibrinogen by murine monoclonal antibodies raised against ClfA

Polyclonal anti-ClfA region A and polyclonal anti-Fbl region A antibodies inhibited both ClfA- and Fbl-mediated bacterial adherence to immobilised fibrinogen. Thus the proteins must share important epitopes. Ten different murine monoclonal antibodies generated by Inhibitex Inc., against rClfA$_{220\text{-}559}$ were used to investigate this further. The inhibitory and displacing activity of each monoclonal antibody is summarized in Table 5.1. Figure 5.6 describes the inhibitory effects of the ten monoclonal antibodies when they were pre-incubated with *S. aureus* Newman *spa* cells.

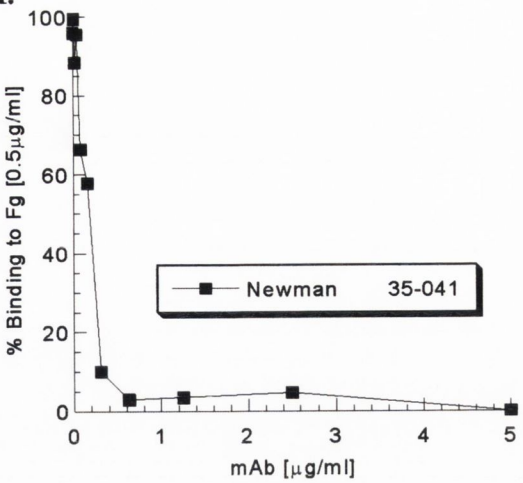
Figure 5.6 The inhibitory effects of murine monoclonal antibodies raised against $rClfA_{220-559}$ on the adherence of *S. aureus* Newman *spa* to an immobilised fibrinogen substrate. **A-J.** *S. aureus* Newman *spa* cells were preincubated with varying concentrations of the murine monoclonal antibodies 12-9, 13-1, 13-2, 15-6, 33-104, 35-006, 35-039, 35-041, 35-052 and 35-220 ranging from 5μg/ml to 9.765ng/ml. The ability of the cells to bind to immobilised fibrinogen was then tested. **K.** Inhibition of binding of *S. aureus* Newman *spa* cells to 0.5mg/ml immobilised fibrinogen by 5mg/ml of murine monoclonal antibodies. Cells that bound to fibrinogen coated microtitre plate wells were stained with crystal violet, washed with PBS and measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages inhibition of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.


A.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 12-9
B.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 13-1
C.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 13-2
D.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 15-6
E.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 33-104
F.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-006

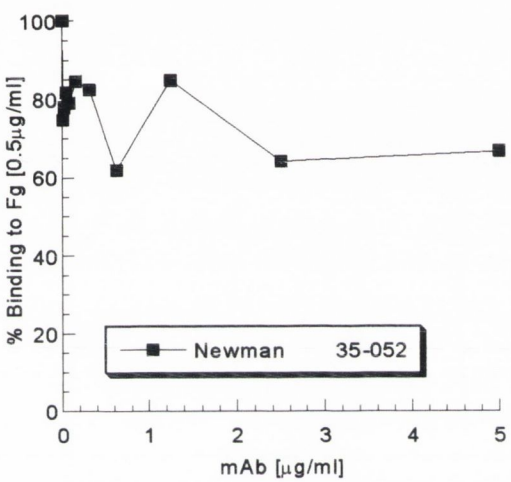
G.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-039

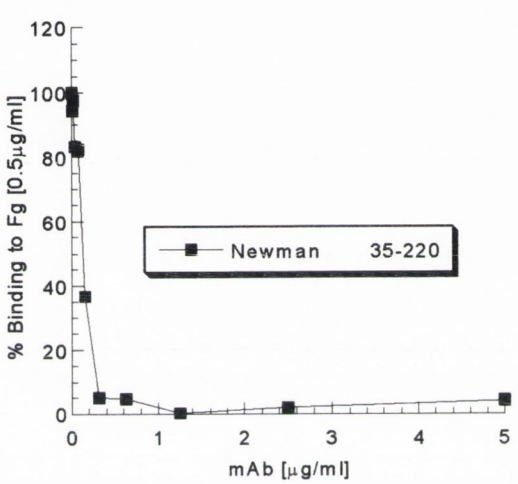
H.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-041

I.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-052

J.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-220

K.

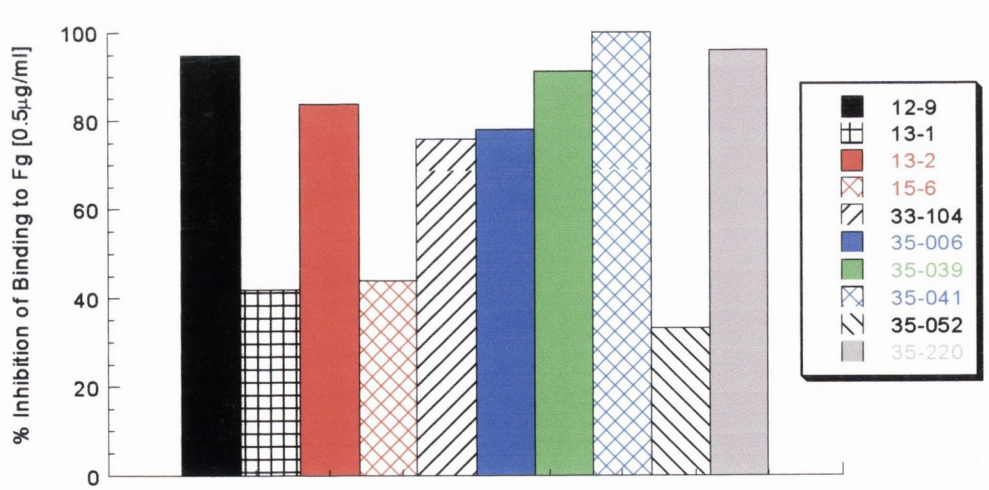

% Inhibition of Binding to Fg [0.5µg/ml]
12-9
13-1
13-2
15-6
33-104
35-006
35-039
35-041
35-052
35-220

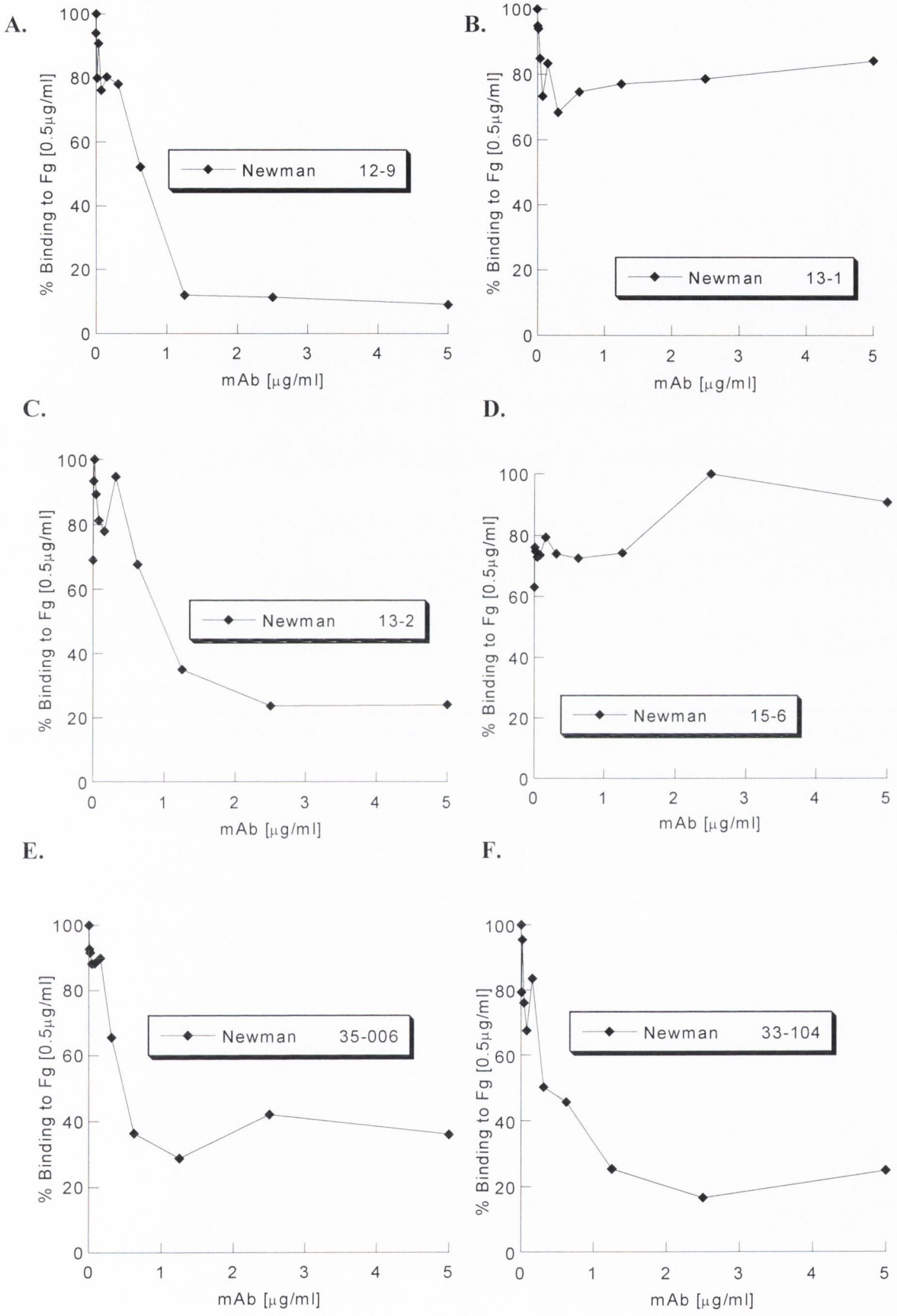
Figure 5.9 shows the inhibitory effects of the ten monoclonal antibodies when they were pre-incubated with *L. lactis* Fbl^{+}_{1003} and *L. lactis* $ClfA^{+}$ cells. The monoclonal antibodies that recognized their cognate antigen ClfA in *S. aureus* Newman *spa* cells and *L. lactis* $ClfA^{+}$ cells, and inhibited ClfA-mediated adherence to immobilised fibrinogen all failed to inhibit Fbl-mediated adherence to fibrinogen. In addition, the antibodies that failed to inhibit ClfA-mediated adherence to immobilised fibrinogen also failed to inhibit Fbl-mediated adherence to fibrinogen. As Fig. 5.8 A shows mAb 12-9 efficiently inhibits *S. aureus* Newman *spa* and *L. lactis* $ClfA^{+}$ adherence to immobilised fibrinogen yet fail to inhibit adherence of *L. lactis* Fbl^{+} and *S. lugdunensis* 1003. This is likely due to the antibodies failing to bind to Fbl and inhibit Fbl-mediated binding to fibrinogen. *L. lactis* $ClfB^{+}$ was used as a control. 12-9 also failed to inhibit ClfB-mediated adherence to fibrinogen or displace adhered *L. lactis* $ClfB^{+}$ from an immobilised fibrinogen substrate.

5.2.7. Displacement of bacteria adhering to immobilised fibrinogen promoted by ClfA and Fbl by murine monoclonal antibodies specific for ClfA

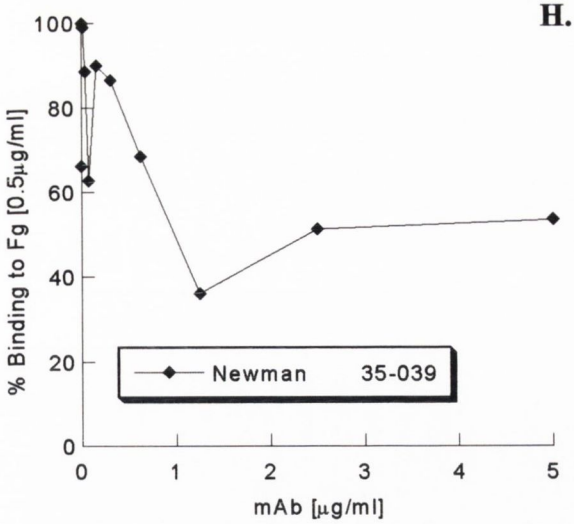
The ability of all ten monoclonal antibodies to displace ClfA and Fbl expressing bacteria from a fibrinogen substrate was analysed. The results of this experiment are illustrated in Figure 5.7, 5.8 and 5.9. Fig 5.8 B describes the ability of mAb 12-9 to displace adherent bacteria from an immobilised fibrinogen substrate. It is clear that mAb 12-9 displacing activity is restricted to ClfA-mediated adherence. *S. aureus* Newman *spa* and *L. lactis* $ClfA^{+}$ are efficiently displaced from an immobilised fibrinogen substrate by mAb 12-9. In contrast *L. lactis* $ClfB^{+}$, *L. lactis* Fbl^{+} and *S. lugdunensis* 1003 are not displaced from an immobilised fibrinogen substrate by mAb 12-9.

Of the monoclonal antibodies analyzed, those that strongly detached *S. aureus* Newman *spa* and *L. lactis* $ClfA^{+}$ cells from a fibrinogen substrate also inhibited the attachment of bacteria to a fibrinogen substrate. Figure 5.7 describes the ability of the ten monoclonal antibodies to displace adherent *S. aureus* Newman *spa* cells from an immobilised fibrinogen substrate. Figure 5.9 describes the ability of the ten monoclonal

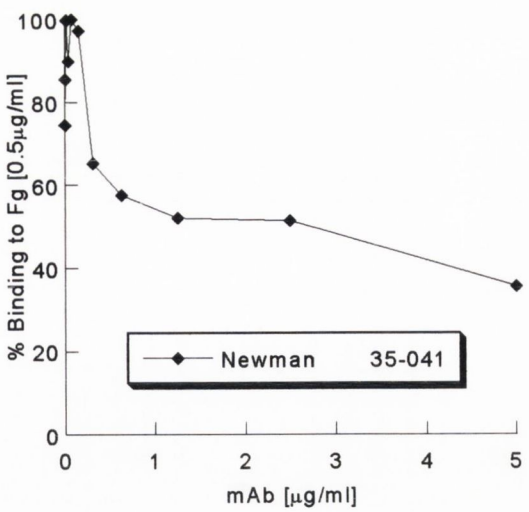
Figure 5.7 The ability of monoclonal antibodies raised against $rClfA_{220-559}$ to displace bound *S. aureus* Newman *spa* cells from an immobilised fibrinogen substrate. **A-J** The displacing effect of varying concentrations of monoclonal antibodies 12-9, 13-1, 13-2, 15-6, 33-104, 35-006, 35-039, 35-041, 35-052 and 35-220 ranging from 5mg/ml to 9.765ng/ml added to microtitre wells containing *S. aureus* Newman *spa* bound to an immobilised fibrinogen substrate. **K.** The displacing effect of 5μg/ml of murine monoclonal antibodies on *S. aureus* Newman *spa* cells bound to 0.5mg/ml immobilised fibrinogen. Cells that remained bound to fibrinogen coated microtitre plate wells after incubation with antibodies were stained with crystal violet and washed with PBS. Adherent cells were measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages displacement of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.


A.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 12-9
B.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 13-1
C.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 13-2
D.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 15-6
E.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-006
F.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 33-104

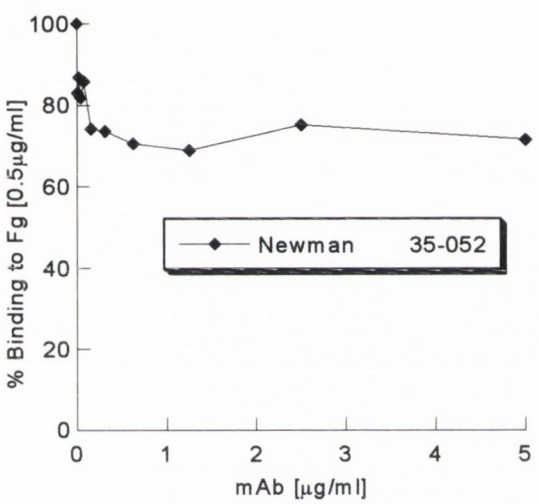
G.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-039

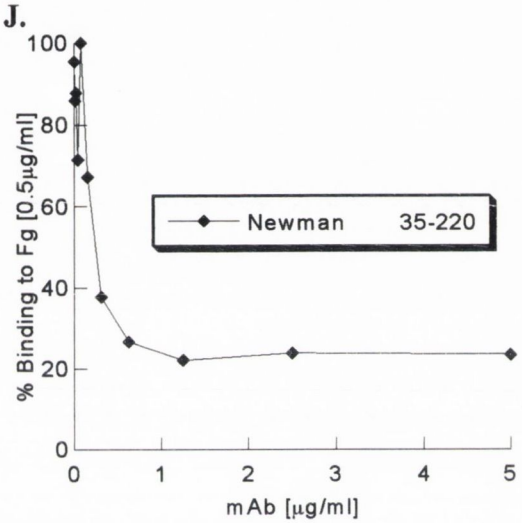
H.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-041

I.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-052

J.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
Newman 35-220

K.

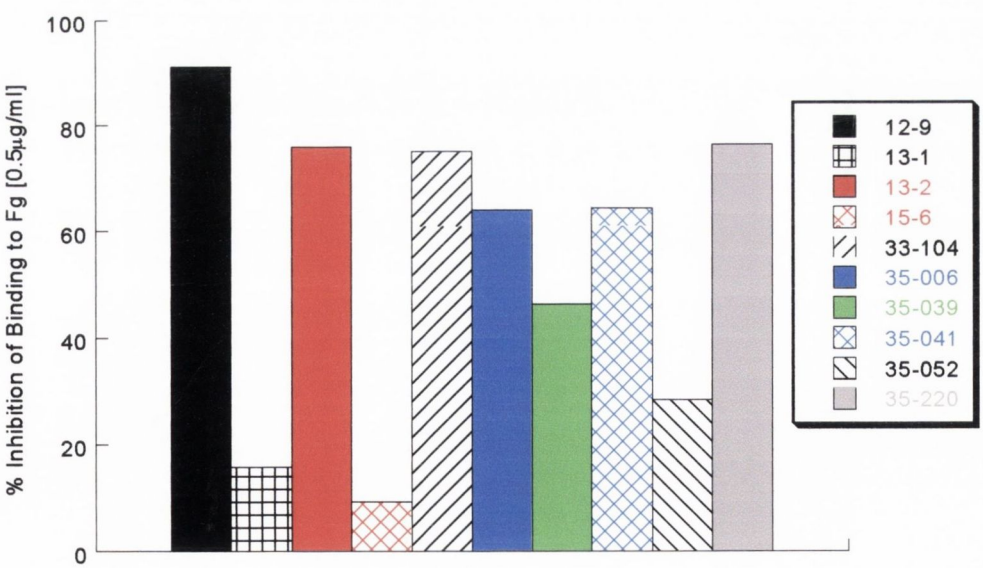
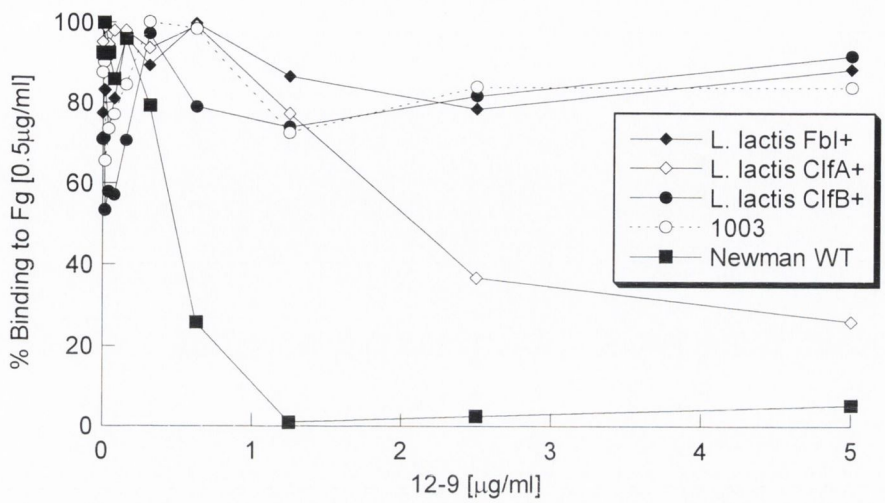

% Inhibition of Binding to Fg [0.5µg/ml]
12-9
13-1
13-2
15-6
33-104
35-006
35-039
35-041
35-052
35-220

Figure 5.8 The inhibitory and displacing effects of murine monoclonal antibody 12-9 raised against $rClfA_{220-559}$ on the Fbl- and ClfA-mediated adherence of bacterial cells to an immobilised fibrinogen substrate. **A.** Varying concentrations of the murine monoclonal antibody 12-9 ranging from 5μg/ml to 9.7ng/ml were tested for their ability to inhibit the binding of *S. lugdunensis* 1003, *S. aureus* Newman *spa*, *L. lactis* Fbl^+ and *L. lactis* $ClfA^+$ and *L. lactis* $ClfB^+$ to immobilised fibrinogen. **B.** Varying concentrations of the murine monoclonal antibody 12-9 ranging from 5μg/ml to 9.7ng/ml were tested for their ability to displace bound *S. lugdunensis* 1003, *S. aureus* Newman *spa*, *L. lactis* Fbl^+ and *L. lactis* $ClfA^+$ and *L. lactis* $ClfB^+$ from an immobilised fibrinogen substrate. Cells that bound to fibrinogen coated microtitre plate wells were stained with crystal violet, washed with PBS and measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages of inhibition or displacement of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.

A.


% Binding to Fg [0.5µg/ml]
12-9 [µg/ml]
L. lactis Fbl+
L. lactis ClfA+
L. lactis ClfB+
1003
Newman WT

B.

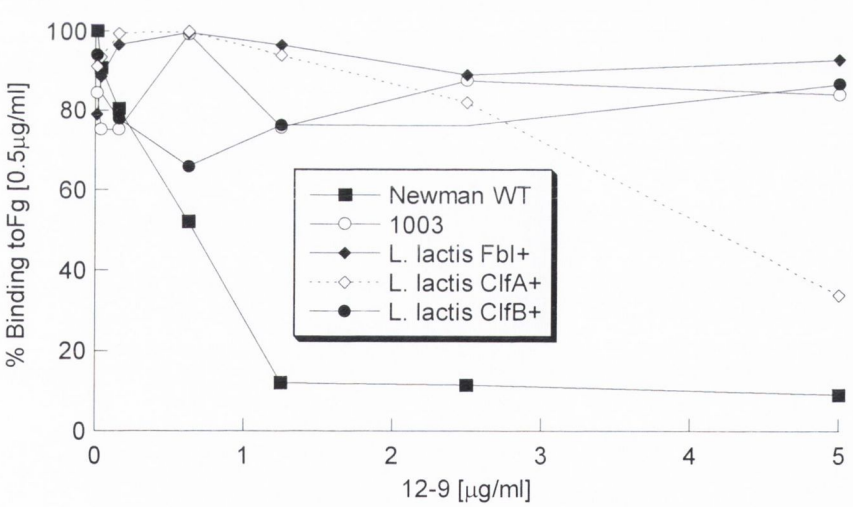

% Binding toFg [0.5µg/ml]
12-9 [µg/ml]
Newman WT
1003
L. lactis Fbl+
L. lactis ClfA+
L. lactis ClfB+

Table 5.1 Summary of the inhibitory and displacing effects of antibodies on Fbl and ClfA mediated adherence to fibrinogen.

	ClfA-mediated Adherence		Fbl-Mediated Adherence	
Antibody	**Inhibition**	**Displacement**	**Inhibition**	**Displacement**
12-9	++	++	-	-
13-1	-	-	-	-
13-2	+	+	-	-
15-6	+/-	-	-	-
33-104	++	++	-	-
35-006	++	++	-	-
35-039	++	++	-	-
35-041	++	+	-	-
35-052	+/-	+/-	-	-
35-220	++	+/-	-	-
Rabbit Polyclonal Anti ClfA	++	++	+	+
Rabbit Polyclonal Anti Fbl	+	+	++	++
Veronate®	++	+	++	+

Key:

- = 0-30%

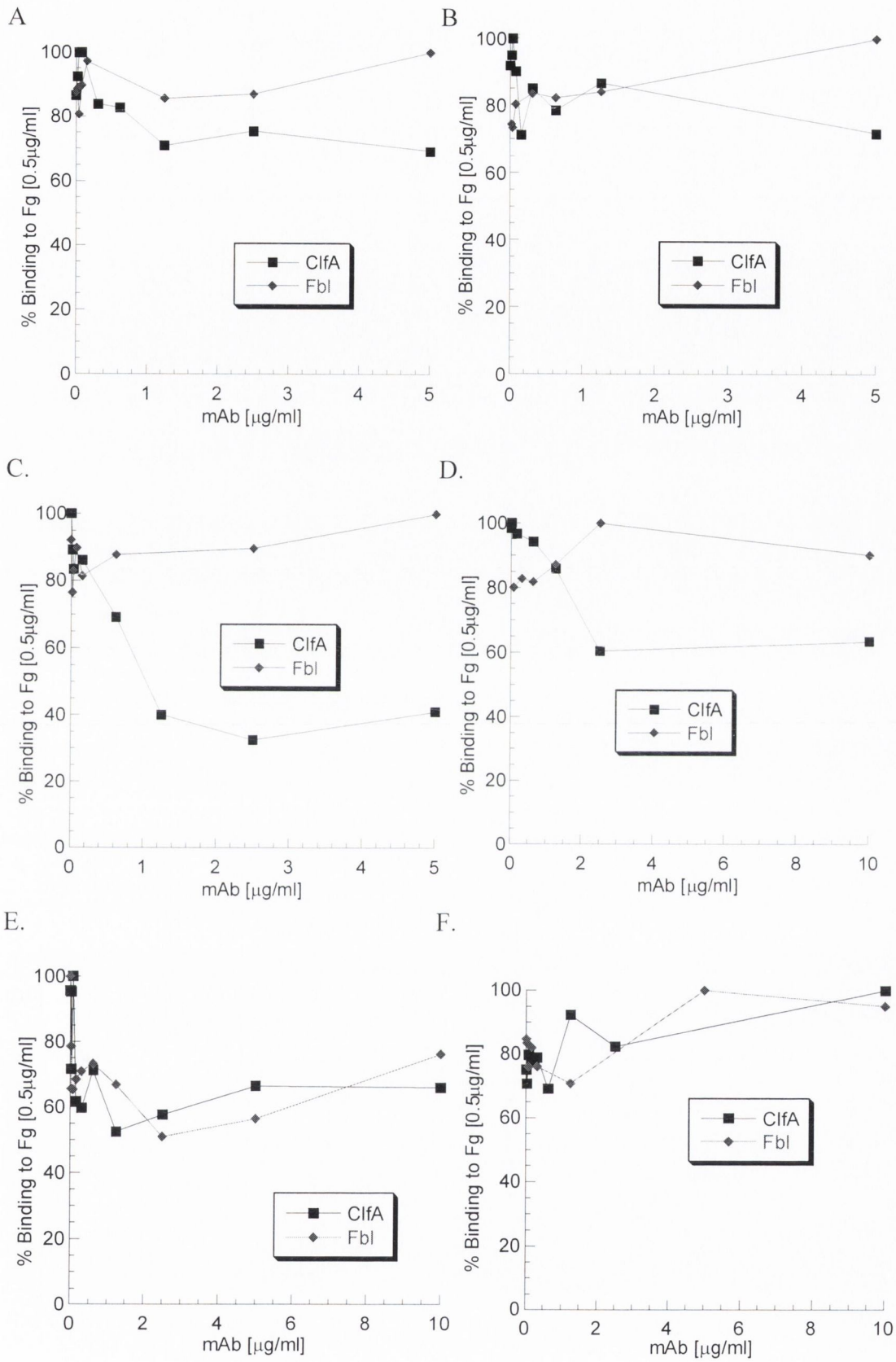
-/+ = 30-50%

+ = 50-60%

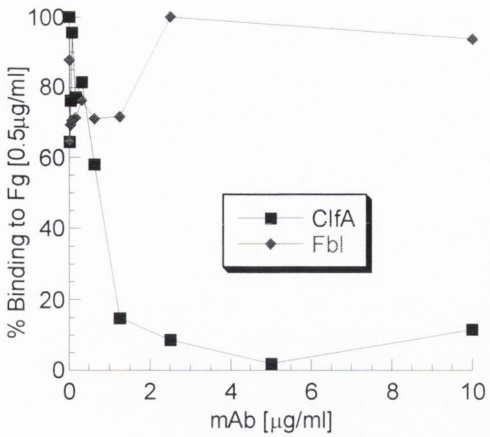
++ = 60-100%

antibodies to displace adherent *L. lactis* Fbl^{+}_{1003} and *L. lactis* $ClfA^{+}$ cells from an immobilised fibrinogen substrate. It is clear that not all of the monoclonal antibodies that potently inhibited binding, effectively detached cells from the fibrinogen substrate. The results of Fig. 5.7, Fig. 5.8 and Fig. 5.9 are summarized in Table 5.1. None of the monoclonal antibodies tested were able to strongly detach Fbl-expressing cells from a fibrinogen substrate. This is likely due to the antibodies failing to bind to Fbl and displace bound Fbl-expressing cells from an immobilised fibrinogen substrate. These results also confirm that different antibodies mediate inhibition alone, or inhibition and displacement as was observed in the differences in the abilities of the polyclonal antibodies to inhibit Fbl- and ClfA-mediated adherence to fibrinogen and to displace bound Fbl- and ClfA-expressing cells from an immobilised fibrinogen substrate.

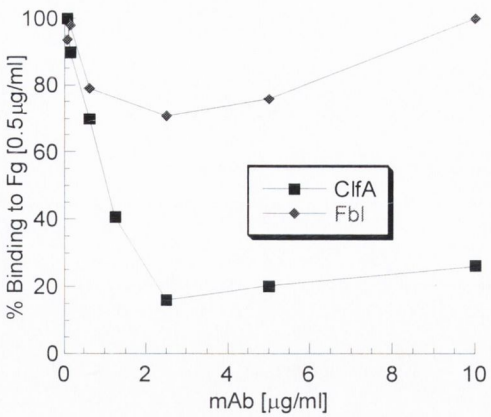
Figure 5.9 The inhibitory and displacing effects of murine monoclonal antibodies raised against $rClfA_{220-559}$ on the adherence of *L. lactis* Fbl^+ and *L. lactis* $ClfA^+$ to an immobilised fibrinogen substrate. **A.-Q.** The inhibitory and displacing effects of varying concentrations of the murine monoclonal antibodies 13-1 (**A**=Inhibition, **B**=Displacement), 13-2 (**C**=Inhibition, **D**=Displacement), 15-6 (**E**=Inhibition, **F**=Displacement), 33-104 (**G**=Inhibition, **H**=Displacement), 35-006 (**I**=Inhibition, **J**=Displacement), 35-039 (**K**=Inhibition, **L**=Displacement), 35-041 (**M**=Inhibition, **N**=Displacement), 35-052 (**O**=Inhibition, **P**=Displacement) and 35-220 (**Q**=Inhibition, **R**=Displacement) ranging from 5mg/ml to 9.7ng/ml on the adherence of *S. aureus* Newman *spa* to fibrinogen. Cells that bound to fibrinogen coated microtitre plate wells were stained with crystal violet, washed with PBS and measured in an ELISA plate reader at 570nm. Values represent the means of triplicate wells and are expressed as the percentages of inhibition or displacement of binding of cells to fibrinogen compared to control (no antibody). This experiment was repeated three times with similar results.


A
B
C.
D.
E.
F.
% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

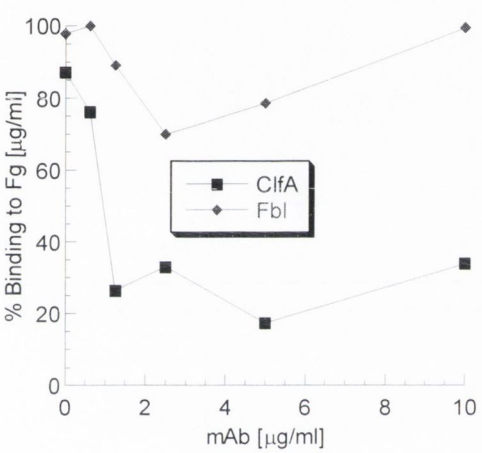
G


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

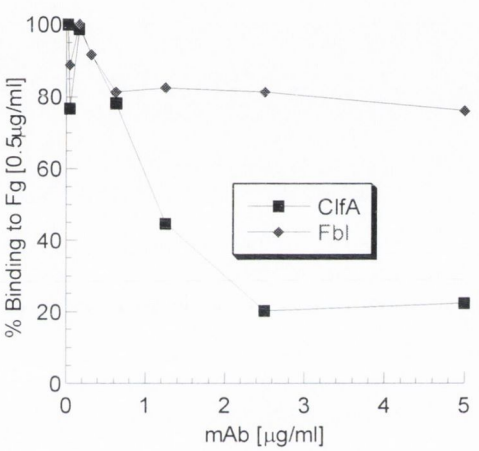
H


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

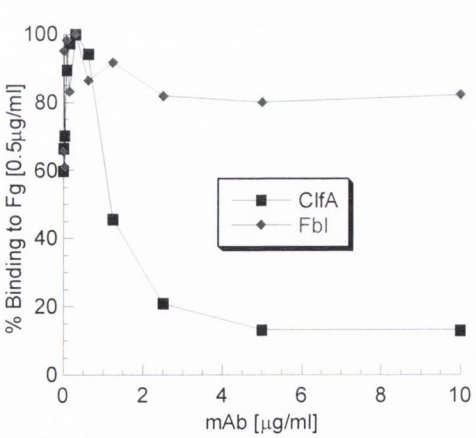
I.


% Binding to Fg [µg/ml]
mAb [µg/ml]
ClfA
Fbl

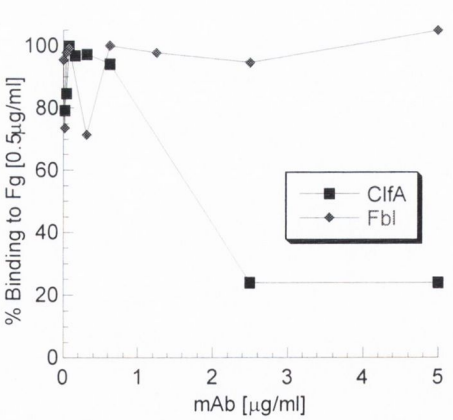
J.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

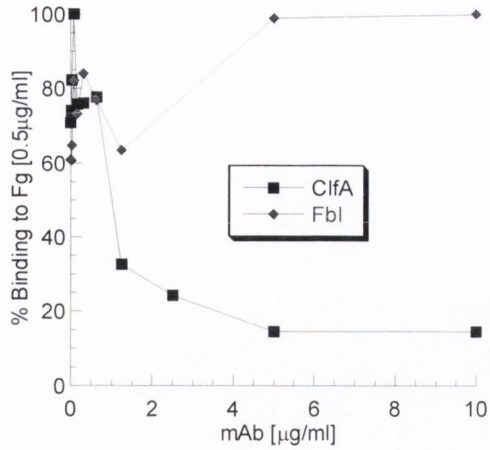
K.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

L.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

M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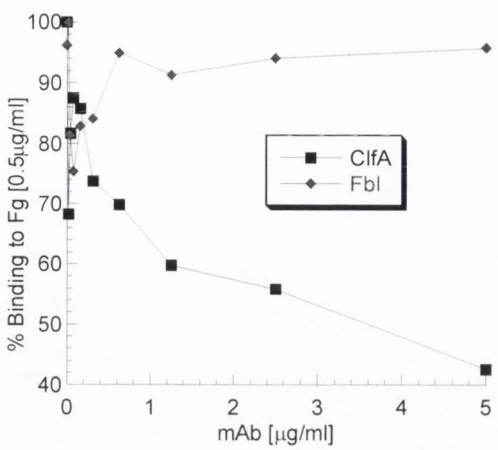
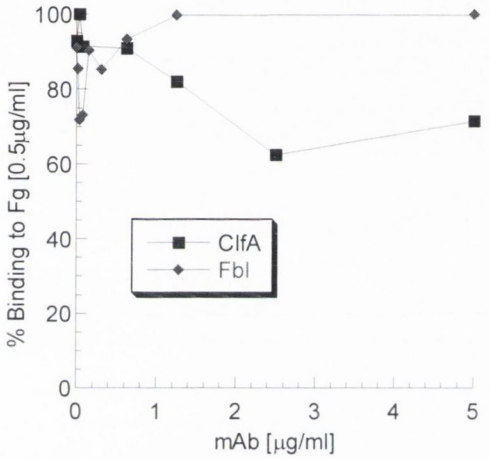
mAb [µg/ml]
ClfA
Fbl

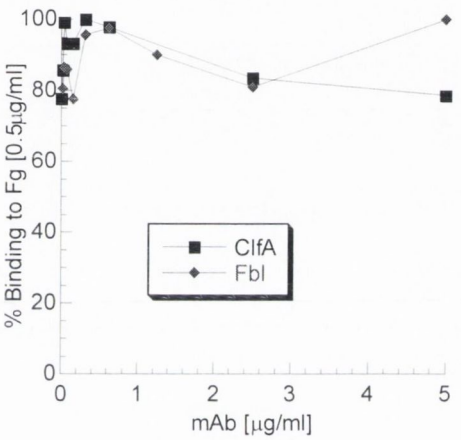
N


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

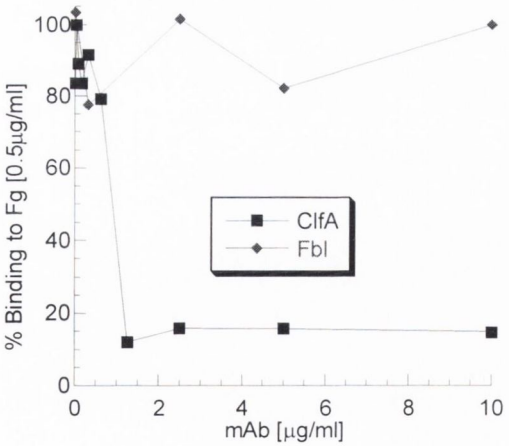
O.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

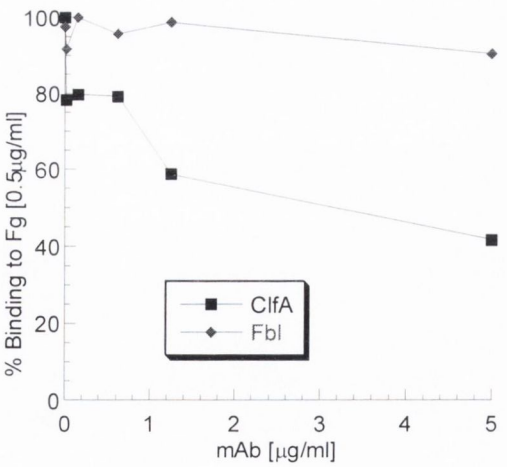
P.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

Q.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

R.


% Binding to Fg [0.5µg/ml]
mAb [µg/ml]
ClfA
Fbl

5.3. Discussion

The initial premise of this study was to elucidate whether ClfA and Fbl were antigenically related as they share a relatively high level of sequence identity at the amino acid level. Vaccination with ClfA/Fbl or passive immunisation with cross-reactive anti-ClfA/anti-Fbl antibodies could spawn therapeutic applications with significant benefit in the clinical setting. Vaccination with recombinant ClfA protein, DNA vaccination with the *clfA* gene and passive immunisation using anti-ClfA region A antibodies have previously been shown to protect against staphylococcal infection in mouse models of staphylococcal disease (Josefsson *et al.*, 2001; Brouillette *et al.*, 2002). It was hoped that immunity against ClfA would also confer a similar immunity against Fbl and thus protection against *S. lugdunensis* disease.

The repertoire of polyclonal antibodies generated against ClfA in rabbits efficiently recognised ClfA on the surface of bacteria and inhibited their interaction with fibrinogen. Presumably this occurs either by steric hindrance, whereby antibody binding obscures the fibrinogen binding site within ClfA preventing the ClfA-fibrinogen interaction, or by binding to the ClfA molecule and thereby changing its three dimensional conformational structure so that it can no longer interact with fibrinogen. Ponnuraj *et al.* (2003) propose that SdrG, the *S. epidermidis* fibrinogen binding protein, interacts with fibrinogen by a "dock, lock and latch" mechanism. If this model of SD-repeat protein-ligand interaction also applies to ClfA, it is possible that a sub-population of antibodies generated against ClfA can bind to ClfA and leave it in an open conformation rendering the ClfA molecule unable to interact with fibrinogen.

Polyclonal rabbit anti-ClfA antibodies were shown to displace bound cells expressing ClfA from a fibrinogen substrate. This observation is relevant in the therapeutic application of both polyclonal and monoclonal anti-ClfA antibodies by passive immunisation. Not only is it possible for antibodies to inhibit free blood-borne cells from interacting with fibrinogen but it is also possible for these antibodies to efficiently detach ClfA from fibrinogen and presumably fibrin clots and thrombi thereby preventing the development of bacterial vegetations at sites of vascular damage and

rendering the bacteria more susceptible to the host immune system and killing by antibiotic chemotherapy. Detachment could involve binding of the antibody to ClfA, and changing its conformation so that it releases its hold on the fibrinogen molecule. Alternatively the antibody could have a higher affinity for ClfA than ClfA has for fibrinogen resulting in the majority of ClfA molecule becoming associated with an antibody which blocks the interaction of ClfA and fibrinogen thus displacing ClfA expressing cells from the fibrinogen substrate.

The polyclonal rabbit anti-ClfA region A antibodies also efficiently inhibited Fbl-mediated bacterial adherence to immobilised fibrinogen in a specific and concentration dependent manner. This indicates that Fbl is sufficiently similar in amino acid sequence and, presumably therefore, in its three dimensional structure such that both proteins share immunogenic epitopes that enable antibodies to inhibit the interaction of Fbl with fibrinogen. Similarly, polyclonal rabbit anti-ClfA region A antibodies detached bound Fbl-expressing cells from a fibrinogen substrate. Polyclonal rabbit anti-Fbl region A antibodies also inhibited both Fbl- and ClfA- mediated bacterial adherence to fibrinogen and efficiently detached Fbl expressing cells from a fibrinogen substrate. Interestingly, however, while the polyclonal rabbit anti-Fbl region A antibodies efficiently detached bound Fbl-expressing bacteria from a fibrinogen substrate, they were significantly less efficient at detaching bound ClfA-expressing bacteria. This could potentially indicate that Fbl and ClfA have subtly different mechanisms of interaction with fibrinogen, as the results in Chapter 4 seem to indicate. However this phenomenon may simply reflect the immunogenic dissimilarity of Fbl and ClfA. Changes in amino acid sequence, and therefore surface topology and charge of the molecules in relation to each other, could lead to reduced recognition of ClfA by anti-Fbl region A antibodies. The antibodies to Fbl were raised in a specific-pathogen-free rabbit whereas the antibodies to ClfA region A were raised in a rabbit that was not specific-pathogen-free. It is possible that the specific-pathogen-free rabbit came in contact with fewer antigens in its short life developing a smaller repertoire of potentially cross-reactive antibodies than the other rabbit, which may contribute to the inability of its antibodies to displace efficiently ClfA-expressing bacteria from fibrinogen.

Veronate™ has been developed by Inhibitex Inc. as a method of protecting very low birth weight infants from potentially life threatening staphylococcal disease. It comprises a purified pool of human IgG enriched for reactivity against the *S. aureus* MSCRAMMS, ClfA and SdrG, the fibrinogen binding protein of *S. epidermidis*. The results of inhibition and displacement experiments, presented herein, showed that not only can Veronate™ inhibit the adherence of ClfA- and Fbl-expressing bacteria to fibrinogen, but it also detached bound bacteria from an immobilised fibrinogen substrate. This is a reassuring result, considering that not only did this product, which is hopefully to be used as an anti-staphylococcal treatment for neonates, efficiently inhibit ClfA-mediated adherence to fibrinogen but it also inhibited Fbl-mediated adherence to fibrinogen and efficiently detached both ClfA and Fbl expressing bacteria from a fibrinogen substrate. Hence, Veronate™ provides a spectrum of anti-staphylococcal antibodies that recognise several disease-causing species of staphylococci, providing a useful therapeutic tool.

In order to determine conclusively whether a single shared epitope in ClfA and Fbl can be exploited to inhibit both of their interactions with fibrinogen, a panel of ten murine monoclonal antibodies, which were generated against $rClfA_{220\text{-}559}$, were examined for their ability to inhibit ClfA- and Fbl-mediated bacterial adherence to a fibrinogen substrate. None of the monoclonal antibodies strongly inhibited the binding of Fbl expressing cells to fibrinogen. None were able to detach Fbl-expressing cells from a fibrinogen substrate. The monoclonal antibodies that recognized the N2 subdomain of ClfA region A, (13-1, 15-6 and 35-220), failed to cause any inhibition of ClfA-mediated binding to fibrinogen. These antibodies were not able to detach ClfA-expressing cells from a fibrinogen substrate. The remaining monoclonal antibodies recognized residues within the N3 subdomain of ClfA region A and all of these antibodies proved able to inhibit the interaction of ClfA and fibrinogen. However, not all of these monoclonal antibodies efficiently detached bound ClfA expressing cells from a fibrinogen, substrate indicating that within this limited panel of monoclonal antibody diversity there were distinct mechanistic differences between inhibition of the ClfA-fibrinogen interaction alone, and the ability to inhibit and detach cells from the fibrinogen substrate. Similar

observations have been made with Cna, the collagen binding protein of *S. aureus* (Visai *et al.*, 2000), whereby monoclonal antibodies were identified that recognized conformation-dependent epitopes that were mapped throughout the Cna-(151-318) domain using a chimeric strategy where segments of Cna were grafted on ACE, a structurally related MSCRAMM from *Enterococcus faecalis*. These monoclonal antibodies were able to inhibit the Cna-collagen interaction and could effectively displace ^{125}I-collagen bound to the bacteria. This study concluded that there were several mechanisms whereby the monoclonal antibodies could interrupt the Cna-collagen interaction including direct steric hindrance of the binding site of Cna by the antibody and that some of the antibodies were binding to the back-side of the Cna molecule and potentially changing the conformation of Cna, resulting in a conformation with a low affinity for collagen. Whether these mechanisms apply to the interruption of the ClfA-fibrinogen interaction by monoclonal antibodies will be discussed in Chapter 6.

Studies are currently underway to identify an antibody that will bind to both Fbl and ClfA and inhibit both of their interactions with fibrinogen. The ideal outcome of studies such as these would be to identify an antibody that recognizes both antigens and inhibits their fibrinogen binding activity. Such an antibody could be used in passive immunization. Alternatively a hybrid ClfA-Fbl molecule could be generated that would provide protective immunity against both proteins if administered as part of a vaccine by identifying the most useful, function blocking immunogenic epitopes present in each protein and combining them in a single recombinant hybrid molecule.

Chapter 6

Identifying the ClfA epitope to which the murine monoclonal antibody 12-9 binds

6.1 Introduction

All the vaccine strategies to combat *S. aureus* infections that have been tested thus far in both animal models and human clinical trials have shown that an increase in specific anti-staphylococcal antibodies has led to a concomitant reduction in morbidity and mortality (Joseffson *et al.*, 2001; Brouillette *et al.*, 2002; Shinefield *et al.*, 2002; Balaban *et al.*, 1998; Senna *et al.*, 2002; Leitner *et al.*, 2003). Therefore the generation of specific antibodies against staphylococcal surface components that lead to functional attenuation and efficient opsonophagocytosis by neutrophils represent a powerful weapon against increasingly antibiotic resistant staphylococci.

The gene encoding the clumping factor of *Staphylococcus aureus*, *clfA*, is encoded by the vast majority of strains thus far tested (100%, Smeltzer *et al.*, 1997; 98-100%, Peacock *et al.*, 2002). *Staphylococcus aureus clfA* mutant strains are less virulent in a mouse model of septic arthritis. Both active immunization with recombinant $ClfA_{40-559}$ protein or a DNA vaccine expressing $ClfA_{40-559}$ from eukaryotic cells, and passive immunization with anti-ClfA region A antibodies protected against *S. aureus* disease and sepsis-induced death (Josefsson *et al.*, 2001; Brouillette *et al.*, 2002). The expression of *clfA* has been demonstrated by differential fluorescence induction in infection-simulating culture (Schneider *et al.*, 2002). Furthermore, anti-ClfA antibodies have been detected in the antiserum of both convalescent and healthy subjects by serological proteome analysis (Vytvytska *et al.*, 2002), peptide display on the surface of *E. coli* (Etz *et al.*, 2002) and immunological screening of whole genome expression libraries (Weichhart *et al.*, 2003). Taken together these data provide strong evidence for the *in vivo* expression of *clfA* and that it is a virulence factor that plays an important role in the pathogenesis of *Staphylococcus aureus* infections.

The use of passive immunotherapy both therapeutically and prophylactically to combat staphylococcal infections is currently being investigated by a number of groups (Weisman *et al.*, 2001; Weisman *et al.*, 2003; Vernachio *et al.*, 2003). Inhibitex Inc. in Georgia, USA, have developed a range of monoclonal antibodies that specifically recognize epitopes within $ClfA_{220-559}$ from strain Newman. Monoclonal antibody 12-9 was chosen for development (Hall *et al.*, 2003; Domanski *et al.*, 2004). These antibodies

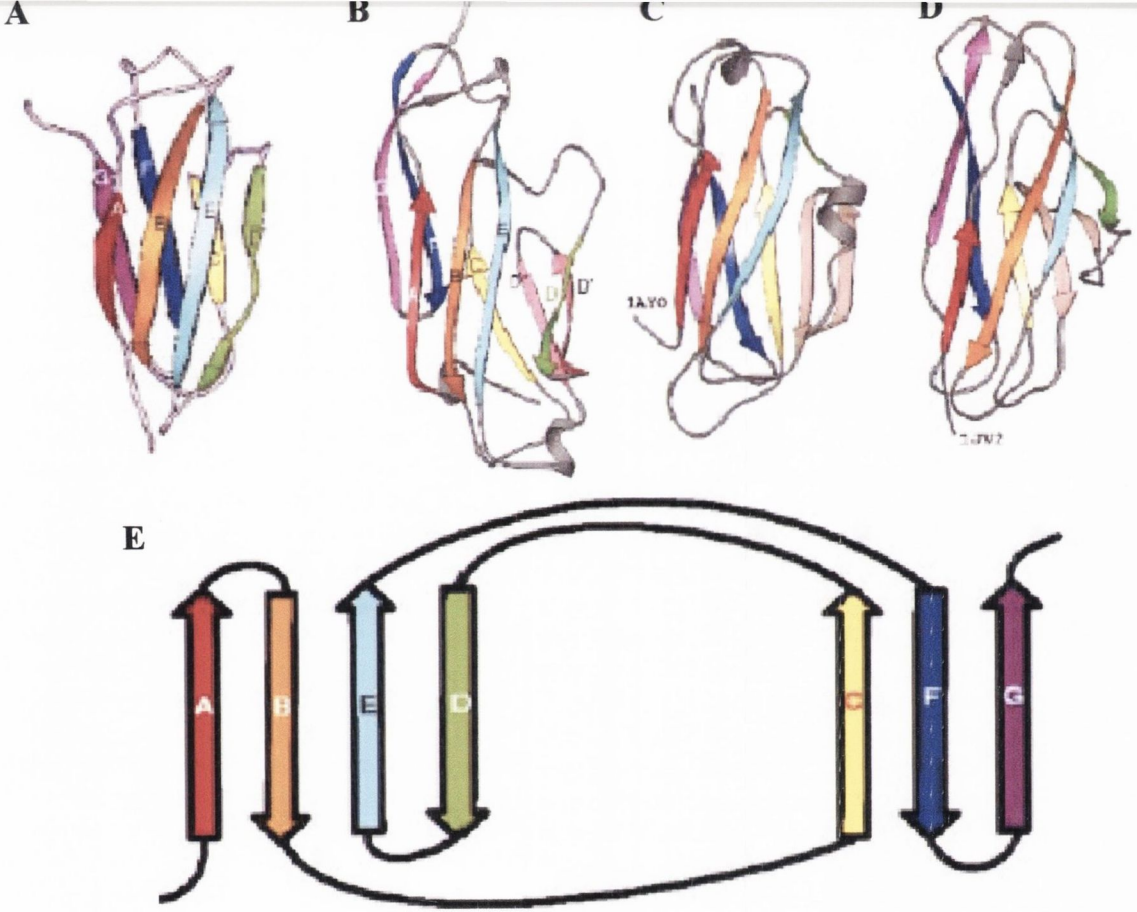


Figure 6.1 Ribbon diagrams of crystal structures with the DEv-IgG fold. A. IgG-C domain. B. N2 domain of ClfA. C. Receptor-binding domain of α2-macroglobulin. D. Calf-2 domain of extracellular segment of the integrin αVβ3. E. Topology of the IgG-C domain. From Deivanayagam *et al.* (2002).

not only enhanced opsonophagocytic uptake of ClfA-coated beads and protected against intravenous *S. aureus* MRSA challenge in a murine sepsis model but they also enhanced the therapeutic efficacy of vancomycin therapy in a model of already-established rabbit infective endocarditis. Also, significantly, 12-9 and its humanized counterpart T1-2 not only appeared to have the potential to neutralize the virulence of ClfA-mediated staphylococcal disease by specifically inhibiting ClfA-mediated adherence of *S. aureus* to fibrinogen, but they also displaced bound *S. aureus* cells that were adhering to an immobilised fibrinogen substrate. Therefore it is clear that passive immunotherapy could be an important weapon against *S. aureus* disease. It is vital to ensure that mutations do not occur which allow *S. aureus* to escape recognition by monoclonal antibodies and threaten efficacy of passive immunotherapy.

The production of antibody is the major contribution of B-cells to adaptive immunity. Antibodies function by binding specifically to their cognate antigens and recruiting other cells and immune effector molecules such as complement, to destroy the pathogen once the antibody is bound. The antigen binding region of antibody is known as the variable (V) region as antibodies vary extensively depending upon the antigen that they recognize. The repertoire of antibodies that an individual elaborates is large enough to ensure that almost any foreign molecule can be recognised. The constant (C) region of the antibody molecule is the region that engages the effector functions of the immune system. Five classes of the C-region engage in different immune effector mechanisms, IgG, IgE, IgM, IgA and IgD.

Each domain of an immunoglobulin molecule has a similar structure called the immunoglobulin fold comprising β-sheets formed by adjacent strands of the polypeptide that pack together to form a particular β-barrel structure known as a jelly-roll barrel. As illustrated in Fig. 6.1, in the constant IgG fold (C-type), strands ABED and GFC are arranged at the front and back sides of the fold, respectively, whereas in its variants (V-, H- and I-type), additional structural elements are found between strands C and D (Bork *et al.*, 1994; Harpaz and Chothia, 1994). Analogous immunoglobulin domains are found in a wide variety of mammalian proteins in the immune system, nervous system and proteins involved in cell-cell recognition and also in bacterial proteins such as ClfA, ClfB and SdrG (Ponnuraj *et al.*, 2003; Deivanayagam *et al.*, 2002) (Fig. 6.1).

The complementarity determining regions that determine antigen specificity of an immunoglobulin molecule are located within hypervariable regions separated by framework regions situated in the variable region. The framework regions form the antiparallel β-strands whereas the hypervariable regions correspond to three loops that connect the sheets and are exposed on the surface of the molecule. When V_H and V_L chains are brought together they form a hypervariable antigen-binding site. The surface and charge characteristics of the antigen-binding site are determined by the sequence of amino acids presented by the hypervariable loops and as a consequence antibodies bind antigens whose surface is complementary to that of the antibody. The region on the surface of an antigen to which an antibody binds is called the antigenic determinant or epitope. An epitope composed of a single segment of a polypeptide chain is called a continuous or linear epitope. However, it is more likely that an antibody will recognize an epitope from the native antigen, whereby different parts of the protein fold together to form a discontinuous or conformational epitope.

The overall structure of a protein molecule is determined by the way in which the individual regions of α-helix and β-sheet fold up. The fold of a protein is its tertiary structure. The tertiary structure is determined and held together by a number of weak bonds including Van der Waals forces, hydrogen bonds, hydrophobic interactions and salt bridges. Additional stability can also be achieved by the presence of covalent disulfide bridges between the sulphur atoms of cysteine residues present at different points in the amino acid sequence, but closely separated spatially.

The interaction between an antibody and its antigen is essentially a protein-protein interaction. The forces that mediate this process are the same as those that direct folding of a polypeptide into its tertiary conformation. These interactions are disrupted by high salt, changes in pH and detergents and so the protein-protein interaction is a reversible non-covalent process. These non-covalent interactions can involve electrostatic interactions between charged amino acid side chains in salt bridges, between electric dipoles as in hydrogen bonds, Van der Waals forces or hydrophobic interactions. When two hydrophobic surfaces come together and exclude water molecules a hydrophobic interaction is formed.

The ability of proteins to form crystals and advances in X-ray crystallography technology has allowed structural biologists to analyze accurately and dissect these interactions. Co-crystallization of proteins with their ligands or antibodies and their antigens allows identification of all the contact residues at the protein-protein interface thus identifying the most important forces that mediate the interaction. Cunningham and Wells (1993) showed that for the interaction of human growth hormone and its receptor, the largest decreases in on rates were observed when positively charged residues were mutated to alanine. The largest increases in on rate resulted from mutagenesis of negatively charged residues to alanine. They also concluded that the majority of interactions between the two proteins were to minimize the off rate and that the contact residues were hydrophobic. This would appear to be a paradigm for most strong protein-protein interactions and it is hypothesized that hydrophobic residues form the strongest contact residues in antibody-antigen interactions though it is difficult to prove without crystallizing the antibody-antigen complexes.

Site-directed mutagenesis has been widely shown to be a useful tool in protein engineering (Burks, 1997), mapping protein-protein (Braistead and Wells, 1996), protein-receptor (Wells, 1996) and protein-substrate interactions (Erlanson *et al.*, 2000; Arkin *et al.*, 2003) and in identifying the antigenic epitopes to which antibodies bind (Parhami-Seren *et al.*, 2003). Alanine scanning mutagenesis is a method whereby each residue targeted for mutagenesis is replaced with an alanine residue thus enabling the analysis of the role of side chain atoms beyond the β-carbon in the structure of the protein or peptide molecule (Ponnuraj *et al.*, 2003; Wells, 1996). Parhami-Seren and colleagues (2003) successfully used this strategy to determine where in streptokinase a particular monoclonal antibody bound that prevented its plasminogen-activating site from operating and that neutralized streptokinase's therapeutic effects.

RasMol is a molecular graphics program intended for the 3-D visualization of proteins, nucleic acids and small molecules (www.umass.edu/microbio/rasmol/). RasMol reads in molecular co-ordinate files in a number of formats and interactively displays the molecule on the screen in a variety of colour schemes and representations. The crystal structures of proteins are stored in PDB file format (Protein Data Bank, from Research Collaboratory for Structural Bioinformatics) which is the standard file format for the

XYZ coordinates of atoms in a molecule. RasMol and more recent and improved programs such as Protein Explorer have proved to be valuable tools in the analysis of the 3D structure of proteins and aid in the prediction of amino acid residues that could be important in protein-protein interactions.

This chapter deals with the identification of the $rClfA_{220-559}$ epitopes to which murine monoclonal antibodies generated against $rClfA_{220-559}$ bind. Initially the antibodies were tested for their ability to recognize the recombinant proteins $rClfA_{40-559}$ and $rFbl_{40-534}$. The antibodies were then tested for their ability to recognize $rClfA_{220-559}$ cloned from different strains of *S. aureus*, which contain naturally occurring mutations that affect the amino acid sequence of the ClfA ligand-binding A region. Using a rational structure-based approach, amino acids were mutated by site-directed mutagenesis to alanine. Residues were chosen on the basis of residues that changed between Fbl and ClfA that were located on the surface of the $rClfA_{220-559}$ molecule as determined by RasMol analysis, but that did not change in the naturally occurring *S. aureus* ClfA variants. One mutated protein $rClfA_{220-559}$ K417A was analysed further with regard to its mAb 12-9 binding ability and Y376A, I517A and R519A were analysed with regard to their mAb 35-039 binding ability.

6.2 Results

6.2.1 Monoclonal antibodies generated against recombinant $Clf_{220-559}$ do not recognise recombinant Fbl_{40-559}

None of the monoclonal antibodies generated against $rClfA_{220-559}$ that were tested in Chapter 5 strongly inhibited Fbl-mediated adherence to fibrinogen. In addition they did not displace bound bacteria expressing Fbl from a fibrinogen substrate. In order to test whether this was due to an inability of the antibodies to recognise the Fbl protein the ten murine monoclonal antibodies were tested for their ability to bind to $rClfA_{40-559}$ and $rFbl_{40-534}$ in an ELISA assay. The monoclonal antibodies bound to either recombinant N2 or recombinant N3 proteins and this is summarised in Table 6.1 (P. Domanski, personal communication.

ELISA plate wells were coated with either $rClfA_{40-559}$ or $rFbl_{40-534}$. Varying amounts of monoclonal antibody ranging from 10μg/ml to 19.5ng/ml were added to the wells and interaction of antigen and antibody was detected using rabbit anti-mouse IgG conjugated to horseradish peroxidase and tetramethylbenzidine substrate. Figure 6.2 clearly shows that the monoclonal antibody 12-9 specifically recognises $rClfA_{40-559}$ in a dose-dependent and saturable manner while it fails to recognise $rFbl_{40-534}$. Furthermore the data presented in Fig. 6.3 indicates that none of the monoclonal antibodies, including mAb 12-9, recognise $rFbl_{40-534}$ whereas they all bind to $rClfA_{40-534}$.

Fig. 6.4 is a RasMol generated diagram which illustrates the differences between the amino acid sequences of the Fbl A domain and the *S. aureus* strain Newman ClfA A domain, as defined by ClustalW analysis, represented on the crystal structure of $rClfA_{220-559}$. This shows that the majority of the 40% of differing residues are located on the outer surface of the ClfA molecule. This presumably contributes to the antigenic differences between the two proteins. It is interesting to note that the residues that have thus far been shown to contribute to binding to fibrinogen (Tyr_{256}, Pro_{336}, Tyr_{338}, Lys_{389}, and Val_{527}) (Hartford *et al.*, 2001; Deivanayagam *et al.*, 2002) are highly conserved in Fbl and ClfA. Additionally none of the variant residues appear to be located at the interface between N2 and N3, proposed to form the binding pocket where fibrinogen binds to ClfA. Therefore

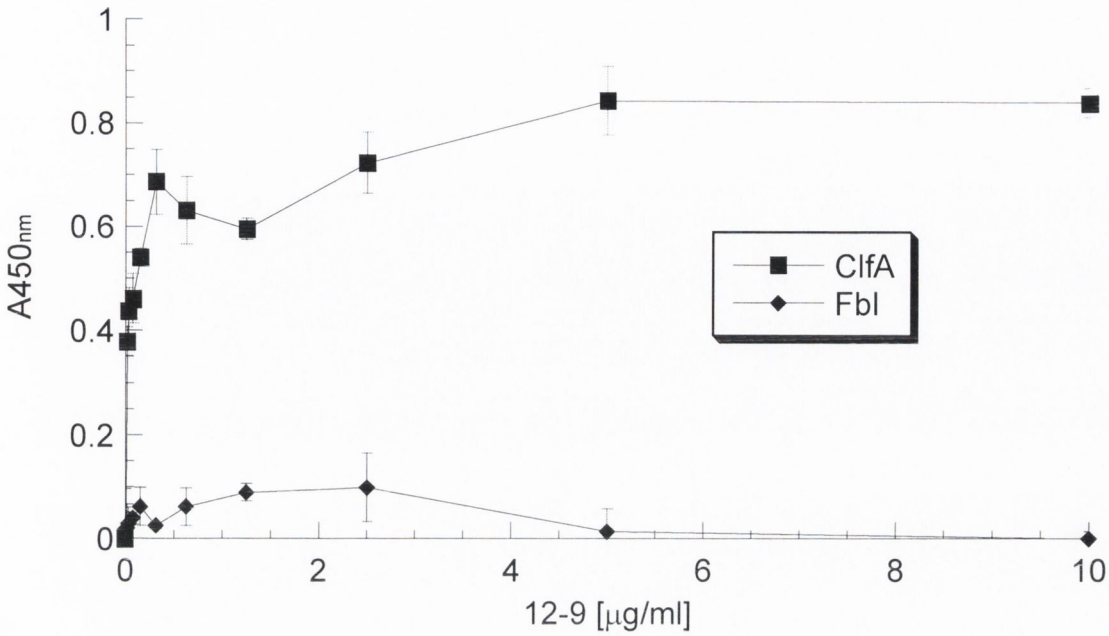
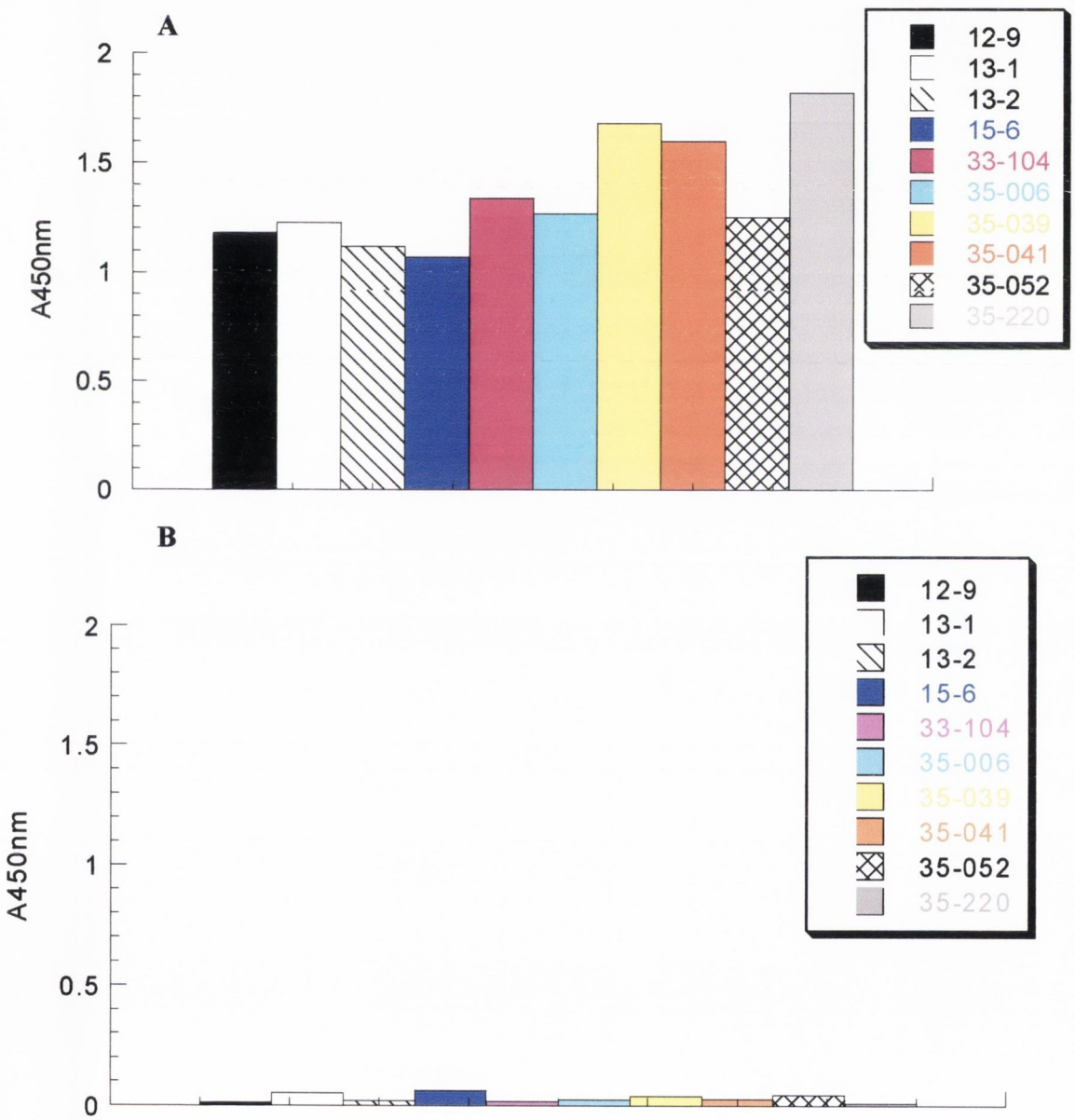


Figure 6.2 Binding of the murine monoclonal antibody 12-9 to recombinant Fbl_{40-534} and $ClfA_{40-559}$. Microtitre wells were coated with 100μl of a 10μg/ml solution of either $rFbl_{40-534}$ or $rClfA_{40-559}$. Increasing concentrations of mAb 12-9 were incubated in the wells for 1 hr at room temperature. Bound antibody was detected by rabbit anti-goat IgG conjugated to horse radish peroxidase followed by development with tetramethylbenzidine substrate. Values represent the means of triplicate wells. This experiment was repeated three times with similar results

Figure 6.3 Binding of a panel of murine monoclonal antibodies to recombinant Fbl_{40-534} and $ClfA_{40-559}$. Microtitre wells were coated with 100µl of a 10µg/ml solution of either $rFbl_{40-534}$ (A) or $rClfA_{40-559}$ (B). 2µg/ml of each antibody was incubated in the wells for 1 hr at room temperature. Bound antibody was detected by rabbit anti-goat HRP-conjugated antibodies followed by development with tetramethylbenzidine substrate. Values represent the means of triplicate wells. This experiment was repeated three times with similar results



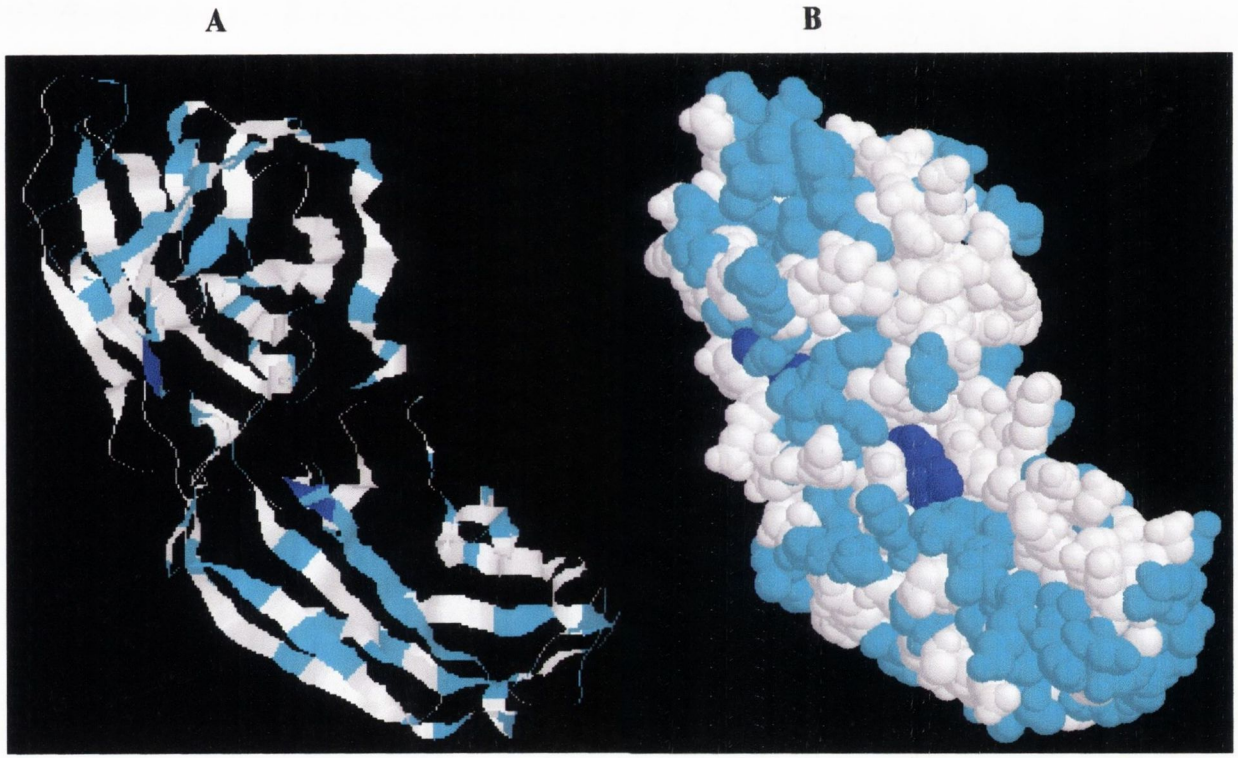


Figure 6.4 Ribbon diagram (A) and space-fill model (B) of the crystal structure $rClfA_{220-559}$ generated by RasMol. The dark blue residues represent the residues that when mutated to alanine abrogates binding of to $rClfA_{220-559}$ fibrinogen. These residues are conserved between Fbl and ClfA. The cyan residues represent the variant residues when the amino acid sequence of $ClfA_{220-559}$ is compared to that of $Fbl_{214-534}$ by ClustalW analysis.

it can be concluded from this data that this bank of ten monoclonal antibodies are highly specific for ClfA and do not recognise any epitopes in Fbl. This is likely to be the reason why, as observed in the data presented in Chapter 5, none of the antibodies either inhibited Fbl-mediated adherence to fibrinogen or displaced Fbl-expressing bacteria from an immobilised fibrinogen substrate.

6.2.2 Cloning and expression of naturally occurring $Clf_{220-559}$ variants

It was important to determine whether the ten murine monoclonal antibodies generated against residues 220-559 of ClfA would recognise ClfA expressed by different strains of *S. aureus*. Hall *et al.* (2003) demonstrated that antibody 12-9 recognised a range of *S. aureus* strains by flow cytometry but this screen failed to take into account differing expression levels of ClfA between strains and other cellular components that could interfere with 12-9 binding to the cell. Therefore it was decided to clone the DNA encoding residues 220-559 of ClfA from a range of *S. aureus* strains from different clonal complexes into the expression vector pQE30 and to analyse the ability of the monoclonal antibodies to recognise the resultant recombinant proteins by ELISA. This would aid in determining whether naturally occurring variations in amino acid sequence from strain to strain affected the ability of the monoclonal antibodies to recognise ClfA.

ClustalW analysis of the DNA sequence encoding residues 220-559 of ClfA was performed on all the available *S. aureus* DNA sequences in the public domain (*S. aureus* strains N315, Mu50, MW2 at http://www.ncbi.nlm.nih.gov/BLAST, *S. aureus* strain COL at http://tigrblast.tigr.org/ufmg/index.cgi?database=s_aureus|seq, *S. aureus* strains MRSA252 and MSSA476 at http://www.sanger.ac.uk/Projects/S_aureus/ and *S. aureus* strain 8325 at http://www.genome.ou.edu/staph.html). Fig. 6.5 shows the results of the ClustalW analysis highlighting the residues that vary. When these variant residues were mapped onto the crystal structure of $rClfA_{220-559}$ the majority of the variant residues were located on the surface of the molecule. None of the residues involved in binding to fibrinogen varied between strains and there were no variant residues on the interface or N2 and N3 that is proposed to form the binding pocket.

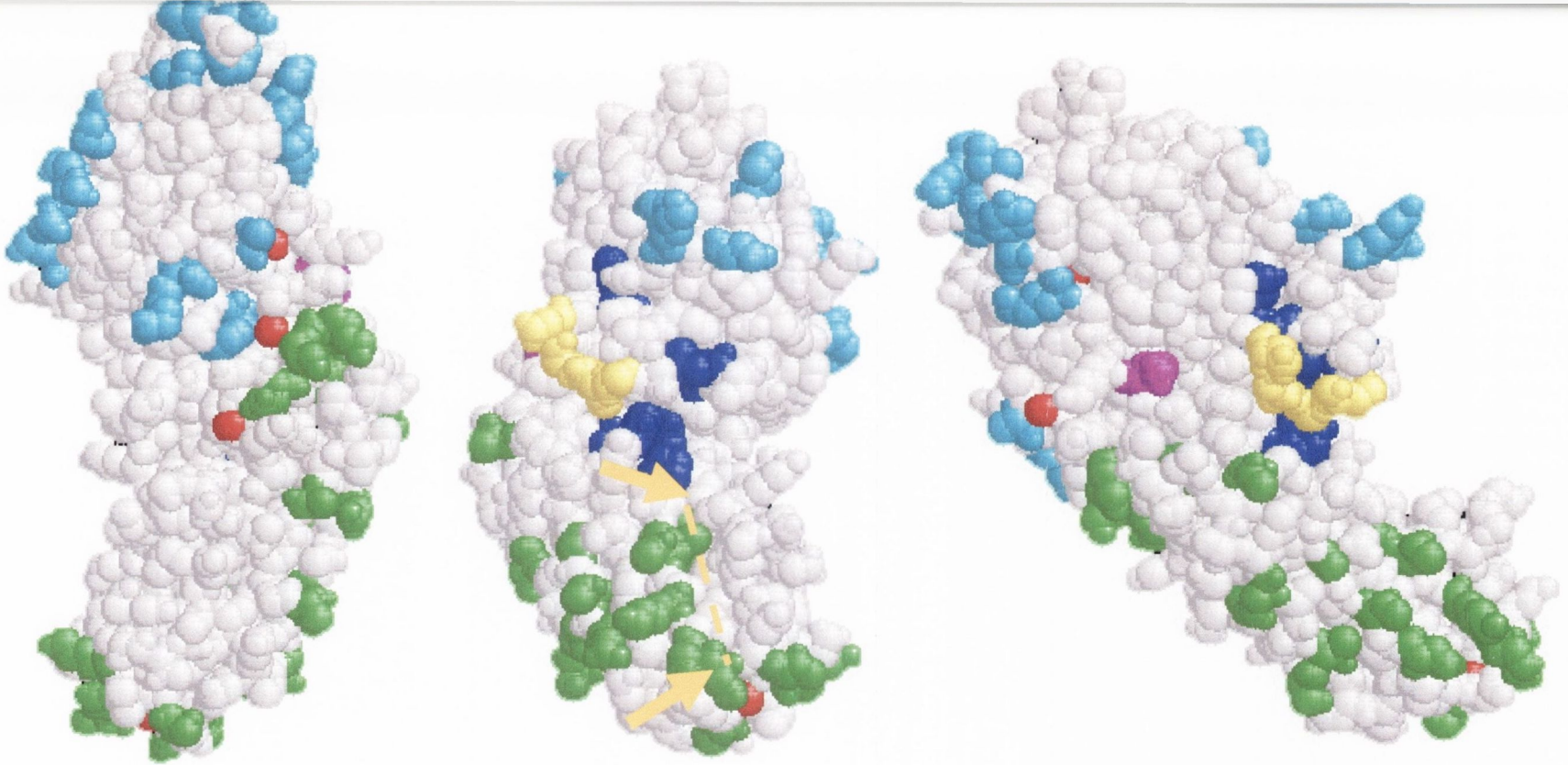
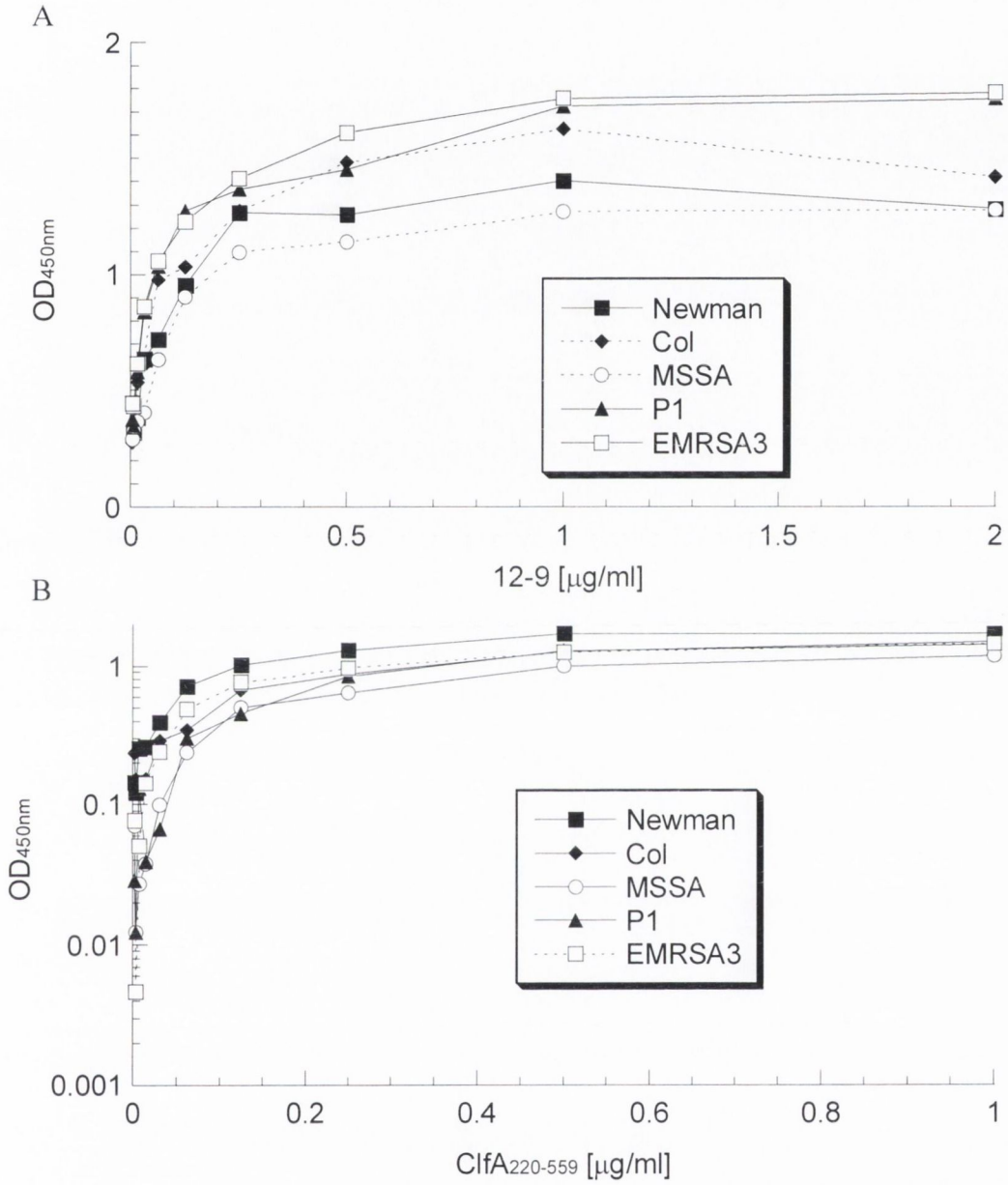


Figure 6.5 Space-filled structural models of three faces of $rClfA_{220-559}$ generated by RasMol. The dark blue residues represent the residues that, when mutated to alanine, abrogate binding of $rClfA_{220-559}$ fibrinogen. The cyan residues represent the variant residues between strains of *S. aureus* in $ClfA_{220-377}$ (N2). The green residues represent the variant residues between strains of *S. aureus* in $ClfA_{377-559}$ (N3). The yellow residues form the proposed latching β-strand and the red residues are the Ca^{2+} ions that coordinate with the $rClfA_{220-559}$ crystal. The orange arrows indicate the groove into which the latching peptide is predicted to enter after ligand binding.

Figure 6.6 The binding of mAb 12-9 to wild-type $rClfA_{220-559}$ and natural $rClfA_{220-559}$ variants. **A**. 96-well ELISA plates wells were coated with 1μg of either wild-type $rClfA_{220-559}$ or a $rClfA_{220-559}$ cloned from different *S. aureus* strains. The plates were blocked with 2% BSA and then probed with two-fold serial doubling dilutions of monoclonal antibody 12-9 ranging from 2μg/ml to 8ng/ml. Bound antibody was detected by rabbit anti-mouse HRP-conjugated antibodies followed by development with tetramethylbenzidine substrate. **B**. ELISA plates wells were coated with 1μg of monoclonal antibody 12-9. The plates were blocked with 2% BSA and then probed with two-fold serial doubling dilutions of $rClfA_{220-559}$ ranging from 1μg/ml to 4ng/ml. Bound protein was detected nickel chelated HRP followed by development with tetramethylbenzidine substrate. Values represent the means of triplicate wells. These experiments were repeated three times with similar results.


A
2
1
0
OD450nm
0
0.5
1
1.5
2
12-9 [μg/ml]
Newman
Col
MSSA
P1
EMRSA3
B
1
0.1
0.01
0.001
OD450nm
0
0.2
0.4
0.6
0.8
1
ClfA220-559 [μg/ml]
Newman
Col
MSSA
P1
EMRSA3

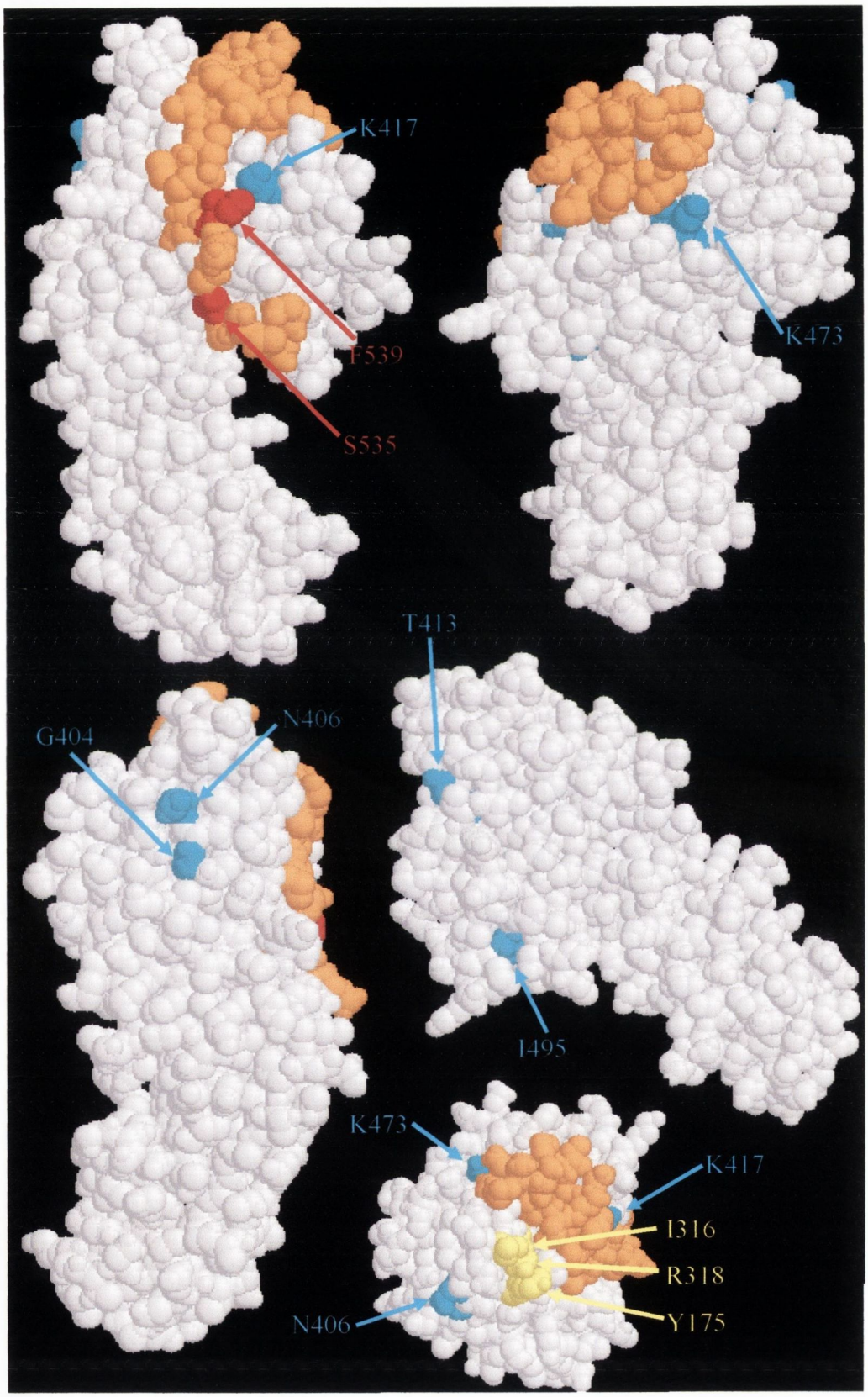
The DNA encoding residues 220-559 of ClfA from seven different strains of *S. aureus* was amplified by PCR and cloned into the pQE30 expression plasmid and transformed into *E. coli* TOPP3. The $rClfA_{220-559}$ proteins were purified from IPTG induced cultures by nickel-chelate affinity chromatography as described in Chapter 2. ELISA plate wells were coated with 100µl of 10µg/ml of each of the variant $rClfA_{220-559}$ proteins. Varying amounts of monoclonal antibody 12-9 were added to the wells. Bound antibody was detected using rabbit anti-mouse IgG conjugated to horseradish peroxidase and tetramethylbenzidine substrate. The data presented in Fig. 6.6 A shows that 12-9 binds to all the variant $rClfA_{220-559}$ proteins and there appears to be no significant differences in affinity.

The reciprocal experiment was also carried out whereby the 96-well microtitre plates were coated overnight with monoclonal antibody 12-9, to which was added varying concentrations of the ClfA variant proteins. Again there appeared to be no significant difference in affinity between wild-type $rClfA_{220-559}$ and the variant $rClfA_{220-559}$ proteins (Fig. 6.6 B). This indicates that coating $rClfA_{220-559}$ or the monoclonal antibody 12-9, onto Sarstedt 96-well microtitre plates does not alter conformation significantly. Furthermore the data presented in this section confirms that 12-9 recognises variant ClfA proteins from different *S. aureus* strains.

6.2.3 Mutagenesis of $Clf_{220-559}$

In order to identify the amino acid residues that comprise the 12-9 epitope, a partial alanine scanning mutagenesis strategy of $rClfA_{220-559}$ was employed. In view of the fact that none of the murine monoclonal antibodies against $rClfA_{220-559}$ recognised $rFbl_{40-534}$, those residues that varied between the two proteins and that were located on the surface of the molecule were chosen as a starting point to identify residues that could comprise the epitope. This list was further narrowed by subtracting those residues that varied between *S. aureus* strains and did not affect the binding affinity of 12-9 for $rClfA_{220-559}$. The study was focused on the N3 domain as mAb 12-9 binds to the N3 domain of $rClfA_{220-559}$ (P. Domanski, personal communication). 62 residues varied between Fbl and ClfA N3 domain. Of these 3 were not surface exposed residues and

Figure 6.7 Positions of residues in $ClfA_{220-559}$ that were mutated to alanine by site directed mutagenesis. Residues K_{417}, K_{473}, T_{413}, I_{495}, N_{406}, and G_{404} are coloured cyan. Residues S_{535} and F_{539} are located within the putative latching peptide and are coloured red. Residues Y_{376}, I_{517} and R_{519} are coloured yellow. The residues comprising the latching peptide are coloured orange.


K417
F539
S535
K473
T413
G404
N406
I495
K473
K417
I316
R318
Y175
N406

therefore not considered for mutagenesis. 19 of the residues also varied between the naturally variant ClfA N3 amino acid sequences. As these 19 did not affect the binding of 12-9 as demonstrated in section 6.2.2, they were not chosen for mutagenesis. 40 residues remained that varied between Fbl and ClfA. Eleven residues were chosen to change to alanine based on their location on the surface of the molecule. Both charged and hydrophobic residues were chosen that were well spaced out on the $rClfA_{220-559}$ crystal structure to cover as many potential epitope locations as possible. I_{517}, R_{519} and Y_{376} were chosen based on their position on the $rClfA_{220-559}$ crystal structure and because the three residues appeared to form a possible epitope. The generation of the site-directed mutations was achieved by overlap-extension PCR. Initially eleven residues were selected to change to alanine: N_{406}, T_{413}, G_{404}, S_{535}, K_{417}, K_{473}, I_{495}, F_{539}, I_{517}, R_{519} and Y_{376} (Fig. 6.7).

The $rClfA_{220-559}$ mutant proteins were purified from IPTG-induced cultures by nickel-chelate affinity chromatography, separated from contaminating proteins by anion-exchange chromatography and then re-purified by nickel chelate chromatography. 1μg of each of the purified $rClfA_{220-559}$ mutant proteins was then dotted onto PVDF membranes. Each protein was then probed with monoclonal antibody. The interaction between antigen and antibody was detected using rabbit anti-mouse IgG conjugated to horseradish peroxidase and New England Biolabs chemiluminescent substrate. The results of this experiment are illustrated in Fig. 6.8. The K417A mutation appeared to bind 12-9 less efficiently than wild-type $rClfA_{220-559}$ and the other single substitution mutants. Therefore the interaction of the K417A mutant $rClfA_{220-559}$ protein and 12-9 was analysed by ELISA in order to analyse whether it formed part of the 12-9 binding epitope (Fig. 6.10).

ELISA plate wells were coated with 100μl of a 10μg/ml solution of each of the variant $rClfA_{220-559}$ proteins. Varying concentrations of monoclonal antibody were added to the wells and the interaction of antigen and antibody was detected using rabbit anti-mouse IgG conjugated to horseradish peroxidase and tetramethylbenzidine substrate. The data presented in Fig. 6.9 indicates that $rClfA_{220-559}$ K417A bound 12-9 with a ~50% lower apparent affinity compared to wild-type $rClfA_{220-559}$. The 13-1 and 15-6 control antibodies recognised both the wild-type and $rClfA_{220-559}$ K417A mutant with a similar

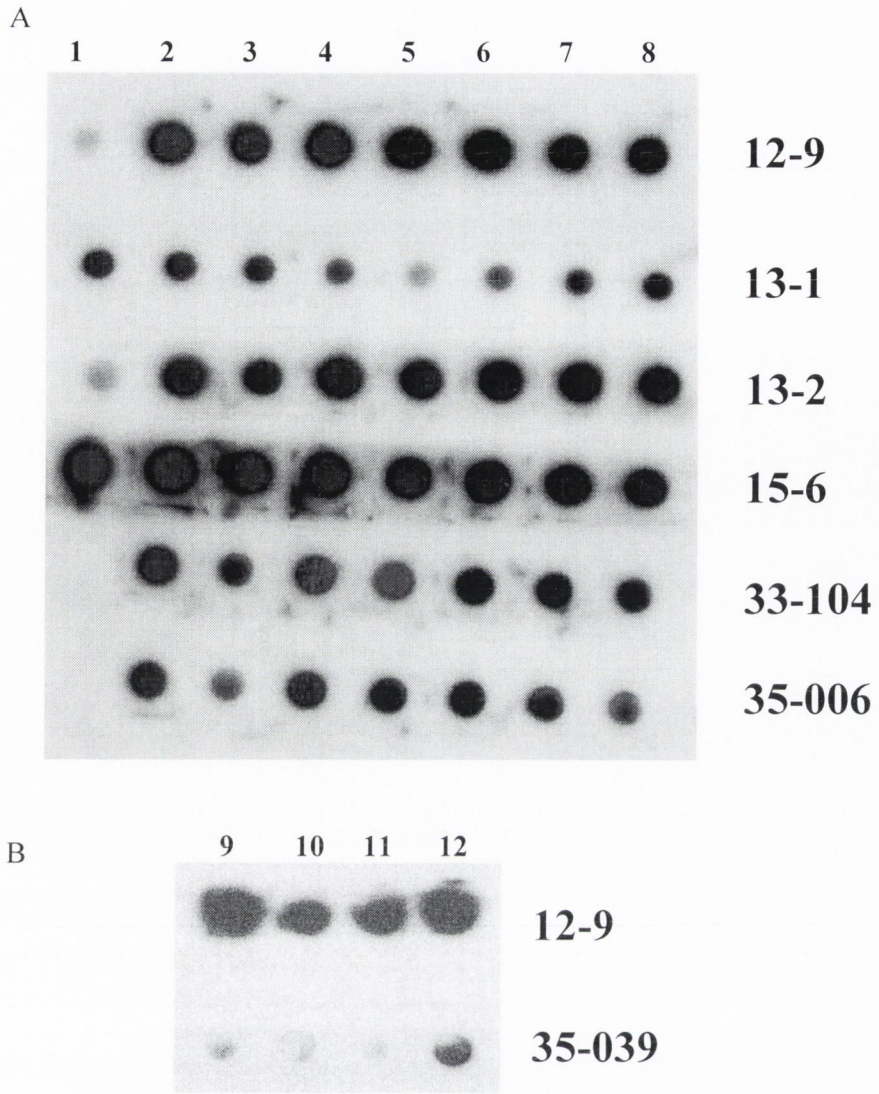
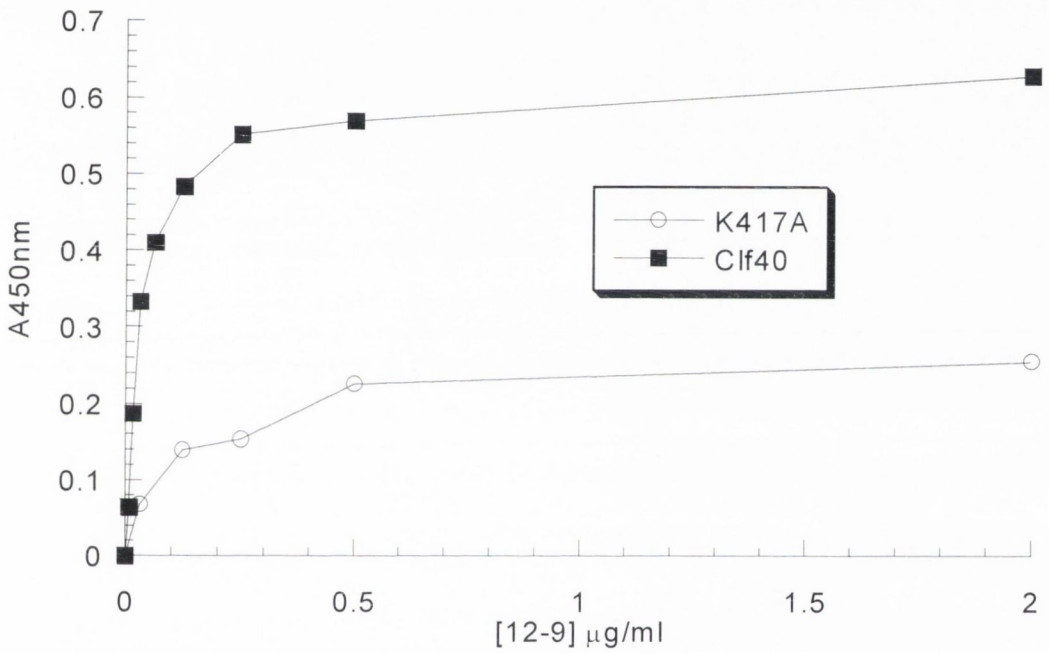


Figure 6.8 Dot immunoblot analyses of mutant rClfA$_{220-559}$ proteins. 1μg of each mutant rClfA$_{220-559}$ protein was dotted out onto PVDF membrane (**1.** K417A, **2.** K473A, **3.** T413A, **4.** S535A, **5.** I495A, **6.** F539A, **7.** N406A, **8.** G404A, **9.** Y376A, **10.** I517A, **11.** R519A, **12.** WT rClfA$_{220-559}$). The membranes were blocked in 10% Marvel for 1hr and then probed with monoclonal antibody. Binding of the mAbs to the mutant rClfA$_{220-559}$ proteins was detected using rabbit anti-mouse IgG-HRP and ECL detection reagents. Membranes in **A** were detected simultaneously and membranes in **B** were detected simultaneously. This experiment was carried out three times with similar results.

Figure 6.9 The binding of mAb 12-9 to wild-type $rClfA_{220-559}$ and $rClfA_{220-559}$ K417A. 96-well ELISA plates wells were coated with 1μg of either wild-type $rClfA_{220-559}$ or $rClfA_{220-559}$ K417A. The plates were blocked with 2% BSA and then probed with two-fold serial doubling dilutions of monoclonal antibody 12-9 ranging from 2μg/ml to 7.8ng/ml. Bound antibody was detected by rabbit anti-mouse HRP-conjugated antibodies followed by development with tetramethylbenzidine substrate. Values represent the means of triplicate wells. This experiment was repeated three times with similar results.



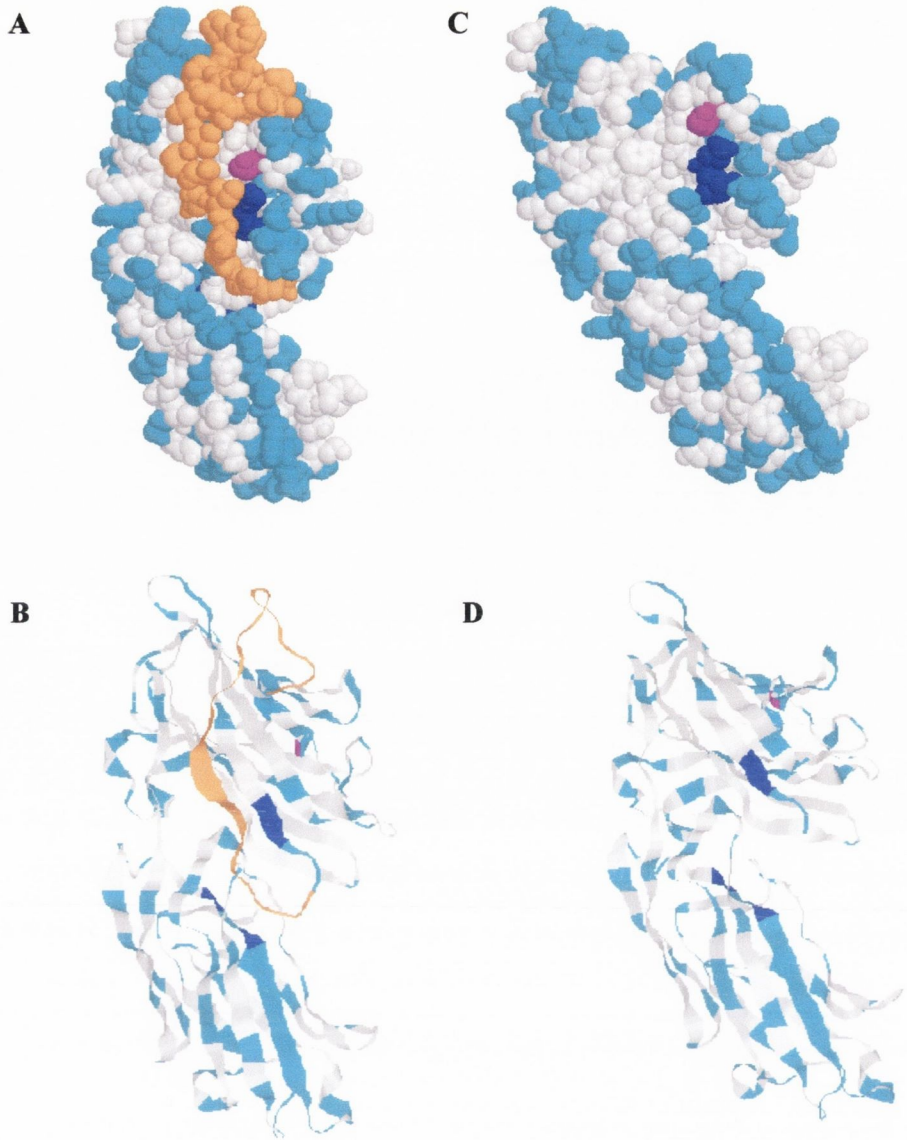


Figure 6.10 The position of the K417A substitution mapped onto the $rClfA_{220-559}$ molecule that has been solved by X-ray crystallography. Space-fill and ribbon diagrams showing the position of K_{417} (magenta) mapped onto the $rClfA_{220-559}$ crystal structure (**A** and **B**). The dark blue residues represent the residues that when mutated to alanine abrogates binding of to $rClfA_{220-559}$ fibrinogen. These residues are conserved between Fbl and ClfA. The cyan residues represent the variant residues when the amino acid sequence of $ClfA_{220-559}$ is compared to that of $Fbl_{214-534}$ by ClustalW analysis. The orange residues represent the latching peptide. When the latching peptide is removed the position of K417 relative to the γ-chain binding trench is apparent (**C** and **D**).

affinity. It also appears that residues I517, R519 and Y376 form part of the binding epitope of mAb 35-039 (Fig. 6.11) as substitution of these residues with alanine reduced the apparent affinity of 35-039 by ~45% as compared to the wild-type $rClfA_{220-559}$ (Fig. 6.12).

Table 6.1 Domain in $ClfA_{220-559}$ to which each monoclonal antibody binds

Monoclonal Antibody	Domain Antibody Binds
12-9	N3
13-1	N2
13-2	N3
15-6	N2
33-104	N3
35-006	N3
35-039	N3
35-041	N3
35-052	N2
35-220	N3

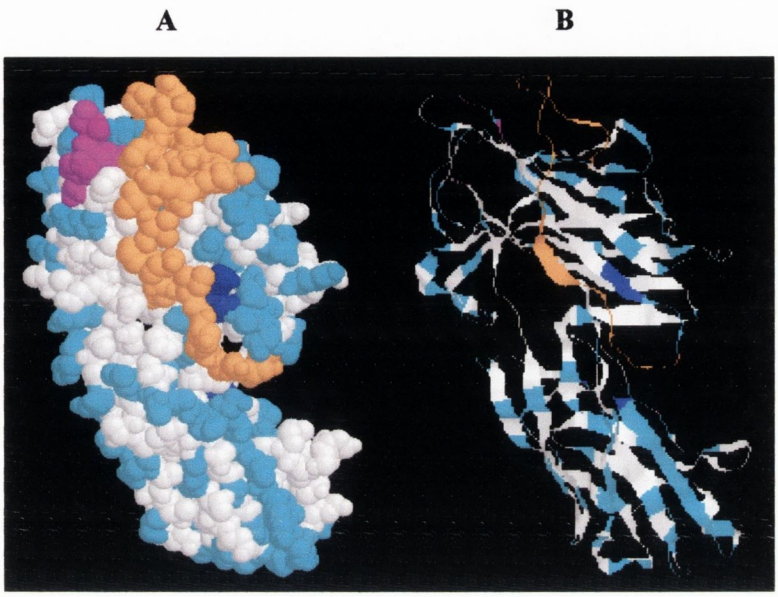
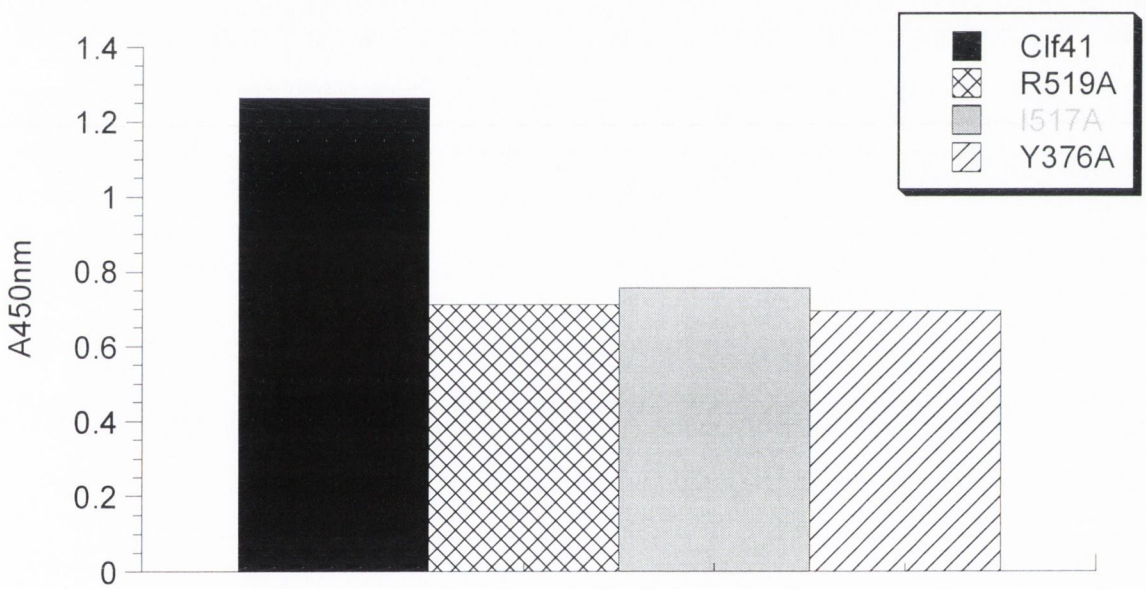


Figure 6.11 The position of the Y376A, I517A and R519A substitutions mapped onto the rClfA$_{220\text{-}559}$ molecule that has been solved by X-ray crystallography. A space-fill (**A**) and ribbon diagram (**B**), generated by RasMol, showing the position of Y_{376}, I_{517} and R_{519} (magenta) mapped onto the rClfA$_{220\text{-}559}$ crystal structure. The dark blue residues represent the residues that when mutated to alanine abrogates binding of to rClfA$_{220\text{-}559}$ fibrinogen. These residues are conserved between Fbl and ClfA. The cyan residues represent the variant residues when the amino acid sequence of ClfA$_{220\text{-}559}$ is compared to that of Fbl$_{214\text{-}534}$ by ClustalW analysis. The orange residues represent the latching peptide.

Figure 6.12 Binding of murine monoclonal antibody 35-039 to wild-type recombinant $ClfA_{220-559}$ or $rClfA_{220-559}$ with single site directed mutations. Microtitre wells were coated with 1μg of $rClfA_{220-559}$. 2μg/ml of antibody was incubated in the wells for 1 hr at room temperature. Bound antibody was detected by rabbit anti-goat HRP-conjugated antibodies followed by development with tetramethylbenzidine substrate. Values represent the means of triplicate wells. This experiment was repeated three times with similar results.



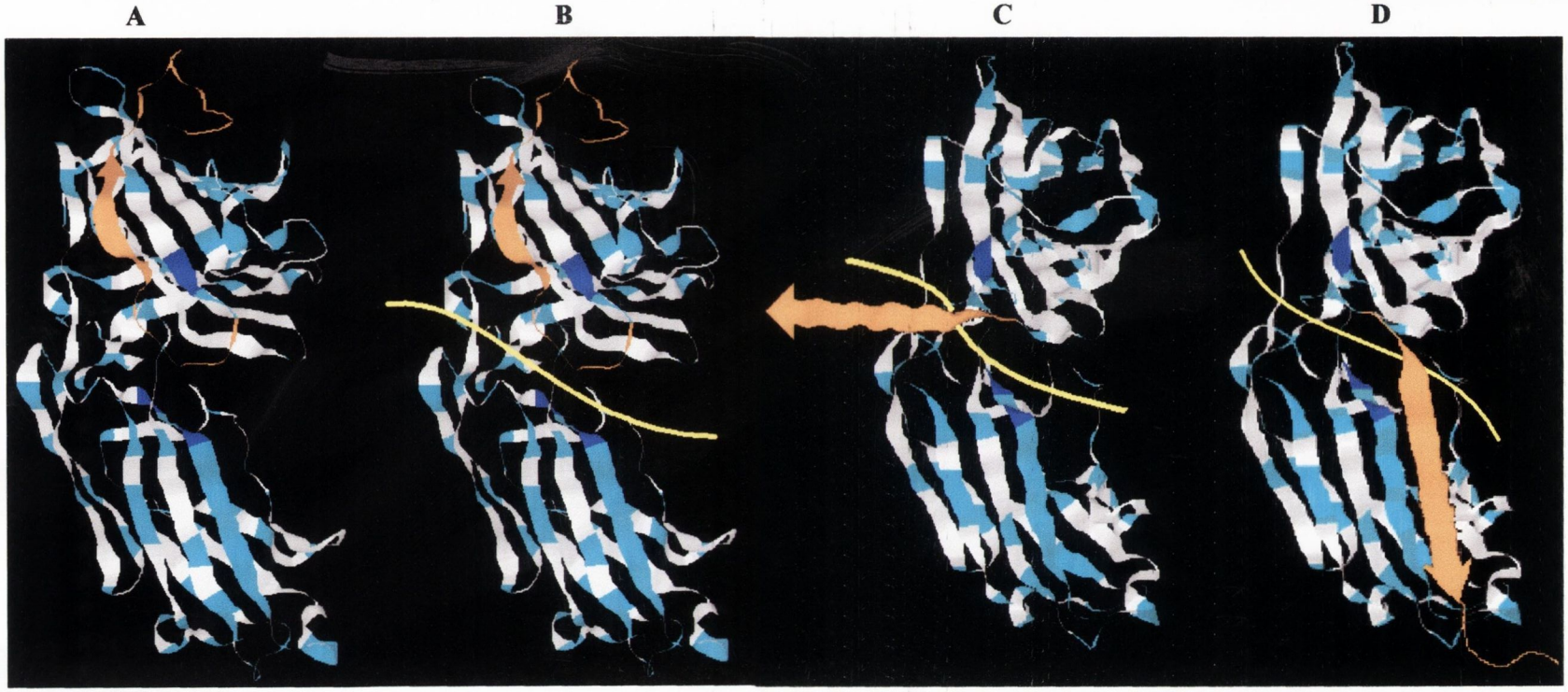
6.3 Discussion

The murine monoclonal antibody 12-9 is one of a panel of monoclonal antibodies raised against $rClfA_{220-559}$ by Inhibitex Inc. However 12-9 is the only mAb to have been chosen to be humanised and submitted for clinical trial studies to evaluate its safety and efficacy as an anti-staphylococcal therapeutic agent. It has the ability to recognise ClfA expressed on the surface of *S. aureus*, to mediate opsonophagocytosis of ClfA-coated beads (and presumably ClfA expressing cells also), to inhibit the binding of *S. aureus* cells to fibrinogen and to detach ClfA-expressing *S. aureus* cells from a fibrinogen substrate (Hall *et al.*, 2003, Domanski *et al.*, submitted). If the humanised counterpart of 12-9, T1-2, passes clinical evaluation it will likely prove to be a valuable anti-*S. aureus* treatment.

Unfortunately neither 12-9 nor a number of other monoclonal antibodies raised against $rClfA_{220-559}$ recognise any epitopes within the closely related homologue Fbl of *S. lugdunensis*. ClfA and Fbl share 60% amino acid identity. The studies in this chapter and chapter 5 show that the 40% difference in amino acid sequence is sufficient to render Fbl unrecognizable to specific murine monoclonal antibodies that recognize either the N2 or N3 region of ClfA. However polyclonal antibodies raised against either ClfA or Fbl recognize both their cognate antigen and the homologue. Therefore the possibility remains that there are shared epitopes between Fbl and ClfA that could be exploited by monoclonal antibody therapy resulting in a treatment that would combat both *S. aureus* and *S. lugdunensis*, especially against native valve endocarditis.

It was important to determine the binding epitope for mAb 12-9 in ClfA and elucidate whether substitutions could occur that render ClfA unrecognizable to 12-9. Firstly, variant ClfA proteins that are expressed by different strains of *S. aureus* were tested. Different *S. aureus* strains express different levels of ClfA, they express proteases, and varying amounts of proteins, capsules and other bacterial factors that could decrease the amount of monoclonal antibody that binds to *S. aureus* cells. The DNA encoding residues 220-559 of the A domain of each variant was cloned into an expression vector, purified and tested for monoclonal antibody binding by dot-immunoblotting and ELISA. This strategy revealed that among the *S. aureus* strains tested none of the

Figure 6.13 Ribbon diagrams of $rClfA_{220-559}$ showing the Dock-Lock and Latch model postulated by Ponnuraj et al. (2003). The apo-$rClfA_{220-559}$ structure that has been solved by X-ray crystallography is seen in A. In this model the proposed latching peptide (orange) is wound around the back of the N3 portion of the molecule and slightly obscures E_{526} and V_{527} which have been shown to abrogate binding to the fibrinogen γ-chain peptide when changed to alanine by site directed mutagenesis of the *clfA* gene. The fibrinogen γ-chain peptide (yellow) is proposed to bind to a trench formed between N2 and N3 (B). The peptide directly contacts residues P_{335}, Y_{337}, E_{526} and V_{527} by binding along this trench. Binding of the fibrinogen γ-chain peptide is proposed to initiate a conformational change in $rClfA_{220-559}$ that releases the latching peptide (C). The latching peptide then closes over the γ-chain peptide, locking it into position and forms β-strand complementation by binding into the groove formed in N2 by residues $_{326}DDVKATLTM_{335}$ (D).


A
B
C
D

variants had a reduced affinity of binding. It must also be borne in mind that a mutation leading to an increase in binding affinity could also identify the epitope as Cunningham and Wells (1993) showed.

One variant protein, $rClfA_{220-559}$ K417A, appeared to reduce the affinity of 12-9 for $rClfA_{220-559}$. This mutation also drastically reduced the binding affinity for other mAbs to $rClfA_{220-559}$. These antibodies had a similar inhibitory affect as 12-9 on the binding of ClfA-expressing bacteria to fibrinogen but were less efficient at inhibiting adherence to fibrinogen and displacing bound bacteria from fibrinogen. However, the fact that this one substitution affects the affinity of several antibodies for $rClfA_{220-559}$ suggests that they all interfere with the fibrinogen binding mechanism of ClfA in a similar manner. It is also likely that the antibodies that both inhibit and displace and that are affected by the K417A mutation all bind to ClfA in close proximity to K417. However the amino acid sequence of the complementarity determining regions of the antibodies probably differs. This would result in a slightly altered epitope structure recognition while still resulting in inhibition of the ClfA-fibrinogen interaction.

The crystal structure of $rClfA_{220-559}$ has been solved without the bound ligand in place (apo-ClfA). This presumably represents the open form of ClfA before fibrinogen binds and before the latching β-strand folds over into the trough in the N2 domain (Fig. 6.13) as is proposed by the SdrG crystal structure (Ponnuraj *et al.*, 2003). $Lysine_{417}$ maps to a position directly above the putative latching peptide sequence and beside $Valine_{527}$ which forms part of the fibrinogen binding pocket of ClfA and when mutated abrogates binding to fibrinogen (Hartford *et al.*, 2001). Therefore there are several ways in which antibody binding to $Lysine_{417}$ and residues adjacent to it could interfere with fibrinogen binding. It is possible that the antibody-antigen interaction could impede latching strand complementation thus preventing locking of the fibrinogen molecule into the ClfA ligand-binding site. If 12-9 has a greater affinity for ClfA than ClfA has for fibrinogen then the antibody could both inhibit the interaction of ClfA with fibrinogen and displace bound ClfA from fibrinogen by preferentially binding ClfA and shifting the ClfA-fibrinogen equilibrium such that less ClfA molecules bind. The effect of 12-9 could also be mediated by obscuring the binding pocket or altering the conformation of the binding pocket.

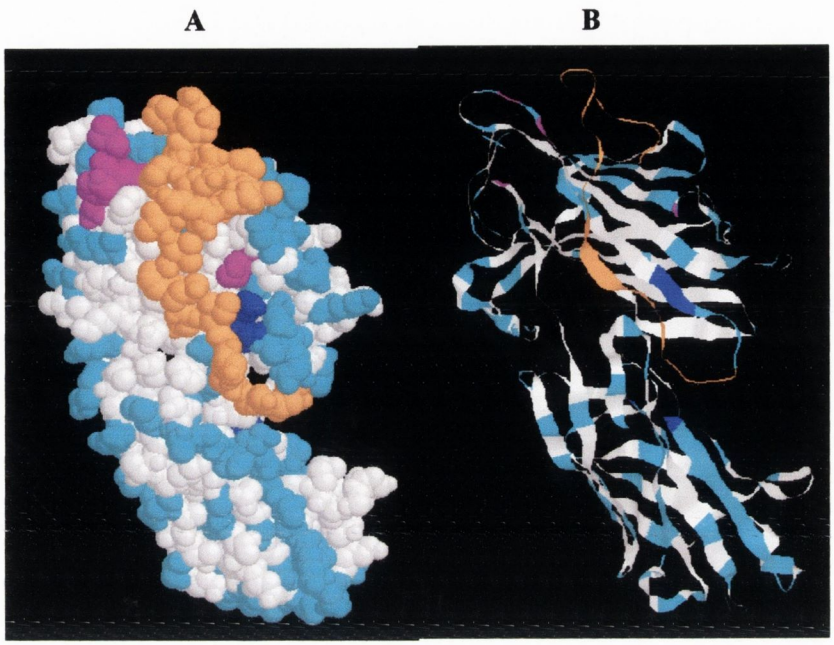


Figure 6.14 The position of the K417, Y376A, I517A and R519A relative to the latching peptide. A space-fill (**A**) and ribbon diagram (**B**), generated by RasMol, showing the position of K_{417}, Y_{376}, I_{517} and R_{519} (magenta) mapped onto the $rClfA_{220-559}$ crystal structure. The dark blue residues represent the residues that when mutated to alanine abrogates binding of to $rClfA_{220-559}$ fibrinogen. These residues are conserved between Fbl and ClfA. The cyan residues represent the variant residues when the amino acid sequence of $ClfA_{220-559}$ is compared to that of $Fbl_{214-534}$ by ClustalW analysis. The orange residues represent the latching peptide.

Visai *et al.* (2000) showed that a monoclonal antibody that bound on the opposite face of the $Cna_{151-318}$ molecule inhibited the interaction of the molecule with collagen and also displaced I^{125} labeled collagen from Cna expressing *S. aureus*. They postulated that the mAb altered the three-dimensional structure of Cna such that it released its hold on collagen. This could explain the observation that the mAb 35-039 epitope maps to the opposite face of the ClfA N3 domain away from the binding cleft (I_{517}, R_{519} and Y_{376}). Because these residues are located some distance away from the binding site it is tempting to speculate that the interaction of mAb 35-039 with its epitope causes a conformational change in the ClfA molecule so that fibrinogen is released from the locking process. However these residues are located adjacent to the C-terminal end of the putative latching strand peptide (but not within the latching peptide) when it is wound around N3 in the apo-$rClfA_{220-559}$ model (Fig. 6.14). It is possible that mAb 35-039 inhibits ClfA binding to fibrinogen by a similar mechanism as mAb 12-9 is proposed to do. Binding of mAb 35-039 could prevent the latching strand peptide from forming the β-strand complementation with the groove in N2 by impeding its movement. This would inhibit efficient fibrinogen binding by ClfA.

However 12-9 interacts with ClfA the net result is the same - the ClfA-fibrinogen interaction is inhibited and ClfA bound to fibrinogen is displaced. Co-crystallization of 12-9 Fab fragments with $ClfA_{220-559}$ would help to determine the exact residues that affect binding and the critical contact residues that mediate the interaction between the two proteins. Further single and combined mutations in close proximity to $Lysine_{417}$ will identify precisely the epitope to which 12-9 and other mAbs bind. This appears to be a valid strategy in verifying the efficacy of monoclonal based anti-staphylococcal therapeutic treatments and could also aid in identifying a common epitope between Fbl and ClfA that could be exploited by either vaccination or passive immunotherapy.

Chapter 7

The SdrH Protein of *Staphylococcus epidermidis*

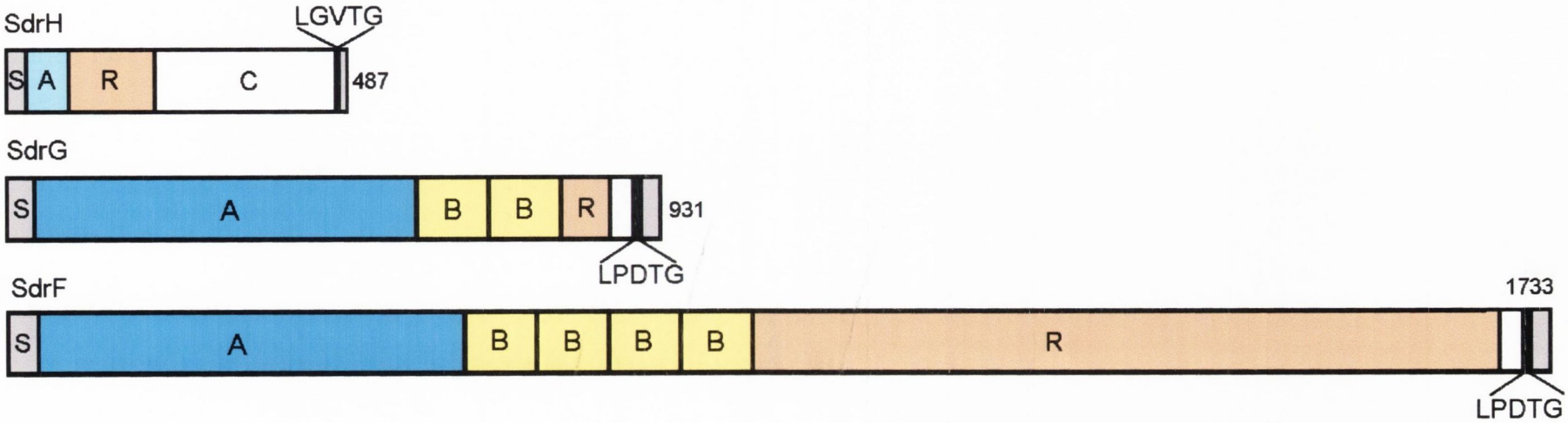
7.1. Introduction

Coagulase-negative staphylococci are the most common causes of infections associated with prosthetic devices such as intravascular catheters, cerebrospinal fluid shunts, prosthetic hearts valves, prosthetic joints, and artificial pacemakers (Crossley and Archer, 1997). The pathogenesis of *Staphylococcus epidermidis* foreign body infections is thought to be based on initial adhesion of the bacteria to the biomaterial surface, followed by colonisation of the medical devices by proliferation of the cells to form a biofilm composed of multilayered clusters embedded in a slime matrix (Tojo *et al.*, 1988; Heilmann *et al.*, 1996). Factors reported to contribute to primary attachment of the bacteria to a polymer include surface hydrophobic interactions, a capsular polysaccharide/adhesin (PI/A), proteinaceous cell surface antigens (SSP-1, SSP-2) and the autolysin AtlE (Heilmann *et al.*, 1997; Klug *et al.*, 2003). Protease treatment of *Staphylococcus epidermidis* has been shown to reduce both hydrophobicity and adherence which implicates surface expressed proteins in primary adhesion (Pascual *et al.*, 1986).

Staphylococcus aureus, a closely related opportunistic pathogen, can also adhere to medical implant devices but this is primarily mediated by bacterial adhesins specific for host fibrinogen and fibronectin which coat biomaterial surfaces shortly after implantation (Vaudaux *et al.*, 1994; Francois *et al.*, 2000). *Staphylococcus epidermidis* also binds host fibrinogen, fibronectin, vitronectin and laminin but little is known about the proteins mediating these interactions or how they would enable the bacteria to adhere to biomaterials coated with host proteins (Heilmann *et al.*, 2003; Williams *et al.*, 2002; Li *et al.*, 2001; Paulsson *et al.*, 1992).

ClfA, the fibrinogen-binding clumping factor protein was the first cell surface-associated protein of *S. aureus* noted to have a serine-aspartate (SD) dipeptide-repeat region (region-R) located between the ligand-binding A region and the C-terminal sequences associated with attachment of the protein to the cell wall (McDevitt *et al.*, 1994, 1995). The SD-repeat region is predicted to span the cell wall and extend the ligand-binding A region from the surface of the bacteria (Hartford *et al.*, 1997).

Figure 7.1 Diagrammatic representations of the SD repeat proteins of *S. epidermidis*. SdrG is a fibrinogen binding protein that binds to the Bβ-chain of human fibrinogen. The functions of SdrF and SdrH are as yet unknown. S, signal sequence; A, A-domain; B, B-repeats; C, C-domain; R, Serine-aspartate dipeptide repeat region.



Subsequently SD-repeat-containing (Sdr) proteins have been identified in several species of staphylococci. Additional members of this family are listed in Table 7.1. The SdrC, SdrD and SdrE proteins of *S. aureus* contain additional repeats, termed B-repeats, located between the A domain and SD-repeat regions. Each B repeat is 110–113 amino acids in length and contains a putative Ca^{2+}-binding EF-hand motif. Ca^{2+} binding has been shown to be required for the structural integrity of the B-repeat regions in SdrD (Josefsson *et al.*, 1998). The functions of SdrC, SdrD and SdrE are unknown. SdrE recognizes an as yet unknown plasma protein which is required to link bacteria to the surface of platelets, causing platelet activation (O'Brien *et al.*, 2002). SdrE is also 70% identical to the bone sialoprotein-binding protein (Tung *et al.*, 2000).

S. epidermidis can express three cell-surface-associated proteins that contain serine-aspartate dipeptide repeats called SdrF, SdrG and SdrH (McCrea *et al.*, 2000) (Fig. 7.1). SdrF and SdrG are similar in sequence and structural organization to the Sdr proteins of *S. aureus* (McCrea *et al.*, 2000; Hartford *et al.*, 2001; Ponnuraj *et al.*, 2003). The C termini of these proteins contain LPXTG sorting signals and hydrophobic amino acid segments characteristic of surface proteins covalently anchored to peptidoglycan. In contrast, SdrH has a short 60-residue A region at its N terminus followed by an SD-repeat region, a unique 277-residue C region and a C-terminal hydrophobic segment. SdrH lacks a sortase recognition LPXTG motif but contains the sequence LGVTG within the hydrophobic C-terminal end of the protein this is possibly a sorting signal but its location makes this unlikely. Unlike other Sdr proteins, the SD-repeat region of SdrH is very close to the N terminus. The SD repeats are separated from the LGVTG sequence by the 270-residue C region. When a collection of *S. epidermidis* strains was tested for *sdrH* by Southern hybridisation, the gene was found in 16/16 strains tested whereas *sdrF* was found in only 12 (McCrea *et al.*, 2000). The presence of antibodies that recognize the small A-region of SdrH in the sera of patients recovering from *S. epidermidis* infections suggests that these proteins are also expressed in the host.

The objective of this study was to determine the function of SdrH. The *sdrH* gene and the SdrH protein were analysed at the molecular level in an attempt to discover its function in *S. epidermidis* and whether it could potentially have a role in

pathogenesis. The *sdrH* gene was cloned into the *L. lactis* expression vector pKS80 and transformed into *L. lactis* strain MG1363. The topology and cellular location of SdrH was analysed in this surrogate expression background and the *L. lactis* $SdrH^+$ strain was used as a tool to screen for a host ligand for SdrH. The expression of SdrH was also analysed under different growth conditions and in different genetic backgrounds.

7.2. Results

7.2.1. *sdrH* homologues in *S. aureus, S. caprae* and *S. capitis*.

The *sdrH* gene is 1464bp in length and the SdrH protein is 487 amino acids in length. It is present in the *Staphylococcus epidermidis* ATCC 12228 genome sequence (Zhang *et al.*, 2003) and the *S. epidermidis* strain RP62A genome sequence (http://tigrblast.tigr.org/ufmg/index.cgi?database=s_epidermidis|seq), the only completely sequenced *S. epidermidis* genomes in the public domain. It is also present in the incomplete *S. epidermidis* genome sequence of strain SR1 (Accession number: AF269654). Three other staphylococcal genes that share significant homology to the *sdrH* gene of *S. epidermidis* were identified by examining the staphylococcal genome sequences available in the NCBI database by BLAST analysis. The three genes were identified on the basis of their amino acid identity to the sequence of the C-terminal 277 amino acids of SdrH. Fig. 7.2 shows an amino acid sequence alignment of all four proteins and Fig. 7.3 indicates the relative sizes of SdrH and its homologues.

The open reading frame SA1839 of *S. aureus* N315 is present in each of the *S. aureus* genome sequences that are available for BLAST analysis (*S. aureus* strains N315, Mu50, MW2 at http://www.ncbi.nlm.nih.gov/BLAST, *S. aureus* strain COL at http://tigrblast.tigr.org/ufmg/index.cgi?database=s_aureus|seq, *S. aureus* strains MRSA252 and MSSA476 at http://www.sanger.ac.uk/Projects/S_aureus/ and *S. aureus* strain 8325 at http://www.genome.ou.edu/staph.html). The sequence of the C-terminal region of this novel protein is 211 amino acids in length. It shares 37% amino acid sequence identity to the C-domain of SdrH. It also shares similar domain organization

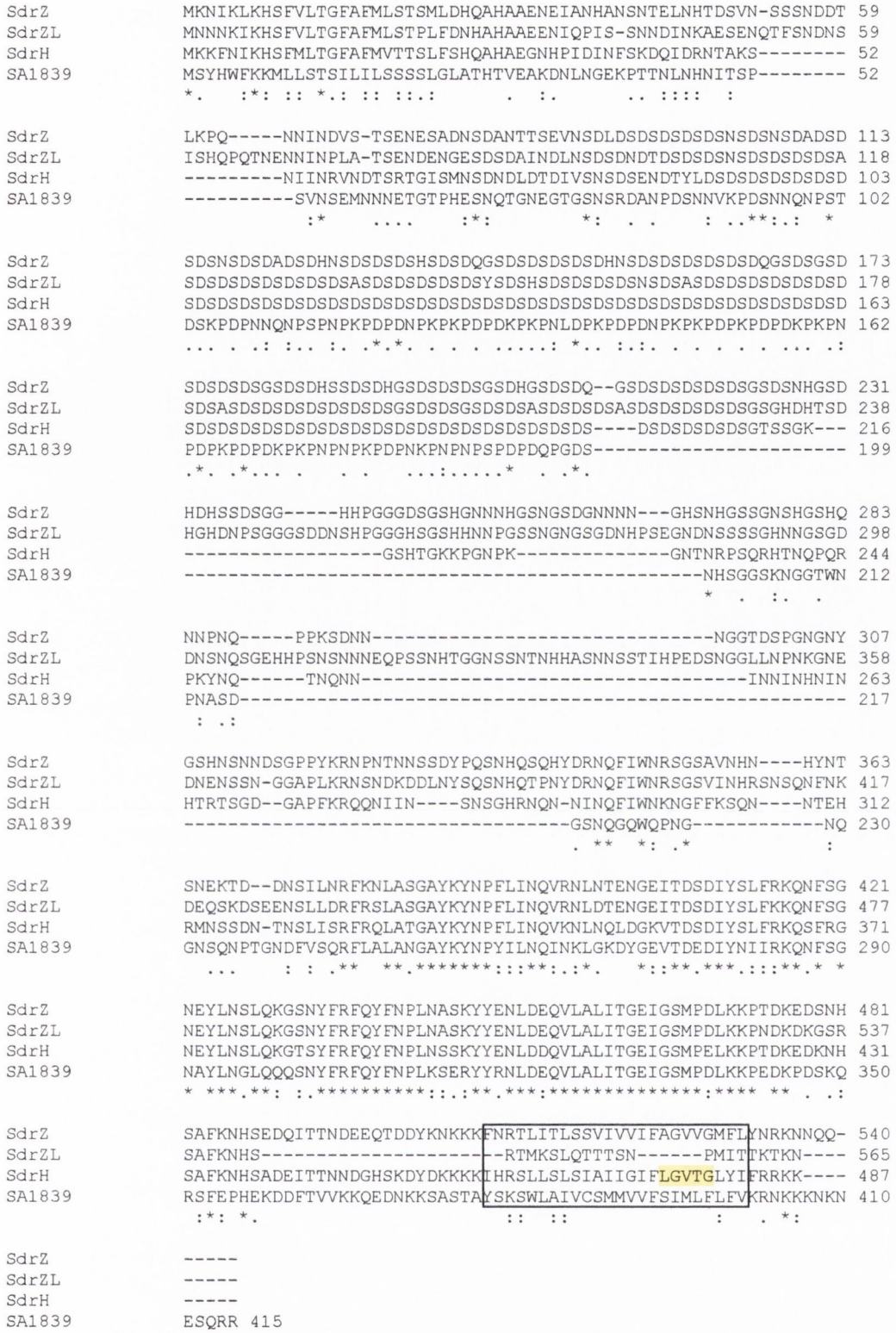


Figure 7.2 ClustalW analysis of homologues of SdrH in staphylococci. An alignment of the amino acid sequences of the SdrH protein of *S. epidermidis* and homologues of SdrH in *S. aureus* (SA1839), *S. caprae* (SdrZ) and *S. capitis* (SdrZL). The potential sortase substrate motif LGVTG is highlighted in yellow. The predicted transmembrane domain is boxed.

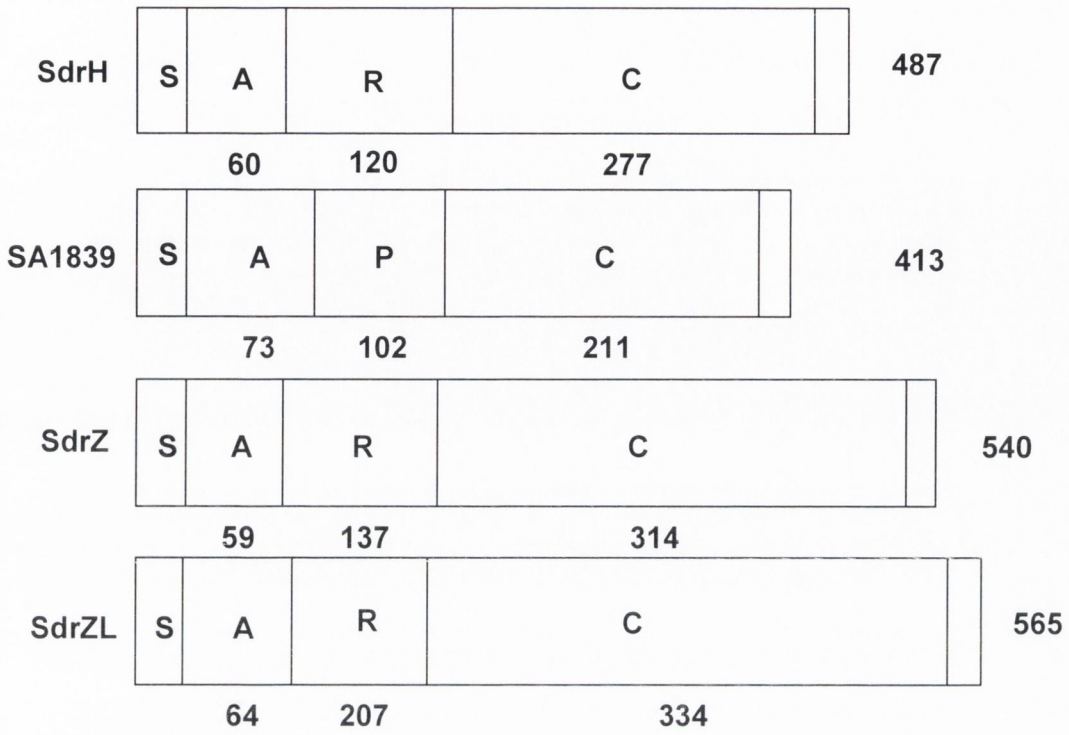


Figure 7.3 Homologues of SdrH in staphylococci. Diagrammatic representation of SdrH of *S. epidermidis* and staphylococcal proteins homologous to SdrH. SA1839 is a *S. aureus* homologue of SdrH that contains a proline rich repeat region in place of a serine-aspartate repeat region. SdrZ is the SdrH homologue in *S. caprae*. SdrZL is the *S. capitis* SdrH homologue. S, signal sequence; A, A-domain; B, B-repeats; C, C-domain; R, Serine-aspartate dipeptide repeat region; P, proline rich repeat region.

with SdrH, being composed of an N-terminal signal sequence followed by a short A-domain, a repeat region, a relatively large C-domain containing putative membrane spanning residues and a positively charged tail at the C-terminus. However the sequences of the A-domains and the repeat regions of SdrH and the *S. aureus* homologues are completely different. The *S. aureus* protein has a repeating PXPXPXPXPXXPX motif whereas SdrH has SD repeats.

The location of the *sdrH* gene and its *S. aureus* homologue, SA1839, within their respective genomes was analysed. The loci of both genes were analysed using Artemis, a program available from the Sanger Center website. Artemis is a free DNA sequence viewer and annotation tool that allows visualization of sequence features and the results of analyses within the context of the sequence, and its six-frame translation. The results of this analysis are shown in Fig. 7.4. The loci bearing both *sdrH* in *S. epidermidis* and SA1839 in *S. aureus* are conserved. They are located downstream from the adjacent *agr* loci and upstream of the *hsp10* and *hsp60* genes. This suggests that both genes might have functional similarity despite their sequence differences.

The SdrZ protein of *S. caprae* (Accession number: AY048595) is more closely related to the SdrH protein and is 67% identical along the entire amino acid sequence. It shows a similar domain organization to SdrH with an N-terminal signal sequence followed by an A-domain 59 amino acids in length which shares 38% amino acid identity with the corresponding A-domain of SdrH. This is followed by a 137 amino acid serine-aspartate repeat region, a large C-domain of 314 amino acids containing putative membrane spanning residues and a positively charged tail at the C-terminus. The C-domain of SdrZ of *S. caprae* is 314 amino acids in length and shares 55% amino acid sequence identity to the C-domain of SdrH.

An SdrZ-like protein of *S. capitis*, SdrZL (Accession number: AAS00714) was identified by BLAST analysis against the C-terminal 277 amino acids of SdrH. SdrZL is 71% identical to the amino acid sequence of SdrZ and 60% identical to the amino acid sequence of SdrH. The SdrZL protein is organized in a similar manner to SdrH and SdrZ. SdrZL has an N-terminal signal sequence followed by an A-domain 64 amino acids in length. This is followed by a 134 amino acid serine-aspartate repeat

Figure 7.4 The *sdrH* locus of *S. epidermidis* compared to the locus of its *S. aureus* homologue. **A.** The position of the *sdrH* gene in the *S. epidermidis* RP62A genome. The *sdrH* gene is downstream of the *agr* locus. *sdrH* is also upstream of the *hsp10* and *hsp60* loci and transcribed in the same direction. **B.** The *sdrH* homologue of *S. aureus* lies in the same position as *sdrH* occupies in the *S. epidermidis* genome.

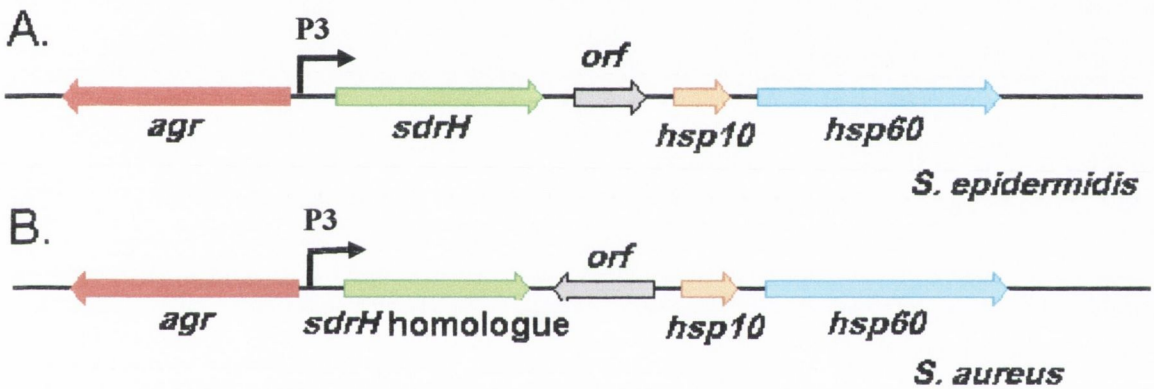
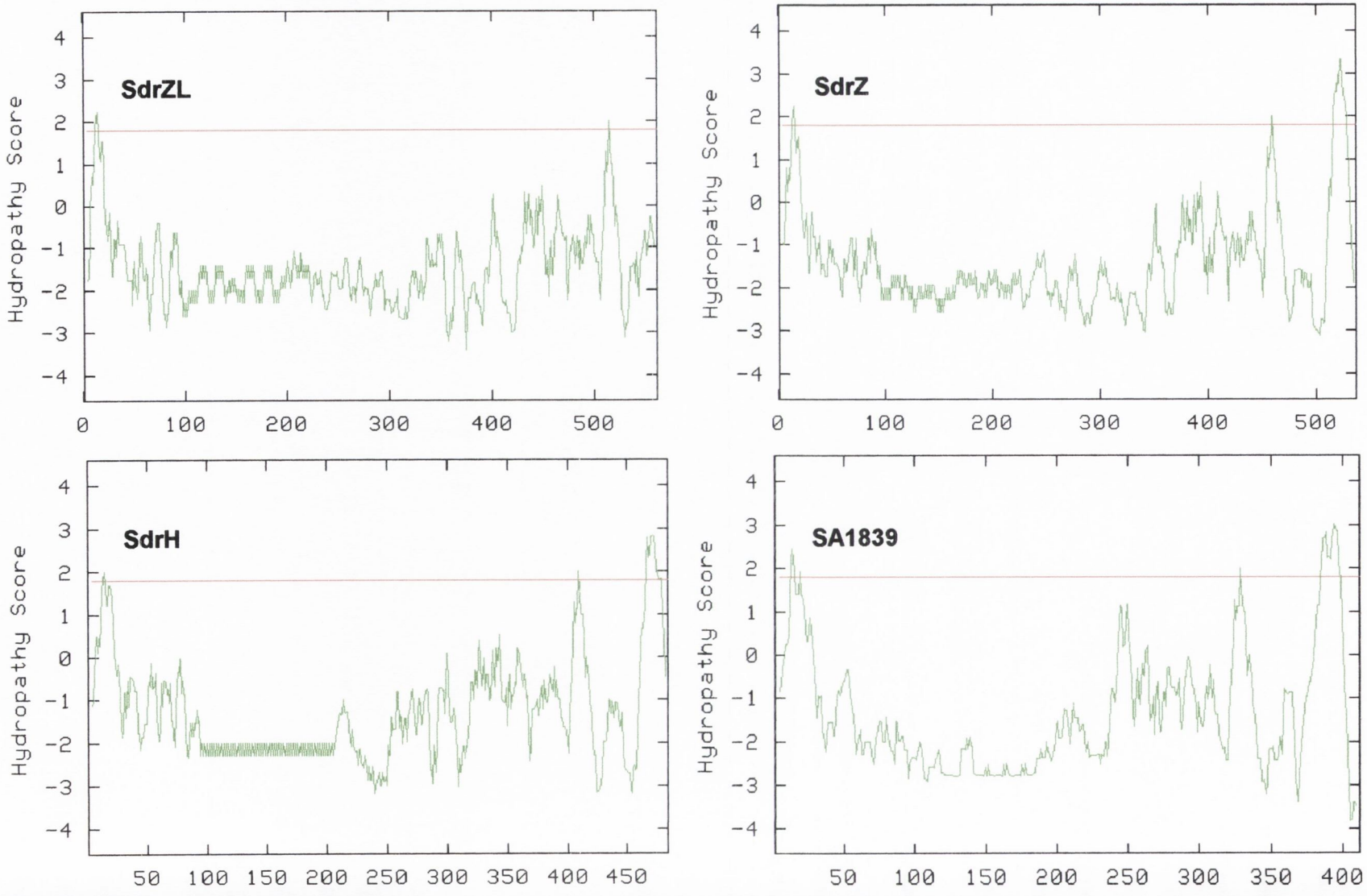


Figure 7.5 Primary structure analysis of the SdrH homologues of staphylococci. Kyte-Doolittle hydropathy plots of the amino acid sequences of SdrH of *S. epidermidis*, SA1839 of *S. aureus*, SdrZ of *S. caprae* and SdrZL of *S. capitis*.


SdrZL
Hydropathy Score
4
3
2
1
0
-1
-2
-3
-4
0
100
200
300
400
500
SdrZ
Hydropathy Score
4
3
2
1
0
-1
-2
-3
-4
0
100
200
300
400
500
SdrH
Hydropathy Score
4
3
2
1
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-2
-3
-4
50
100
150
200
250
300
350
400
450
SA1839
Hydropathy Score
4
3
2
1
0
-1
-2
-3
-4
50
100
150
200
250
300
350
400

region and a large C-domain of 337 amino acids. Figure 7.5 shows Kyte-Doolittle hydropathy plots of all four homologues which indicates that the proteins share similar primary amino acid sequence structure properties.

The C domain of SdrH shares the highest amino acid identity with its three homologues. The amino acid sequence $_{405}$QVLALITGEIGSEMPL$_{420}$ was subjected to BLASTP analysis against bacterial proteins in the NCBI database. This analysis identified that this sequence or similar sequences are conserved in staphylococci and several other bacterial genome sequences. A periplasmic protein of *Corynebacterium diptheriae* and an outer membrane protein of *Chlamydia trachomatis* both contain sequences that share amino acid sequence identity to this peptide sequence.

7.2.2. Attempted construction of an *sdrH* mutant by allele replacement

The construction of an allele replacement mutant of the *sdrH* gene in *S. epidermidis* strain 9491 was attempted as described in section 2.25. The pROJ:*sdrH::tet* plasmid was constructed by O. Hartford in *S. aureus* RN4220 (Fig. 7.6). This plasmid contains the pE194*tsrep* mutation which results in a plasmid that cannot replicate at 43°C. It also incorporates the pC221 *mob* site which allows efficient conjugational mobilization from *S. aureus* RN4220 in the presence of both pG01 and pC221. pC221 is a mobilizable plasmid and pG01 is a conjugational plasmid. pC221 provides trans acting mob proteins that interact with the *mob* site in pROJ6448.

The pROJ:*sdrH::tet* plasmid was then introduced into *S. aureus* pG01 pC221. The pG01 and pC221 plasmids allowed for conjugational mobilization of the pROJ:*sdrH::tet* plasmid into *S. epidermidis* strains. The *S. aureus* pG01 pC221 pROJ:*sdrH::tet* strain was mated with *S. epidermidis* strain 9491. Transconjugants were selected on the basis of the presence of the pROJ:*sdrH::tet* plasmid (tet^R) and absence of pG01 and pC221. Strain 9491 is resistant to norfloxacin therefore norfloxacin resistant colonies that were also resistant to tetracycline were selected as putative transconjugants.

Figure 7.7 Scheme for construction of an *sdrH* mutant by allele replacement mutagenesis. Schematic diagram illustrating the intended double crossover event between the pROJ6448:*sdrH*::*tet* plasmid and the chromosomal copy of *sdrH*. The plasmid is expected to integrate 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 opposite side of the chromosomal locus results in the exchange of mutant and wild-type alleles. If the integration event occurred, the presence of the *tet* gene in the centre of the *sdrH* gene would lead to an altered restriction profile. This could be detected by Southern blotting.

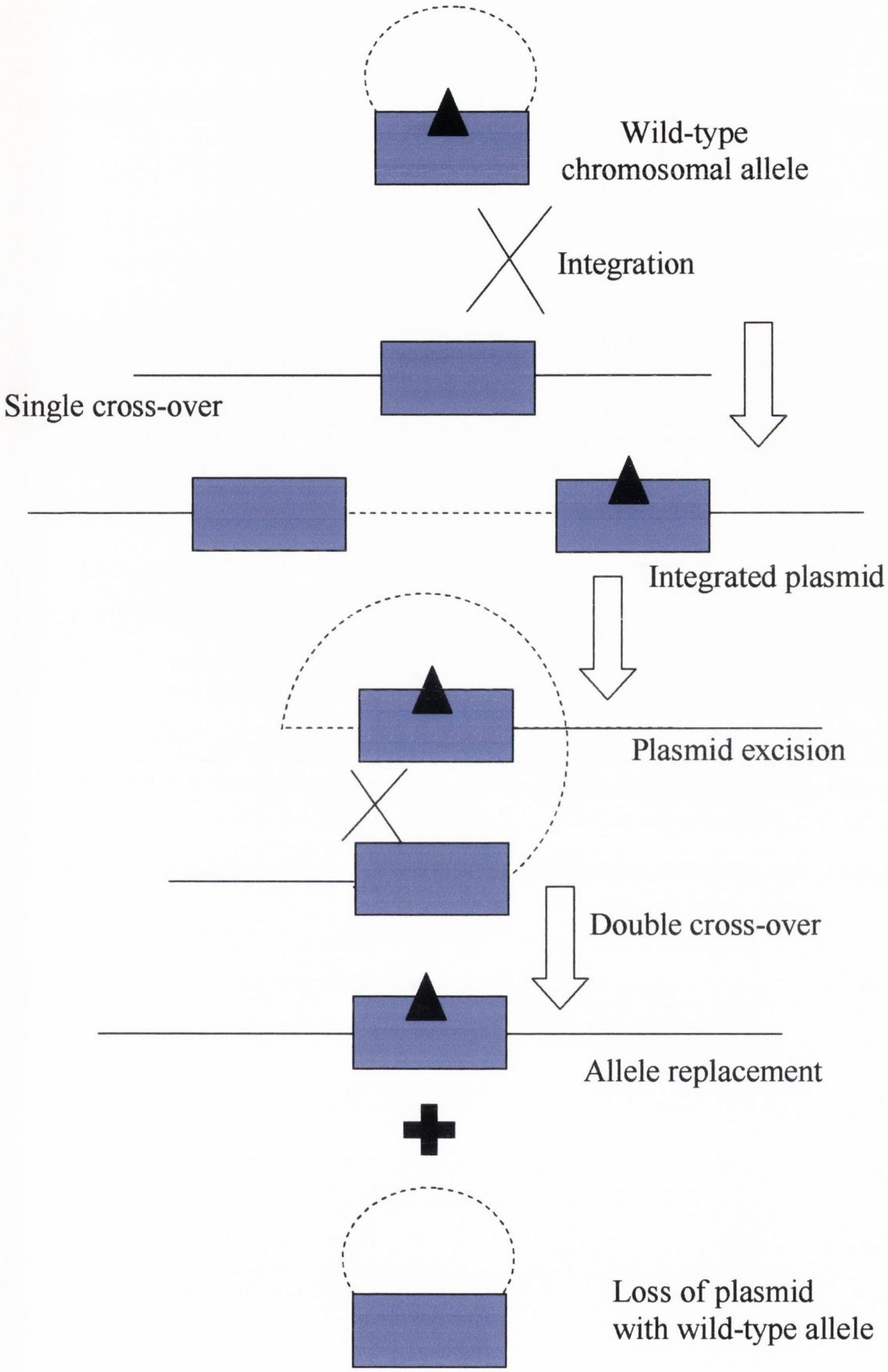
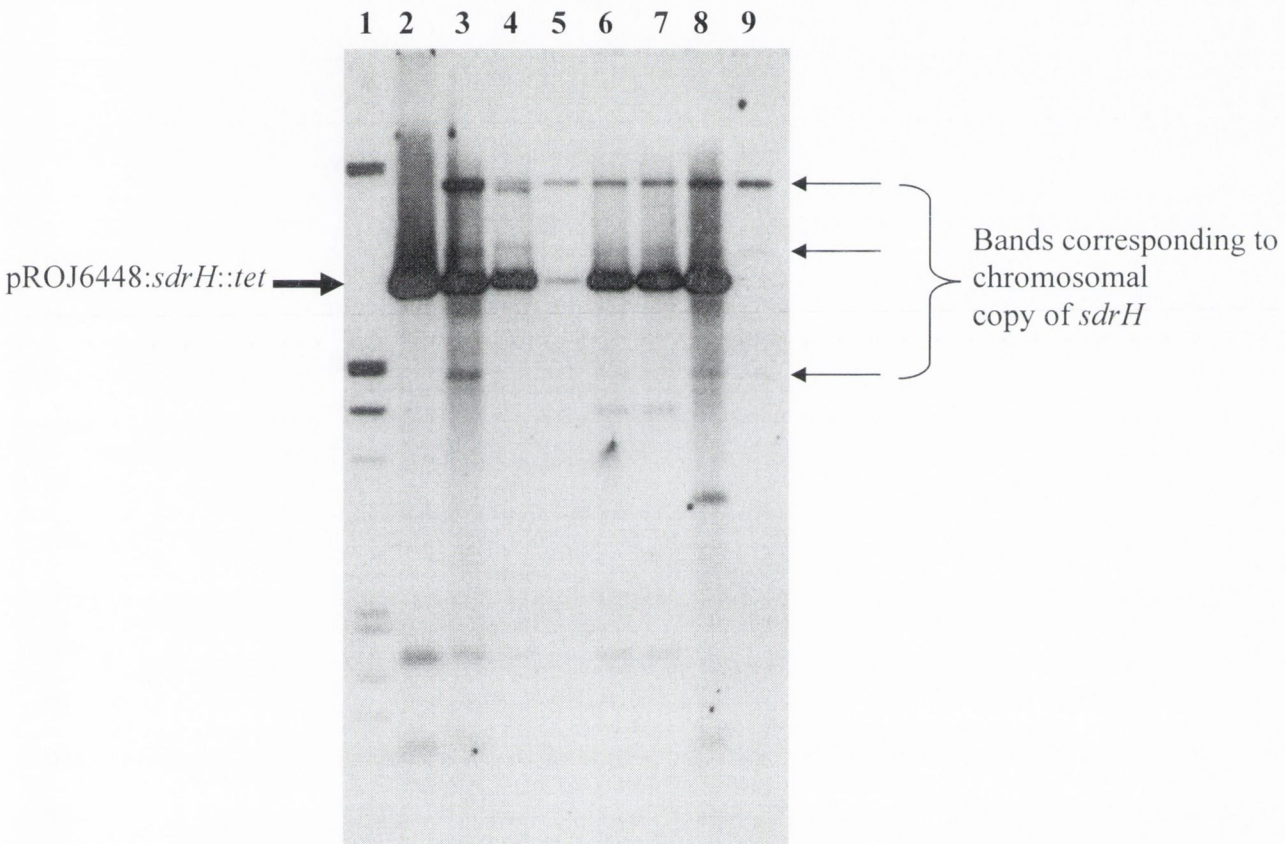

Wild-type
chromosomal allele
Integration
Single cross-over
Integrated plasmid
Plasmid excision
Double cross-over
Allele replacement
Loss of plasmid
with wild-type allele

Figure 7.8 Southern blotting of putative *sdrH* mutants. *Xba*I digested chromosomal DNA of *S. epidermidis* 9491 and putative integrants. The chromosomal copies of SdrH remain intact in all strains while the pROJ6448:*sdrH*::*tet* plasmid has remained extra-chromosomally located. Lane 1. DIG-labeled DNA molecular weight marker. Lane 2. pROJ6448:*sdrH*::*tet* plasmid. Lane 3-8. Putative *S. epidermidis* 9491 pROJ6448:*sdrH*::*tet* integrants. Lane 9. *S. epidermidis* 9491. Arrows mark the bands corresponding to the *Xba*I digested bands representing wild type chromosomal copy of *sdrH* (lane 9) and pROJ6448:*sdrH*::*tet* plasmid (lane 2). In some lanes the bands are weak due to insufficient amounts of DNA. Overexposure of the autorad allowed them to be detected.


1 2 3 4 5 6 7 8 9
pROJ6448:*sdrH*::*tet*
Bands corresponding to chromosomal copy of *sdrH*

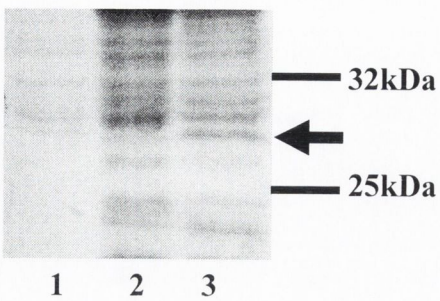
Temperature sensitive plasmids are used to introduce mutations into chromosomal genes in *S. aureus* (Fig. 7.7). Following temperature shifting experiments that had been carried out on putative transconjugants, the genomic DNA of the surviving colonies were analysed for insertion of the temperature sensitive plasmid by Southern blotting, probing with DIG-labeled DNA encoding the C-region of SdrH. The results of this experiment showed that the pROJ:*sdrH::tet* plasmid had not integrated into the chromosome of *S. epidermidis* 9491 (Fig. 7.8). The temperature sensitive *rep* mutation in the vector must have reverted to wild-type as the plasmid persisted extra-chromosomally at the restrictive temperature. It is likely that the *sdrH* gene had proven recalcitrant to disruption by allele replacement mutagenesis. No further attempts were made to construct an *sdrH* mutant of *S. epidermidis*.

7.2.3. Production of polyclonal antibodies to region C of SdrH

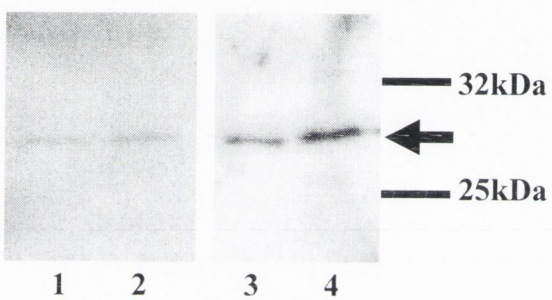
In order to generate a recombinant C-domain of SdrH, the DNA encoding this region was amplified by PCR from *S. epidermidis* strain 9491 genomic DNA. The resultant fragment was cloned into the pQE30 expression vector, forming plasmid pQE30SdrHC and transformed into *E. coli* strain XL-1 blue. This plasmid was subsequently transformed into the protease-deficient *E. coli* strain TOPP3 and a 30kDa protein with a hexahistidine tag was expressed following induction with IPTG (Fig. 7.9 A). Expression was monitored by separation of cell lysates by SDS-PAGE and Western immunoblotting probing with a murine monoclonal antibody specific for the hexahistidine peptide. The hexahistidine tagged protein was purified from cell lysates by immobilised metal chelate affinity chromatography (Fig. 7.9 B). This protein was used to immunize rabbits as described in section 2.21. A series of dilutions of the antibody from 1:2,000 to 1:20,000 reacted with 20μg of the recombinant C domain protein in a strip Western immunoblotting experiment (Fig. 7.9 C) indicating that the antibodies had a high affinity for the immunizing antigen.

Figure 7.9 Expression of the C-domain of SdrH and production of anti-C domain antibodies. **A.** IPTG inducible expression of SdrH C domain. Lane 1, uninduced XL-1 Blue (pQE30:SdrHC). Lane 2, XL-1 Blue (pQE30:SdrHC) after 2 hours induction with 0.1mM IPTG. Lane 3, XL-1 Blue (pQE30:SdrHC) after 4 hours induction with 0.1mM IPTG. Proteins were separated by SDS-PAGE on a 10% acrylamide gel stained with Coomassie blue. **B.** Lysate of XL-1 Blue (pQE30:SdrHC) cells induced for 4 hours with 0.1mM IPTG was added to a charged Ni^{2+} sepharose column. Lanes 1 and 2, fractions eluted from the column with 100mM imidazole, separated by SDS-PAGE on a 10% acrylamide gel and stained with Coomassie. Lanes 3 and 4, Samples from lanes 1 and 2, transferred to PVDF membrane and probed with monoclonal antibodies specific for the hexahistidine tag. **C.** New Zealand white rabbits were immunized with purified C-domain of SdrH. Western immunoblot of 20μg SdrH region C probed with anti-SdrH region C antibody: Lane 1, 1:2,000 dilution. Lane2, 1:5,000 dilution. Lane 3, 1:10,000 dilution. Lane 4, 1:20,000 dilution. Bound antibody was detected with goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase.

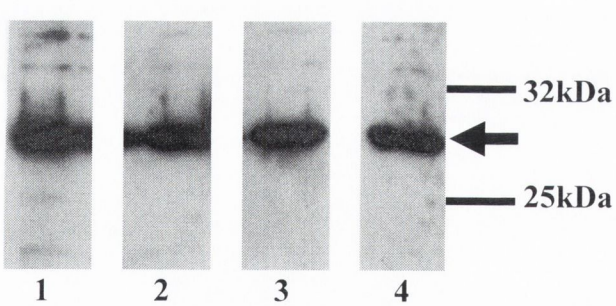
A.



B.



C.



7.2.4. Cloning the *sdrH* gene into pKS80 in *L. lactis* MG1363

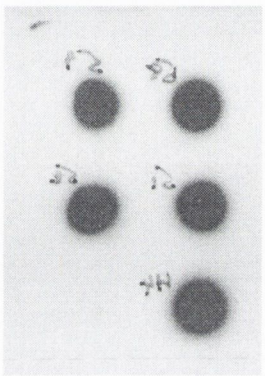
In order to express SdrH at a high level on the surface of a surrogate host bacterium, the *sdrH* gene from *S. epidermidis* strains 9491 was cloned into the *L. lactis* expression vector pKS80 and transformed into *L. lactis* strain MG1363 (Chapter 3) (Fig. 7.10). Positive clones were identified using polyclonal anti-SdrH region A antibodies by screening transformants in a whole cell dot-immunoblot experiment. These antibodies had been generated against residues 31-91 of SdrH which was fused to a Glutathione-S-Transferase (GST) affinity tag (Dr. O. Hartford). The antibodies were affinity purified with sepharose coupled to GST-SdrH A domain as the antibodies cross-reacted with *L. lactis* MG1363. Affinity purification of the polyclonal anti-SdrH region A antibodies eliminated this cross-reactivity. Positive clones were confirmed by PCR and restriction digest analysis of the plasmid DNA. The transformants were also probed with anti-SdrH region C antibodies. None of the positive clones reacted with anti-SdrH region C antibodies in a whole cell dot immunoblot. The whole cell dot-immunoblots show that the A-domain of SdrH is expressed on the surface of *L. lactis* $SdrH^+$ (Fig 7.10 A) whereas the C-Domain does not appear to be surface-exposed (Fig 7.10 B).

7.2.5. Topology of SdrH

It was important to determine whether the SdrH protein is anchored to the cell wall peptidoglycan despite its degenerate LXXTG sortase motif and whether any domain of the protein is exposed on the cell surface. This would clarify its potential role as a cell-wall anchored adhesin. Therefore the expression of SdrH in both *S. epidermidis* and *L. lactis* $SdrH^+$ was analysed by whole cell dot blotting and by SDS-PAGE and Western immunoblotting analysis of solublised cell wall proteins and proteins isolated from the protoplast. The protein content of culture supernatants of both *S. epidermidis* and *L. lactis* $SdrH^+$ was precipitated by treatment with trichloracetic

Figure 7.10 Cloning and Expression of *sdrH* in *L. lactis* MG1363. **A.** *L. lactis* transformants expressing the SdrH protein were identified by whole cell dot-blotting probing with affinity-purified anti-SdrH region A antibodies. *L. lactis* carrying the pKS80 plasmid without an insert was used as a control. Five positively reacting transformants are shown. **B.** Anti-SdrH region C antibodies were applied to the same group of *L. lactis* pKS80 cells and pKS80 SdrH^{+} cells immobilised on nitrocellulose membranes. *L. lactis* carrying the pKS80 plasmid without an insert was used as a control.

A.



L. lactis pKS80 SdrH +ve clone

B.

L. lactis pKS80 SdrH +ve clone

acid and analysed by SDS-PAGE and Western immunoblotting but no SdrH was detected.

7.2.5.1.Expression of the SdrH protein by *S. epidermidis*

Lysostaphin-solublised cell wall extracts and detergent lysed stabilized protoplasts of *S. epidermidis* were separated by SDS-PAGE through a 10% acrylamide gel. The separated proteins were then transferred to PVDF membranes and probed using either anti-SdrH region A antibodies or anti-SdrH region C antibodies. An immunoreactive band with an apparent molecular weight of 70kDa was detected in the protoplast fraction but not in the lysostaphin-solubilised cell wall fraction (Fig. 7.11). This most likely corresponds to the SdrH protein. However the failure to isolate an *sdrH* mutant prevented unequivocal identification of this band as SdrH. The putative SdrH protein was associated with the protoplast fraction indicating that SdrH is not sorted to the cell wall in *S. epidermidis* and is most likely retained in the cell membrane.

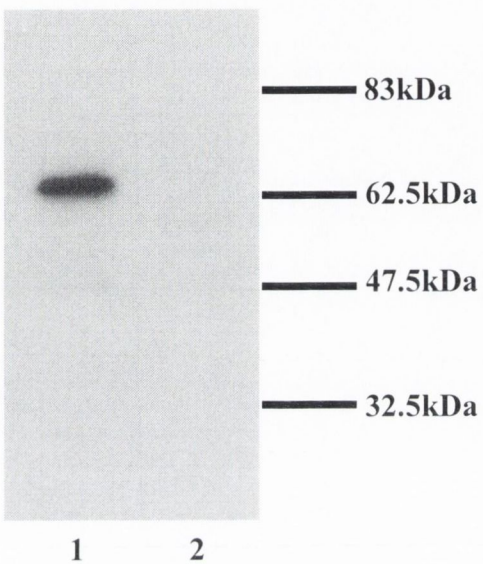
7.2.5.2.Expression of the SdrH protein by *L. lactis*

Whole cell dot immunoblotting with *L. lactis* SdrH$^+$ cells showed that the A domain is surface exposed while the C-domain is not (Fig. 7.10). In order to investigate this further, *L. lactis* SdrH$^+$ cells were treated with trypsin, washed three times to remove the protease before being spotted out onto a nitrocellulose membrane for dot immunoblotting analysis. Trypsin treated cells did not react with anti-SdrH region A antibodies indicating that the A domain must be surface exposed and accessible to the protease (Fig. 7.12 A).

Western immunoblotting of the *L. lactis* SdrH$^+$ cell wall fraction solubilised using lysozyme and mutanolysin confirmed that SdrH is not sorted to the cell wall. When the protoplasts of *L. lactis* SdrH$^+$ were analysed, two strongly immunoreactive bands of 70kDa and 50kDa were detected which were absent in the MG1363 pKS80 control (Fig. 7.12 B). The largest corresponded in size to the single immunoreactive

Figure 7.11 Expression of the SdrH protein by *S. epidermidis*. **A.** Lane 1, stabilized protoplasts of *S. epidermidis*. Lane 2, lysostaphin solublised cell wall fraction of *S. epidermidis*. Proteins were separated by SDS-PAGE (10% acrylamide gels), transferred to PVDF membrane and probed with affinity-purified anti-SdrH region A antibodies. **B.** Lane 1, stabilized protoplasts of *S. epidermidis*. Lane 2, lysostaphin solublised cell wall fraction of *S. epidermidis* separated by SDS- PAGE (10% acrylamide gels), transferred to PVDF membrane and probed with anti-SdrH region C antibodies.

A.


83kDa
62.5kDa
47.5kDa
32.5kDa
1
2

B.

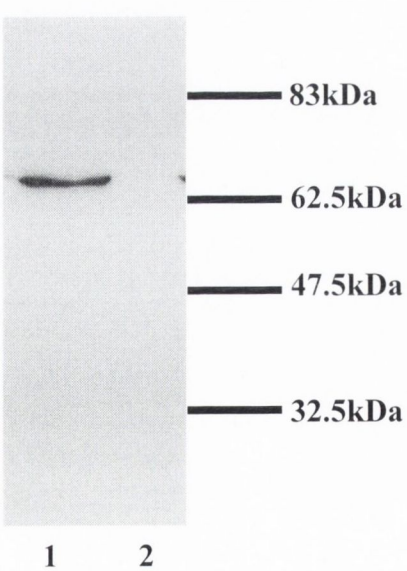
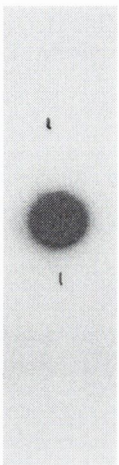

83kDa
62.5kDa
47.5kDa
32.5kDa
1
2

Figure 7.12 Expression of the SdrH protein in the heterologous expression host *L. lactis* MG1363. **A.** Trypsin treatment of whole *L. lactis* $SdrH^+$ cells. **B.** Lane 1, mutanolysin/lysozyme solubilised cell wall fraction of *L. lactis* pKS80 cells. Lane 2, stabilized protoplast of *L. lactis* pKS80 cells. Lane 3, mutanolysin/lysozyme solubilised cell wall fraction of *L. lactis* $SdrH^+$. Lane 4, stabilized protoplast of *L. lactis* $SdrH^+$. Proteins were separated by SDS-PAGE (10% acrylamide gels), transferred to PVDF membranes and probed with affinity-purified anti-SdrH region A antibodies.

A.

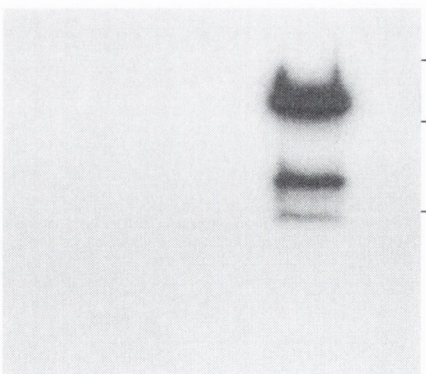


L. lactis pKS80

L. lactis SdrH^{+}

L. lactis SdrH^{+} Trypsin

B.



band in *S. epidermidis* cell wall extracts. The smaller bands are most likely products of proteolytic degradation. It can be concluded that SdrH is expressed on the surface of *L. lactis* but that it appears that the protein is not sorted to the cell wall. Further fractionation into cytoplasmic membrane and cytosol fractions was not carried out.

7.2.6. Identifying a ligand for SdrH

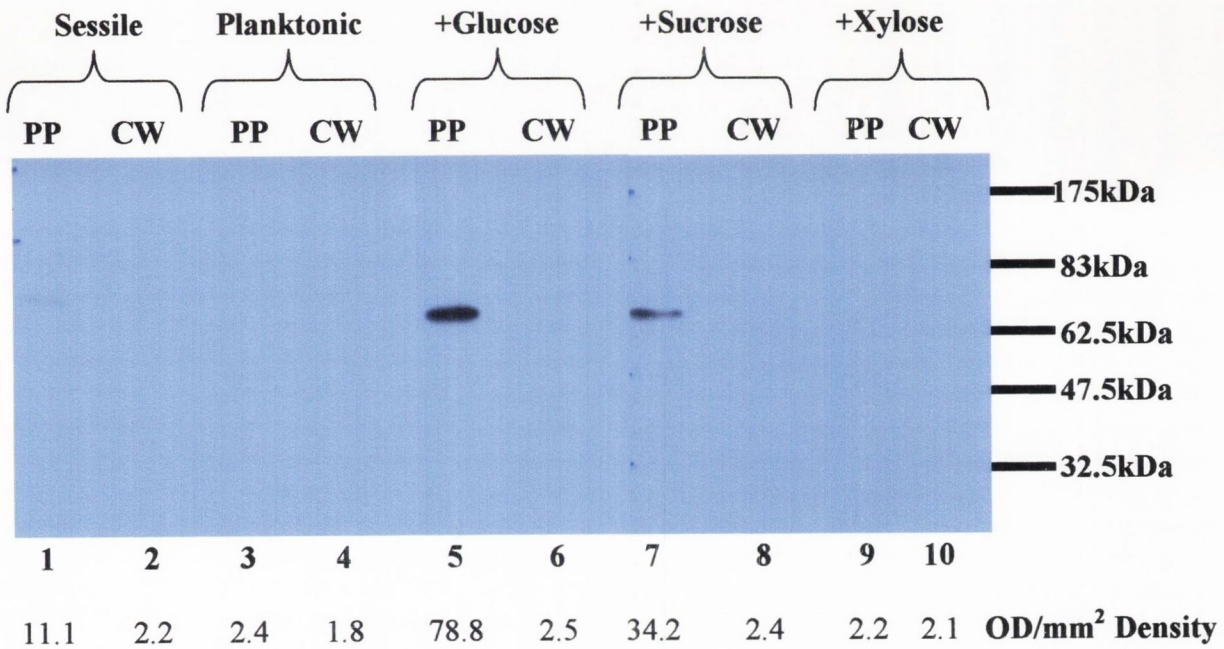
The *L. lactis* $SdrH^+$ strain was used to determine whether SdrH interacted with any host molecules similar to other members of the Sdr family of staphylococcal surface proteins. The following host proteins were tested using a modified ELISA-type bacterial cell adhesion assay as described in section 2.11: fibrinogen, fibronectin, collagen, laminin, keratin, mucin and human plasma. Proteins were immobilised in 96-well ELISA dishes at a concentration of 1mg/well and *L. lactis* $SdrH^+$ cells were added at an OD_{600nm} of 1. No evidence for binding to host proteins was detected. *S. aureus* cells previously shown to interact with the ligands were used as controls. Binding to bacteria by ligands in solution was not performed. *L. lactis* cells expressing SdrH were also tested for its ability to bind to desquamated nasal epithelial cells but showed no significant increase in binding to the cells above that showed by *L. lactis* carrying the empty vector pKS80. Therefore SdrH is unlikely to be directly involved in colonizing the skin.

7.2.7. Regulation of the expression of *sdrH*

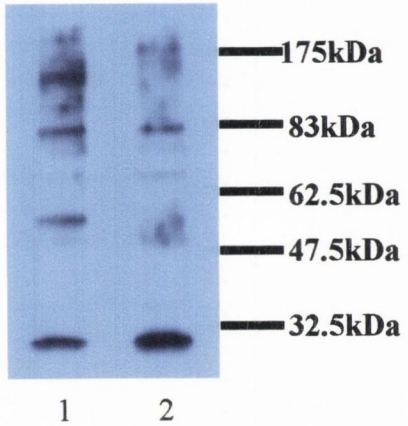
The *sdrH* gene was analysed by the tBLASTn program in the http://www.ncbi.nlm.nih.gov server. A gene for a bifunctional xylanase of *Ruminococcus flavifasciens, xynA*, showed 30% homology to the DNA sequence encoding the C domain of SdrH. This gene is involved in the digestion of cellulose by *R. flavifasciens*. It was therefore hypothesized that SdrH could have a role in sugar metabolism in *S. epidermidis*. This was tested by growing *S. epidermidis* in the presence of glucose, sucrose and xylose (Fig. 7.13). The growth of *S. epidermidis* in

Figure 7.13 The effect of growth conditions on expression of the SdrH protein in *S. epidermidis*. **A.** *S. epidermidis* strain 9491 was grown on the surface of Trypticase Soy agar plates (lanes 1 and 2), in Trypticase Soy broth with aeration (lanes 3 and 4), and in Trypticase Soy broth with aeration supplemented with either Glucose (lanes 5 and 6), sucrose (lanes 7 and 8) or xylose (lanes 9 and 10). Cells grown to stationary phase were washed and resuspended to an OD_{600nm} of 40 and digested with lysostaphin. 10µl of the cell wall fraction or detergent lysed stabilized protoplast fraction was analysed by SDS-PAGE (10% acrylamide), transferred to PVDF membrane and probed with anti-SdrH region A antibodies. PP, Stabilized protoplast fraction. CW, Cell wall fraction of cells digested with lysostaphin. This experiment was performed three times with similar results. **B.** Protoplasts of *S. epidermidis* strain 9491 grown in Trypticase Soy broth alone (1) or supplemented with glucose (2) probed with anti-SdrG region A antibodies as a control.

A


Sessile
Planktonic
+Glucose
+Sucrose
+Xylose
PP
CW
PP
CW
PP
CW
PP
CW
PP
CW
175kDa
83kDa
62.5kDa
47.5kDa
32.5kDa
1
2
3
4
5
6
7
8
9
10
11.1
2.2
2.4
1.8
78.8
2.5
34.2
2.4
2.2
2.1
OD/mm² Density

B


175kDa
83kDa
62.5kDa
47.5kDa
32.5kDa
1
2

TSB supplemented with 0.5% glucose led to an approximate 40-fold increase in the expression of SdrH protein in comparison to *S. epidermidis* grown in TSB alone. This was determined by analyzing the Western immunoblot in Fig. 7.11 by comparative densitometry.

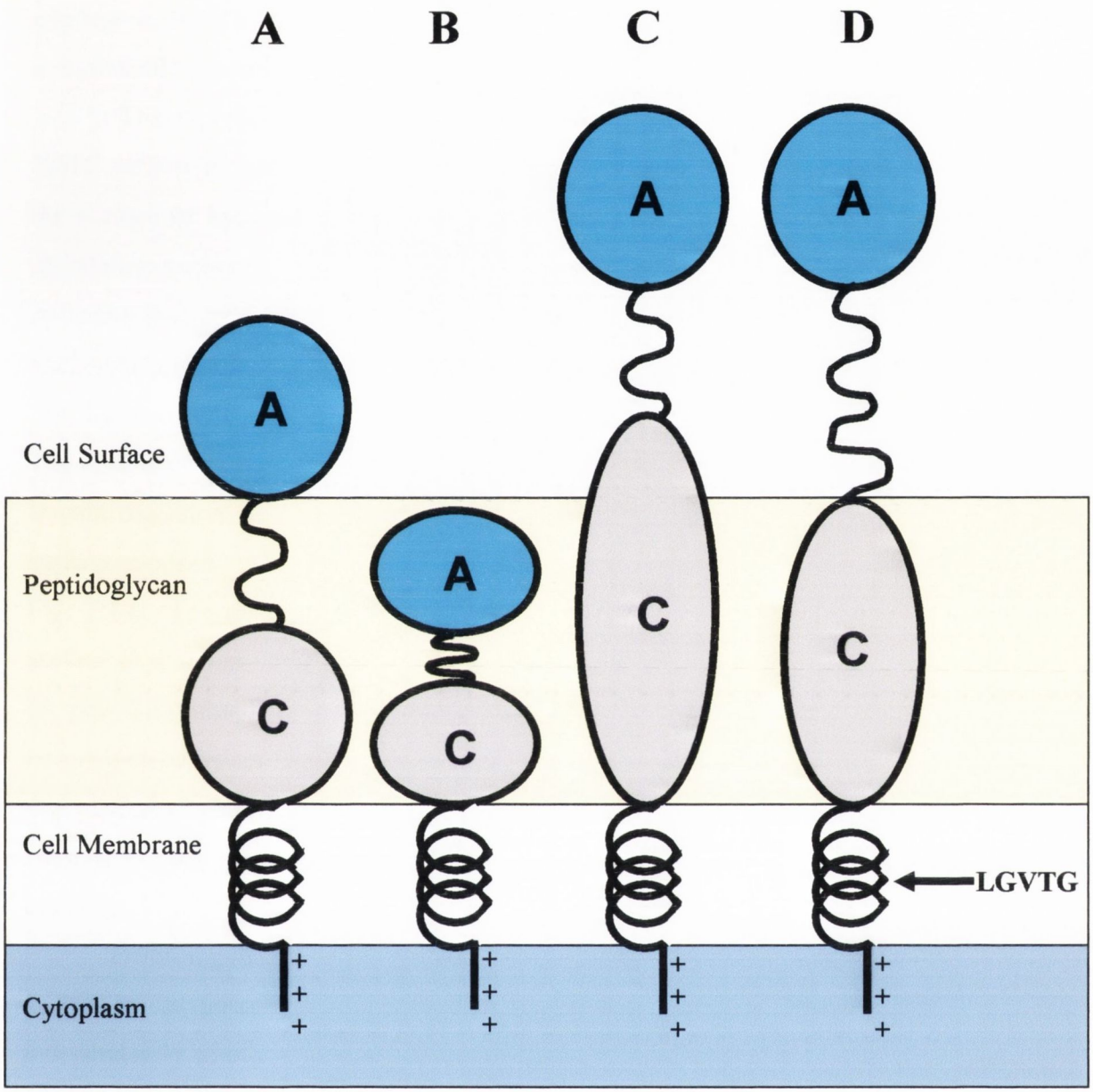
The conditions that maximized SdrH expression were also observed to maximize biofilm production. The expression of SdrH in isogenic *ica*$^{+}$ and *ica*$^{-}$ strains was analysed by SDS-PAGE and Western immunoblotting of stabilized *S. epidermidis* protoplasts in the presence and absence of glucose. However, no significant difference in expression was observed. SdrH expression was also upregulated when grown as a sessile culture on TSA agar plates as opposed to growth as planktonic cells in TSB with aeration (Fig. 7.13). These conditions have also been reported to maximize biofilm production in previous studies thus suggesting a possible link between SdrH expression and biofilm production in *S. epidermidis.*

7.3. Discussion

SdrH is a member of the SD repeat protein family of *Staphylococcus epidermidis*. Analysis of the *S. epidermidis* RP62A genome sequence revealed that the *sdrH* gene is located between the *agr* locus and the heat shock protein genes *hsp10* and *hsp60*. Upon tBLASTn analysis of the *sdrH* gene against the non-redundant sequences at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi two closely homologous staphylococcal genes, *sdrZ* of *S. caprae* and the SA2019 of *S. aureus*, and one more distant gene, *xynA* of *Ruminococcus flavifasciens* were identified. The complete genome for *S. caprae* is unavailable but the *S. aureus* homologue occupies the equivalent locus as *sdrH*, in-between the *agr* locus and the *hsp10* and *hsp60* genes. It is reasonable to surmise that all three genes occupy the same locus on their respective genomes and that they are likely to have the same function in different staphylococci. Analysis of other staphylococcal species, by Southern blotting and sequence analysis, will indicate whether the *sdrH*-like genes are conserved across the genus. The XynA protein shares only 30% identity with SdrH yet suggested a link between SdrH and carbohydrate metabolism. This lends credibility to genomic sequence analysis as a viable method for suggesting possible functions for genes. A caveat remains, however, as one annotated genome sequence attributed a fibrinogen binding function to SdrH due to its association with SdrG. Therefore bioinformatic analysis of genomic sequences is only useful if it is performed properly and accompanied by corroborating experimental work.

Previous studies by McCrea *et al.* (2000) indicated that SdrH must be expressed *in vivo* during infections as it elicited a strong immune response in convalescent patients' sera. This is a strong indication that it might play a role in *S. epidermidis* infections but does not provide any further information as to whether it is a virulence factor of *S. epidermidis* or has a housekeeping function. Attempts to generate a *sdrH* negative *S. epidermidis* mutant both in this study and in a previous investigation (O. Hartford, unpublished data) indicated that the *sdrH* gene is recalcitrant to disruption by either plasmid integration or allele replacement gene deletion. However, this does not mean that *sdrH* is an essential gene. The *sdrE* gene of *S. aureus* could not be disrupted

Figure 7.14 Schematic diagram showing the possible topological organization of SdrH.



by plasmid integration, but was deleted from the *S. aureus* chromosome by allele replacement of the entire *sdrCDE* locus (O'Brien *et al.*, 2002). Another useful strategy to try to determine the essentiality of the *sdrH* gene would be to titrate down its expression using inducible antisense RNA (Ji *et al.*, 2000) or fusion of the sdrH gene to a regulatable promoter (Zhang *et al.*, 2000; Xia *et al.*, 1999).

The expression of the SdrH protein on the surface of *S. epidermidis* and *L. lactis* $SdrH^+$ cells was analysed in order to ascertain its topology with regard to exposure on the surface of bacteria and whether it was either covalently sorted to the cell wall or membrane-anchored. Neither strain released SdrH after cell wall solublisation. This indicates that the LGVTG sequence at the C-terminal end of the protein is not a cell wall sorting motif. Digestion of the cell wall of both *S. epidermidis* strains and *L. lactis* Fbl^+ by murolytic enzymes also revealed that the protein was retained by the protoplast. The A-domain of SdrH was surface exposed, as shown in the whole cell dot blotting and trypsin digestion experiments. Consequently SdrH is membrane-anchored and partly surface-exposed. A model for the orientation of SdrH in the cell surface is shown in Fig. 7.14. The most likely scenario is where the A-domain is the only part that is surface exposed or where it is exposed along with the SD-repeats. One cannot rule out the possibility that a small part of the C domain is exposed and that it is insufficient to be recognised by anti-C domain antibodies. The fact that the homologues of SdrH in other species (SdrZ, SdrZL and SA1839) do not contain any LPXTG motifs suggest that none of the proteins are sorted to the cell wall and that they are all membrane-anchored proteins.

Heterologous expression of staphylococcal genes in *L. lactis* is a useful tool for analyzing the potential function of the gene product by expression of the protein independently of other surface factors. *L. lactis* does not express many surface proteins as judged from Coomassie blue stained SDS-PAGE of proteins isolated from the cell wall of the control strain, *L. lactis* MG1363 containing the empty vector pKS80. The expression of SdrH in *L. lactis* allowed the screening of several human matrix and serum proteins as possible ligands for SdrH but none were found to promote *L. lactis* $SdrH^+$ adherence. *L. lactis* $SdrH^+$ cells did not adhere to desquamated nasal epithelial

cells. However, this was not an exhaustive search for a ligand and several other strategies could have been employed. Further analysis using *L. lactis* expressing SdrH and the recombinant A domain of SdrH will identify a ligand and suggest a possible function for SdrH.

Clues to a potential function for SdrH came with the discovery that it has a distant homologue in *R. flavifasciens*, a symbiont of ruminants. The XynA protein is a xylanase involved in degradation of xylan residues in cellulose. Hence, it was reasonable to hypothesize that SdrH could have some role in *S. epidermidis* that involved sugar metabolism or interaction with sugar residues (e.g. peptidoglycan). It was shown that growth of *S. epidermidis* in glucose increased SdrH expression 50-fold but did not affect the expression of SdrG. Growth of *S. epidermidis* in glucose also increased biofilm production. Therefore the expression of SdrH was compared under sessile and planktonic growth conditions. This showed that SdrH was also upregulated under sessile growth conditions. It is tempting to postulate that SdrH is involved in biofilm formation and it is possible that SdrH aids in biofilm formation or has some ancillary role, such as the anchoring of biofilm to the cell surface. The *hsp10* and *hsp60* genes that lie downstream of *sdrH* have also previously shown to be upregulated by growth in glucose (Vandecasteele *et al.*, 2001) while the expression of *agr*, which is transcribed in the opposite direction to *sdrH*, is downregulated by glucose at least in *S. aureus* (Regassa *et al.*, 1992). The expression of the *ica* locus, which is responsible for the production of PIA, the major component of *S. epidermidis* biofilm, is down-regulated by the presence of glucose. Conversely PIA production is increased in the presence of glucose indicating an Ica-independent, glucose-dependent mechanism for biofilm formation (Dobinsky et al., 2003). SigB has been shown to be the major regulator of biofilm production in *S. epidermidis* (Rachid *et al.*, 2000; Knobloch *et al.*, 2001). Analysis of a *sigB* mutant with regard to its effect on *sdrH* transcription would help to clarify the link between biofilm production and SdrH expression.

Genome Therapeutics is developing ramoplanin as a novel glycolipodepsipeptide antibiotic with potent bactericidal activity for many Gram-positive bacterial species for the prevention, treatment and control of serious

bloodstream infections. Currently, ramoplanin is in Phase III development for the prevention of bloodstream infections caused by vancomycin resistant enterococci (VRE) in VRE-colonized, neutropenic cancer patients. The mechanism of action of ramoplanin involves disruption of cell wall biosynthesis resulting in cell lysis. The antibiotic has been shown to bind to lipid I and to lipid II and to inhibit MurG and the transglycosylase PBP1b indicating that ramoplanin acts at a late stage of peptidoglycan biosynthesis. A study by Genome Therapeutics revealed that transcription of *sdrH* was upregulated up to eight-fold in a dose-dependent manner by treatment of *S. epidermidis* cells with sub-lethal concentrations of ramoplanin by expression profiling using a set of spotted PCR product microarrays (http://www.genomecorp.com/programs/pdf/IDSA_2002_Microarrays.pdf). The array represents most of the predicted genes in *Staphylococcus epidermidis* and identifies genes whose expression level is altered upon treatment of cells with ramoplanin. This method of expression profiling would be ideal to identify the conditions in which SdrH is expressed and perhaps shed light on its function and whether its expression is not only linked to that of biofilm but also peptidoglycan synthesis.

The *sdrH* gene has been detected in both sequenced strains of *S. epidermidis* and all strains of *S. epidermidis* tested by Southern blotting (McCrea *et al.*, 2000). Its *S. aureus* homologue is present in all seven of the *S. aureus* strains that have been sequenced and 30 strains tested by PCR (Etz *et al.*, 2002). A third member of the SdrH family is present in strains of *S. caprae*. The expression of SdrH and its *S. aureus* homologue SA1839 in vivo has been identified by screening convalescent patient sera thus both genes are expressed during infections (McCrea *et al.*, 2000, Etz *et al.*, 2002). If SdrH is an essential protein, the common C-domain of SdrH and its homologues could provide a useful anti-staphylococcal drug target. The expression of the SdrH homologues in *S. aureus*, *S. caprae* and *S. capitis* in the *L. lactis* heterologous gene expression system would complement any further investigations into the function of SdrH and its homologues.

Thus the potential for development of therapeutic strategies that would potentially target the entire staphylococcal genus would be of great benefit to immuno-

compromised patients at risk from opportunistic infections by both *S. aureus* and coagulase negative staphylococci and warrants further investigation into this intriguing protein.

Chapter 8

General Discussion

8.1 Discussion

Staphylococci are opportunistic pathogens and a major cause of community acquired and nosocomial infections. They asymptomatically colonize the squamous epithelia of the anterior nares and the skin of healthy humans but can cause infection when the skin or mucosal barriers are breached, for example following insertion of a foreign body and in hosts with compromised immune systems. Staphylococci have the ability to initiate wound infections and subsequently cause diseases such as osteomyelitis, infective endocarditis, and bacteraemia, leading to secondary infections in any of the major organ systems. *S. epidermidis* is the leading cause of prosthetic valve endocarditis. However, fewer than 5% of these organisms are associated with native valve endocarditis (Whitener *et al.*, 1993). Instead *S. aureus* and *S. lugdunensis* are more frequently associated with cases of native valve endocarditis (Shuttleworth *et al.*, 1992). MSCRAMM protein adhesins are implicated in enabling the bacteria to both colonize the host and to initiate metastatic foci of infection by recognizing and specifically binding to extracellular components of host tissues or to serum-conditioned implanted biomaterials such as catheters, artificial joints, and vascular grafts. Further tissue damage around the site of an infection is caused by elaboration of bacterial virulence factors such as exoenzymes and exotoxins and an aggressive host immune response.

S. aureus possesses a veritable arsenal of weaponry that serve to thwart host immune systems and antimicrobial chemotherapies and play a part in the pathogenic process. These include surface-associated adhesins, capsular polysaccharide, exoenzymes and exotoxins which allow staphylococci to adhere to eukaryotic tissue and extracellular matrices, to resist opsonophagocytosis, to lyse eukaryotic cells, and to trigger the production of a cascade of host immunomodulating molecules. Antibiotic resistance mechanisms other than acquired resistance mechanisms include biofilm production, small colony variants and the persister state (Bigger, 1945). Staphylococcal ligand binding proteins have the capability to mask the surface of the bacterial cell against recognition by phagocytic cells. In this way adhesins not only contribute to the pathogenicity of staphylococci by their colonizing and tissue adhesive properties but also by concealing the bacterium from immune recognition so that an infection can proceed unhindered.

The data presented herein shows that Fbl is a fibrinogen-binding protein and clumping factor of *S. lugdunensis*. Fbl shares 60% amino acid identity with its *S. aureus* homologue ClfA and behaves in a similar functional manner. Fbl expression on the surface of bacteria promotes adherence to an immobilised fibrinogen substrate by binding to the γ-chain. As with ClfA the Fbl-fibrinogen interaction can be disrupted by high Ca^{2+} concentrations. Deivanayagam *et al.* (2002) discounted the role of the EF-hand in Ca^{2+} binding of ClfA because the calcium ions that co-crystallized with $rClfA_{220-559}$ did not co-ordinate with the residues of the proposed EF-hand. However $rClfA_{220-559}$ was not crystallized in the presence of Ca^{2+} ions. Therefore the residues identified in this paper represent high-affinity Ca^{2+} binding sites. Perhaps the inhibition of fibrinogen binding by high levels of Ca^{2+} that is observed for both Fbl and ClfA is mediated by low affinity binding sites that will only be identified by mutagenesis and crystallization of $rClfA_{220-559}$ in the presence of Ca^{2+} ions. It is possible that this will confirm the role of the proposed EF-hand motif in Ca^{2+} binding.

The major difference between Fbl and ClfA is the completely divergent sequence of the respective N1 domains of the ligand binding A regions. The reason for this remains unclear. Presumably N1 contributes to the antigenic differences of the proteins as it does not appear to affect ligand specificity. The construction of hybrid ClfA-Fbl N1-N2-N3 molecules with the alternate N1 replacing the native N1 might suggest what role N1 plays in the function of ClfA and Fbl if any.

Polyclonal anti-ClfA region A antibodies also recognized Fbl epitopes. The Fbl protein from *S. lugdunensis* and purified recombinant Fbl_{40-534} were both recognized by polyclonal anti-ClfA region A antibodies. These antibodies also inhibited the adherence of Fbl-expressing bacteria to fibrinogen and displaced bound bacteria from an immobilised fibrinogen substrate. Correspondingly, polyclonal antibodies raised against $rFbl_{40-534}$ also inhibited the adherence of Fbl-expressing bacteria to fibrinogen and displaced bound bacteria from an immobilised fibrinogen substrate. However these antibodies were less efficient at inhibiting ClfA-mediated adherence of bacteria to fibrinogen and poorly displaced ClfA-expressing bacteria from an immobilised fibrinogen substrate. Nonetheless polyclonal anti-Fbl region A antibodies recognized ClfA by Western immunoblotting. An attempt to eliminate Fbl expression in *S. lugdunensis* would be a worthwhile strategy that would unequivocally

attribute *S. lugdunensis* fibrinogen binding to the expression of Fbl. An Fbl$^-$ *S. lugdunensis* mutant would also aid in testing whether Fbl$^-$ mutants are attenuated in infection models. It is also important to determine whether vaccination with recombinant Fbl protein could protect against *S. lugdunensis* infection. Passive immunization with either polyclonal or Monoclonal antibodies that protect against both Fbl- and ClfA-mediated staphylococcal infection is a desirable and feasible goal for counteracting both *S. lugdunensis* and *S. aureus* infections. monoclonal antibodies against $rFbl_{40\text{-}534}$ might cross react with ClfA.

Fbl also mediates the initiation of platelet aggregation when expressed on the surface of either *S. lugdunensis* or *L. lactis*. This phenomenon appears to be dependent on the copy number of Fbl molecules expressed on the surface of the cell. Serial two-fold dilutions of *S. lugdunensis* or *L. lactis* cells probed with anti-Fbl antibodies showed that there was approximately 64-fold more Fbl expressed on the surface of *L. lactis* than *S. lugdunensis*. *L. lactis* Fbl$^+$ also caused platelet activation much more efficiently than *S. lugdunensis*. Controlled expression of Fbl in conjunction with accurate estimation of the copy number by flow cytometry would help to determine the optimum level of Fbl expression that results in platelet aggregation. Separation of platelet proteins and probing with biotin-labeled Fbl or affinity chromatography of platelet components using Fbl coupled to sepharose might enable identification of ligand(s) on the surface of platelets to which Fbl binds.

It is very interesting that the 40% of amino acid residues that vary between Fbl and ClfA N2 and N3 domains do not change ligand binding specificity. Both proteins still bind to the γ-chain of fibrinogen and the residues implicated in contacting with the γ-chain peptide are conserved. However, ClfA definitely has a higher affinity for fibrinogen than Fbl. Perhaps the amino acid changes in the latching peptide trough affect the on and off rates of Fbl and fibrinogen. There are a greater proportion of charged residues in ClfA than Fbl in this region. This could also reflect a different disease progression between *S. aureus* and *S. lugdunensis*. *S. lugdunensis* is not equipped with as extensive an array of proteases and extracellular proteins as *S. aureus*. Proteins such as staphylokinase activate plasminogen that degrades fibrin clots and could theoretically aid in releasing *S. aureus* cells from platelet-bacteria thrombi allowing the infection to metastasize. Perhaps the lower affinity of Fbl for fibrinogen circumvents its lower range of accessory proteins and avoids the need for

extensive proteolytic degradation to release *S. lugdunensis* cells from thrombi. It is possible that Fbl has a higher off rate when interacting with fibrinogen and that this alone helps *S. lugdunensis* release itself from a thrombus and allows cells to metastasize and spread *S. lugdunensis* infections systemically.

In order to test the theory that the different kinetics of binding to fibrinogen is due to the variation in the latching peptide and trough, rather than the ligand binding specificity it would be necessary to mutate sequentially the residues in the latching trough in ClfA, critically $_{277}$DDVK$_{280}$, to determine whether this affects the kinetics of interaction of ClfA with fibrinogen. Surface plasmon resonance and isothermal titration calorimetry should be used to determine the precise kinetics of binding and detachment (k_{on} and k_{off}). If the differences suggested by ELISA can be quantified more precisely then the mutant proteins would also be tested by surface plasmon resonance and isothermal titration calorimetry in comparison to both wild-type ClfA and Fbl. Any increase in off rate observed would be consistent with the theory that Fbl has a higher off rate when interacting with fibrinogen. Mutation of these trough residues would also determine whether the specificity for the γ-chain peptides is altered as is seen in Fbl. Perhaps this is why Fbl has such a markedly different profile of variant peptide binding as compared to ClfA. There is also preliminary data that suggests that like ClfB, ClfA and Fbl both interact with cytokeratin 10, but with a lower affinity. Changing trough residues to residues similar to that of those in the ClfB trough could alter the specificity of ClfA so that it would bind to Keratin with a higher affinity. ClfB interacts with the tail residues of keratin which are rich in GS, similar to the complimentary β-strand that locks into the trough, GSGSGD. .Reciprocal changes of trough residues in ClfB to those in ClfA and Fbl would indicate whether this theory is correct.

The differences in amino acid sequence between the two proteins also affects the presentation of antigenic epitopes. While polyclonal antibodies to Fbl and ClfA recognize both Fbl and ClfA, and cross-inhibit adherence to fibrinogen, none of the murine monoclonal antibodies raised against rClfA$_{220\text{-}559}$ recognized Fbl epitopes. The majority of variant residues that affect antibody binding are on the surface of the molecule. Saturation of the variant surface residues of ClfA with alanine mutations will further aid in the identification of antibody epitopes. However it still remains important to determine whether a monoclonal antibody can be found which

recognises a common epitope between ClfA and Fbl. This would putatively improve the immunotherapy for patients suffering from native valve infective endocarditis where a small but significant number of cases are caused by *S. lugdunensis* and would presumably not respond to Aurexis treatment.

There is a possibility that the use of monoclonal antibodies in the treatment of *S. aureus* infection could act as a selection pressure for the evolution of ClfA mutants that escape recognition by monoclonal antibodies. This trend could be anticipated by searching for escape mutants in a bacterial population by fluorescent cell sorting using fluorescently labeled monoclonal antibodies as discussed by Daugherty *et al.* (2000). Cells with no signal would be sorted first as they would not be recognised by the fluorescently labeled monoclonal antibodies. These cells would be subsequently tested to determine whether they bound fibrinogen to show that they still express functional ClfA. Variants would then have to be sequenced to identify the mutation(s) responsible for lack of antibody recognition. Functional tests would then be performed by flow cytometry, and inhibition and displacement in ELISA-type assays. It would also be important to test a collection of *S. aureus* strains to correlate antibody recognition with surface protein expression. The ClfA amino acid sequences of more strains should be sequenced, in particular strains that produce a large amount of ClfA but only bind a small amount of monoclonal antibody.

The K417A substitution reduced the binding of the monoclonal antibody 12-9 and others to $rClfA_{220\text{-}559}$. It is important to carry out surface plasmon resonance to determine whether the on or off rates of antigen: antibody interactions are affected by K417A substitution and to analyze whether, as Cunningham and Wells (1993) concluded, that avid protein-protein interactions minimize off rates rather than increase on rates. Co-crystallization of $rClfA_{220\text{-}559}$ with the mAb Fab fragments in situ will confirm the essential contact residues. Site-directed mutagenesis will identify all the residues that contribute to the interaction of antibody and antigen. It would also be important to sequence the hypervariable regions of the complementarity determining regions of the different monoclonal antibodies that bind around K_{417} in order to determine variations in amino acid sequence and how this might lead to slightly altered recognition of the same epitope region while still providing similar functional attenuation by binding to ClfA.

The Y376A, I517A and R519A mutations affected the binding of another mAb, 35-039 to $rClfA_{220\text{-}559}$. This antibody also inhibited the binding of ClfA-

expressing bacteria to fibrinogen and displaced bound bacteria from an immobilised fibrinogen substrate. It is interesting that the potential epitopes of both 12-9 and 35-039 both lie adjacent to the putative latching peptide in the apo-form of $rClfA_{220-559}$. It seems likely that the mAbs function by restricting the movement of the latching peptide so that the fibrinogen γ-chain is never locked into position. Perhaps the ClfA-fibrinogen interaction is dynamic whereby the latch opens and closes and this is why the mAbs can both inhibit and displace. If the mAbs have a higher affinity for ClfA than ClfA has for fibrinogen then this would explain why bacteria bound to fibrinogen can be displaced. This could be confirmed by surface plasmon resonance to analyze the K_{on} and K_{off} rates. Complete saturation mutagenesis of the residues that vary between Fbl and ClfA will aid in determining where all of the monoclonal antibodies bind and give a better picture of how these antibodies inhibit the function of ClfA. It will also help analyze how ClfA interacts with fibrinogen and the structural changes that are involved in the transition from ClfA apo-form to the ClfA-fibrinogen complex. A monoclonal antibody recognizing the latching peptide or the latching groove would provide valuable information on the role of the β-strand complementation in the stabilization of the ClfA-fibrinogen complex. If either antibody inhibits the ClfA-fibrinogen interaction then it would confirm that β-strand complementation between the latching peptide and the latching groove is vital for the formation of a stable ClfA-fibrinogen complex.

Fibrinogen binding proteins might be able to avoid inactivation by neutralizing antibodies by subtly altering their molecular surface such that they can still bind to fibrinogen and yet avoid antigenic recognition. This is presumably a widespread phenomenon that affects immunological inter-species recognition. It would be interesting to test other *S. aureus* ligand binding proteins that have homologues in other species such as ClfB of *S. aureus* and SdrY of *S. caprae* and determine what level of amino acid difference can be tolerated before homologues become antigenically distinct.

SdrH of *S. epidermidis* is another surface-located and membrane-anchored protein. Further work is needed to identify the function of SdrH and its homologues in other staphylococcal species. Attempted disruption of the *sdrH* gene was unsuccessful suggesting that it might be an essential gene. However this must be verified by antisense RNA or promoter titration. Southern blotting hybridisation of

representative strains of all staphylococcal species will help to determine if the incidence of *sdrH* gene or a homologue is a genus-wide occurrence.

SdrH is a membrane anchored surface exposed protein that is upregulated by sessile growth conditions and by addition of glucose and sucrose to the growth medium. It is unlikely that changes in osmolarity affect the expression of SdrH as addition of xylose to the growth medium had no affect. Further analysis of SdrH expression under differing growth conditions will confirm this. The expression of SdrH has also been observed to be significantly upregulated in the presence of the glycopeptide antibiotic ramoplanin. An ideal way to determine the expression profile of SdrH would be to analyze expression of *S. epidermidis* genes by microarray transcription analysis and 2D PAGE electrophoresis of total cell proteins. The effects of ramoplanin and the functionally related peptide nisin, as well as growth in glucose and sucrose on global gene expression could then be analysed simultaneously by proteomics and transcriptomics. This will identify other genes that are regulated by the same growth conditions and might give a clue about the function of SdrH.

In order to determine the molecular function of SdrH and its homologues the recombinant C and A regions of each of the proteins could be purified and tested to see whether they bind to peptidoglycan, lipidII or complex carbohydrates. This might involve coupling the recombinant proteins to CNBr-activated sepharose and analyzing whether the ligands adhere to the protein by affinity chromatography.

As with Fbl, SdrH appears to be membrane-anchored and surface exposed protein and not cell wall anchored via an LPXTG motif. Confirmation of this requires fractionation studies. The purified A- and C-domains could also be analysed to see whether they dimerise or oligomerise under non-reducing and/or non-denaturing conditions. SdrH appears to be a membrane-bound protein and could require oligomerisation to function properly. Whatever the function of SdrH transpires to be, it is intriguing to contemplate why the staphylococcal genus requires this gene to be functionally conserved at the C-terminus and yet obviously antigenically distinct at the N-terminus.

In conclusion, it is evident that while *S. aureus* mounts a devastating attack on the host immune system, several defensive strategies have been developed in order to counteract the wide variety of staphylococcal virulence factors. It is reassuring that while staphylococci constantly mutate and adapt in order to avoid antibiotics immunological approaches can be used to thwart staphylococcal diseases. However,

more research is needed into mechanisms and treatments that protect immunocompromised individuals against antibiotic resistant staphylococcal disease when their weak immune systems are overwhelmed.

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