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Molecular Characterisation of the Interactions Between *Staphylococcus aureus* and Elastin

A thesis submitted for the degree of Doctor of Philosophy

by

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April 2002

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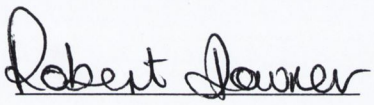

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Summary

Previous studies have shown that a cell-surface 83 kDa elastin-binding protein of *Staphylococcus aureus* (EbpS) mediates binding to soluble elastin. Antibodies were produced to the N terminus and C terminus of EbpS. Western immunoblotting identified EbpS as an 83 kDa protein in whole cell lysates of *S. aureus*. Release of EbpS from purified *S. aureus* cell envelopes, with either lithium chloride or sodium dodecyl sulphate, revealed that the protein is associated with the cell surface by a different mechanism to that of the typical cell-wall-associated protein ClfA, which belongs to a family of staphylococcal extracellular matrix-binding proteins that are covalently anchored to the cell wall and are known as microbial surface components recognising adhesive matrix molecules (MSCRAMMs). EbpS was localized to the cytoplasmic membrane of *S. aureus* and *Lactococcus lactis* expressing EbpS by cellular fractionation using the stabilised protoplast method whereby cell-wall-associated proteins were released from the cell surface by enzymatic digestion of the cell wall peptidoglycan while maintaining an osmotically stable protoplast. In addition, EbpS was detected in the purified membrane fraction of *S. aureus* that had been prepared by mechanically smashing the cells and separating fractions by differential centrifugation.

In silico analysis of the primary amino acid sequence predicted that EbpS is an integral membrane protein. A series of hybrid proteins was formed between EbpS at the N-terminus and either alkaline phosphatase or β-galactosidase at the C-terminus (EbpS-PhoA, EbpS-LacZ). PhoA and LacZ were fused to EbpS between hydrophobic domains H1-H2, H2-H3 and distal to H3. Expression of enzymatic activity in *Escherichia coli* showed that EbpS is an integral membrane protein with two membrane spanning domains H1 and H3. N-terminal residues 1–205 and C-terminal residues 343–486 were predicted to be exposed on the outer face of the cytoplasmic membrane. This topological model was supported by the absorption of polyclonal antibodies to the surface exposed regions of EbpS onto the surface of stabilised *S. aureus* protoplasts. Cross-linking studies, using the homobifunctional cross-linking reagent dithiobis(succinimidyl proprionate) (DSP),

suggested that EbpS is part of a heteromeric or homomeric complex in the cytoplasmic membrane.

Expression of EbpS was correlated with the ability of cells to grow to a higher density in liquid culture, suggesting that EbpS may have a role in regulating cell growth. *S. aureus* wild-type dominated when grown in mixed culture with an isogenic *ebpS* mutant and cultures of *ebpS* mutants grew to a lower density than cultures of the parental strain as determined by optical density and bacterial dry weight yields. The ability of truncated derivatives of EbpS expressed in an *ebpS* deficient background to complement the growth defect was assessed and indicated that an EbpS derivative lacking the LysM motif at the C terminus of the protein could not complement the growth phenotype.

EbpS was unable to promote bacterial adherence to immobilised elastin, as shown by the ability of a derivative of *S. aureus* strain P1, in which the *ebpS* gene had been interrupted by allelic replacement, to adhere to the immobilised ligand. The elastin-binding capacity of strain P1 was lost when the genes for both the fibronectin-binding proteins, FnBPA and FnBPB, were interrupted by allelic replacement. Expression of FnBPA or FnBPB from multicopy plasmids restored the elastin-binding phenotype. The regions of the FnBP proteins responsible for elastin binding were identified by inhibition studies using polyclonal antibodies and recombinant truncated derivatives of the FnBP proteins. The purity of commercially available elastin preparations was assessed and the relevance of using elastin peptides dried onto the wells of microtitre plates as representative of elastin tissue was assessed in an ELISA-based assay.

Publications originating or anticipated from the present work

Downer, R., Roche, F., Park, P. W., Mecham, R. P. and Foster, T. J. (2002). The elastin-binding protein of *Staphylococcus aureus* (EbpS) is expressed at the cell surface as an integral membrane protein and not as a cell wall-associated protein. *J. Biol. Chem.* **277**, 243-250.

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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
C	cytosine
G	guanine
T	thymine

Antibiotics

Ap	ampicillin
Cm	chloramphenicol
Erm	erythromycin
Kan	kanamycin
Rif	rifampicin
Spc	spectinomycin
Tet	tetracycline

Other Abbreviations

σ^B	sigma factor B
σ^S	sigma factor S
agr	accessory gene regulator
AMP	adenine monophosphate
ATP	adenine triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate
BSA	bovine serum albumin
CDM	chemically defined medium
CFU	colony forming unit
ClfA	clumping factor A
ClfB	clumping factor B
Cna	collagen-binding protein
CNBr	cyanogen bromide
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSP	dithiobis(succinimidyl proprionate)
Eap	extracellular adherence protein
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Efb	extracellular fibrinogen-binding protein

ELISA	enzyme linked immunosorbent assay
Emp	extracellular matrix-binding protein
ER	endoplasmic recticulum
ET	exfoliative toxin
Fab	fragment of antibody
Fc	crystallisable fragment of IgG
FnBP	fibronectin-binding protein
FSB	final sample buffer
HRP	horseradish peroxidase
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobases
kDa	kilodalton
LacZ	β-galactosidase
LysM	lysin motif
Map	MHCII analogous protein
MLS	macrolide, lincosamide and streptogramin B
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
nt	nucleotide
ONPG	o-nitrophenyl-β-D-galactopyranoside
orf	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PhoA	alkaline phophatase
PI-Phospholipase	phosphatidylinositol phospholipase
PNSG	poly-N-succinyl β-1-6 glucosamine
POD	peroxidase
ppGpp	guanine tetraphosphate
psi	pounds per square inch

PVDF	polyvinylidene diflouride
RNA	ribonucleic acid
Rot	repressor of toxins
rpm	revolutions per minute
rRNA	ribosomal RNA
sar	staphylococcal accessory regulator
SCV	small colony variant
Sdr	serine-aspartate repeat
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	staphylococcal enterotoxin
Sig	sigma factor
Spl	serine protease-like
Ssp	staphylococcus serine protease
TCA	trichloroacetic acid
Tris	trishydroxymethylaminomethane
TSA	trypticase soy agar
TSB	trypticase soy broth
TSST-1	toxic shock syndrome toxin 1
U/ml	units per ml
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Tada gan iarracht.

Clonkeen College, Blackrock. 1987-1993.

FOR MY GRANDMOTHER

Chapter 1

Introduction

1.1 The biology of *Staphylococcus aureus*

1.1.1 Classification and identification

Staphylococcus aureus is a Gram-positive non-motile, non-sporing, catalase-positive coccus that forms smooth, entire, raised colonies that often contain a golden pigment. Bacterial taxonomy places the genus *Staphylococcus* in the *Bacillus-Lactobacillus-Streptococcus* cluster of the family *Micrococcaceae* (Ludwig *et al.*, 1985; Schleifer, 1986). The genera within this family exhibit a number of different molecular features, including susceptibility to the lytic enzyme lysostaphin, GC content of genomic DNA and cell wall composition. DNA-DNA hybridisation and analysis of 16S and 23S rRNA sequences has revealed that the staphylococci form a well-defined genus consisting of at least 26 species, 13 of which are associated with humans (Schleifer and Kroppenstedt, 1990).

Genomic DNA of *S. aureus* has a low G and C content (36%). The cell wall is composed of peptidoglycan, which has lysine as the diamino acid, a pentaglycine cross-bridge and ribitol teichoic acid that is substituted with N-acetyl glucosamine (Schleifer and Kandler, 1972; Ward, 1981). *S. aureus* is halotolerant and can grow on agar containing up to 1.7 M (10% w/v) sodium chloride, a characteristic that can selectively separate staphylococci from mixed cultures. The production of the extracellular enzyme coagulase distinguishes *S. aureus* from the less pathogenic coagulase negative staphylococcal species. In clinical microbiology laboratories commercially available agglutination tests are used for the identification of *S. aureus*. These test for the presence of coagulase (a fibrinogen-binding protein loosely associated with the cell wall), ClfA (a cell-wall-associated fibrinogen-binding protein) and protein A (the immunoglobulin-binding protein) using particles coated with IgG and fibrinogen. The accuracy of these tests has been improved by the addition of monoclonal antibodies against capsular antigens of *S. aureus*. Other characteristics that are used to identify *S. aureus* are its ability to express heat stable DNase and to produce acid from carbohydrate substrates.

S. aureus is an opportunistic pathogen of humans that permanently colonises approximately 30% of the population and transiently colonises another 30-50%

(Kluytmans *et al.*, 1997). The natural habitat of the organism in humans is the skin and the anterior nares. If the natural barriers are damaged *S. aureus* can infect host tissue and cause a localised infection from where it can diseminate via the bloodstream to internal organs and cause invasive diseases resulting in high morbidity and mortality.

1.1.2 Small colony variants

S. aureus small colony variants (SCVs) have been isolated from patients with persistent, antibiotic-resistant and recurring infections (Proctor *et al.*, 1995). The majority of clinical *S. aureus* SCV isolates are characterised by defects in menadione and hemin biosynthesis, resulting in defective electron transport. SCVs produce persistent infections due to their slow growth, intracellular location and decreased antibiotic uptake (Proctor *et al.*, 1995; von Eiff *et al.*, 1997a; Proctor and Peters, 1998). All aspects of this phenotype, such as slower growth, reduced production of lytic toxins, no pigmentation, failure to ferment mannitol and resistance to aminoglycoside antibiotics and host antimicrobial peptides, can be attributed to loss of one or another of the enzymes critical to the biosynthesis of the electron transport chain components (Proctor, 2000). SCVs are inherently resistant to the positively charged aminoglycoside antibiotics and host antimicrobial peptides due to the loss of membrane potential resulting from defects in electron transport (Lewis *et al.*, 1990; Vesga *et al.*, 1996). SCVs are able to persist within cultured endothelial cells because they do not lyse the cells due to decreased α-toxin production (von Eiff *et al.*, 1997b).

A proportion of *S. aureus* SCVs have defects in unsaturated fatty acid biosynthesis (Goldenbaum and White, 1974). Menaquinone, a component of the electron transport chain, is formed from menadione plus a repeating isoprenoid tail (Goldenbaum and White, 1974). This fatty acid tail is required for association with the bacterial membrane and the formation of the electron transport chain (Frerman and White, 1967). Defects in fatty acid biosynthesis can hinder this process, resulting in a defective electron transport chain and the SCV phenotype.

SCVs present a challenge for diagnosis and treatment. The altered colony morphology and biochemical profile may lead to the erroneous identification of *S. aureus* SCVs as viridans streptococci or coagulase-negative staphylococci (Kahl *et al.*, 1996).

The slow growth and antibiotic resistance may lead to the belief that a new, rather than recurrent, infection is present.

1.1.3 Stress resistance

S. aureus is capable of surviving inside the host and in a wide variety of environmental niches. The organism can colonise the nasopharynx and skin of healthy individuals as well as persist outside the host in dust, soil, water and a number of other environments (Clements and Foster, 1999). *S. aureus* possesses a number of mechanisms that control its response to environmental stresses. It is the adaptability and resistance to these stresses that allow the organism to be such a successful opportunistic pathogen.

The ability of *S. aureus* to survive and grow in environments not associated with pathogenesis promotes spread and transmission. Staphylococci are extremely desiccation tolerant and halotolerant. The mechanism of desiccation tolerance is poorly understood. The organism can grow on agar containing up to 1.7 M (10% w/v) sodium chloride. When grown in high concentrations of salt, *S. aureus* accumulates osmoprotectant solutes such as glycine, proline and betaine in the cytoplasm, shortens the interpeptide bridges of cell wall peptidoglycan to confer mechanical strength to the cell wall, and represses the expression of virulence factors (Townsend and Wilkinson, 1992; Vijarankul *et al.*, 1995; Chan and Foster, 1998). Recently, analysis of the genome sequence of the methicillin-resistant *S. aureus* strains Mu50 and N315 identified an operon with similarity to the Kdp operon of *Clostridium acetobutylicum*. In *C. acetobutylicum* the Kdp operon encodes an ATP-dependent transport system involved in osmolarity resistance (Kuroda *et al.*, 2001). Osmotic stress also induces the expression of alkyl oxide synthase (AhpC), an enzyme believed to be involved in bacterial resistance to reactive nitrogen intermediates, suggesting that osmotic stress induces protection against other stresses (Armstrong-Buisseret *et al.*, 1995).

In the environment or on the skin of the host, nutrient availability becomes a limiting factor. Glucose and multiple-nutrient limitation results in the loss of viability of approximately 99% of the population within two days. A population of cells will remain viable for several months and develop an increased survival potential (Watson *et al.*, 1998a). The surviving cells become smaller and develop increased resistance to acid and

oxidative stress (Watson *et al.*, 1998a). Several genes involved in establishing, maintaining and exiting the starvation survival state have been identified. These include the major superoxide dismutase, SodA, which is also involved in resistance to oxidative and acid stress, a heme synthase, CtaA, and a component of the SOS response (*umuC*) (Clements *et al.*, 1999a; Watson *et al.*, 1998b).

S. aureus usually enter the body via a wound, caused accidentally or by surgery. Neutrophils and macrophages migrate to the site of infection and engulf bacteria into a phagolysosome. In the phagolysosome bacteria encounter acid pH conditions and oxidative stress from reactive oxygen species, such as superoxide anions, hydrogen peroxide and peroxynitrite (Hampton *et al.*, 1998). *S. aureus* produces at least two superoxide dismutases (SODs) for the destruction of superoxide anions (Clements *et al.*, 1999b). *S. aureus* also produces a single major catalase (KatA), which accumulates extracellularly and may be responsible for limiting the effects of hydrogen peroxide in the phagolysosome (Watson *et al.*, 1998b). The *S. aureus* thiol peroxidase (Bcp) recently characterised by Jeong *et al.* (2000) was shown to reduce linoleic acid hydroperoxide and hydrogen peroxide with the use of thioredoxin as an *in vivo* immediate electron donor. The bactericidal effects of peroxynitrite, generated within neutrophils and macrophages, may be limited by the activity of an alkyl oxide reductase (AhpC) produced by *S. aureus* (Armstrong-Buisseret *et al.*, 1995). The mechanism of acid tolerance in *S. aureus* is unknown, but the organism can develop resistance to the lethal effects of low pH if first exposed to a higher, non-lethal pH (Chan *et al.*, 1998). This suggests the existence of an acid-inducible tolerance system.

The stationary phase sigma factor (σ^B) is required for acid and oxidative stress resistance but not the starvation survival response (Chan *et al.*, 1998; Kullik *et al.*, 1998). Recently, PerR has been identified as a transcriptional regulator of oxidative stress resistance. PerR was found to upregulate expression of KatA, AhpC and Bcp when cells were exposed to high concentrations of hydrogen peroxide (Horsburgh *et al.*, 2001).

1.2 Virulence factors

1.2.1 Extracellular matrix-binding proteins of *S. aureus*

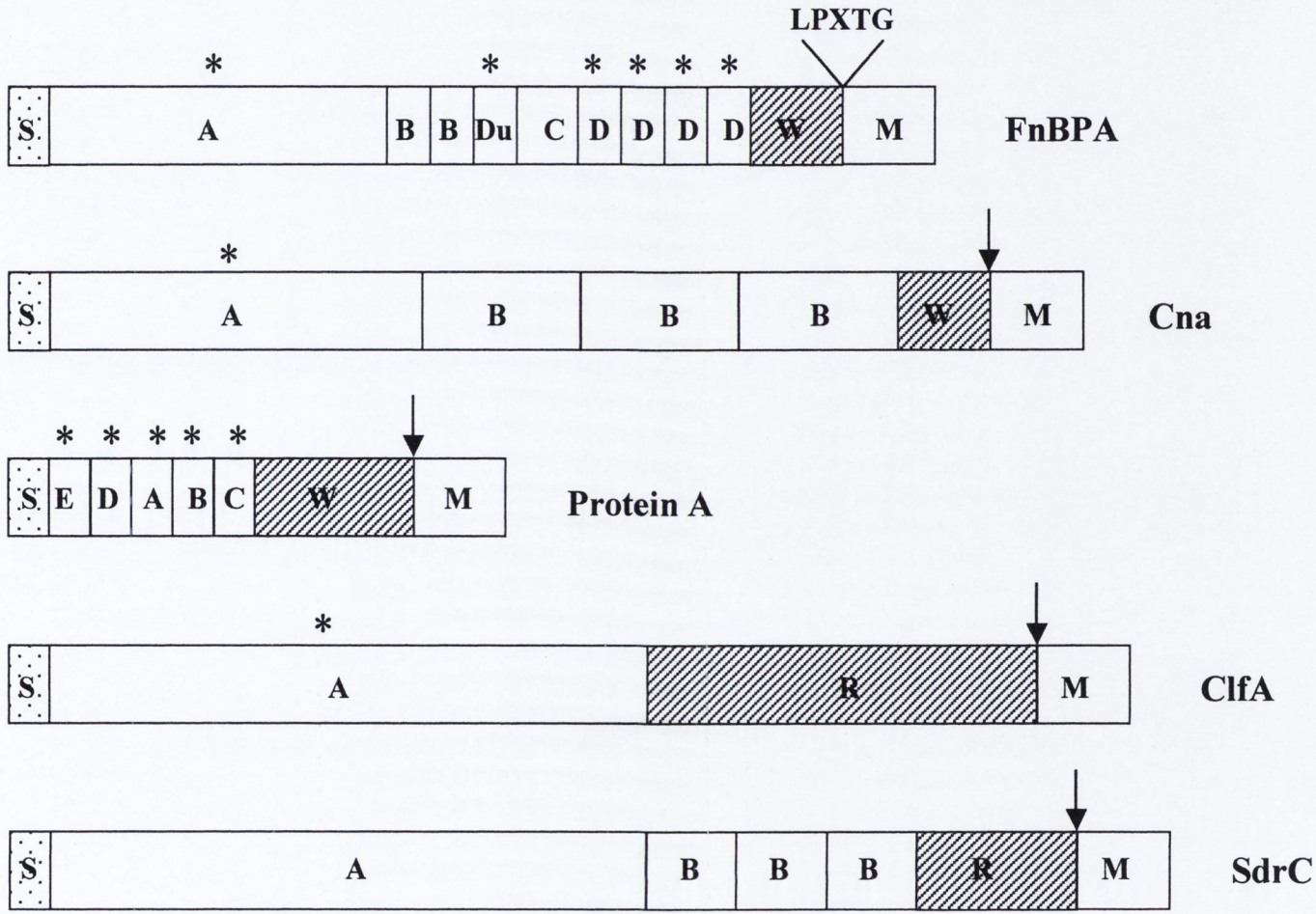
S. aureus is responsible for a large number of community acquired and nosocomial infections. The ability of this organism to cause disease depends largely on two types of virulence determinants, namely the production of extracellular toxins and the expression of surface associated proteins. The latter bind to components of the mammalian extracellular matrix (ECM) and are known collectively as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). The binding of MSCRAMMs to the ECM of vertebrate tissues is a fundamental process required for colonisation, invasion and ultimately for causing disease. A number of MSCRAMMs of *S. aureus* have been characterised both structurally and functionally. These include the collagen-binding protein, Cna, fibrinogen-binding proteins, ClfA and ClfB, the fibronectin-binding proteins, FnBPA and FnBPB, and the immunoglobulin- and von Willebrand factor-binding protein, protein A (Table 1.1).

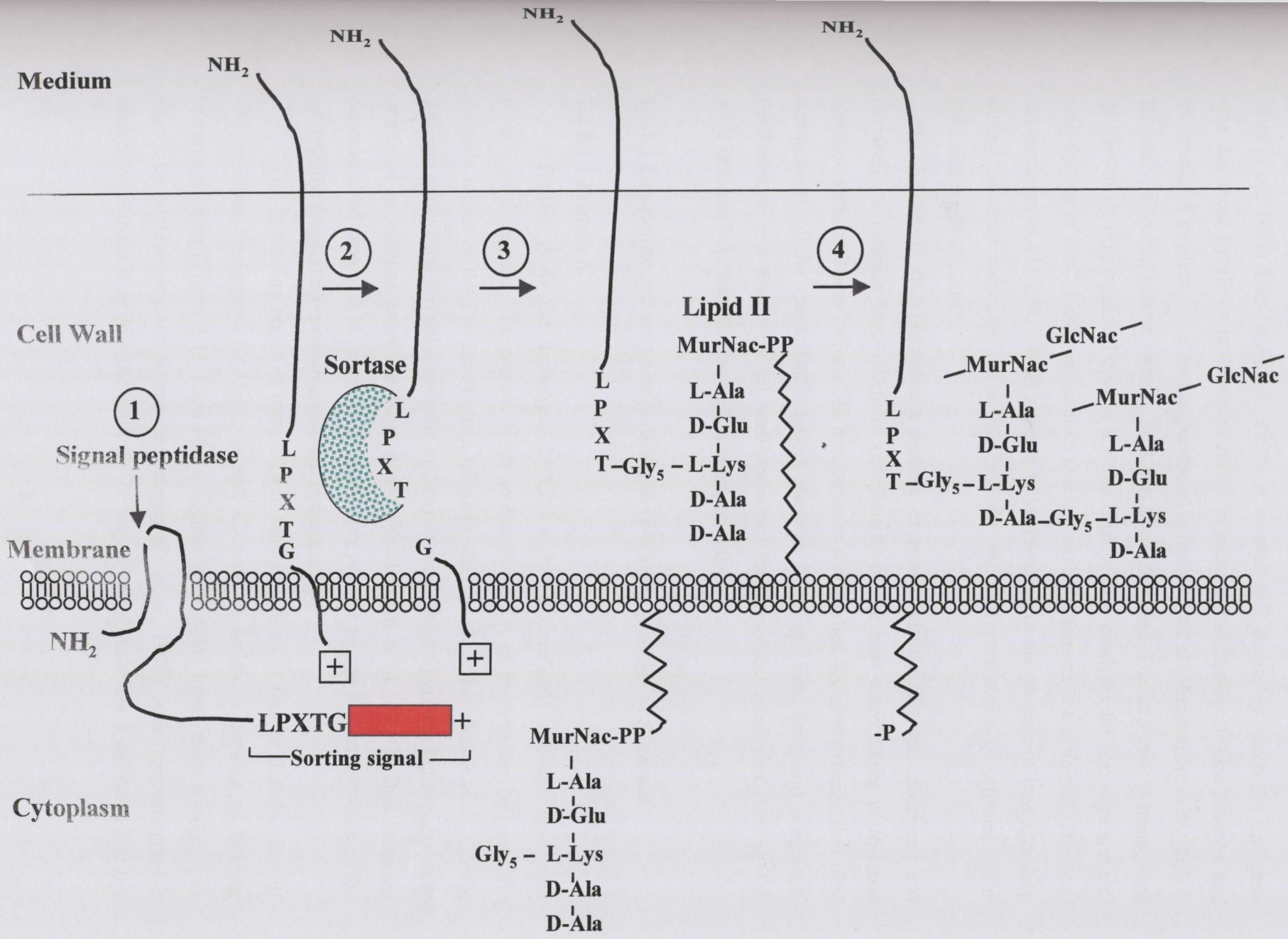
The staphylococcal MSCRAMMs are covalently attached to the uncross-linked pentaglycine bridge of lipid II that is incorporated into the growing peptidoglycan via the transpeptidation and transglycosylation reactions of cell wall synthesis (Perry *et al.*, 2002). This occurs at the C terminus where the proteins contain conserved features required for cell wall sorting. These include a positively charged tail at the extreme C terminus, a membrane-spanning domain, an LPXTG motif and a wall-spanning domain either rich in proline and glycine residues or composed of serine and aspartate dipeptide repeats (Schneewind *et al.*, 1993; Narvarre *et al.*, 1998) (Figure 1.1). The membrane bound staphylococcal enzyme SrtA cleaves the proteins between the threonine and glycine residue of the LPXTG motif, thus releasing the proteins from their membrane anchor (Ton-That *et al.*, 1999). SrtA then catalyses the covalent linkage of the carboxy end of the threonine residue to the amino end of glycine in the nascent pentaglycine cross-bridge by transpeptidation (Mazmanian *et al.*, 1999; Ton-That *et al.*, 1999) (Figure 1.2). Both the cleavage function and the transpeptidation function of SrtA require a conserved cysteine residue at position 184 in the catalytic domain of the protein (Ton-

Table 1.1 Characterised surface proteins of *S. aureus*.

Protein	**Ligand/Function**
MSCRAMMs	
Protein A	IgG, IgM, von Willebrand Factor
ClfA (clumping factor A)	Fibrinogen/Involved in platelet aggregation
ClfB (clumping factor B)	Fibrinogen, Epidermal Cytokeratins/ Involved in platelet aggregation
FnBPA (fibronectin-binding protein A)	Fibronectin, Fibrinogen
FnBPB (fibronectin-binding protein B)	Fibronectin, Fibrinogen
Cna (collagen-binding protein)	Collagen
Other Surface Proteins	
SdrC (serine-aspartate repeat protein C)	Unknown
SdrD (serine-aspartate repeat protein D)	Unknown
SdrE (serine-aspartate repeat protein E)	Unknown/Involved in platelet aggregation
Pls (plasmin-sensitive protein)	Unknown
Bap (biofilm-associated protein)	Unknown/Involved in biofilm formation
IsdC (iron responsive surface determinant protein C)	Unknown/Iron aquisition

Figure 1.1 Schematic diagram of the structural organisation of the fibronectin-binding protein (FnBPA), the collagen-binding protein (Cna), protein A, the fibrinogen-binding protein (ClfA) and SdrC of *S. aureus*. S represents secretion signal sequences, W represents the proline- and glycine-rich cell wall-spanning domain, R represents the serine-aspartate dipeptide repeats and M represents the membrane-spanning region followed by the positively charged tail. Ligand-binding domains are indicated by asterisks. Downward pointing arrows represent an LPXTG motif.


LPXTG
S
A
B
B
Du
C
D
D
D
D
W
M
FnBPA
S
A
B
B
B
W
M
Cna
S
E
D
A
B
C
W
M
Protein A
S
A
R
M
ClfA
S
A
B
B
B
R
M
SdrC


Medium
Cell Wall
Membrane
Cytoplasm
NH2
1
Signal peptidase
2
Sortase
3
4
L P X T G
LPXTG
Sorting signal
+
Lipid II
MurNac-PP
L-Ala
D-Glu
L-Lys
D-Ala
D-Ala
Gly5
GlcNac
MurNac
-P

That *et al.*, 1999; Ton-That *et al.*, 2000). A mutant of *S. aureus* in which the *srtA* gene was disrupted by allelic replacement failed to process and display surface proteins and had reduced virulence in a mouse peritoneal infection model (Mazmanian *et al.*, 2000). Recently, a second sortase protein, SrtB, has been identified. The *srtB* gene is transcribed as part of an iron-regulated operon and the SrtB protein catalyses the cleavage and cell wall sorting of IsdC, a cell wall protein that is cleaved by the enzyme at an NPQTN motif (Mazmanian *et al.*, 2002). SrtB appears to be involved in the process of iron aquistion during bacterial infection and is required for bacterial persistence in infected tissue (Mazmanian *et al.*, 2002).

S. aureus also possesses a number of non-covalently cell-wall-associated and extracellular proteins that adhere to components of the host extracellular matrix and immune system. These include Sbi that binds to mammalian IgG and proteins that bind components of the extracellular matrix such as Map/Eap, Efb, Emp and coagulase. Furthermore, non-covalently cell-wall-associated staphylococcal autolysins such as Atl, AtlE and Aas have adhesive as well as autolytic activity.

1.2.1.1 Covalently cell-wall-associated matrix-binding proteins of *S. aureus*

1.2.1.1.1 Protein A

Protein A is the archetypal LPXTG-anchored covalently cell-wall-associated protein of *S. aureus*. The protein binds to the Fc region of mammalian IgG and the variable region of the Fab heavy chain of IgM (Ulhén *et al.*, 1984; Graille *et al.*, 2000). The *spa* gene is transcribed during the mid-exponential phase of growth but expression is repressed as cultures enter stationary phase (Vandenesch *et al.*, 1991). This temporal expression of protein A is regulated in a complex manner by *agr* and components of the *sar* regulon (regulation is covered in detail in section 1.3).

The Ig-binding region is composed of tandem repeats of four or five domains of ~58 residues, designated E, D, A, B and C, each of which is capable of binding immunoglobulin (Figure 1.1). Each of the Ig-binding domains forms three α-helices, as shown by nuclear magnetic resonance (Starovasnik *et al.*, 1996). X-ray crystallographic studies of domain B in complex with the Fc region of IgG subclass 1 indicated that the

interaction between the two molecules involves 11 residues in the Fc region and 9 residues in the B domain (Gouda *et al.*, 1998). Similarly, X-ray studies of a crystal of a Fab fragment of IgM and the D domain of protein A revealed that the interaction involves helices II and III of the D domain and framework residues of the Fab fragment of IgM, without the involvement of hypervariable regions on the Fab molecule (Graille *et al.*, 2000).

Protein A binds IgG in such a way that that it blocks the Fc receptors on phagocytic cells binding to their target in the Fc region of bound IgG. This could inhibit opsonophagocytosis of *S. aureus* cells by phagocytic cells and promote immune evasion. This theory is supported by the fact that a *spa* mutant of *S. aureus* was taken up more efficiently by PMNs than the parental strain (Gemmell *et al.*, 1991).

Recently protein A has been implicated in the pathogenesis of infective endocarditis. Bacterial adherence to platelets and adhesive endovascular foci are important steps in the colonisation of damaged heart valves. Protein A binds to von Willebrand factor, a large multimeric glycoprotein that mediates platelet adhesion at sites of endothelial damage, and to the gC1qR/p33 receptor on the surface of activated platelets (Hartleib *et al.*, 2000; Nguyen *et al.*, 2000). Either of these interactions could promote bacterial colonisation of damaged endovascular tissue and establish a focus of infection.

Kawasaki disease is an acute vasculitis of young children that is characterised immunologically by increased amounts of circulating activated T cells, B cells, macrophages and monocytes, and physically by symptoms similar to those caused by exposure to a superantigen (Kawasaki, 1967). Toxic shock syndrome toxin of *S. aureus* has been implicated in Kawasaki syndrome (Leung *et al.*, 1993). Recent studies have shown that *S. aureus* isolated from the rectum or pharynx of patients with Kawasaki disease expressed significantly higher levels of protein A than isolates from patient with toxic shock syndrome (Wann *et al.*, 1999). The diverse immunological properties of protein A, including the ability to activate B cells (Vasquez *et al.*, 1994) and complement (Kozlowski *et al.*, 1996) may account for some of the immune activation associated with the disease.

In vivo studies using *S. aureus spa* mutants revealed that protein A is a virulence factor in mouse cutaneous abscess and peritonitis models (Patel *et al.*, 1987) but does not contribute to virulence in rabbit ocular keratitis (O'Callaghan *et al.*, 1994). An important factor in the development of infective endocarditis is the formation of platelet-bacteria thrombi on the surface of heart valves (Sullam *et al.*, 1996). Bacterial induced platelet aggregation may be important in the pathogenesis of the disease. Recently, it has been shown that although protein A could not promote platelet aggregation independently, it enhanced the aggregation response when expressed on the surface of *S. aureus* (O'Brien *et al.*, 2002a).

1.2.1.1.2 Fibronectin-binding MSCRAMMs

Fibronectin is a dimeric glycoprotein that occurs in soluble form in human plasma and in insoluble form in the extracellular matrix (Hynes, 1993). The fibronectin monomer is between 235 and 270 kDa in size and forms dimers by the formation of disulphide bonds near the C terminus before secretion. The protein is composed of different combinations of type I, type II and type III modules that are clustered into functional domains that bind ligands such as heparin, fibrin, collagen, glycosaminoglycans and thrombospondin (Ruoslahti, 1988) (Figure 1.3). The primary function of the protein is to mediate the adhesion of various cell types through a centrally located RGD integrin receptor (Yamada, 1989). It is also involved in processes such as cell migration, cell differentiation and wound healing (Hynes, 1993).

S. aureus is a major cause of infections associated with indwelling catheters and cardiovascular and orthopaedic devices (Maki, 1982; Kristinsson, 1989). Bacterial adherence to indwelling devices is a critical step for the initiation of infection (Vaudaux *et al.*, 1989). Indwelling devices are coated with host matrix proteins such as fibrinogen and fibronectin. Fibrnonectin coating the surface of biomaterial is proteolytically degraded but still retains the ability to promote bacterial attachment (Vaudaux *et al.*, 1995).

The primary binding site for *S. aureus* is located in the 27 kDa N-terminal peptide of fibronectin formed by plasmin degradation. This region binds to *S. aureus* with the same affinity as the native protein (Bozzini *et al.*, 1992; Sottile *et al.*, 1991). It is

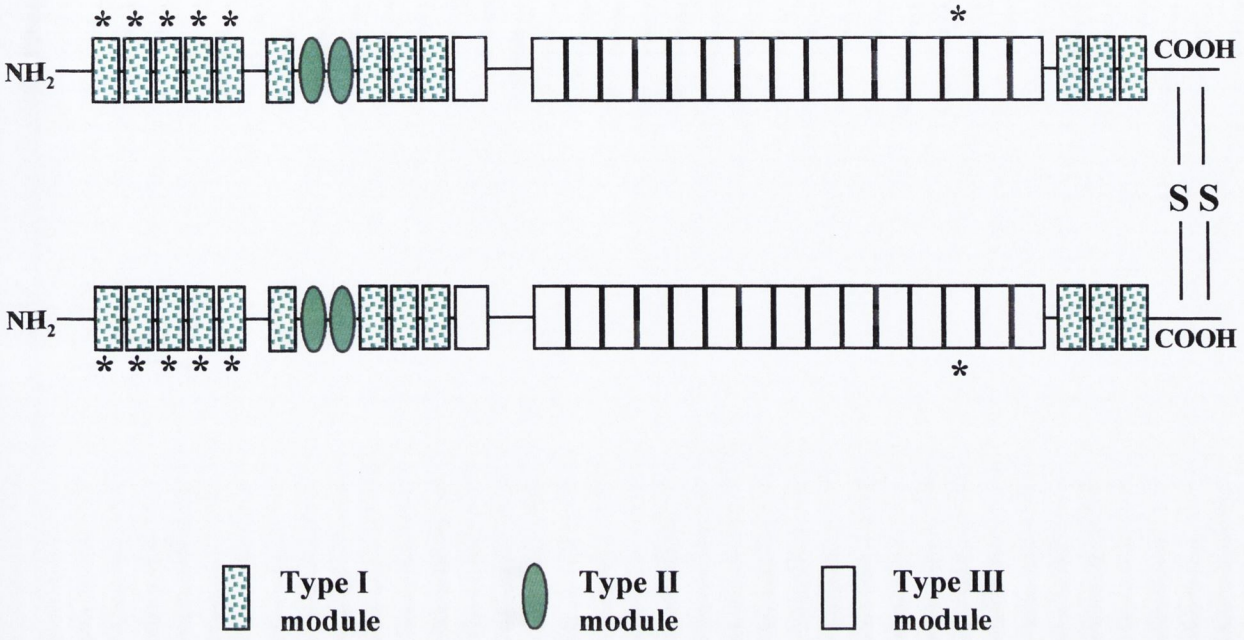


Figure 1.3 Structure of fibronectin. * indicates *S. aureus*-binding sites.

composed of five type I modules, each of which is essential for binding (Figure 1.3). A second lower affinity binding site has also been identified in the single C-terminal type III_{14} heparin-binding module (Bozzini *et al.*, 1992). This domain is only exposed on fibronectin when it has taken on a fibrillar conformation and may act synergistically with the N-terminal type I sites.

The fibronectin-binding proteins, FnBPA and FnBPB, are two cell-wall-associated proteins that belong to the MSCRAMM family of *S. aureus* proteins and promote bacterial adherence to immobilised fibronectin *in vitro* (Greene *et al.*, 1995), to plasma clots and to *ex vivo* coverslips removed from subcutaneous tissue cages in guinea pigs (Vaudaux *et al.*, 1993, Vaudaux *et al.*, 1995). In *S. aureus* strain 8325-4 they are encoded by two closely linked genes, *fnbA* and *fnbB*, which are tandemly arrayed but transcribed separately (Jönsson *et al.*, 1991; Greene *et al.*, 1995). Studies with 163 *S. aureus* clinical isolates revealed that 77% of strains had both the *fnbA* and *fnbB* genes and that 23% had only one of the genes, but there was no difference in adherence to fibronectin between isolates with one or two genes (Peacock *et al.*, 2000). Transcription of the *fnb* genes is restricted to the early exponential phase of growth (Greene *et al.*, 1995). Wolz *et al.* (2000) demonstrated that *fnbA* transcription is negatively regulated by *agr* and positively regulated by *sar* and that an *agr*-independent mechanism is involved in restricting *fnbA* transcription to early exponential phase (global regulation is described in section 1.3). The fibronectin-binding ability of the FnBP proteins is lost as cultures enter stationary phase. This is thought to be a result of the degradation of the proteins by *S. aureus* V8 serine protease (McGavin *et al.*, 1997) and masking of the proteins by capsular polysaccharide (Pöhlmann-Dietze *et al.*, 2000).

The FnBP proteins have a structural organisation similar to that of the fibronectin-binding proteins of several species of *Streptococcus* (Patti *et al.*, 1994a). The amino acid sequences of FnBPA and FnBPB are quite distinct at their N-terminal region A (45% identical), but the C terminus bearing the D repeats and the cell wall sorting domains are 95% identical. The D repeats at the C terminus of FnBPA and FnBPA are the primary ligand-binding domains of the proteins. They consist of 3-5 repeats of a 38-40 amino-acid motif (Figure 1.1). The consensus sequence EDT/S-(X 9, 10)-GG-(X 3, 4)-I/VDF, where X is any residue, is common to most D domains and to the fibronectin-binding domains

of FnBA and FnBB of *Streptococcus dysgalatiae* and binds to the type I modules of fibronectin (McGavin *et al.*, 1993a; House-Pompeo *et al.*, 1996). The D repeats of the fibronectin-binding proteins lack a folded secondary structure until they bind their ligand. Binding of the D repeats to fibronectin induces the formation of a discernible secondary structure and ligand-induced binding site (LIBS) epitopes (House-Pompeo *et al.*, 1996). This is demonstrated by the fact that monoclonal antibodies to the ligand-binding domain of a fibronectin-binding protein of *S. dysgalatiae* only recognise the bound form of the protein (Speziale *et al.*, 1996). Similarly, antibodies to the D repeats of FnBPA isolated from patients with staphylococcal infections recognised the ligand-binding domain when complexed with fibronectin but could not inhibit fibronectin binding (Casolini *et al.*, 1998). This may be a method of evading the host immune response because antibodies to the immunodominant D repeats only recognise the proteins after they bind their ligand and cannot inhibit bacterial adhesion.

Recent evidence has revealed that other regions of FnBPA are involved in ligand binding. Expression of truncated derivates of FnBPA on the surface of *S. aureus* indicated that the B repeats contain a fibronectin-binding site (Massey *et al.* 2001) and a third fibronectin-binding site has been identified in a 40 amino-acid region of the C region termed Du (Joh *et al.*, 1998). Region A of FnBPA shares ~25% sequence identity with the ligand binding regions of ClfA and ClfB from *S. aureus* and SdrG (Fbe) of *Staphylococcus epidermidis*, all of which bind fibrinogen. Recombinant region A of FnBPA has been shown to bind to C-terminal residues of the γ-chain peptide of fibrinogen, the same site that ClfA (Section 1.2.1.1.3) binds to. Indeed, recombinant FnBPA inhibits ClfA binding (Wann *et al.*, 2000).

Studies of the role played by the fibronectin-binding proteins in a rat endocarditis infection model produced conflicting results (Kuypers and Proctor, 1989; Flock *et al.*, 1996). However, recent studies have shown that FnBPA expressed on the surface of the heterologous host *Lactococcus lactis* is sufficient to produce endocarditis in rats with catheter induced aortic vegetations (Que *et al.*, 2001). In addition, immunization with the D2-domain of FnBPA expressed on the surface of cow-pea mosaic virus induced antibody-mediated protection against endocarditis in a rat infection model (Rennermalm *et al.*, 2001). Other functions of the fibronectin-binding proteins include the activation of

human T lymphocytes (Miyamoto *et al.*, 2001), the promotion of bacterial adherence to the skin of patients with atopic dermatitis (Cho, *et al.*, 2001) and bacterial adherence to human airway epithelium (Mongodin *et al.*, 2002).

Until recently *S. aureus* has been considered an exclusively extracellular pathogen. Recent studies have shown that the organism can invade a range of non-professional phagocytes *in vitro*, including epithelial cells (Dziewanowska *et al.*, 1999; Lammers *et al.*, 1999), endothelial cells (Peacock *et al.*, 1999; Massey *et al.*, 2001), fibroblasts (Fowler *et al.*, 2000), a human embryonic cell line (Sinha *et al.*, 1999) and osteoblasts (Ahmed *et al.*, 2001) in an FnBP-dependent manner. Bacterial internalization involves stimulation of a signal transduction event by the host tyrosine kinases, which results in actin rearrangement and phagocytosis of the bacteria (Sinha *et al.*, 1999; Dziewanowska *et al.*, 1999). The host cell fibronectin receptor, integrin $\alpha_5\beta_1$, has been shown to mediate invasion for mouse fibroblasts (Fowler *et al.*, 2000), human 293 epithelial cells (Sinha *et al.*, 1999) and HEp-2 epithelial cells (Dziewanowska *et al.*, 2000) via a fibronectin bridge. The heat shock protein Hsp60 has been reported as a host cell receptor on the surface of bovine mammary epithelial cells for the fibronectin-binding proteins (Dziewanowska *et al.*, 2000).

1.2.1.1.3 Fibrinogen-binding MSCRAMMs

Fibrinogen is a blood-borne glycoprotein of 340 kDa that is involved in plasma clot formation and platelet aggregation, and is one of the main proteins deposited on implanted biomaterials. The protein is composed of three pairs of non-identical polypeptide chains (Aα, Bβ and γ) that are assembled by their N termini through a number of disulphide bonds to form a symmetrical dimeric structure (Doolittle, 1984; Ruggeri, 1993). The fibrinogen molecule has three distinct domains, including two terminal D domains and a central E domain (Figure 1.4). Fibrinogen is cleaved by thrombin to form insoluble fibrin, a major component of blood clots. Following fibrin formation the clot is stabilised by the covalent linkage of the α- and γ-chains at their C termini by the enzyme factor XIII. Fibrinogen also mediates adherence and aggregation of platelets at sites of injury by interacting with the integrin $\alpha_{IIb}\beta_3$ on the surface of platelets via the C terminus of the γ-chain and the RGD motif on the α-chain (Hawiger *et*

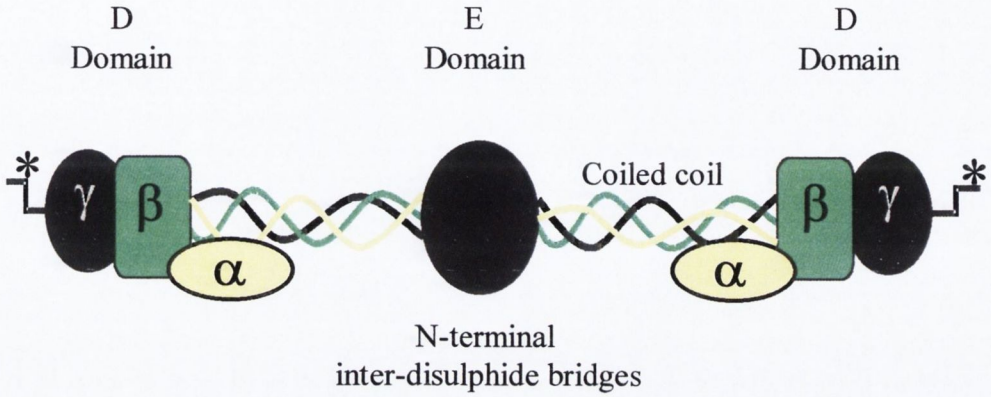


Figure 1.4 Structure of fibrinogen. * indicates binding site for *S. aureus* ClfA.

al., 1982). Fibrinogen and fibrin cleavage products are also involved in the regulation of cell adhesion and spreading, display vasoconstrictive and chemotactic activities, and are mitogens for a variety of cell types (Herrick *et al.*, 1999).

Clumping factor A (ClfA) and clumping factor B (ClfB) are the cell surface MSCRAMMs responsible for the clumping of *S. aureus* cells when suspended in soluble fibrinogen and for bacterial adherence to immobilised fibrinogen *in vitro* (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998). They also mediate bacterial adherence to fibrinogen-coated *ex vivo* biomaterial after short-term conditioning (Vaudaux *et al.*, 1993; Vaudaux *et al.*, 1995; Ní Eidhin *et al.*, 1998). Transcription of the *clfA* gene is regulated in an *agr*-independent and *sar*-independent manner and occurs maximally in the postexponential phase of growth (Wolz *et al.*, 1996; Dunman *et al.*, 2001). The ClfA protein is expressed on the surface of bacteria throughout the growth cycle (McDevitt *et al.*, 1994) and is found on the cell surface at the highest levels during stationary phase (J. Higgins, unpublished data). Transcription of the *clfB* gene stops before the end of exponential phase and is not regulated by the global regulators *agr* or *sar* (McAleese *et al.*, 2001). Transcription of the *clfB* gene increased in a mutant of the *sarA* homolog, *rot,* suggesting that Rot may be a repressor of ClfB expression (F. McAleese, unpublished data).

Both the ClfA and ClfB protein have typical cell wall attachment regions comprising an LPXTG motif, membrane anchor and positively charged C terminus. Both proteins contain repeat regions comprising alternating serine and aspartate residues (region R) (Figure 1.1). In ClfA this region varies between 193 to 440 residues in length between isolates (McDevitt and Foster, 1995a). This region of ClfA functions as a stalk to display the ligand-binding region of the protein on the cell surface (Hartford *et al.*, 1997). The fibrinogen-binding region of ClfA is contained in region A of the protein between residues 221 and 559 (McDevitt *et al.*, 1995b). Mutational analysis of ClfA expressed on the surface of *S. aureus* cells and of the purified recombinant form of the protein revealed that residues E526 and V527 are involved in ligand recognition (Hartford *et al.*, 2001a). The binding site for ClfA in fibrinogen has been localized to the 15 residues at the C-terminal end of the γ-chain, a site that is also recognised by the platelet integrin $\alpha_{IIb}\beta_3$ (McDevitt *et al.*, 1997). The binding of fibrinogen to the integrin $\alpha_{IIb}\beta_3$ on the surface of activated platelets results in platelet aggregation *in vitro* and the

formation of platelet-fibrin thrombi *in vivo*. Recombinant region A of ClfA is an effective inhibitor of fibrinogen-dependent platelet aggregation (McDevitt *et al.*, 1997). Since platelet aggregation stimulates the secretion of antimicrobial peptides, ClfA mediated inhibition of platelet aggregation may be a mechanism of evading host defences. Similar to the interaction between fibrinogen and the platelet integrin $\alpha_{IIb}\beta_3$ the binding of ClfA to fibrinogen is inhibited by divalent cations ions. Region A of ClfA contains a Ca^{2+}-binding EF-hand-like motif that is required both for Ca^{2+} regulation and ligand binding (O'Connell *et al.*, 1998).

Clumping factor B primarily binds to the α-chain of fibrinogen (Ní Eidhin *et al.*, 1998). Two forms of the ClfB protein occur on the bacterial cell surface, the smaller of which is generated by the proteolytic degradation at the N terminus of the protein by the *S. aureus* metalloprotease, aureolysin. The proportion of the smaller form of the protein, which cannot bind to fibrinogen, increases as cells enter stationary phase (McAleese *et al.*, 2001). Inhibition of *S. aureus* binding to immobilised fibrinogen using polyclonal antibodies revealed that the ligand-binding activity of the protein is located in the N-terminal region A of the protein between residues 44 and 542 (Ní Eidhin *et al.*, 1998). Further studies with purified recombinant ClfB truncates indicated that region A of the protein is composed of three independently folded subdomains, named N1, N2 and N3 (Perkins *et al.*, 2001). The minimal fibrinogen-binding activity of ClfB was located in a recombinant from of the protein comprising subdomains N2 and N3 of region A (Perkins *et al.*, 2001).

ClfA is a virulence factor in a rat endocarditis model, as demonstrated by the lower infection rate of a *clfA* mutant strain compared to the parental strain (Moreillon *et al.*, 1995). Recent studies have shown that ClfA expressed on the surface of the heterologous host *Lactococcus lactis* is sufficient to produce endocarditis in rats with catheter induced aortic vegetations (Que *et al.*, 2001). This may be mediated by the interaction between ClfA and platelets. Recombinant ClfA binds to a 118 kDa membrane receptor on the surface of platelets (Siboo *et al.*, 2001). Adherence to platelets may be an important mechanism for initiating endocarditis infections, since platelets attached to damaged heart valves may act as a foci for bacterial attachment. ClfA is also a virulence factor in a mouse septic arthritis model (Josefsson *et al.*, 2001). A *clfA* mutant strain

caused less severe arthritis than the parental strain. In addition, active immunization with recombinant ClfA and passive immunization of mice with rat and rabbit anti-ClfA antibodies protected against *S. aureus* arthritis. Recently, using *S. aureus* allelic replacement mutants and heterologous protein expression in *L. lactis*, it has been shown that both ClfA and ClfB promote platelet aggregation, a phenomenon that may be important in the pathogenesis of infective endocarditis (O'Brien *et al.*, 2002a).

The role of ClfB in a rat endocarditis model has been examined using a *clfB* mutant strain and a complemented strain overexpressing ClfB from a multicopy plasmid (Entenza *et al.*, 2000). These studies indicated that ClfB significantly increased infectivity when overexpressed from a multicopy plasmid but that a mutant of *S. aureus* with an insertion mutation in the *clfB* gene only marginally decreased the infection rate. Recently, studies have shown that ClfB on the surface of *S. aureus* and *L. lactis* adhere to epidermal cytokeratins and that the protein is probably a major determinant in *S. aureus* nasal colonisation (O'Brien *et al.*, 2002b).

1.2.1.1.4 Collagen-binding MSCRAMM

Collagen is a major structural protein of mammals that provides tensile strength to connective tissues such as bone, ligament, cartilage and tendon, and to the fibrous matrices of skin and blood vessels (Olsen and Ninomiya, 1994). There are four distinct types of collagen, some of which display tissue specific tropism. Type I collagen is found in skin, bone, ligaments and tendons. Type II collagen is preferentially found in cartilage while type III collagen in associated with blood and skin. Type IV collagen is found almost exclusively in basement membranes. All collagen types are composed of a triple helix of polypeptide chains known as α-chains. These chain wind around each other to form a right handed superhelical structure. Each chain contains multiple repeats of the sequence motif GXY, where X is often proline and Y is often hydroxyproline.

The *S. aureus* collagen-binding protein, Cna, promotes bacterial adherence to several types of collagen, including types I, II, III and IV and to collagenous tissues such as cartilage (Switalski *et al.*, 1989; Switalski *et al.*, 1993). Unlike other staphylococcal MSCRAMMs the *cna* gene is not found in the majority of *S. aureus* strains (Gillaspy *et al.*, 1997; Smeltzer *et al.*, 1997). Contradictory evidence for the role of Cna in *S. aureus*

bone and joint infections has been presented. In one study nearly all infection-causing strains possessed the *cna* gene (Switalski *et al.*, 1993), whereas in another study the prevalence of the *cna* gene in infection-causing strains was proportional to the prevalence of the gene in a control sample (Ryding *et al.*, 1997). Expression of the *cna* gene occurs maximally during the exponential phase of growth and diminishes to almost undetectable levels during stationary phase (Gillaspy *et al.*, 1998). The global regulatory protein SarA binds directly to DNA sequence upstream of the *cna* gene and negatively regulates its expression (Blevins *et al.*, 1999). This *sar*-mediated regulation of expression was shown to be independent of *agr* (Blevins *et al.*, 1999).

The N terminus of the Cna protein consists of a ~500-residue unique region called region A. This is followed by region B, which is a tandem array of a 187-residue domain that is repeated one to four times, depending on the strain (Gillaspy *et al.*, 1997) (Figure 1.1). Analysis of the structure of the recombinant forms of region A and the B repeats of Cna suggest that the N terminus of the protein forms independently folded subdomains that may have different functions (Rich *et al.*, 1998). At the C terminus Cna possesses the typical cell wall attachment regions comprising an LPXTG motif, membrane anchor and positively charged C terminus. The ligand-binding region of Cna was localized between residues 151 and 318 of region A and critical residues for binding were identified (N232 and Y233) using a series of overlapping synthetic peptides (Patti *et al.*, 1993; Patti *et al.*, 1995). The crystal structure of the collagen-binding domain revealed that the region folds like a "jelly roll" in two β-sheets connected by a short α-helix (Symersky *et al.*, 1997). A trench, capable of accommodating the collagen triple helix, traverses one of the β-sheets. The role of this trench in collagen binding was demonstrated by site-directed mutagenesis, in which changes to residues forming the walls of the trench resulted in decreased collagen-binding activity (Symersky *et al.*, 1997). This mechanism of Cna mediated collagen binding is similar to that of the mammalian collagen receptor $\alpha_1\beta_1$ integrin (Rich *et al.*, 1999). The function of the B repeats is still unclear. They do not bind to collagen and do not affect the collagen-binding activity of region A (Rich *et al.*, 1998). Studies with capsule producing strains of *S. aureus* have shown that the presence of a capsule can mask the collagen-binding activity of Cna (Gillaspy *et al.*, 1998). It was proposed that the B repeats could act as a stalk and project the ligand-binding region of

Cna through the capsule to perform its ligand-binding function. However, the expression of Cna on the surface of *S. aureus* with multiple B repeats did not restore collagen binding to a capsule producing strain (Snodgrass *et al.*, 1999).

The role of Cna in *S. aureus* septic arthritis and osteomylitis has been studied. Patti *et al.* (1994b) reported that 70% of mice injected with a clinical *S. aureus* developed clinical signs of arthritis whereas this occurred with only 27% of mice injected with a *cna* mutant of the same strain. Recent studies have contradicted this finding with no significant difference in the ability of a *cna* mutant to cause septic arthritis compared to the parental strain (Elasri *et al.*, 2002). However, it was shown that Cna makes an important contribution to the ability of *S. aureus* to cause osteomylitis through hematogenous spread (Elasri *et al.*, 2002). In the same study it was also found that the *cna* mutant used in the study by Patti *et al.* had a reduced capacity to bind fibronectin (69.8%) when compared to the parental strain. This could account for the results observed in this study. Recent studies have shown that Cna is a virulence factor in soft contact lens-associated bacterial keratitis (Rhem *et al.*, 2000).

1.2.1.1.5 The Sdr proteins

Southern blot analysis of the chromosomal DNA of *S. aureus* strain Newman revealed that the organism possesses three more genes that encode the serine-aspartate repeat region (region R) found in ClfA and ClfB. The corresponding proteins were named SdrC, SdrD and SdrE (Josefsson *et al.*, 1998a). The *sdr* genes are tandemly arrayed and the *sdr* locus was detected in all 31 strains of *S. aureus* tested, although strain 8325-4 does not possess *sdrE* while strain Phillips does not contain the *sdrC* gene (Josefsson *et al.*, 1998a).

The Sdr proteins have a structural organisation similar to that of ClfA and ClfB. They have a secretory signal sequence at the N terminus and a large region A of approximately 500 residues. The B region, containing two to five 110-113 residue B-motifs, separates region A from region R. Each of the proteins has sequences at the C terminus necessary for sorting to the cell wall and each protein has been detected in the lysostaphin-solubilised cell wall fraction of *S. aureus* (O'Brien, 2001) (Figure 1.1). The A regions of the Clf proteins and the Sdr proteins exhibit 20-30% identity at the amino

acid level and the only conserved sequence is a TYTFTDYVD motif (Josefsson *et al.*, 1998a). Each B repeat contains a Ca^{2+}-binding EF-hand. Removal of Ca^{2+} from this site in the B repeats of recombinant SdrC dramatically changed the conformation of the protein from a globular form to an elongated structure (Josefsson *et al.*, 1998b). Recently, using *S. aureus* allelic replacement mutants and heterologous protein expression in *L. lactis*, it has been shown that SdrE promotes platelet aggregation, a phenomenom that may be important in the pathogenesis of infective endocarditis (O'Brien *et al.*, 2002a).

Tung *et al.*, (2000) described a protein of *S. aureus* that binds to bone sialoprotein that displays 76% identity with region A of SdrE. Three proteins from *Staphylococcus epidermidis*, designated SdrF, SdrG and SdrH, that share homology to the Sdr proteins of *S. aureus* have been described (McCrea *et al.*, 2000). SdrF and SdrG have a structural organisation similar to that of the other Sdr proteins. SdrH has a shorter region A, a unique 277-residue region C and no LPXTG motif. SdrG, also called Fbe, promotes bacterial adherence to immobilised fibrinogen and fibrinogen-coated catheters (Pei and Flock, 2001; Hartford *et al.*, 2001b). Studies using recombinant SdrG and recombinant truncates of fibrinogen have shown that region A of SdrG binds to the Bβ-chain of fibrinogen and inhibits thrombin-induced fibrinogen clotting (Davis *et al.*, 2001). The functions of the other Sdr proteins of *S. aureus* and *S. epidermidis* have yet to be determined.

1.2.1.1.6 Other LPXTG proteins

The plasmin-sensitive protein (Pls) is a large cell-wall-associated protein of *S. aureus*. The *pls* gene is only found in methicillin-resistant strains of *S. aureus* with the type I SCC *mec* element and is closely associated with the *mecA* gene (Savolainen *et al.*, 2001). The protein contains three repeat regions, one of which is a serine-aspartate repeat region characteristic of the Clf-Sdr family of proteins. Pls expressed on the cell surface blocked fibronectin and IgG binding *in vitro* and may be involved in the spread of bacteria to other sites of infection (Savolainen *et al.*, 2001).

Recently, a novel cell-wall-associated protein involved in biofilm formation in bovine mastitis *S. aureus* strains has been indentified and characterised. The C terminus of Bap (biofilm-associated protein) contains an LPXTG motif, cell-wall-spanning domain

and membrane anchor. The core region of the protein consists of 13 sucessive nearly identical repeats each containing 86 amino acids (Cucarella *et al.*, 2001). The *bap* gene was present in 5% of bovine mastitis strains tested and was absent from 75 human clinical isolates (Cucarella *et al.*, 2001). Expression of Bap in *S. aureus* strain Newman resulted in biofilm formation and interfered with the ligand-binding functions of ClfA and FnBPA (J. Penadés, personal communication).

In silico analysis of the complete genome sequences of the methicillin-resistant *S. aureus* strains N315 (www.bio.nite.go.jp/cgi-bin/dogan/genome_top.cgi?'n315') and Mu50 (www.w3.grt.kyushu-u.ac.jp/VRSA/Sequencing) (Kuroda *et al.*, 2001), and of the incomplete genome sequences of *S. aureus* strains COL (www.tigr.org/tdb/mdb/mdbinprogress.html), NCTC 8325 (www.genome.ou.edu./staph.html), EMRSA-16 and MSSA (www.sanger.ac.uk/Projects/S_aureus) has identified the genes of 9 previously uncharacterised proteins that have LPXTG motifs, cell-wall-spanning domains and membrane anchors (F. Roche, personal communication). PCR analysis of 344 *S. aureus* clinical isolates has revealed that 2 of the genes are significantly associated with *S. aureus* strains that caused invasive disease. Western immunoblotting using purified IgG from patients recovering from *S. aureus* infections revealed that 5 of the proteins reacted significantly stronger with patient's sera than sera from healthy individuals (F. Roche, personal communication). This suggests that that these proteins are expressed *in vivo* and are immunogenic during human infection. The role of these proteins during infection has yet to be elucidated.

1.2.1.2 Non-covalently cell-surface-associated matrix-binding proteins of *S. aureus*

1.2.1.2.1 Sbi: a second IgG-binding protein

A second IgG-binding protein has been identified on the surface of *S. aureus* cells. Sbi is a 436-residue protein that binds to IgG from various species via a single IgG binding domain of approximately 52 residues that shares significant homology with the IgG-binding domain of protein A (Zhang *et al.*, 1998). The protein contains a typical secretion signal sequence at the N terminus but lacks a cell-wall-sorting LPXTG motif

(Zhang *et al.*, 1998). Sbi was released from the cell surface by boiling cells in SDS, suggesting that it is associated with the cell surface in a non-covalent manner. Further studies revealed that synthesis of Sbi is upregulated by the binding of IgG to Sbi on the cell surface and that the protein can also bind the serum component apolipoprotein H via a 57-residue motif distinct from the IgG-binding domain (Zhang *et al.*, 1999; Zhang *et al.*, 2000). However, the function of Sbi in staphylococcal pathogenesis has yet to be determined.

1.2.1.2.2 Coagulase

Coagulase is a predominantly extracellular protein that activates the conversion of fibrinogen to fibrin. The protein binds to human prothrombin in a 1:1 molar ratio to form a complex called staphylothrombin, which converts fibrinogen to fibrin in a process that does not involve proteolytic cleavage of prothrombin (Kawabata *et al.*, 1985). A fraction of the protein remains associated with the cell surface where it can activate prothrombin but cannot promote bacterial clumping in the presence of soluble fibrinogen (Bóden and Flock, 1989; McDevitt *et al.*, 1992). However, cell-surface-associated coagulase can promote binding to soluble fibrinogen, especially in an *agr* mutant where the protein is overexpressed (Wolz *et al.*, 1996). The prothrombin-binding region of coagulase is in the variable N terminus of the protein, while the fibrinogen-binding region of the protein is located at the C terminus, which contains 5, 6 or 8 tandem repeats of 27 amino acid residues (McDevitt *et al.*, 1992).

Coagulase production is the main characteristic used to distinguish *S. aureus* from the less virulent coagulase-negative staphylococci in clinical laboratories. However, the role of coagulase in bacterial pathogenesis is unclear. Coagulase-negative mutants of *S. aureus* were less virulent in a mouse model of blood-borne staphylococcal pneumonia (Sawai *et al.*, 1997), but no difference in virulence was observed between wild-type and coagulase mutants in mouse models of experimental endocarditis, subcutaneous infections or mammary infections.

1.2.1.2.3 MHCII analogous protein (Map)/Extracellular adherence protein (Eap)

Map is a 72 kDa protein of *S. aureus* that does not possess a membrane anchor or an LPXTG motif required for covalent attachment to the cell wall (Jönsson *et al.*, 1995). The protein was released from the surface of *S. aureus* cells treated with 1M LiCl and bound a variety of host matrix proteins, including fibrinogen, fibronectin, bone sialoprotein, vitronectin and thrombospondin (Homonylo-McGavin *et al.*, 1993). Map has six repeated domains of 110 residues that share significant sequence homology with the peptide-binding groove of the β–chain of the major histocompatibility complex class II (MHCII) involved in antigen presentation to B cells, hence the name Map (MHCII analogous protein) (Jönsson *et al.*, 1995).

Palma *et al.* (1999) identified a similar protein of 60 kDa called Eap that binds to a wide variety of mammalian matrix proteins and acts as both an adhesin and a ligand. Studies of the localisation of Eap revealed that 70% of the protein was located in the culture supernatant while 30% remained associated with the cell wall. Purified Eap enhanced *S. aureus* adherence to fibroblasts and epithelial cells (Palma *et al.*, 1999). This is due to the ability of Eap to rebind *S. aureus* cells via a surface located neutral phosphatase and to act as a bridging molecule between *S. aureus* and eukaryotic cells (Flock and Flock, 2001). The roles of Map and Eap in bacterial pathogenesis have yet to be elucidated but the presence of *map/eap* homologues in 97.9% of a panel of 240 *S. aureus* clinical isolates suggests that these proteins play an important role (Hussain *et al.*, 2001a).

1.2.1.2.4 Extracellular fibrinogen-binding protein (Efb)

The extracellular fibrinogen-binding protein (Efb, previously Fib) is a 15.8 kDa extracellular protein produced by *S. aureus* mainly during the post-exponential phase of growth (Palma *et al.*, 1998). The *efb* gene is highly conserved and is unique to *S. aureus* (Bóden-Wäsfelt, 1995). The protein binds to fibrinogen but does not promote bacterial adherence to immobilised fibrinogen *in vitro* (Palma *et al.*, 1996). The binding of Efb to fibrinogen is divalent, with one binding site at the N terminus of Efb and the other at the C terminus of the protein and is dependent on Ca^{2+} concentration (Palma *et al.*, 1998; Wade *et al.*, 1998). Efb also inhibits platelet aggregation by binding to the α-chain of

fibrinogen (Palma *et al.*, 2001). The protein has been shown to be a potential virulence factor in a rat wound infection model (Palma *et al.*, 1996). In addition, vaccination with recombinant Efb reduced the number of bacteria colonising the mammary gland in a mouse mastitis model (Mamo *et al.*, 1994). Further evidence for Efb as a virulence factor was demonstrated by the fact that 49% of serum from patients suffering from *S. aureus* infections had elevated levels of anti-Efb antibodies (Colque-Navarro *et al.*, 2000).

1.2.1.2.5 Extracellular matrix-binding protein (Emp)

The extracellular matrix-binding protein (Emp) is a 38.5 kDa protein that is closely associated with the cell surface but is extractable by SDS. The protein is expressed during the stationary phase of growth and promotes binding to fibrinogen, fibronectin, vitronectin and collagen as judged by Western ligand blotting and bacterial adherence to fibrinogen and fibronectin coated coverslips (Hussain *et al.*, 2001b). The *emp* gene was present in all 240 *S. aureus* clinical isolates tested but was absent from the chromosomal DNA of 10 *S. epidermidis* strains as determined by PCR analysis (Hussain *et al.*, 2001b). The role of Emp in *S. aureus* pathogenesis has yet to be determined.

1.2.1.2.6 Autolysins

Bacterial autolysins are cell-wall-associated proteins that are thought to function during cell separation by cleavage of the cell wall peptidoglycan. The autolysins of different staphylococcal species, such as AtlE of *S. epidermidis*, Aas of *S. saprophyticus* and Atl of *S. aureus* are structurally and organisationally related (Hell *et al.*, 1998). Each protein displays both *N*-acetyl-muramyl-L-alanine amidase and endo-β-*N*-acetyl-D-glucosaminidase activity (Oshida *et al.*; 1995; Hell *et al.*, 1998). None of the proteins possess the motifs required for covalent cell wall attachment such an LPXTG motif, wall-spanning domain or a membrane anchor, but recent studies have shown that Atl of *S. aureus* is anchored to the cell surface via ionic interactions with anionic cell wall polymers such as teichoic acid and lipoteichoic acid (Takano *et al.*, 2000). In addition to their autolytic activites, staphylococcal autolysins display adhesive properties. Aas of *S. saprophyticus* binds to fibronectin and sheep erythrocytes (Hell *et al.*, 1998) and AtlE of *S. epidermidis* adheres to vitronectin and promotes attachment to polystyrene (Heilmann

et al., 1997). Recently it has been shown that AtlE promotes bacterial adherence to central venous catheters and is a virulence factor in a rat central venous catheter infection model (Rupp *et al.*, 2001). Although no adhesive properties have been reported for Atl of *S. aureus*, autolysins may represent a new class of matrix- and indwelling device-binding proteins.

1.2.2 Exotoxins

A list of the toxins secreted by *S. aureus* and the diseases with which they are associated is shown in Table 1.2. The *S. aureus* exotoxins can be classified in to two groups: (i) membrane active toxins and (ii) toxins with superantigenic activity. The membrane active toxins contribute to the pathogenesis of *S. aureus*, but none of them are exclusively responsible for a particular clinical syndrome. The cytolytic pore-forming toxin, α-toxin, is the best-characterised membrane active toxin of *S. aureus*. *In vitro* and *in vivo* the toxin is hemolytic, cytotoxic, dermonecrotic and lethal. Studies with *hla* deficient mutants in animal infection models have implicated α-toxin in the pathogenesis of cutaneous and brain abscess, ocular keratitis and septic arthritis. Recently, it has been shown that *S. aureus* strains harboring the gene for Panton-Valentine leukocidin were associated with lethal cases of staphylococcal pneumonia in young patients, characterised by necrotic ulceration of tracheal and bronchial mucosa (Gillet *et al.*, 2002).

S. aureus superantigenic toxins include the pyrogenic toxins and the exfoliative toxins. The pyrogenic toxins are mostly associated with diseases such as toxic shock syndrome and food poisoning. The exfoliative toxins are known to induce scalded skin syndrome, particularly in infants.

1.2.3 Extracellular enzymes

A list of the characterised extracellular enzymes of *S. aureus* is shown in Table 1.3. *S. aureus* secretes a number of enzymes that interact with and degrade host proteins such as fibrinogen, fibrin and elastin, and a number of lipases. The significance of these enzymes in the pathogenesis of *S. aureus* is unclear but they are generally thought to be involved in processes such as nutrient uptake, tissue damage and avoidance of host immune defenses. Bioinformatic analysis of the *S. aureus* genome has revealed that *S.*

Table 1.2 Toxins secreted by *S. aureus*.

Toxin/gene name	Mechanism of virulence	Location of gene	Disease association
Membrane active toxins			
α-toxin (*hla*)	Cytolytic pore-forming toxin. Lyses erythrocytes,mononuclear immune cells,epithelial cells, endothelial cells and platelets.	Chromosomal	Cutaneous abscess (Patel *et al.*, 1987). Ocular keratitis (O'Callaghan *et al.*, 1997). Arthritis (Nilsson *et al.*, 1999a). Brain abscess (Kielian *et al.*, 2001).
β-toxin (*hlb*)	Mg^{2+}-dependent hot-cold sphingomyelinase. Kills erythrocytes and leukocytes. Stimulates cytokine release.	Chromosomal	Murine mastitis (Foster *et al.*, 1990). Ocular keratitis (O'Callaghan *et al.*, 1997).
δ-toxin (*hld*)	Cytolytic pore-forming toxin. Affects a variety of membranes including erythrocytes, leukocytes, tissue culture cells and bacterial protoplasts.	Chromosomal. Located at the 5′ end of *agr* RNAIII transcript.	
Bicomponent toxins			
γ-toxin (*hlg*)	Haemolytic and leukotoxic pore-forming toxin.	Chromosomal. The *hlg* locus encodes *hlgA*, *hlgB* and *hlgC*.	Endophthalmitis (Supersac *et al.*, 1997). Arthritis (Nilsson *et al.*, 1999a).
Panton-Valentine leukocidin (*luk-PV*)	Non-haemolytic, leukotoxic and tissue necrotic. Lysis of human PMNLs.	Phage encoded. The *luk-PV* locus encodes *lukS-PV* and *lukF-PV*.	Cutaneous infections (Lina *et al.*, 1999). Pneumonia (Gillet *et al.*, 2002).
Superantigenic toxins			
Pyrogenic toxins			
TSST-1 (*tsst*-1)	Hyper-stimulation of T lymphocyte proliferation and cytokine release.	Chromosomal, on pathogenicity islands SAP-1and SAP-2.	Toxic shock syndrome (Reingold *et al.*, 1982). Kawasaki disease (Leung *et al.*, 1993).
Enterotoxins (SEA-O) (*sea-seo*)	Stimulation of the emetic center of the brain. Hyper-stimulation of T lymphocyte proliferation and cytokine release.	Chromosome, plasmid or bacteriophage-depending on gene.	Food poisoning (Dinges *et al.*, 2000). Non-menstral toxic shock syndrome (Reingold *et al.*, 1982).
Exfoliative toxins			
ETA, ETB (*eta, etb*)	Esterase activity causing intraepidermal skin peeling. Targets desmoglein, a desmosomal cadherin (Amagai *et al.*, 2000)	*eta* is chromosomal and *etb* is on a plasmid.	Scalded skin syndrome (Melish and Glasgow, 1970).

Table 1.3 Enzymes secreted by *S. aureus*.

Enzyme/gene name	Activity/Substrate	Disease/Putative role in virulence
Coagulase (*coa*)	Activates prothrombin, to convert fibrinogen into fibrin (Kawabata *et al.*, 1985).	Blood-borne pneumonia (Sawai *et al.*, 1997). Induction of fibrin clot may protect against host defenses.
Staphylokinase (*sak*)	Activates plasminogen to degrade fibrin clots (Collen, 1998).	May promote the release of bacteria from fibrin clots or abscesses surrounded by fibrin.
Nuclease (*nuc*)	Hydrolyzes single- or double-stranded DNA and RNA at 5′ end of phosphodiester bonds (Weber *et al.*, 1992).	
Hyaluronate lyase (*hysA*)	Depolymerization of hyaluronic acid (Farrell *et al.*, 1995).	May be involved in tissue damage.
Proteases		
V8 serine protease (*sspA*)	Cleaves glutamoyl peptide bonds. Cleaves heavy chain of human Ig. Inactivates neutrophil elastase inhibitor (Potempa *et al.*, 1986). Degrades FnBPs on the surface of *S. aureus* (McGavin *et al.*, 1997). Cleaves SspB to mature form and controls bacterial autolytic activity (Rice *et al.*, 2001).	Required for *in vivo* growth and survival (Coulter *et al.*, 1998).
SspB (*sspB*)	Cysteine protease (Rice *et al.*, 2001).	
Spl A-F (*splA-F*)	Six trypsin-like serine proteases encoded by an operon and share homology to V8 protease (Reed *et al.*, 2001).	
Thiol protease (3 Types)	Broad substrate specificity, similar to papain. Type I degrades elastin (Potempa *et al.*, 1988).	May promote tissue damage.
Metalloprotease (aureolysin) (*aur*)	Zinc-requiring endopeptidase that cleaves before bulky hydrophobic amino acids. Activates V8 protease and prothrombin (Drapeau, 1978). Cleaves plasma proteinase inhibitors. Degrades ClfB on the surface of *S. aureus* (McAleese *et al.*, 2001).	May promote the spread of bacteria to other sites of infection.
Lipases		
Glycerol ester hydrolase (*geh*)	Cleaves long-chain glycerol esters. Impairs granulocyte function (Rollof and Normark, 1992). Generate free fatty acids that impair immune system.	Impairs functions of the immune system and may be important for bacterial nutrition.
Lipase (*lip*)	Cleaves short-chain glycerol esters. Impairs granulocyte function (Rollof and Normark, 1992). Generate free fatty acids that impair immune system.	Impairs functions of the immune system and may be important for bacterial nutrition.

Table 1.3 Enzymes secreted by *S. aureus*, continued.

PI-Phospholipase C (*plc*)	Degrades membrane associated inositol phospholipids and releases glycan-PI-anchored proteins on the surface of eukaryotic cells (Marques *et al.*, 1989).	May compromise host cell functions.
Fatty acid-modifying enzyme (FAME)	Catalyses the esterfication of bacteriocidal lipids to alcohols (Kapral *et al.*, 1992).	Protects against the bacteriocidal effects of free fatty acids.

aureus may possess as many as forty membrane-bound, cell-wall-associated and extracellular proteases (J. Potempa, personal communication).

1.2.4 Capsules

More than 90% of *S. aureus* clinical isolates produce capsular polysaccharide (CP). These have been divided into 11 serogroups, which can also be divided into two distinct groups based on colony morphology. Mucoid-type capsule strains (serogroups 1 and 2) are heavily encapsulated and colonies have a mucoid appearance on solid medium. Microcapsule strains (serogroups 3-11) possess a thin layer of capsular polysaccharide and are non-mucoid in appearance on solid medium. Serotypes 5 and 8 are most prevalent among clinical isolates of *S. aureus* (Hochkeppel *et al.*, 1987). CP5 and CP8 share the same sugar composition of a trisaccharide repeat of a single *N*-acetylmannosaminuronic acid followed by two *N*-acetylfucosamine sugars (Murthy *et al.*, 1983). They only differ in the linkages between the amino sugars and the position of the O acetylation. The *cap5* and *cap8* gene clusters are composed of 16 genes, 12 of which are almost identical whereas the other 4 are type specific (Sau *et al.*, 1997).

Expression of the mucoid type I capsule is constitutive and is only modestly affected by environmental factors (Lee *et al.*, 1993; Ouyang and Lee, 1997). Expression of the microcapsule serotypes 5 and 8 is regulated by environmental factors. Lee *et al.* (1993) demonstrated enhanced expression of type 8 capsule when *S. aureus* was grown on agar, in iron depleted medium or *in vivo*. Expression of the type 5 capsule was enhanced under conditions of high oxygen tension but was reduced under alkaline growth conditions (Dassy *et al.*, 1991). Type 5 and type 8 capsules have been shown to be positively regulated in the post-exponential phase of growth by *agr*, a global regulator of virulence factor expression (Dassy *et al.*, 1993). Later studies revealed that *agr* positively regulated the expression of *cap8* at the level of transcription and that SarA affected the expression of capsular polysaccharide both at the level of transcription and posttranslationally (Luong *et al.*, 2002).

The importance of capsules to the pathogenesis of *S. aureus* is demonstrated by the prevalence of capsule expression in clinical isolates. The role of mucoid capsules in experimental infections is well established. It has been shown that the type 1 has an

antiphagocytic affect by masking the complement component C3b deposited on the cell wall surface (Peterson *et al.*, 1978). $CP5^+$ strains of *S. aureus* have been shown to be more virulent than $CP5^-$ mutants in mouse models of septic arthritis, renal abscess formation and subcutaneous abscess formation (Nilsson *et al.*, 1997; Portoles *et al.*, 2001). Specific antibodies against CP5 and CP8 have been shown to be protective against *S. aureus* infections in animal infection models (Fattom *et al.*, 1996; Lee *et al.*, 1997).

The *in vivo* expressed capsular polysaccharide/adhesin of *S. epidermidis* PNSG (poly-N-succinyl β-1-6 glucosamine) is the predominant chemical component of the extracelluar biofilm layer of coagulase-negative staphylococci and promotes bacterial adherence to catheters (Muller *et al.*, 1993). Recently it has been shown to be a virulence factor in a rat central venous catheter infection model (Rupp *et al.*, 2001). In addition, PNSG has been identified on the surface of *S. aureus* strains isolated from the lungs of cystic fibrosis patients (McKenny *et al.*, 1998; McKenny *et al.*, 1999). The polysaccharide is distinct from the serologically and structurally defined capsular polysaccharide serotypes. Growth of $PNSG^+$ *S. aureus* lung and sputum isolates in trypticase soy broth under aerobic conditions resulted in decreased expression of PNSG, indicating that the polysaccharide is an *in vivo*-expressed factor in human infection (McKenny *et al.*, 1999). In *S. epidermidis* the protein products of the *ica* operon (*icaADBC*) synthesize PNSG. The *ica* locus was detected by PCR in all eight *S. aureus* isolates tested and is present in the genomes of strains COL and 8325-4, that are currently being sequenced and in the genome of the recently sequenced methicillin-resistant *S. aureus* strains Mu50 and N315 (McKenny *et al.*, 1999; Kuroda *et al.*, 2001). In addition, vaccinating mice with PNSG provided protection against *S. aureus*-induced metastatic kidney infection and mortality (McKenny *et al.*, 1999).

1.3 Regulation of virulence factor expression

The regulation of virulence factor expression is a complex and incompletely understood process. The expression of a single virulence factor may be regulated directly or indirectly by as many as four regulatory factors. The interactions between individual regulatory mechanisms and regulatory proteins, and the subtle differences between the regulatory circuits of different *S. aureus* strains are just beginning to be uncovered (Blevins *et al.*, 2002).

1.3.1 The accessory gene regulator system (*agr*)

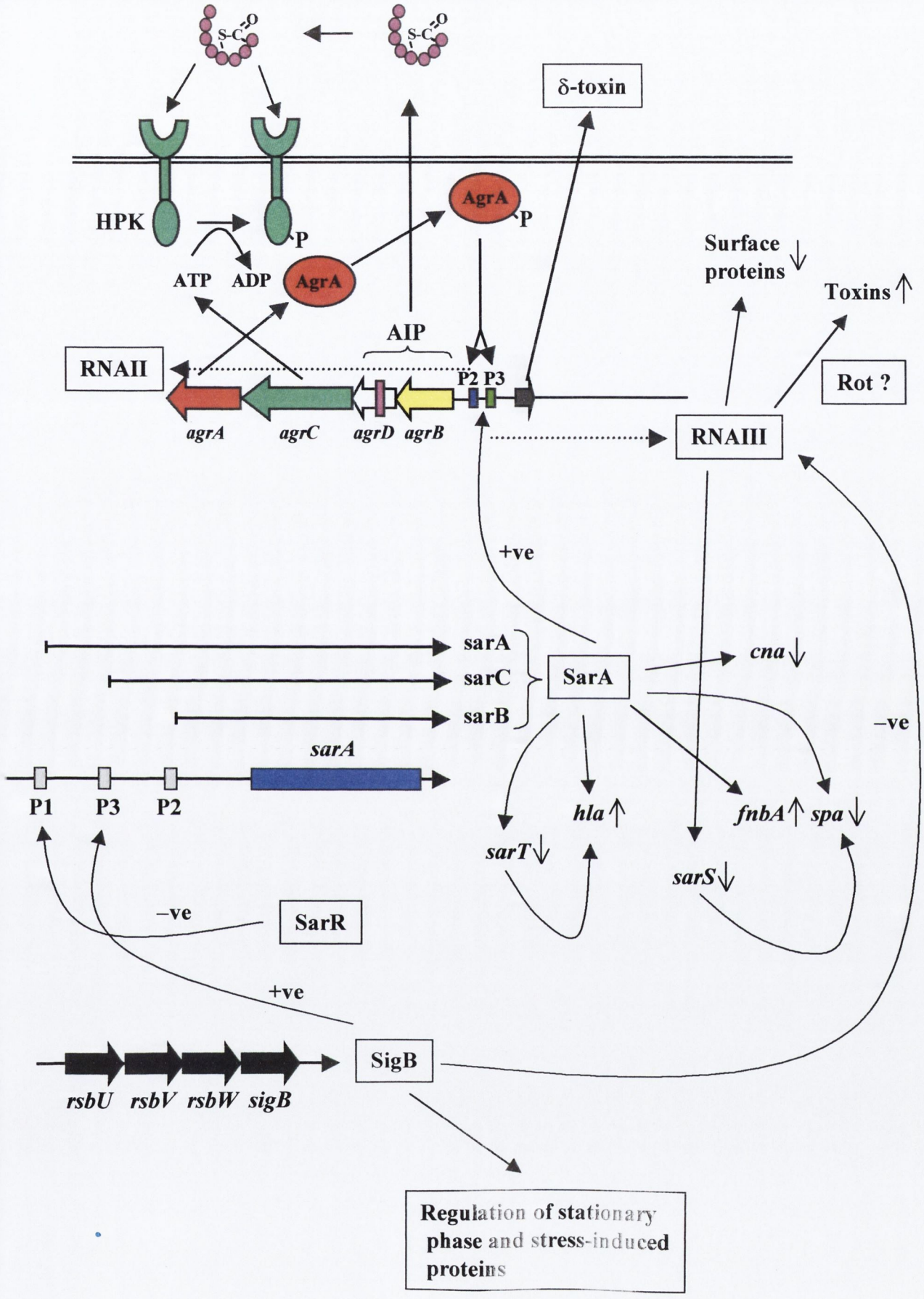
The *agr* locus of *S. aureus* coordinates the expression of most virulence factors (Recsei *et al.*, 1986). Mutants defective in *agr* exhibited increased expression of cell-surface-associated matrix-binding proteins such as protein A, fibronectin-binding proteins and coagulase and decreased levels of synthesis of extracellular toxins such as α-toxin, β-toxin, δ-toxin, enterotoxin B, DNase and TSST-1 (Recsei *et al.*, 1986; Kornblum *et al.*, 1990). Therefore, the expression of some cell surface matrix-binding proteins is negatively regulated by *agr* and exoprotein synthesis is positively regulated by *agr*. The activity of *agr* is low during the early stages of infection, which allows for the expression of cell surface matrix-binding proteins that promote bacterial adherence at sites of infection. As the infection progresses the expression of surface adhesins is suppressed and the expression of extracellular toxins is induced. This is likely to promote the spread of bacteria to other sites of infection, evasion of host immune responses and the release of internalized bacteria from the host endosome (Novick and Muir, 1999; Qazi *et al.*, 2001).

The *agr* locus consists of two divergent transcripts RNAII and RNAIII, transcribed from two distinct promoters, P2 and P3, respectively (Peng *et al.*, 1988) (Figure 1.5). RNAII, which is transcribed from promoter P2, encodes a four-gene operon, *agrB*, *agrD*, *agrC* and *agrA*, each of which is required for transcriptional activation of the *agr* P2 operon and the P3 promoter (Ji *et al.*, 1995). AgrC is the histidine kinase part of a classic two-component regulatory system and is supposed to activate the cytoplasmically located response regulator, AgrA, via phosphorylation of a conserved aspartate residue

Figure 1.5 Schematic diagram of the regulatory loci *agr*, *sarA*, *sigB*, *rot*, *sarS*, *sarT* and *sarR*. The *agr* locus consists of two divergent transcripts RNAII and RNAIII, transcribed from two distinct promoters, P2 and P3, respectively. RNAII, which is transcribed from promoter P2, encodes a four-gene operon, *agrB*, *agrD*, *agrC* and *agrA*. AgrC is the histidine kinase part of a classic two-component regulatory system and is supposed to activate the cytoplasmically located response regulator, AgrA, via phosphorylation of a conserved aspartate residue. Phosphorylated AgrA promotes transcription of the P2 and P3 promoters driving expression of RNAIII. RNAIII is the effector molecule of the *agr* system that is responsible for the downregulation of the expression of some cell-wall-associated adhesins and the upregulation of exoprotein synthesis. The positive effect of RNAIII expression on exoprotein synthesis may be mediated by repression of Rot protein, a repressor of toxin synthesis. It is likely that the *agr* system represses protein A (*spa*) expression by downregulating expression of SarS, a positive regulator of *spa* expression.

The *sarA* locus contains three overlapping transcripts *sarA*, *sarB* and *sarC* that initiate from three promoters P1, P2 and P3, respectively. Expression of the transcripts *sarA* and *sarB* from promoters P1 and P2, respectively, is mediated by the primary sigma factor σ^A. The P3 *sar* promoter is activated in stationary phase by the alternative sigma factor, σ^B. The SarA homologue, SarR, has been found to be a repressor of SarA expression. SarA positively regulates the expression of the *agr* system and directly regulates the transcription of virulence factor genes by binding to the promoter regions. SarA also positively regulates expression of α-toxin (*hla*) by repressing SarT expression, a repressor of α-toxin production.

SigB is the stationary phase sigma factor that regulates the expression of stationary phase and stress-induced proteins. The *sigB* locus consists of a four gene operon. The SigB protein is maximally expressed during stationary phase and positively regulates transcription of the *sarC* transcript from the P3 promoter. SigB also negatively regulates the expression of RNAIII.


δ-toxin
HPK
P
ATP
ADP
AgrA
AgrA
P
AIP
RNAII
P2 P3
agrA
agrC
agrD
agrB
Surface proteins
Toxins
Rot ?
RNAIII
+ve
sarA
sarC
sarB
SarA
cna
–ve
sarA
P1
P3
P2
hla
fnbA
spa
sarT
sarS
–ve
SarR
+ve
SigB
rsbU
rsbV
rsbW
sigB
Regulation of stationary phase and stress-induced proteins

(Lina *et al.*, 1998). Phosphorylated AgrA is believed to promote transcription of the P2 and P3 promoters, although binding of the protein to the promoters has yet to be demonstrated (Morfeldt *et al.*, 1996). Transcription of the P2 and P3 promoters is also dependent on the dimeric DNA-binding protein SarA (Chien and Cheung, 1998) (Figure 1.5).

The stimulus for the expression of the *agr* system is cell density (Ji *et al.*, 1995). AgrD encodes an autoinducer propeptide that is processed to the active autoinducing peptide and/or exported from the cell by membrane bound AgrB (Ji *et al.*, 1997; Saenz *et al.*, 2000). When the autoinducing peptide reaches a critical concentration it activates the protein kinase activity of AgrC (Lina *et al.*, 1998) (Figure 1.5). Thus P2 is autocatalytic. The ligand-binding site of AgrC is proposed to be located in the third extracellular loop of the protein (Lina *et al.*, 1998).

The autoinducing activity of the AgrD peptide was found in stationary phase cultures (Ji *et al.*, 1995). Edman degradation N-terminal sequencing revealed that the autoinducing peptide was an octa- or heptapeptide whose activity was lost after treatment with neutral hydroxylamine. The AgrD peptide contains a thiolactone bond between a conserved cysteine residue and the carboxy-terminal residue forming a thioester ring structure, which is essential for *agr* activation, and a linear N-terminal tail (Ji *et al.*, 1997). Linear synthetic peptides and lactam or lactone derivatives did not show any biological activity (Mayville *et al.*, 1999). Agr deletion strains harboring *agrB* and *agrD* on plasmids produced biologically active peptide pheromone, suggesting that AgrB is responsible for the production of mature pheromone (Saenz *et al.*, 2000). In *S. aureus*, 4 different *agr* subgroups have been found that have been classified according to the primary amino acid sequence of the AgrD peptide pheromone (Ji *et al.*, 1997). The addition of a culture supernatant from one group led to the inhibition of *agr* activity of the other groups, but did not inhibit growth (Ji *et al.*, 1997). Mutational analysis of the *S. aureus* subgroup 2 pheromone revealed that both the amino acids in the thiolactone ring and those in the N-terminal tail are important for both activation the *agr* system but that amino acids in the N-terminal tail of the pheromone are not important for inhibition (Mayville *et al.*, 1999). This suggests that activation and inhibition of the *agr* system occur by different mechanisms. It has been proposed that activation occurs by a covalent

interaction of the pheromone with AgrC occurs via a trans-acylation reaction, whereas inhibition may occur by non-covalent interactions with AgrC (Mayville *et al.*, 1999; Lyon *et al.*, 2000).

The effector of both positive and negative *agr*-dependent regulation is RNAIII, a 514 nt RNA molecule transcribed from the P3 promoter (Novick *et al.*, 1993) (Figure 1.5). RNAIII contains a small open reading frame encoding the 26-amino acid residue δ-toxin. δ-toxin plays no significant role in the regulation of virulence gene expression. During the exponential phase of growth the extracellular concentration of the autoinducing peptide increases until a critical concentration is reached and the synthesis of RNAIII is induced. The induction of RNAIII results in the downregulation of some surface protein genes and the upregulation of exoprotein genes. RNAIII regulates most genes in the *agr* regulon at the transcriptional level by an unknown mechanism. It has been suggested that RNAIII indirectly regulates gene expression by interacting with regulatory proteins from outside the *agr* locus. Recently, a number of proteins that regulate exoprotein and surface protein synthesis, such as Rot and SarS (also known as SarH1), whose expression is regulated by *agr* have been identified (McNamara *et al.*, 2000; Tegmark *et al.*, 2000; Cheung *et al.*, 2001). RNAIII also regulates the expression of at least one protein at the translational level. The binding of RNAIII to a 300 nt untranslated 5′ region of the *hla* transcript (α-toxin) prevents the formation of intramolecular base pairing and allows translation to be initiated (Novick *et al.*, 1993; Morefeldt *et al.*, 1995). Recent studies using gene chips revealed that the *agr* locus could regulate greater than 140 genes (Dunman *et al.*, 2001).

A model of the secondary structure of RNAIII revealed that the molecule is characterised by 14 hairpin structures and that the 5′ end and the 3′ end are in close proximity (Benito *et al.*, 2000). The 3′ end is important for the repression of protein A expression and the 5′ side of the central domain is involved in the activation of α-toxin translation (Benito *et al.*, 2000).

The importance of *agr* in bacterial virulence has been studied in several animal models. *agr* mutants showed reduced virulence in models of subcutaneous abscess formation (Mayville *et al.*, 1999) and arthritis (Abdelnour *et al.*, 1993) in mice, osteomylitis (Gillaspy *et al.*, 1995) and endocarditis (Cheung *et al.*, 1994) in rabbits and

were impaired in internalization and induction of apoptosis in mammary epithelial cells (Wesson *et al.*, 1998). Recent studies have shown that *agr* is expressed prior to lysis of the host endosome after *S. aureus* is taken up into endothelial cells and that *agr*-regulated proteins are required prior to the release and replication of *S. aureus* (Qazi *et al.*, 2001). These studies suggest that targeting of the *agr* system is an excellent candidate for targeting by novel antimicrobial agents.

1.3.2 The *sarA* locus

The *S. aureus* accessory regulator (SarA) protein also influences both exoprotein and cell surface protein expression (Cheung *et al.*, 1992). The *sarA* locus contains three overlapping transcripts *sarA*, *sarB* and *sarC* that initiate transcription from three promoters P1, P2 and P3, respectively (Bayer *et al.*, 1996) (Figure 1.5). Each of the transcripts has a common 3′ end encoding a 14.7 kDa DNA-binding protein known as SarA (Bayer *et al.*, 1996). Expression of the three transcripts is regulated temporally to ensure that a *sar* transcript is present in both exponential and stationary phase. Expression of the transcripts *sarA* and *sarC* from promoters P1 and P2, respectively, is mediated by the primary sigma factor σ^A and is maximal during the exponential phase of growth (Manna *et al.*, 1998). The P3 *sar* promoter is activated in stationary phase by the alternative sigma factor, σ^B, which is stationary phase-specific and stress-inducible (Deora *et al.*, 1997; Manna *et al.*, 1998). SarR, a homologue of SarA, is a regulatory protein that acts upstream of *sarA* to downregulate transcription from the *sarA* P1 promoter by binding to the promoter region (see section 1.3.3).

SarA exists as a dimer that binds to multiple AT-rich regions of variable lengths within the promoters of genes (Chien *et al.*, 1999; Schumacher *et al.*, 2001). SarA binds to the intergenic region of the P2 and P3 promoters of the *agr* locus and increases both RNAII and RNAIII transcription and therefore contributes to virulence factor expression indirectly (Morefeldt *et al.*, 1996; Chien and Cheung, 1998) (Figure 1.5). SarA has also been shown to directly regulate the expression of virulence factors such as *hla* (α-toxin), *spa* (protein A), *fnb* (fibronectin-binding proteins) and *sec* (enterotoxin C), by binding to a conserved sequence in the promoter regions (Chien *et al.*, 1999; Wolz *et al.*, 2000). Recent studies using gene chips and 2-D gel electrophoresis revealed that the *sarA* locus

could regulate greater than 100 genes (Ziebandt *et al.*, 2001; Dunman *et al.*, 2001). Further evidence for the role of SarA in the regulation of virulence factor expression comes from the observation that an *agr sarA* double mutant had more greatly reduced virulence that an *agr* mutant in a rabbit model of infective endocarditis (Cheung *et al.*, 1994).

Recently, biochemical and bioinformatic analysis has identified a number of SarA homologues that are also involved in the regulation of virulence factor expression.

1.3.3 SarA homologues in *S. aureus*

While mutations in *agr* and *sarA* have been shown to reduce virulence in animal infection models, these mutations did not render the bacteria completely avirulent. This suggested that other regulatory factors might be involved in the regulation of virulence factor expression. Recently, a number of regulatory proteins that share homology with the SarA protein of *S. aureus*, including SarS, Rot, SarR and SarT, have been identified and characterised and their role in the regulation of virulence factor expression has been examined.

Biochemical and *in silico* analysis identified a SarA homologue designated SarS (also known as SarH1) (Tegmark *et al.*, 2000; Cheung *et al.*, 2001). The *sarS* locus comprises three transcripts of 952, 1549 and approximately 3000 nt, each of which is thought to encode a single product, SarS (Tegmark *et al.*, 2000). SarS is a 250-residue protein with two SarA-like domains of 125 residues each. The SarS protein acts downstream of *agr* and *sarA* to activate the transcription of the protein A gene, *spa*. Gel shift analysis revealed that SarS bound specifically to the 158 bp *spa* promoter and that transcription of the gene was down-regulated in a *sarS* mutant, indicating that SarS is a positive regulator of *spa* expression (Cheung *et al.*, 2001). Northern blot analysis of *sarS* transcription in *agr* and *sarA* mutant backgrounds revealed that *sarS* transcription is repressed by both *agr* and *sarA* gene products (Cheung *et al.*, 2001). Both *agr* and *sarA* gene products are known to repress protein A expression. It is likely that *agr* mediates *spa* repression by down-regulating *sarS* expression. However, repression of *spa* expression by SarA was found to be independent of SarS.

Protease activity and α-toxin activity are low in an *agr* mutant because *agr* gene products induce the expression of exoproteins (Kornblum *et al.*, 1990). Transposon mutagenesis of an *agr* mutant of *S. aureus* identified an *orf* that, when inactivated, partially restored the expression of protease and α-toxin (McNamara *et al.*, 2000). The transposon-inactived gene was designated *rot*, for repressor of toxins. Analysis of the predicted amino acid sequence of the Rot protein revealed that it shares some homology with SarA and AgrA (McNamara *et al.*, 2000). The mechanism of Rot mediated toxin repression has yet to be elucidated.

Previously, a 12 kDa protein of *S. aureus* that shares homology with SarA was partially purified using a DNA-specific column containing a 49 bp sequence upstream of the *sar* P2 promoter (Manna *et al.*, 1998). N-terminal sequencing of the partially purified protein identified a 345 bp gene, designated *sarR*, encoding a protein with a predicted molecular mass of 13.6 kDa (Manna and Cheung, 2001). Purified recombinant SarR was found to bind to the *sarA* P1, P2 and P3 promoters, as confirmed by gel shift analysis and DNA footprinting studies. Allelic replacement of the *sarR* gene with an *ermC* marker revealed that transcription of the *sarA* P1 promoter was increased in a *sarR* mutant compared to the parental strain and that this increase in transcription was accompanied by an increase in the expression of the SarA protein (Manna and Cheung, 2001). These data suggest that SarR is a regulatory protein that acts upstream of *sarA* to down-regulate transcription from the *sarA* P1 promoter by binding to the promoter region.

In silico analysis of the *S. aureus* genome identified another gene, *sarT*, whose predicted gene product shared homology with both SarA and SarR (Schmidt *et al.*, 2001). SarT was found to repress transcription of the α-toxin gene, *hla*, as determined by Northern blotting, Western immunoblotting and a rabbit erythrocyte hemolytic assay. Analysis of *hla* transcription in a *sarA sarT* double mutant revealed that SarA activates *hla* transcription by repressing *sarT* expression (Schmidt *et al.*, 2001).

The functions of these proteins under different growth conditions and their interactions with each other and other regulatory systems have yet to be examined. Analysis of the complete genome sequence of the methicillin-resistant *S. aureus* strains Mu50 and N315 identified at least one more SarA homologue and 89 uncharacterised putative transcription regulators (Kuroda *et al.*, 2001).

1.3.4 SigB

The alternative sigma factor of *S. aureus*, σ^B, plays a role in stress responses and the regulation of virulence factor expression. While *B. subtilis* possesses as many as 20 sigma factors, analysis of the *S. aureus* genome revealed that the organism has only two, *sigA* and *sigB* (Kuroda *et al.*, 2001). The *sigA* and *sigB* gene products correspond to the housekeeping Sigma 70 and the general stress transcription factor, respectively.

The *sigB* gene is encoded in a four-gene operon (*rsbU*, *rsbV*, *rsbW*, *sigB*) (Figure 1.5) that shares amino acid and organisational similarity to an analogous operon in *B. subtilis* (Kullik and Giachino, 1997). In *S. aureus* RsbW has been shown to function as an anti-sigma factor that binds to SigB and inhibits SigB-dependent transcription (Miyazaki *et al.*, 1999; Palma and Cheung, 2001). In *B. subtilis*, RsbV, in its dephosphorylated form, competes with SigB to bind to RsbW, resulting in the release of SigB to initiate transcription of SigB-dependent genes. The ability of RsbV to bind to RsbW depends on the phosphorylation status of RsbV, which is controlled by the phosphatase RsbU (Voelker *et al.*, 1995). Allelic replacement of the *rsbU* or *rsbV* gene in *S. aureus* resulted in a reduction in the SigB protein level, suggesting that RsbU and RsbV of *S. aureus* have functions similar to those of their *B. subtilis* homologues (Giachino *et al.*, 2001; Palma and Cheung, 2001).

σ^B is mainly expressed during the stationary phase of growth and has been shown to be involved in recovery from heat shock and in acid and hydrogen peroxide resistance (Kullick and Giachino, 1997; Chan *et al.*, 1998). In addition, strains with a *sigB* mutation demonstrated increased production of α-toxin, a reduction in yellow pigmentation and increased sensitivity to hydrogen peroxide (Cheung *et al.*, 1999; Kullick *et al.*, 1998). However, they did not differ significantly in pathogenicity from the parental strains and did not show defective starvation-survival kinetics compared to the parental strain (Chan *et al.*, 1998). SigB interacts with the global regulators, *agr* and *sarA*, to regulate virulence factor expression. In a *sigB* mutant, *sarC* was no longer transcribed (Cheung *et al.*, 1999; Bischoff *et al.*, 2001). This indicated that SigB positively regulates the expression of the *sarC* transcript, but the mechanism of activation is not understood. Recently it has been shown that SigB negatively regulates the expression of RNAIII (Bischoff *et al.*, 2001). 2-D gel and Northern blotting analysis of cytoplasmic and extracellular proteins from *sigB*

mutants and their parental strains revealed that at least 23 cytoplasmically located proteins, including SarA, are positively regulated by σ^B and that 11 extracellular proteins are negatively regulated by σ^B (Gertz *et al.*, 2000; Ziebrandt *et al.*, 2001).

1.3.5 Two-component regulatory systems

Two-component regulatory systems, consisting of a membrane sensor (histidine kinase) and a cytoplasmic response regulator, enable bacteria to sense their environment and to adjust target gene expression accordingly. In response to a signal, the membrane sensor becomes autophosphorylated at a histidine residue in the cytoplasmic domain of the sensor. This phosphate group is then transferred to an aspartate residue of the cytoplasmic response regulator, which in turn stimulates or represses expression of target genes (Dziejman and Mekalanos, 1995).

Seven two-component regulatory systems have been characterised in *S. aureus* (Summarised in Table 1.4). These systems are involved in the regulation of a diverse range of cellular processes, including the regulation of virulence factor expression, autolysis, sensitivity to penicillin, energy transduction in response to changes in oxygen availability and the regulation of bacterial cell wall or membrane composition.

Recently, *in silico* analysis of the completed genome sequence of the methicillin-resistant *S. aureus* strains Mu50 and N315 identified a total of 17 gene pairs that share homology to two-component regulatory systems (Kuroda *et al.*, 2001). The functions of these systems and these interactions with other regulatory systems have yet to be elucidated.

1.3.6 Posttranslational regulation

There is mounting evidence that the ligand-binding activity of a number of *S. aureus* MSCRAMMs is regulated by digestion of these proteins on the cell surface by staphylococcal extracellular proteases. McGavin *et al.*, (1997) reported that incubating cells with purified *S. aureus* V8 serine protease reduced the amount of fibronectin-binding proteins on the cell surface and reduced adherence to immobilised fibronection and to the extracellular matrix produced by fetal rabbit lung fibroblasts. Karlsson *et al.* (2001) reported that the levels of cell-wall-associated fibronectin-binding proteins and

Table 1.4 Characterised two-component regulatory systems of *S. aureus*.

Name of system	Function	Reference
AgrA-AgrC	Regulates expression of exotoxins and cell surface adhesins by a quorum sensing mechanism.	Novick, 2000.
SaeS-SaeR	Positively regulates the expression of α-toxin, β-toxin and coagulase.	Giraudo *et al.* 1999.
YycG-YycF	Regulation of cell wall or membrane composition. Disruption of *yycG* or *yycF* resulted in hypersensitivity to macrolide and lincosamide antibiotics.	Martin *et al.*, 1999.
ArlS-ArlR	Regulates the expression of α-toxin, β-toxin, lipase, coagulase, serine protease (Ssp) and protein A by interacting with both the *agr* and *sar* systems.	Fournier *et al.*, 2001.
LytS-LytR	Controls the rate of autolysis by affecting murein hydrolase activity and promotes penicillin tolerance.	Groicher *et al.*, 2000.
SrhS-SrhR	Regulates the expression of proteins involved in energy metabolism in response to oxygen availability.	Throup *et al.*, 2001.
SrrA-SrrB	Regulates the expression of TSST-1 and protein A via the *agr* system. May act in anaerobic repression of *S. aureus* virulence factors.	Yarwood *et al.*, 2001.

protein A were very low in a *sarA* mutant, which produces high amounts of extracellular proteases. This decrease in cell-bound proteins was independent of transcription levels of the *fnbA*, *fnbB* and *spa* genes. Studies with various protease gene deficient mutants revealed that the serine protease, V8, is the most important protease in the release of cell-wall-associated FnBPs and protein A (Karlsson *et al.*, 2001). Recently, it has been shown that the fibrinogen-binding protein, ClfB, is cleaved on the cell surface to a truncated form that cannot promote bacterial adherence to immobilised fibrinogen (McAleese *et al.*, 2001). Cleavage of recombinant ClfB was promoted by the *S. aureus* metalloprotease, aureolysin, at a SLAVA motif (McAleese *et al.*, 2001).

There are a number of reasons why the ligand-binding functions of *S. aureus* MSCRAMMs are modified by proteases. Proteases are regulated in a growth phase-dependent manner, and are maximally expressed during the postexponential and stationary phases of growth (McAleese *et al.*, 2001). Cleavage of matrix-binding proteins on the bacterial cell surface may promote the detachment of cells from the initial site of infection and shedding into the bloodstream. This would promote bacterial spread to other sites of infection. Alternatively, protease cleavage may release immunogenic regions of surface proteins to which antibodies have been raised. This could protect *S. aureus* cells from the opsonic and complement activating effects of bound host antibodies. Another possibility is that cleavage of surface proteins by extracellular proteases could release biologically active fragments of the MSCRAMMs, which may play a role in pathogenesis.

1.4 Approaches to combating *S. aureus* infections

S. aureus is responsible for a large number of community-acquired infections and accounts for 270,000 hospital-acquired infections in the United States alone, each year. It is the leading cause of surgical wound infections and hospital-acquired infections worldwide. Staphylococcal infections are classically treated with antibiotics, which are usually effective. However, over the past two decades, multi-drug resistant strains of *S. aureus* have spread worldwide and recent reports have shown that up to 40-60% of staphylococci isolated from patients who acquired staphylococcal diseases while in hospital were resistant to multiple antibiotics (Flock, 1999). Most strains are only susceptible to the glycopeptide, vancomycin. Recently, there have been reports of vancomycin-resistant clinical isolates, which emphasizes the need for fresh approaches to the prevention and treatment of staphylococcal diseases (Hiramatsu, 1998).

1.4.1 Unsuccessful *S. aureus* vaccines and experimental vaccines that provided protection in animals

The emergence of multi-drug resistant *S. aureus* has led to an increased interest in research for vaccines to combat *S. aureus* diseases. Vaccination with killed *S. aureus* cells and toxoided α-toxin failed to protect patients from peritonitis or catheter-related infections despite the stimulation of anti-*S. aureus* and anti α-toxin antibodies (Poole-Warren *et al.*, 1991). Passive immunization with anti-protein A antibodies did not protect infant rats against systemic staphylococcal infection (Greenberg *et al.*, 1987).

There are a large number of reports of successful protection of animals from *S. aureus* infections using vaccines containing purified surface components or inactivated toxins. Purified fibronectin-binding protein provided protection in models of mastitis and endocarditis (Schennings *et al.*, 1993; Mamo *et al.*, 1994). In addition, immunization with the D2-domain of FnBPA expressed on the surface of cow-pea mosaic virus induced antibody-mediated protection against endocarditis in a rat infection model (Rennermalm *et al.*, 2001). Immunization with a recombinant fragment of the collagen-binding protein protected against sepsis-induced death in mice (Nilsson *et al.*, 1998). Recently,

immunization with a recombinant fragment of region A of the fibrinogen-binding protein, ClfA, protected mice from severe septic arthritis and passive immunization with antibodies against the same protein protected mice against *S. aureus* arthritis and sepsis-induced death (Josefsson *et al.*, 2001). Inhibitex Inc. (www.inhibitex.com) is developing a human monoclonal antibody, Aurexix™, that recognises ClfA, as well as a plasma-based product enriched for anti-ClfA (*S. aureus*) and anti-SdrG (*S. epidermidis*) antibodies for treating low birth weight neonates with staphylococcal infections (Veronate™).

Immunization with mutant forms of staphylococcal toxins and antibodies against toxins have proved effective in combating *S. aureus* infections in animal models. A mutant form of enterotoxin A lacking superantigenicity protected against septic death in mice (Nilsson *et al.*, 1999b). Similarly, a vaccine containing an attenuated recombinant form of staphylococcal enterotoxin B protected mice against a lethal challenge with the native toxin (Coffman *et al.*, 2002). An inactivated mutant form of α-toxin raised neutralising antibodies that passively protected against lethal infection in mice (Menzies and Kernoldle, 1996). Recently, a mutated recombinant from of TSST-1 toxin purified from *E. coli* protected rabbits against challenge with lethal doses of native TSST-1 (Gampfer *et al.*, 2002).

Other surface components that are effective antigens in animal model infections include capsular polysaccharides and the *in vivo*-expressed capsular polysaccharide adhesin, PNSG (poly-N-succinyl β-1-6 glucosamine). Immunization with polysaccharide from the serotype 1 macrocapsule protected mice against infection in a renal abscess model (Lee *et al.*, 1988). Active immunization with the serotype 5 capsular polysaccharide (CP5) and passive immunization with anti-CP5 antibodies protected mice against bacterial challenge (Fattom *et al.*, 1996). Similarly, antibodies against serotype CP8 have been shown to be protective in a rat model of endocarditis (Lee *et al.*, 1997). PNSG is a polysaccharide expressed at high levels *in vivo* by *S. aureus* and clinically isolates of coagulase negative staphylococci and is distinct from the serologically and structurally defined *S. aureus* capsular polysaccharides (McKenny *et al.*, 1999). Vaccinating mice with PNSG provided protection against *S. aureus*-induced metastatic kidney infection and mortality (McKenny *et al.*, 1999).

1.4.2 Novel approaches for the identification of virulence factors and novel vaccine targets

Recently, a number of approaches have been used to identify genes that are expressed *in vivo* or are required for *in vivo* growth, such as *in vivo* expression technology (IVET), signature tagged mutagenesis (STM) and antisense RNA. STM allows the analysis of a large number of individual transposon-generated individually tagged mutants in a single animal (Hensel *et al.*, 1995). Mutants that are viable *in vitro* (infecting pool) but not *in vivo* (recovered pool) can be identified and the inactivated gene can be cloned and sequenced. IVET identifies genes that are preferentially expressed *in vivo* (Mahan *et al*, 1993). This technology relies on a promoter trap that uses genetic recombination as a reporter for the activation of genes *in vivo*. Antisense RNA technology has been used to inhibit gene expression in both eukaryotic and bacterial systems (Harth *et al.*, 2000). Recently the technology has been applied to *S. aureus* to identify genes essential for bacterial growth *in vitro* and *in vivo*.

These approaches have been tested in a number of animal infection models and have identified genes that are expressed *in vivo* and are required for *S. aureus* growth and survival in the host. When applied to a *S. aureus* murine renal abscess model, IVET identified 45 staphylococcal genes that were induced *in vivo* (Lowe *et al.*, 1998). These included the *agr* global regulatory system, genes involved in capsule production and a leader peptidase involved in protein secretion. 28 of the genes identified were of unknown function. STM screens of *S. aureus* mutants in murine abscess, bacteraemia and wound models and rabbit endocarditis identified genes involved in *S. aureus* virulence (Mei *et al.*, 1997; Coulter *et al.*, 1998). The majority of the genes identified were involved in amino acid biosynthesis and transport, cell wall biosynthesis and enzymes involved in cellular biosynthetic pathways. Recently, a study using tetracycline-inducible *S. aureus* antisense RNA library identified a number of *S. aureus* genes that are essential for growth *in vitro* and are required for bacterial survival *in vivo* (Ji *et al.*, 2001). These included genes involved in DNA metabolism, transcription, translation and the fibronectin-binding proteins.

Only a small proportion of the genes identified in these screens were classical virulence factors, such as toxins, proteases and adhesins. This may be attributed to a

number of factors, including (i) in complex heterogeneous pools used in these types of study virulence factors, such as toxins and proteases, may be provided *in trans* by other pool members, (ii) with cell-surface matrix-binding proteins there is degeneracy of function (e.g. FnBPA and FnBPB), (iii) virulence factors are temporally expressed and the mutants defective in *in vivo* growth and virulence may be reflective of the *in vivo* growth state achieved at the time of mutant recovery.

The availability of completed genome sequences of the methicillin-resistant *S. aureus* strains Mu50 and N315 and the incomplete genome sequences of strains COL, NCTC 8325, EMRSA-16 and MSSA provides excellent tools for the study of bacterial virulence factors. Bioinformatic analysis has already identified hundreds of uncharacterised genes that share homology to known proteins of other organisms that are involved in a diverse range of cellular processes, including transport, stress resistance, antibiotic resistance and the regulation of gene expression (Kuroda *et al.*, 2001). The small size of bacterial genomes has facilitated the study of gene expression using microarray technology. This kind of technology has already been used to identify members of the *agr* and *sarA* regulons and could be used to study gene expression *in vivo* and under different growth conditions (Dunman *et al.*, 2001). The availability of such information will undoubtedly contribute to the study of bacterial virulence and the identification of novel antimicrobial targets and vaccine targets.

1.4.3 Novel targets for antimicrobial drugs

Antisense and conditional mutant essentiality testing has identified many essential genes with the potential to be developed as drug targets (McDevitt and Rosenberg, 2001). As well as the promising results obtained with vaccines containing surface components of *S. aureus*, a number of potential targets for novel staphylococcal drugs have been proposed. Although there is some evidence that these targets could be excellent candidates for anti-staphylococcal therapy, these targets have not yet been investigated in full and require further research.

The *S. aureus* protein sortase (SrtA) is required for the sorting of cell-wall-associated extracellular matrix-binding proteins to the cell surface (Mazmanian *et al.*, 1999). Inactivation of the *srtA* gene by allelic replacement abolished the Ig-binding,

fibrinogen-binding and fibronectin-binding capacity of *S. aureus*. In addition, after peritoneal infection in a mouse model, the LD_{50} of the *srtA* mutant was 100 times higher than the parental strain (Mazmanian *et al.*, 2000). A SrtA homologue, SrtB, has been identified in *S. aureus*. This protein catalyses the cleavage and cell wall sorting of IsdC, a cell wall protein that is cleaved by the enzyme at an NPQTN motif (Mazmanian *et al.*, 2002). SrtB appears to be involved in the process of iron aquistion during bacterial infection and is required for bacterial persistence in infected tissue (Mazmanian *et al.*, 2002). Pallen *et al.* (2001) reported that Gram-positive bacterial genomes encode at least two, and in some cases several, different sortase genes. These data suggest that an inhibitor of sortase could not only be an excellent anti-*S. aureus* therapy but could be of general use against Gram-positive bacteria.

Another potential target for an anti-staphylococcal therapy is the global regulatory locus, *agr*. Products of the *agr* locus regulate the expression of *S. aureus* virulence factors using a cell density quorum sensing mechanism (Novick, 2000). An autoinducing peptide, composed of seven of eight residues and a thiolactone ring, is responsible for the autophosphorylation of the histidine protein kinase of the Agr two-component regulatory system, which ultimately leads to expression of the Agr effector molecule, RNAIII (Novick, 2000). Lyon *et al.* (2000) demonstrated that the histidine protein kinase of the Agr two-component regulatory system, AgrC, is responsible for the activation and inhibition of the Agr system. This suggests that AgrC could be an excellent target for an inhibitor of *S. aureus* virulence. In the same study a derivate of the autoinducing peptide, composed of a thiolactone ring but lacking the N-terminal linear section, inhibited the *agr* response in all *S. aureus* strains tested, in *S. warneri*, and to some extent in *S. epidermidis*. A derivative of the autoinducing peptide with broad specificity could be a candidate for an anti-staphylococcal therapy.

Two-component regulatory systems are ubiquitous in both Gram-positive and Gram-negative bacteria and are involved in adaptive regulatory processes that enable bacteria to sense their environment, regulate virulence factor expression and are involved in antibiotic resistance (Dziejman and Mekalanos, 1995). Sensor kinases and response regulators are highly conserved across bacterial species, particularly at the active sites (Hoch and Varughese, 2001). In addition, bioinformatic analysis has revealed that the

residues making up the surface interaction between sensor kinases and response regulators are conserved across related bacterial species (Hoch and Varughese, 2001). Structure based design is being used to identify potential inhibitors of the active sites and interaction sites of two-component regulatory systems. Such inhibitors could be used to inhibit the function of *S. aureus* two-component systems involved in the regulation of virulence factor expression, such as Agr, Arl, Sae and Srr. All these targets differ from traditional drug targets in that they are not essential for growth *in vitro*.

1.5 Aims and rationale of this study

Elastic fibers, composed of elastin and microfibrillar components, are a major component of the mammalian extracellular matrix (ECM) (Mecham and Davies, 1994). These fibers play a crucial role in maintaining the structural integrity and function of tissues in which reversible extensibility or deformability is required. Thus elastin and elastic fibers are present in abundance in tissues that require elasticity such as the lung, skin, and major blood vessels, but elastin is widely expressed at lower levels in most mammalian tissues. Elastic fibers are complex structures that exist in the body as three distinct morphological forms (Mecham and Davies, 1994). In elastic ligaments, lung and skin, the fibers are small, ropelike, and variable in length. In major blood vessels, such as the aorta, elastic fibers form concentric sheets while in elastic cartilage a honeycomb morphology is adopted.

Elastin is an extremely hydrophobic and insoluble protein that is the major component of elastic fibers. The protein has a low content of acidic and basic amino acids and is rich in hydrophobic amino acids, with glycine making up 30% of the residues (Uitto, 1979). The elastic nature of elastin is derived from several lysine derivatives that serve as covalent cross-linkages between protein monomers (Bressan and Prockop, 1977).

Tropoelastin, the soluble precursor of elastin, is produced by a large number of mammalian cells, including smooth muscle cells, endothelial cells, chondrocytes and fibroblasts (Mecham and Davies, 1994). Tropoelastin is highly conserved across mammalian species at both the DNA and amino acid level (Sandberg and Davidson, 1984). The protein is composed of two major types of domains (i) hydrophobic domains of three to six hydrophobic residues and (ii) hydrophilic domains often consisting of lysine residues that are involved in cross-linking. In general these hydrophobic and hydrophilic domains alternate (Indik *et al.*, 1989). The C terminus of the protein is highly basic and is strongly conserved among all species (Indik *et al.*, 1987) (Figure 1.6). Tropoelastin is secreted into the extracellular space as a 72 kDa protein at specific regions on the cell surface (Bressan and Prockop, 1977). A thermodynamically controlled

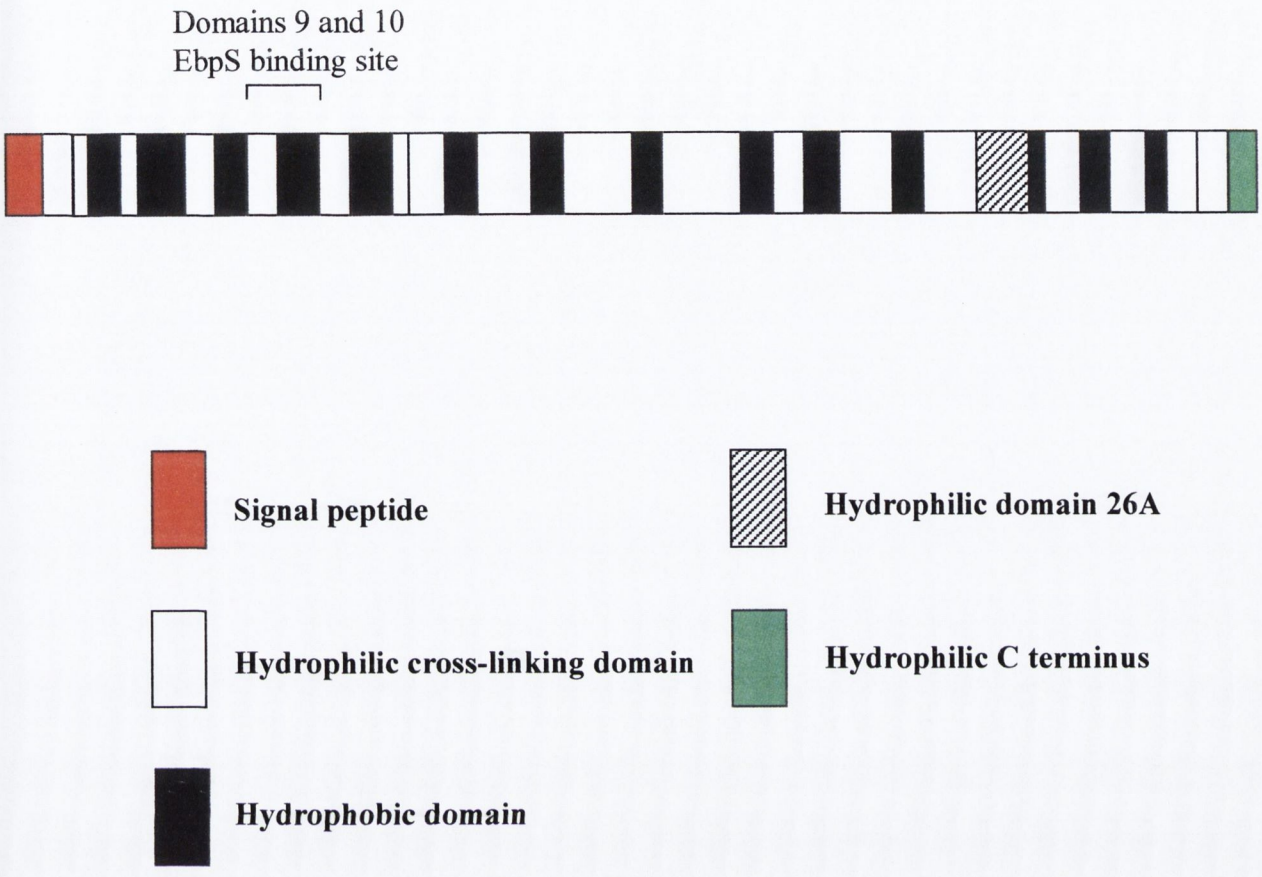


Figure 1.6 Schematic diagram of human tropoelastin. The secretion signal sequence, hydrophilic cross-linking domains and hydrophobic domains are indicated. Domain 26A is an unusually hydrophilic domain that is only found in human tropoelastin and whose function is unknown. The hydrophilic C terminus is basic in nature and is highly conserved amongst species.

process called coacervation orientates the tropoelastin monomers into an ordered fibrillar structure via interactions between the hydrophobic domains of neighbouring molecules (Cox *et al.*, 1974). Lysine residues in the hydrophilic cross-linking domains of secreted tropoelastin are then rapidly cross-linked both inter- and intramolecularly by the enzyme lysyl oxidase, to form mature elastin (Kagan and Trackman, 1991).

Elastin is difficult to purify and is generally isolated from elastic tissues by removing all other connective tissue components by denaturing or degradation. Purification procedures rely upon the resistance of elastin to protein solvents and hydrolysis by dilute acids or alkali. Oxalic acid is used to degrade elastin to form α-elastin peptides and KOH is used to form κ-elastin peptides (Mecham and Lange, 1982).

The invasive nature of *S. aureus* suggests that the bacteria have a high probability of interacting with the elastin matrix at sites of infection. Park *et al.* (1991) demonstrated a specific interaction between *S. aureus* strain Cowan and soluble elastin peptides. Affinity chromatography with *S. aureus* lysostaphin extracts, using elastin peptides as the active ligand, identified a 25 kDa surface protein as the elastin-binding protein of *S. aureus* (EbpS). N-terminal sequencing of native EbpS expressed on the surface of *S. aureus* enabled the construction of a degenerate oligonucleotide probe to identify the *ebpS* gene. The *ebpS* gene was cloned and sequenced and was found to be an open reading frame of 606 bp encoding a protein with a predicted molecular mass of 23.4 kDa (Park *et al.*, 1996). Genetic and physical mapping of the chromosome of *S. aureus* strains of different phage groups has shown that the *ebpS* gene is ubiquitous and conserved and maps to the same chromosomal locus (Smeltzer *et al.*, 1997). Recombinant EbpS and polyclonal antibodies raised against the recombinant form of the protein inhibited staphylococcal elastin binding, suggesting that EbpS is the surface factor mediating *S. aureus* binding to soluble elastin (Park *et al.*, 1996). Using a series of EbpS truncated derivatives and synthetic peptides, later studies localised the ligand-binding site of EbpS to a 21-amino acid region of the protein contained within residues 14-34 (Park *et al.*, 1999).

DNA sequencing of the *ebpS* gene from *S. aureus* strains Cowan and 8325-4 revealed that a sequencing error occurred in the original study and that the open reading frame is actually 1,461 bp in length encoding a 486-residue protein (Roche, 2000). The

protein isolated by Park *et al.* (1991) corresponds to the N-terminal 202-residues of the full-length protein. Analysis of the primary amino acid sequence of the predicted EbpS protein revealed that it does not bear a secretion signal sequence or any of the features of the family of surface proteins that are covalently associated with the cell wall peptidoglycan, including an LPXTG motif, a membrane-spanning domain and a positively charged C terminus. However, the protein does possess three centrally located hydrophobic domains, designated H1 (residues 205–224), H2 (residues 265–280) and H3 (residues 315–342) and at the extreme C terminus of EbpS there is a 50-residue domain that shares homology with the LysM motif of cell wall hydrolases of other bacterial species (Roche, 2000) (Figure 1.7). This is a domain that is generally regarded to promote binding to cell wall peptidoglycan although this has not been formally demonstrated (Joris *et al.*, 1992).

A site-specific mutation in the *ebpS* gene was isolated by allelic replacement (Roche, 2000). Subsequent studies demonstrated that an *ebpS* mutant showed a 72% reduction in soluble tropoelastin binding compared to the parental strain. Binding was reduced to background levels by incubation with excess cold elastin peptides, reaching the same level as the *ebpS* mutant incubated with cold peptides. The complementing plasmid pCU-*ebpS*$^{+}$ restored elastin binding to the same level as the parental strain (Roche, 2000). In addition, a recombinant N-terminal truncate of EbpS was shown to bind in a dose-dependent manner to immobilised elastin peptides in an ELISA-based assay (Roche, 2000). However, EbpS did not promote bacterial adherence to immobilised elastin peptides, as shown by the ability of an *S. aureus ebpS* mutant to adhere to the immobilised ligand (Roche, 2000). The fibronectin-binding proteins, FnBPA and FnBPB, were tentatively identified as the surface proteins responsible for bacterial adherence to immobilised elastin peptides (Roche, 2000).

This thesis examines the mechanism by which EbpS is associated with the bacterial cell surface and the role played by the protein in bacterial growth. Further evidence for the role of the fibronectin-binding proteins in bacterial adherence to immobilised elastin peptides is presented and the regions of the staphylococcal MSCRAMMs responsible for adherence are identified by inhibition studies using polyclonal antibodies and recombinant truncated derivatives of the FnBP proteins.

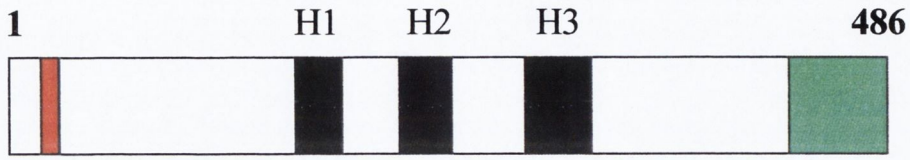


Figure 1.7 Schematic representation of the primary structure of the full length 486-residue elastin-binding protein of strain 8325-4 (Roche, 2000) with the elastin binding domain ( ) three hydrophobic domains ( ), H1 (residues 205–224), H2 (residues 265–280) and H3 (residues 315–342) and the LysM motif ( ).

Chapter 3 describes the production of polyclonal antibodies against the N terminus and C terminus of EbpS and the localisation of EbpS to the cytoplasmic membrane by detailed cellular fractionation analysis. Chapter 4 illustrated that EbpS is an integral membrane protein, with both the N terminus and C terminus on the extracellular face of the cytoplasmic membrane, by the expression of EbpS fusions to the enzymes alkaline phosphatase (PhoA) and β-galactosidase (LacZ) in *E. coli*. This topology is confirmed in *S. aureus* using antibody absorption experiments. Cross-linking studies, using the homobifunctional cross-linking reagent dithiobis(succinimidyl proprionate) (DSP), suggested that EbpS exists as part of a heteromeric or homomeric complex in the cytoplasmic membrane. Chapter 5 confirmed that EbpS does not promote bacterial adherence to immobilised elastin peptides in an ELISA-based assay. An *ebpS* mutant of *S. aureus* exhibited a growth defect that could be complemented by the expression of EbpS from an integrated plasmid. The region of EbpS responsible for this growth defect was identified by expressing truncated derivatives of the protein in an *ebpS* deficient strain. Chapter 6 examined the role of the of the fibronectin-binding proteins in bacterial adherence to immobilised peptides purified from human and bovine sources using *S. aureus* FnBP-deficient mutants and complementation analysis. The purity of commercially available elastin preparations was assessed and the relevance of using elastin peptides dried onto the wells of microtitre plates as representative of elastin tissue was assessed in an ELISA-based assay. The regions of the staphylococcal MSCRAMMs responsible for elastin binding were identified by inhibition studies using polyclonal antibodies and recombinant truncated derivatives of the FnBP proteins.

Chapter 2

Materials and Methods

2.1 Bacterial growth conditions and plasmids

E. coli strains harbouring plasmids were routinely grown in L broth and L agar supplemented with appropriate antibiotics, ampicillin (Ap) (100 μg/ml) and spectinomycin (Spc) (25 μg/ml). *S. aureus* strains were grown in trypticase soy broth (TSB) or agar (TSA) incorporating antibiotics chloramphenicol (Cm) (10 μg/ml), erythromycin (Em) (10 μg/ml), kanamycin (Kan) (50 μg/ml) or tetracycline (Tet) (3 μg/ml) where appropriate, unless otherwise stated. *E. coli* and *S. aureus* strains were routinely grown at 37°C at 200 rpm, unless otherwise stated. *L. lactis* strain MG1363 was statically grown at 30°C in M17 broth or agar supplemented with 0.5% (w/v) glucose. *L. lactis* transformants were selected on M17 agar with 0.5% (w/v) glucose and erythromycin (5 μg/ml). Bacterial strains are listed in Table 2.1 and plasmids used or constructed are listed in Table 2.2.

2.2 Transductions, transformations and electrotransformations

Transduction in *S. aureus* was performed by the method of Asheshov (1966) using bacteriophage 85. Plasmids were electroporated into *S. aureus* strain RN4220 or CYL316 as described by McDevitt *et al.* (1993). *E. coli* strains were transformed by the calcium chloride method of Sambrook *et al.* (1989). Electrotransformation of *L. lactis* strain MG1363 was performed according to Wells *et al.* (1993).

2.3 DNA manipulations

Standard methods were used for DNA manipulation in constructing plasmids (Sambrook *et al.*, 1989). Plasmid DNA for cloning was purified by WizardPlus™ minipreps (Promega), with the addition of either mutanolysin (500 U/ml) (Sigma) or lysostaphin (200 μg/ml) (AMBI) to lyse *L. lactis* and *S. aureus* respectively. *S. aureus* genomic DNA was isolated using the AGTC bacterial genomic DNA purification kit (Edge BioSystems) adapted for use with staphylococci by incorporation of lysostaphin (200 μg/ml) (Foster, 1998). Restriction enzymes were purchased from Roche and used as recommended by the suppliers.

Table 2.1 Bacterial strains.

Strain	Relevant genotype	Relevant properties	Source/ Reference
S. aureus			
RN4220		ATCC 12598 Restriction deficient derivative of 8325-4	Kreiswirth *et al.* (1983)
CYL316		Derivative of RN4220 that supplies bacteriophage L54a integrase *in trans*	Lee *et al.* (1991)
Cowan		NCTC 8530	
MSSA		Community-acquired hyper virulent strain	Enright *et al.* (2000)
EMRSA-16	methicillin resistant	Epidemic UK hospital-acquired MRSA strain	Enright *et al.* (2000)
COL		MRSA isolate	
P1		Rabbit virulent strain	Sherertz *et al.*(1993)
P1 *ebpS*	*ebpS*::*erm*	*ebpS*-defective mutant of P1	Roche (2000)
P1 *fnbA fnbB*	*fnbA*::*tet fnbB*::*erm*	*fnbA* and *fnbB*-defective double mutant of P1	This laboratory (C. Greene, unpublished)
P1 *fnbA fnbB* (pFNBA4$^+$)	*fnbA*::*tet fnbB*::*erm*	Derivative of P1 *fnbA fnbB* expressing FnBPA from plasmid pFNBA4	This study
P1 *fnbA fnbB* (pFNBB4$^+$)	*fnbA*::*tet fnbB*::*erm*	Derivative of P1 *fnbA fnbB* expressing FnBPB from plasmid pFNBB4	This study
Newman		NCTC8178	Duthie and Lorenz (1952)
Newman *spa*	*spa*::*kan*	*spa*-defective mutant of Newman	This laboratory (F. Roche, unpublished)
Newman *spa ebpS*	*spa*::*tet ebpS*::*erm*	*spa* and *ebpS*-defective mutant of Newman	Roche (2000)
8325-4		NCTC 8325 cured of prophages	Novick (1967)
8325-4 *ebpS*	*ebpS*::*erm*	*ebpS*-defective mutant of 8325-4	Roche (2000)
8325-4 *fnbB*	*fnbB*::*erm*	*fnbB*-defective mutant of 8325-4	Greene *et al.* (1995)

Table 2.1 Bacterial strains, continued

Strain	Relevant genotype	Relevant properties	Source/ Reference
8325-4 *fnbA fnbB*	*fnbA*::*tet fnbB*::*erm*	*fnbA*- and *fnbB*-defective mutant of 8325-4	Greene *et al.* (1995)
8325-4 *fnbA fnbB* (pFNBA4$^+$)	*fnbA*::*tet fnbB*::*erm*	Derivative of 8325-4 *fnbA fnbB* expressing FnBPA from plasmid pFNBA4	Greene *et al.* (1995)
8325-4 *fnbA fnbB* (pFNBB4$^+$)	*fnbA*::*tet fnbB*::*erm*	Derivative of 8325-4 *fnbA fnbB* expressing FnBPB from plasmid pFNBB4	Greene *et al.* (1995)
8325-4 *ebpS* (pCL84)	*ebpS*::*erm geh*::*tet*	Derivative of 8325-4 *ebpS* with plasmid pCL84 integrated into the lipase gene (*geh*)	This study
8325-4 *ebpS* (pCL84-*ebpS*$^+$)	*ebpS*::*erm geh*::*tet*	Derivative of 8325-4 *ebpS* expressing EbpS from the integrated plasmid pCL84	This study
8325-4 *ebpS* ($EbpS_{\Delta 14-34}$)	*ebpS*::*erm geh*::*tet*	Derivative of 8325-4 *ebpS* expressing EbpS lacking residues 14-34 from the integrated plasmid pCL84	This study
8325-4 *ebpS* ($EbpS_{\Delta 365-389}$)	*ebpS*::*erm geh*::*tet*	Derivative of 8325-4 *ebpS* expressing EbpS lacking residues 365-389 from the integrated plasmid pCL84	This study
8325-4 *ebpS* ($EbpS_{\Delta 402-431}$)	*ebpS*::*erm geh*::*tet*	Derivative of 8325-4 *ebpS* expressing EbpS lacking residues 402-431 from the integrated plasmid pCL84	This study
8325-4 *ebpS* ($EbpS_{\Delta 440-479}$)	*ebpS*::*erm geh*::*tet*	Derivative of 8325-4 *ebpS* expressing EbpS lacking residues 440-479 from the integrated plasmid pCL84	This study
E. coli			
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^r)]	Highly transformable strain	Stratagene
M15 (pREP4)	*lacI* Kan^r on pREP4, F$^-$ *recA uvr*$^+$ *lon*$^+$ *lac*	Strain M15 expressing the LacI repressor from plasmid pREP4	Qiagen

Table 2.1 Bacterial strains, continued

Strain	Relevant genotype	Relevant properties	Source/ Reference
CC118	*araD139* Δ(*ara,leu*)*7697* Δ*lacX74 phoA20 galE galK thi rpsE argE*(Am) *recA1*	*phoA*-defective strain	Manoil and Beckwith (1986)
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	*lacZ*-defective strain	Sambrook *et al.* (1989)
TG1 (pDIA17)	*lacI* Kan^r on pDIA17	Strain TG1 expressing the LacI repressor from plasmid pDIA17	This study
Topp 3	Rif^r [F′ *proAB lacI*^{q} ZΔM15 Tn10 (Tet^r) (Kan^r)]	Protease-deficient strain of *E. coli*	Stratagene
L. lactis			
MG1363		Plasmid free derivative of strain NCDO712	Gasson *et al.* (1983)
MG1363 (pKS80-*ebpS*$^+$)		Strain MG1363 expressing EbpS from plasmid pKS80	This study
MG1363 (pKS80-*fnbA*$^+$)		Strain MG1363 expressing FnBPA from plasmid pKS80	This study
MG1363 (pKS80-*fnbB*$^+$)		Strain MG1363 expressing FnBPB from plasmid pKS80	This study
MG1363 (pKS80-*clfA*$^+$)		Strain MG1363 expressing ClfA from plasmid pKS80	O'Brien (2001)
MG1363 (pKS80-*sdrC*$^+$)		Strain MG1363 expressing SdrC from plasmid pKS80	O'Brien (2001)

Table 2.2 List of plasmids.

Plasmid	Features	Marker(s)	Source/ Reference
pQE-30	Expression vector, recombinant proteins fused to polyhistidine tag (N-terminal His-tag)	Ap^r	Qiagen Inc.
pQE-*ebpS*$_{343-486}$	pQE30 containing the 3′end of the *ebpS* gene from strain 8325-4, encoding residues 343-486	Ap^r	This study
pQE-*fnbA*$_{37-605}$	pQE30 containing the 5′end of the *fnbA* gene from strain 8325-4, encoding residues 37-605	Ap^r	This study
pQE-*fnbA*$_{745-878}$	pQE30 containing the 3′end of the *fnbA* gene from strain 8325-4, encoding residues 745-878	Ap^r	This study
pQE-*fnbB*$_{37-540}$	pQE30 containing the 5′end of the *fnbB* gene from strain 8325-4, encoding residues 37-540	Ap^r	This study
pKK233-2	ATG vector for the expression of proteins in *E. coli*	Ap^r	Amann and Brosius (1985)
pKK233-*ebpS*	pKK233-2 containing the *ebpS* gene from strain 8325-4	Ap^r	This study
pKS5.2	pBluescript KS^+ containing *ebpS* on a 5.2 kb 8325-4 genomic fragment	Ap^r	Roche (2000)
pFNBA4	Multicopy plasmid containing the *fnbA* gene from strain 8325-4	Ap^r in *E. coli* Cm^r in *S. aureus*	Greene *et al.* (1995)
pFNBB4	Multicopy plasmid containing the *fnbB* gene from strain 8325-4	Ap^r in *E. coli* Cm^r in *S. aureus*	Greene *et al.* (1995)
pDIA17	Derivative of pACYC184 with the *lacI* gene cloned into the *Bam*HI site allowing expression from the *tet* promoter	Cm^r	P. Bertin (unpublished)
pCU1	Shuttle vector derived from pC194 and pCU19, maintaining high copy number in *E. coli* and *S. aureus*	Ap^r in *E. coli* Cm^r in *S. aureus*	Augustin *et al.* (1992)
pCU-*ebpS*$^+$	pCU1 containing 8325-4 *ebpS* gene plus 300 bp upstream and 200 bp downstream sequence	Ap^r in *E. coli* Cm^r in *S. aureus*	Roche (2000)

Table 2.2 List of plasmids, continued.

Plasmid	Features	Marker(s)	Source/ Reference
pCL84	*S. aureus* expression vector that integrates into the lipase gene (*geh*) using the bacteriophage L54a integration system	Spc^r in *E. coli* Tet^r in *S. aureus*	Lee *et al.* (1991)
pCL84-*ebpS*	pCL84 containing the 8325-4 *ebpS* gene plus 300 bp upstream and 200 bp downstream sequence	Spc^r in *E. coli* Tet^r in *S. aureus*	This study
pCL84-$ebpS_{\Delta 14-34}$	Derivative of pCL84-*ebpS* lacking the coding region for residues 14–34	Spc^r in *E. coli* Tet^r in *S. aureus*	This study
pCL84-$ebpS_{\Delta 365-389}$	Derivative of pCL84-*ebpS* lacking the coding region for residues 365–389	Spc^r in *E. coli* Tet^r in *S. aureus*	This study
pCL84-$ebpS_{\Delta 402-431}$	Derivative of pCL84-*ebpS* lacking the coding region for residues 402–431	Spc^r in *E. coli* Tet^r in *S. aureus*	This study
pCL84-$ebpS_{\Delta 440-479}$	Derivative of pCL84-*ebpS* lacking the coding region for residues 440–479	Spc^r in *E. coli* Tet^r in *S. aureus*	This study
pBAF	PhoA fusion expression vector	Ap^r	Fulkerson and Mobley (2001)
pBAF*ebpS-phoA*	Derivatives of pBAF containing 5′ truncates of the 8325-4 *ebpS* gene. Fusion positions between EbpS and PhoA were at A234, T247, S256, S285, A296, H306, D369 and D402 of EbpS	Ap^r	This study
pLKC480	LacZ fusion expression vector	Ap^r Kan^r	Tiedeman and Smith (1988)
pLKC480*ebpS-lacZ*	Derivatives of pLKC480 containing 5′ truncates of the 8325-4 *ebpS* gene. Fusion positions between EbpS and LacZ were at A234, T247, S256, S285, A296, H306, D369 and D402 of EbpS	Ap^r Kan^r	This study
pKS80	*L lactis* expression vector	MLS resistance	Wells *et al.* (1993)
pKS80-$ebpS^+$	pKS80 containing the 8325-4 *ebpS* gene	MLS resistance	This study
pKS80-$fnbA^+$	pKS80 containing the 8325-4 *fnbA* gene	MLS resistance	This study
pKS80-$fnbB^+$	pKS80 containing the 8325-4 *fnbB* gene	MLS resistance	This study

2.4 Expression and purification of $rEbpS_{343-486}$, $rEbpS_{1-267}$, $rFnBPA_{37-605}$, $rFnBPA_{745-878}$ and $rFnBPB_{37-540}$

The 3′ end of the *ebpS* gene encoding residues 343 to 486 was PCR-amplified from plasmid pKS5.2 using the forward primer RD-F1 and the reverse primer RD-R1 (Table 2.3). The region of the *fnbA* gene encoding residues 37 to 605 was PCR amplified from plasmid pFNBA4 using the forward primer FAA-Fwd and the reverse primer FAA-Rev. The region of the *fnbA* gene encoding residues 745 to 878 was PCR-amplified from plasmid pFNBA4 using the forward primer FAD-Fwd and the reverse primer FAD-Rev. The region of the *fnbB* gene encoding residues 37 to 540 was PCR-amplified from plasmid pFNBB4 using the forward primer FAB-Fwd and the reverse primer FAB-Rev. The PCR conditions were 30 cycles of 94°C for 1 min, 48-50°C for 1 min and 72°C for 1-4 min using *pfu* polymerase (Promega). The resulting PCR products were cloned into the N-terminal six-histidine tag expression vector pQE-30. The resulting constructs were transformed into *E. coli* M15 ($rEbpS_{343-486}$) or *E. coli* Topp 3 ($rFnBPA_{37-605}$, $rFnBPA_{745-878}$ and $rFnBPB_{37-540}$).

The transformants were grown in 4 litres of L broth supplemented with Ap (100 μg/ml) to an OD_{600nm} of between 0.7 and 0.9. Expression was then induced by addition of 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown at 37°C for 4 h at 200 rpm. Cells were pelleted at 7,000×*g* for 15 min. The pellet was washed once with L broth. The cells were again pelleted and resuspended in 50 ml of binding buffer (Tris-HCl, 5 mM imidiazole, 0.5 M NaCl, pH 7.9). The cells were lysed by three passes through a French press (SIM-Aminco Spectronic Instruments) at 1,500 psi. The lysate was centrifuged at 20,000×*g* for 50 min at 4°C to remove cell debris. A 5 ml iminodiacetic acid-sepharose 6B Fast Flow column (Pharmacia) was charged with 87.5 M Ni^{2+} and equilibrated with binding buffer. The cleared lysate was filtered through a 0.45μm filter and applied to the Ni^{2+} affinity column with a flow rate of 1 ml/min and washed with binding buffer until the absorbance at 280 nm of the eluate was <0.001. A linear gradient of imidazole (5-200 mM, total volume 100 ml) in 0.5 M NaCl and 20 mM Tris-HCl (pH 7.9) was applied. Fractions containing protein were confirmed by Coomassie blue staining of SDS-PAGE gels. Protein containing fractions were pooled and dialysed in two changes of phosphate

Table 2.3 Primers used to express recombinant proteins in *E. coli*.

Primer Name	Primer sequence
RD-F1	5′-CGCGGATCCAACAATCATAATAATGGTAC–3′
RD-R1	5′-CCCAAGCTTTTATGGAATAACGATTTG TTG-3′
FAA-Fwd	5′-AAAGGATCCGCATCAGAACAAAAGACAAC-3′
FAA-Rev	5′-AGAGTCGACCTACTCAGAGGACTCAGTG -3′
FAD-Fwd	5′-CGCGGATCCGGCCAAAATAGCGGTAAC -3′
FAD-Rev	5′-CCCAAGCTTCTATGGCACGATTGGAGGTG -3′
FBA-Fwd	5′-CGCGGATCCGCATCGGAACAAAACAATAC -3′
FBA-Rev	5′-AGAGTCGACCTATTCAGTTTCAATGGTACCTTC -3′

- The following restriction sites are underlined: *Bam*HI GGATCC, *Hin*dIII AAGCTT, *Sal*I GTCGAC.

buffered saline (PBS) at 4°C for 16 h. Presence of the six-histidine tag on the recombinant protein was confirmed using India™ HisProbe-HRP (Pierce) according to the manufacturers instructions.

rEbpS$_{1-267}$ was insoluble in the *E. coli* cytoplasm and formed inclusion bodies. The protein was purified under denaturing conditions (gifted by F. Roche).

2.5 Generation of rabbit anti-rEbpS$_{343-486}$ and anti-rEbpS$_{1-267}$ polyclonal antibodies

Preimmune sera were collected and New England White rabbits were injected with 1 ml of purified rEbpS$_{1-267}$ or rEbpS$_{343-486}$ (50 μg) mixed 1:1 with complete Freunds adjuvant. Booster injections (50 μg) mixed 1:1 with incomplete Freunds adjuvant were given at 2 and 4 weeks. Sera were tested by Western immunoblotting using whole cell lysates of *S. aureus* strains Newman *spa* and Newman *spa ebpS*. The immunoglobulin fraction was purified by the method of Owen (1985).

2.6 Affinity purification of antisera to rEbpS$_{343-486}$

5 μg of rEbpS$_{343-486}$ was dialysed overnight at 4°C in coating buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). 1 g of CNBr-activated sepharose™ (Amersham Pharmacia Biotech) was washed 10 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 settle on ice for 1 h. 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 left at room temperature for 3 h to block any remaining active gaps. The gel was then washed 6 times alternating between blocking buffer and wash buffer (0.1 M CH_3CO_2Na, 0.5 M NaCl, pH 3.9) and left overnight at room temperature 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 30 ml 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 280 nm 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).

2.7 SDS-PAGE, Western immunoblotting and Western ligand affinity blotting

Samples were boiled for 5 min in an equal volume of final sample buffer [0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.002% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol] before electrophoresis. SDS-PAGE was performed as described by Laemmli (1970) through a 4.5% stacking and 10% polyacrylamide separating gel. Proteins were transferred electrophoretically to polyvinylidene diflouride (PVDF) Western immunoblotting membrane (Boehringer-Mannheim) by the wet transfer system (Bio-Rad) in transfer buffer [20 mM Tris, 0.15 M glycine, 20% (v/v) methanol] at 100 V for 1 h. Membranes were incubated for 15 h at 4°C in blocking reagent [10 mM Tris-HCl, 0.9% (w/v) NaCl, 5% (w/v) skimmed milk (Marvel), pH 7.4]. Rabbit anti-$ClfA_{220-559}$ antibodies (gifted by O. Hartford) were used at a dilution of 1:2,000. Rabbit anti-$rEbpS_{1-267}$ and anti-$rEbpS_{343-486}$ antibodies were used at dilutions of 1:1,500. Rabbit anti-*Micrococcus luteus* F_1/F_0-ATPase antisera, which cross-reacts with the *S. aureus* protein was a gift of Prof. P. Owen, Trinity College Dublin and was used at a dilution of 1:2,000. Mouse anti-β-galactosidase polyclonal antibodies (Sigma) were used at a dilution of 1:50. Rabbit polyclonal antibodies to bacterial alkaline phosphatase (Abcam Ltd.) were used at a dilution of 1:10,000. Rabbit anti-$rFnBPA_{37-605}$ antibodies were a gift of Dr. E. Wann, Texas A&M University and were used at a dilution of 1:2,000. Rabbit polyclonal anti-human fibrinogen-HRP antibodies (Dako) were used at a dilution of 1:4,000. Goat polyclonal anti-human fibronectin antibodies (Dako) were used at a dilution of 1:2,000. Protein A conjugated to horseradish peroxidase (Sigma) was used to detect proteins using the LumiGLO chemiluminescence substrate (New England Biolabs) according to the manufacturer's instructions. India™ HisProbe-HRP (Pierce) was used at a dilution of 1:5,000 and detected using the LumiGLO chemiluminescence substrate according to the manufacturer's instructions.

1mg of human fibronectin (Calbiochem) was biotinylated using the EZ-Link™ sulpho-NHS-LC-biotinylation kit (Pierce) according to the manufacturer's instructions. Biotinylated fibronectin was used at a concentration of 50 μg/ml and detected using a 1:2,000 dilution of streptavidin-POD conjugate (Roche).

2.8 Preparation of staphylococcal whole cell lysates

Overnight cultures of *S. aureus* strain Newman *spa* and Newman *spa ebpS* were washed in PBS and resuspended to an OD_{600nm} of 100 in PBS containing protease inhibitors (40 µl/ml)(Mini-Complete, Boehringer-Mannheim) and DNase (80 µg/ml). Cell walls were digested by addition of lysostaphin (200µg/ml) and incubated at 37°C for 10 min. An equal volume of final sample buffer [0.125 M Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β–mercaptoethanol, 0.002% (w/v) bromophenol blue, pH 6.8] was added to each digest and boiled for 10 min. 10 µl of each sample was analysed by SDS-PAGE and Western immunoblotting.

2.9 Preparation of crude staphylococcal cell envelopes for LiCl and SDS treatment

100 OD_{600nm} units of an overnight culture of *S. aureus* strain Newman *spa* were washed in ice-cold Tris-buffered saline (TBS) [50 mM Tris-HCl, 0.1 M NaCl, pH 7.5]. Cells were harvested by centrifugation at 7,000×*g* for 10 min and resuspended in 1.5 ml ice-cold buffer A [TBS, 0.5M phenylmethylsulfonyl fluoride (PMSF), iodoacetamide (1 mg/ml)] containing DNase (80 µg/ml). The suspension was then placed in a blue cap FastRNA tube (Savant) and shaken in a FP120 FastPrep™ cell disrupter (Savant) at speed 6 for 40 sec. This treatment was repeated 10 times with cooling of the tube, on ice for 1 min, between each cycle. Cell lysis was confirmed by phase contrast microscopy. Another 5 ml of ice-cold buffer A was added to the lysate. This was then centrifuged at 2,000×*g* for 5 min at 4°C to sediment any remaining cells. Another 5 ml of ice-cold buffer A was added to the supernatant, which was then centrifuged at 15,000×*g* for 15 min at 4°C. The insoluble material (crude cell envelopes) was washed twice in ice-cold buffer A.

2.10 LiCl and SDS extraction of proteins from crude staphylococcal cell envelopes

Cell envelopes were resuspended in either 1 ml of 3 M LiCl or 1 ml of final sample buffer (FSB) [50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 5.6% (v/v) β-mercaptoethanol]. The LiCl treated envelopes were centrifuged at 37°C at 200 rpm for 1½ h. The insoluble material was then

pelleted by centrifugation at 13,000×*g* and the supernatant was dialysed into PBS overnight at 4°C. The insoluble pellet was washed 3 times in ice-cold buffer A [TBS, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), iodoacetamide (1mg/ml)] and then resuspended in buffer A containing lysostaphin (200 μg/ml) and incubated at 37°C for 30 min to release any proteins that remained associated with the pelleted fraction.

Similarly, SDS envelopes were boiled for 3 min and pelleted as above. The supernatant was taken as proteins released by SDS. The insoluble pellet was washed and treated with lysostaphin, as above, to release any remaining cell wall associated proteins.

2.11 Cloning and expression of EbpS, FnBPA and FnBPB in *L. lactis*

The *ebpS orf* was PCR-amplified from plasmid pKS5.2 using primers pKS80-F3 5′-GCGGGATCCTCAATAATTTTAAAGATGACTTG-3′ and pTREX-Rev 5′-GCGG GATCCTTATGGAATAACGATTTGTTG-3′ (*Bam*HI restriction site underlined). The *fnbA orf* was PCR-amplified from chromosomal DNA of *S. aureus* strain P1 using primers FA-Fwd 5′-CGCTGATCAAAAACAATCTTAGGTACGGC-3 and F-Rev 5′-CG CTGATCATTATGCTTTGTGATTCTTTTTATTTC-3′ (*Bcl*I restriction site underlined). The *fnbB orf* was PCR-amplified from chromosomal DNA of *S. aureus* strain P1 using primers FB-Fwd 5′-CGCTGATCAAAAGCAATCTTAGATACGGC-3′ and F-Rev 5′-CGCTGATCATTATGCTTTGTGATTCTTTTTATTTC-3′ (*Bcl*I restriction site underlined). PCR was performed using *pfu* polymerase (Promega). PCR conditions were 30 cycles of 94°C for 1 min, 49-55°C for 1 min and 72°C for 3-7 min. The PCR products were cloned into the *Bcl*I site of the *L. lactis* expression vector pKS80 (Hartford *et al.*, 2001b). Due to the nature of the *Bcl*I site the second residue (serine) of EbpS had to be substituted with two residues, isoleucine and leucine. This changed the N terminus of EbpS expressed *in L. lactis* from MSNNFK to MILNNFK. The N terminus of FnBPA expressed in *L. lactis* was changed from MKNNL to MIKNNL and the N terminus of FnBPB was changed from MKSNL to MIKSNL. *L. lactis* strains MG1363 (pKS80-*clfA*$^{+}$) and MG1363 (pKS80-*sdrC*$^{+}$) were supplied by Dr. L. O'Brien, Trinity College Dublin.

2.12 Cell fractionation

2.12.1 Fractionation of *S. aureus* and *Lactococcus lactis* (Stabilised protoplasts)

50 ml of *S. aureus* stationary phase cultures were pelleted by centrifugation at 7,000×*g* and washed with 10 ml of PBS. The cell suspensions were adjusted to 100 OD_{600nm} units and washed again in PBS. Cells were then resuspended in 1 ml digestion buffer [50 mM Tris-HCl, 20 mM $MgCl_2$, 30% (w/v) raffinose, pH 7.5]. Cell wall proteins were released from stabilised protoplasts by digestion with lysostaphin (200 μg/ml) for 30 min with occasional shaking in the presence of protease inhibitors (40 μl/ml) (Mini-Complete protease inhibitors, Boehringer-Mannheim). Protoplasts were then harvested by centrifugation at 6,000×*g* for 20 min. The supernatant was taken as the cell wall fraction. The protoplasts were resuspended in 1ml 50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5 with 40 μl/ml protease inhibitors. An equivalent of 1 OD_{600nm} unit of each fraction was analysed by SDS-PAGE and Western immunoblotting.

For complete fractionation of *S. aureus* Newman *spa* stationary phase (OD_{600nm} 12) or late exponential phase (OD_{600nm} 1.5) cultures protoplasts were further fractionated into membrane and cytoplasmic fractions. Protoplasts were washed once in digestion buffer and resuspended in ice-cold lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5) with protease inhibitors and DNase (80 μg/ml). Protoplasts were lysed on ice by vortexing and repeated pipetting. Complete lysis was confirmed by phase contrast microscopy. Membrane and cytoplasmic fractions were separated by centrifugation at 4°C for 1 h at 40,000×*g*. The supernatant (cytoplasmic fraction) was removed and the pellet (membrane fraction) was washed with ice-cold lysis buffer. Both fractions were centrifuged again under the same conditions. The membrane pellet was finally resuspended in lysis buffer. Membrane and cytoplasmic fractions were assayed for isocitrate dehydrogenase activity (Ells, 1959).

To obtain concentrated extracellular proteins 10 ml of the culture supernatant was filtered through a 0.45 μm filter. Proteins were precipitated from the media by addition of a 1:20 volume of ice-cold 100% (w/v) trichloroacetic acid (TCA). The mixture was left on ice for 30 min. Precipitated proteins were pelleted by centrifugation at 20,000×*g* for 15 min at 4°C. The pellet was washed once with ice-cold acetone and pelleted as above.

The resulting pellet was air dried and resuspended in 1 ml of final sample buffer. An equivalent of 1 OD_{600nm} unit of each fraction was analysed by SDS-PAGE and Western immunoblotting.

Similarly, 50 ml of stationary phase *L. lactis* cultures expressing EbpS, ClfA, SdrC, FnBPA or FnBPB were pelleted by centrifugation and washed in 10 ml of M17 broth. Cell suspensions were adjusted to 40 OD_{600nm} units pelleted and washed in 1 ml of sterile distilled water containing EDTA (5 mM) and protease inhibitors (75µl). Cells were then resuspended in 1 ml of 26% (w/v) raffinose prepared in 50 mM Tris-HCl, 20 mM $MgCl_2$, pH 8.0. Cell wall proteins were released by digestion with mutanolysin (500 U/ml) (Sigma) and lysozyme (200 µg/ml) (Sigma) for 15 min at 37°C with occasional shaking in the presence of protease inhibitors (75 µl/ml). Protoplasts were harvested at 6,000×*g* for 20 min. The supernatant was taken as the cell wall fraction. Protoplasts were resuspended in 1ml of 50 mM Tris-HCl, 20 mM $MgCl_2$, pH 8.0. A TCA precipitate of each cultures media was prepared as above. The equilavent of 0.4 OD_{600nm} units of each fraction was analysed by SDS-PAGE and Western immunoblotting.

2.12.2 Mechanical disintegration

Staphylococcal cytoplasmic membranes were also prepared by cell disruption. A stationary phase culture of strain Newman *spa* was adjusted to 150 OD_{600} units and washed in PBS. Cells were resuspended in 1.5 ml of lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5) containing protease inhibitors (40 µl/ml) (Mini-Complete, Boehringer-Mannheim) and DNase (80 µg/ml). The cell suspension was transferred to a blue cap FastRNA tube and shaken in a FP120 Fastprep™ cell disrupter (Savant) at speed 6 for 40 sec. This was repeated 10 times with cooling on ice for 1 min between cycles. Cell lysis was monitored by phase contrast microscopy. Lysates were then centrifuged at 4°C for 2 min at 3,000g to pellet any remaining whole cells. The supernatant was centrifuged at 4°C for 10 min at 15,000×*g* to sediment cell walls. The soluble fraction, containing cytoplasmic membranes and cytoplasm, was removed to a fresh tube and centrifuged again under the same conditions to remove any remaining cell wall contamination. The resulting supernatant was then centrifuged at 4°C for 1 h at 40,000×*g*

to sediment cytoplasmic membranes. The membrane pellet was washed in ice-cold lysis buffer, pelleted again under the same conditions and finally resuspended in 1ml of lysis buffer.

2.13 Cloning and expression of EbpS from plasmid pKK233-2

The *ebpS orf* was PCR-amplified from plasmid pKS5.2 using primers Top-F1 5′-CATGCCATGGCTAATAATTTTAAAGATGACTTTG-3′ and RD-R1 5′-CCCAAGCTTTTATGGAATAACGATTTGTTG-3′ with *pfu* polymerase (Promega). PCR conditions were 30 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 3 min. The forward primer incorporated a *Nco*I restriction site (underlined) and the reverse primer incorporated a *Hin*dIII restriction site (underlined). The nature of the *Nco*I restiction site (CCATGG) demanded that the first base of the second codon must be guanine. To accommodate this the forward primer designed to clone *ebpS* into the *Nco*I site changed the N terminus of the resulting EbpS protein from MSNNFKD to MANNFKD. The PCR product was cloned into the *Nco*I/*Hin*dIII restriction sites of plasmid pKK233-2 and transformed into *E. coli* strain XL1-blue.

Strain XL1-blue expressing EbpS was grown to an OD_{600nm} of 0.4 in 20 ml of Luria broth containing 100 μg/ml of Ap at 37°C at 200 rpm. Cultures were then induced with 0.1 mM, 0.5 mM or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for a further 2 h under the same growth conditions. One culture was left uninduced. A culture of XL1-blue containing the empty vector pKK233-2 was induced with 1 mM IPTG. 0.3 OD_{600nm} units of each culture was pelleted by centrifugation at 14,000×*g* for 5 min and resuspended in 20 μl of PBS before boiling in FSB and Western immunoblotting analysis.

2.14 Fractionation of *E. coli* into inner and outer membrane fractions

E. coli strain XL1-blue expressing EbpS was grown to an OD_{600nm} of 0.5 in Luria broth containing 100 μg/ml of Ap at 37°C at 200 rpm. Cells were pelleted by centrifugation at 6,000×*g* for 15 min. To form sphaeroplasts cells were resuspended in 4 ml of ice-cold Tris-HCl pH 8.0. An equal volume of ice-cold sucrose (1 M) made up in

200 mM Tris-HCl (pH 8.0) was added to the suspension and Na_2EDTA (pH 7.5) was added to a final concentration of 500 μM. 140 μl of lysozyme solution (12 mg/ml lysozyme, 0.5 M sucrose, 100 mM Tris-HCl, pH 8.0) was added and the suspension was incubated on ice for 5 min. DNase and RNase were added to final concentrations of 50 μg/ml and the suspension was incubated at room temperature for 30 min. Sphaeroplasts were lysed by one pass through a French press (SIM-Aminco Spectronic Instruments) at 1,000 psi. The lysate was collected in 4×volumes ice-cold dH_2O. Whole cells were removed by centrifugation at 3,000×*g* for 15 min. The total membrane fraction was harvested by centrifugation at 40,000×*g* for 90 min at 4°C. The pellet was washed once in Wash buffer (50 mM sucrose, 3.3 mM Tris-acetate, 1 mM EDTA, pH 7.8) and pelleted under the same conditions. The membrane pellet was resuspended in 2.5 ml of 30% (w/w) sucrose made up in 5 mM EDTA (pH 7.5). A sucrose gradient was produced using sucrose solutions of 52.5, 50, 47.5, 45,42.5, 40, 37.5 and 35% (w/w) made up in 5 mM EDTA (pH 7.5). The membrane solution was centrifuged through the sucrose gradient at 218,000×*g* for 44 h in a Beckman L8 ultracentrifuge. The gradient was tapped and separated into 24 fractions. Each fraction was measured at A_{280} and fractions containing inner or outer membranes were determined. Membrane containing fractions were centrifuged at 40,000×*g* for 90 min at 4°C and the pellets resuspended in equal volumes 50 mM Tris-HCl (pH 7.5). Equal volumes of each fraction were analysed by SDS-PAGE and Western immunoblotting with anti-rEbpS$_{343-486}$ antibodies.

2.15 Construction of *ebpS-phoA* and *ebpS-lacZ* fusions

5′ truncates of *ebpS* incorporating 263 bp of sequence upstream of the ATG start codon of plasmid pKK233-2 were PCR-amplified from plasmid pKK233-*ebpS* using the same forward primer LacZ/PhoA-Fwd and the relevant reverse primers (Table 2.4) using *pfu* polymerase (Promega). PCR conditions were 30 cycles of 94°C for 1 min, 50-53°C for 1 min and 72°C for 3 min. The PCR products were amplified incorporating an *Eco*RI restriction site at the 5′ end and a *Sal*I restriction site at the 3′ end and cloned into the *phoA* vector pBAF (Fulkerson and Mobley, 2001) and the *lacZ* vector pLKC480 (Tiedeman and Smith, 1988) to produce in-frame *ebpS-phoA* and *ebpS-lacZ* fusions,

Table 2.4 List of primers used to construct *ebpS-phoA* and *ebpS-lacZ* fusions.

Primer Name	Primer Sequence
LacZ/PhoA-Fwd	5′-CCGGAATTCATGACAGCTTATCATCGACTG-3′
A234-Rev	5′-ACGCGTCGACCCAGCCTCATTTGAATGTTTC-3′
T247-Rev	5′-ACGCGTCGACCCAGTCGAGTTATTCGCCTTG-3′
S256-Rev	5′-ACGCGTCGACCCAGACTTATCTTGAGACGC-3′
S285-Rev	5′-ACGCGTCGACCCAGAAGCACTTTTACTTGCTG-3′
A296-Rev	5′-ACGCGTCGACCCTGCATTATAGAGGCATGTG-3′
H306-Rev	5′-ACGCGTCGACCCATGATTGTCATGTTCATCATG-3′
D369-Rev	5′-ACGCGTCGACCCGTCTTTGTCTTTACTTTCATC-3′
D402-Rev	5′-ACGCGTCGACCCATCATTATCAGATTCATCTTTAG-3′

- The following restriction sites are underlined: *Eco*RI GAATTC, *Sal*I GTCGAC.

respectively. Positive clones were selected in competent *E. coli* strain XL1-blue and then transformed into *E. coli* strain CC118 (Manoil and Beckwith, 1986) (*ebpS-phoA* fusions) or *E. coli* strain TG1 (Sambrook *et al.*, 1989) (pDIA17) (*ebpS-lacZ* fusions). pDIA17 is a derivative of pACYC184 with the *lacI* gene cloned into the *Bam*HI site allowing expression from the *tet* promoter (P. Bertin, Institute Pasteur, personal communication).

2.16 Assays for alkaline phosphatase and β-galactosidase

Alkaline phosphatase (AP) activity was initially tested on L agar plates incorporating 100 μg/ml Ap, 1 mM IPTG and 40 μg/ml of the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate (Sigma). β-galactosidase activities were detected on L agar incorporating 100 μg/ml Ap, 25 μg/ml Cm, 2 mM IPTG and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Melford Laboratories). To quantify AP activity the strain CC118 harbouring pBAF*ebpS-phoA* fusions were grown to OD_{600nm} of 0.4 at 30°C with aeration in L broth containing 100 μg/ml Ap and 0.4% (w/v) glucose. Similarly, to quantify β-galactosidase activity, strain TG1 (pDIA17) also harbouring plasmid pLKC480*ebpS-lacZ* fusions were grown in Luria broth incorporating 100 μg/ml Ap and 25 μg/ml Cm. IPTG was added to a final concentration of 3 mM (*phoA* fusions) or 2 mM (*lacZ* fusions) and cultures were grown for a further 4 h. AP activity was assayed by the method of Brickman and Beckwith (1975) with the addition of iodoacetamide (4 mg/ml) to the Tris-HCl buffer pH 8.0 used to resuspend cells. LacZ activity was measured by the method of Miller (1972).

2.17 Preparation of membrane fraction from *E. coli* strains expressing EbpS-PhoA or EbpS-LacZ fusion proteins

E. coli strains harbouring *ebpS-phoA* and *ebpS-LacZ* fusions were grown and induced as described in section 2.16. Cells were pelleted by centrifugation at 6,000×*g* for 10 min and resuspended in PBS with 10 mM $MgCl_2$, Mini-Complete Protease inhibitors (40 μl/ml) and lysozyme (200 μg/ml) to an OD_{600nm} of 10. Samples were then transferred to a blue cap Fast RNA tube and shaken in a FP120 FastPrep™ cell disrupter (Savant) at speed 4.5 for 40 seconds. This was repeated five times with cooling on ice between

cycles. The lysates were left on ice for 10 min and the supernatants were spun at 4°C for 1 h at 40,000×*g*. The membrane pellets were washed in PBS containing 10 mM $MgCl_2$ and pelleted, under the same conditions, and finally resuspended in PBS. Equal volumes of each sample were analysed by SDS-PAGE and Western immunoblotting.

2.18 Absorption of antibodies with staphylococcal protoplasts

Stabilised protoplasts were prepared from strains Newman *spa ebpS* and Newman *spa ebpS* (pCU-*ebpS*$^+$) grown to exponential phase (OD_{600} = 1.5). Protoplasts were washed once in digestion buffer [50 mM Tris-HCl, 20 mM $MgCl_2$, 30% (w/v) raffinose, pH 7.5], recovered by centrifugation at 6,000×*g* for 20 min and resuspended in digestion buffer containing a 1:5000 dilution of anti-$ClfA_{220-559}$ antibodies. Samples were left to incubate at room temperature for 15 min to saturate an uncharacterised membrane associated immunoglobulin binding activity of *S. aureus*. Protoplasts were washed again in digestion buffer and resuspended in the same buffer to an OD_{600nm} of 100. Doubling dilutions of protoplasts were incubated to a final volume of 400 µl of digestion buffer containing protease inhibitors (Mini-Complete, Boehringer-Mannheim) and either a 1:32,000 dilution of anti-$rEbpS_{343-486}$ antibodies or a 1:128,000 dilution of anti-$rEbpS_{1-267}$ antibodies. Samples were incubated at room temperature for 30 min. Protoplasts were then pelleted at 6,000×*g* for 20 min. Protoplasts were observed by phase contrast microscopy after every wash and incubation step to ensure that no significant lysis had occurred. Supernatants containing unabsorbed antibodies were used to detect immobilised $rEbpS_{1-267}$ or $rEbpS_{343-486}$ by dot immunoblotting.

1 µg of $rEbpS_{1-267}$ or $rEbpS_{343-486}$ was transferred to polyvinylidene difluoride (PVDF) Western immunoblotting membrane (Boehringer-Mannheim) using the Bio-Rad dot blot apparatus. Each well was incubated for 30 min at room temperature in blocking reagent [10 mM Tris-HCl, 0.9% (w/v) NaCl, 5% (w/v) skimmed milk (Marvel), pH 7.4]. Supernatants from antibody absorption with doubling dilutions of staphylococcal protoplasts were incubated in relevant wells of the dot blot apparatus for 30 min. After incubation the PVDF membrane was removed from the apparatus and washed three times for 10 min in wash buffer [10 mM Tris-HCl, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20, pH

7.4]. Goat anti-rabbit antibodies conjugated to horseradish peroxidase (Dako) were used at a 1:2,000 dilution to detect bound antibodies using the LumiGLO chemiluminescence substrate (New England Biolabs).

2.19 Cross-linking studies

Cytoplasmic membranes from cells of a stationary phase culture of *S. aureus* strain Newman *spa* were prepared as described in section 2.12.2 with the exception that the membrane pellet was finally resuspended in 200 mM triethanolamine. The protein concentration of the membrane sample was determined using the Bio-Rad Protein Assay kit according to the manufacturers instructions. A stock solution of dithiobis(succinimidyl proprionate) (DSP) was prepared at a concentration of 1mg/ml in dimethyl sulfoxide (DMSO). The membrane sample and DSP were mixed to a concentration of protein to DSP of 50:1 and made up to a final volume of 100 µl with triethanolamine. The mixture was then incubated at 25°C for 1 min and the reaction was quenched by the addition of Tris-HCl (pH 8.0) to a final concentration of 200 mM. The sample was then boiled for 10 min in non-reducing FSB (without β-mercaptoethanol) before SDS-PAGE and Western immunoblotting analysis with anti-rEbpS$_{343-486}$ antibodies. As a control membranes were treated in exactly the same way without DSP.

2.20 Preparation of chemically defined medium

The constituents of chemically defined media (CDM) are listed in Table 2.5. Each group was made up separately. Group 1 was dissolved in 700 ml of distilled water. Groups 3, 4 and 5 were made up in distilled water as ×100, ×20 and ×100 stock solutions, respectively. Groups 1, 3, 4 and 5 were mixed together, made up to 900 ml with distilled water and autoclaved. Group 2 was dissolved in 100 ml of distilled water, autoclaved and added to the rest of the media after cooling. Final pH was adjusted to 7.2.

2.21 Competition growth experiments

S. aureus strains were grown at 37°C for 18 h in 3 ml of CDM in a test tube in an orbital shaking incubator at 200 rpm. 3 ml of fresh CDM was inoculated to an OD_{600nm} of 0.03 with each strain and grown under the same conditions for 24 h. At time points of 2

Table 2.5 Composition of chemically defined medium (CDM).

Ingredients	mg/L	Ingredients	mg/L
Group 1		*Group 2*	
$Na_2HPO_4 \cdot 2H_2O$	10000	Glucose	10000
KH_2PO_4	3000	$MgSO_4 \cdot 7H_2O$	500
L-Aspartic acid	150		
L-Alanine	100	*Group 3*	
L-Arginine	100	Biotin	0.1
L-Cystine	50	Nicotinic acid	2
Glycine	100	D-Pantothenic acid, Ca salt	2
L-Glutamic acid	150	Pyridoxal	4
L-Histidine	100	Pyridoxamine dihydrochloride	4
L-Isoleucine	150	Riboflavin	2
L-Lysine	100	Thiamin hydrochloride	2
L-Leucine	150		
L-Methionine	100	*Group 4*	
L-Phenylalanine	100	Adenine sulphate	20
L-Proline	150	Guanine hydrochloride	20
L-Serine	100		
L-Threonine	150	*Group 5*	
L-Tryptophan	100	$CaCl_2 \cdot 6H_2O$	10
L-Tyrosine	100	$MnSO_4$	5
L-Valine	150	$(NH_4)_2SO_4$	6
		$FeSO_4$	6

h, 8 h and 24 h a sample of the culture was diluted in sterile phosphate buffered saline (PBS) and plated on TSA and the total number of colony forming units (CFU) in the culture was determined. Plates were then replica plated onto TSA containing Em (10 μg/ml). The number of Em resistant colonies was then expressed as a percentage of the total CFU.

2.22 Bacterial dry weight determination

S. aureus strains were grown at 37°C for 18 h in 20 ml of CDM in a 250-ml conical flask in an orbital shaking incubator at 200 rpm. Cells from an 8-ml volume of culture were washed in 50 mM Tris-HCl (pH 7.5) and twice in distilled water before being transferred to pre-weighed glass tubes. The cells were dried in an oven at 105°C for 24 h. Then the tubes were weighed again and the dry weight of the cells obtained by subtracting the weight of the tube.

2.23 Cloning of *ebpS* in plasmid pCL84 and production of truncated derivatives

The *ebpS orf* was PCR-amplified from plasmid pKS5.2 with 300 bp of upstream sequence and 200 bp of downstream sequence using primers Comp-Fwd and Comp2-Rev with *pfu* polymerase (Promega). PCR conditions were 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 4 min. The resulting PCR-product was cloned into the *Eco*RI restriction site of plasmid pCL84 (Lee *et al.* 1991).

To produce truncated derivatives of the *ebpS* gene on plasmid pCL84, PCR was performed from plasmid pKS5.2 between primers Comp-Fwd and a reverse primer complementary to sequence within the *ebpS* coding sequence incorporating a *Bam*HI restriction site and between Comp2-Rev and a forward primer complementary to sequence within the *ebpS* coding sequence incorporating a *Bam*HI restriction site (Table 2.6) using *pfu* polymerase. PCR conditions were 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 4 min.

Plasmid pCL84 derivatives were electroporated into *S. aureus* strain CYL316 and integrants were selected on TSA incorporating 3 μg/ml Tc. The integrated plasmids were then transduced into strain 8325-4 *ebpS*. Transductants were grown on TSA incorporating 5% (v/v) egg yolk (Oxoid) to check for loss of lipase activity.

Table 2.6 Primers used to produce *ebpS* truncated derivatives in plasmid pCL84.

Primer	Primer Sequence	Protein
Comp-Fwd	5′-CCGGAATTCGGATCAATGTAGACGGAAG-3′	
Comp2-Rev	5′-CGCGAATTCGACAAGACCTTGTTAAATAAC-3′	
EBD-Fwd	5′-CGCGGATCCTTAGAACATCAGGATACAATAG-3′	$EbpS_{\Delta 14-34}$
EBD-Rev	5′-CGCGGATCCACGATTTTTTTCAAAGTCATC-3′	
SKD-Fwd	5′-CGCGGATCCCAAGACAAAGCGACTAAAG-	$EbpS_{\Delta 365-389}$
SKD-Rev	5′-CGCGGATCCTTCATCAGCATTATTTTTATTTG-3′	
QN-Fwd	5′-CGCGGATCCCGTCAAGGTGGTGGCC-3′	$EbpS_{\Delta 402-431}$
QN-Rev	5′-CGCGGATCCATCATTATCAGATTCATCTTTAG-3′	
LysM-Fwd	5′-CGCGGATCCGGTCAACAAATCGTTATTCC-3′	$EbpS_{\Delta 440-479}$
LysM-Rev	5′-CGCGGATCCTCTTTGGCCACCACCTTG-3′	

- The following restriction sites are underlined: *Eco*RI GAATTC, *Bam*HI GGATCC.

2.24 Adhesion assay to immobilised elastin peptides

The procedure to coat the wells of microtitre plates with elastin peptides is derived from the methods used by Hinek *et al.* (1999) and Roche (2000). To coat the microtitre plate wells (Povair) κ-elastin from bovine *ligamentum nuchae*, human aortic elastin or human lung elastin (Elastin Products Company) was dissolved in coating buffer (0.1M sodium bicarbonate, pH 9.4) and added at a 50-µl volume to the wells at a range of concentrations. The elastin peptides were air dried under UV light (366 nm) at room temperature for 18 h. Human fibronectin (Calbiochem) was coated onto the wells of microtitre plates by dissolving in coating buffer and incubating at 4°C for 18 h. Control wells contained only coating buffer. Wells were then blocked for 2 h at room temperature with 5% (w/v) bovine serum albumin (BSA) made up in PBS that had been filtered through a 0.45 µm filter (Gelman Laboratories). *S. aureus* cultures were grown at 37°C to an OD_{600nm} of 0.5 (early exponential phase) or 12 (stationary phase) in 20 ml of TSB at 200 rpm. Cells were then washed in PBS and resuspended to an OD_{600nm} of 1 (1×10^8 CFU/ml). Bacterial cell adherence was measured using a nucleic acid binding probe, SYTO-13, (Molecular Probes). SYTO-13 was supplied by the manufacturer as a 5 mM solution in dimethyl sulfoxide, which was diluted 1:10 in 10 mM Tris-HCl (pH 7.5) to give a working solution of 0.5 mM. Washed bacterial cultures were incubated with SYTO-13 at a final concentration of 2.5 µM at room temperature for 15 min in the dark. Elastin coated wells of the micotitre plates were washed three times with PBS and 100 µl of stained cells were added to each well and incubated in the dark at room temperature for 1½ h. After incubation, wells were washed three times with PBS and the amount of cells bound was determined using a LS-50B spectrometer with excitation at 488 nm and emission at 509 nm.

2.25 Aggregation of *S. aureus* in the presence of elastin peptides

Overnight cultures of *S. aureus* strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) were diluted to an OD_{600nm} of 0.03 into 3 ml of fresh TSB, TSB containing 50 µg/ml κ-elastin from bovine *ligamentum nuchae* and TSB containing 50 µg/ml BSA (filtered) in 30-ml sterilin tubes with the caps loose (high aeration). Cultures were grown at 37°C at 200 rpm and observed at regular intervals for

the formation of macroscopic clumps. Upon the formation of clumps the optical densities of the cultures were read at 600 nm.

2.26 ELISA

To coat the microtitre plate wells (Nunc-Immuno™ plate MaxiSorb™ surface, Nalge Nunc Int.) human aortic elastin (Elastin Products Company) was dissolved in coating buffer (0.1 M sodium bicarbonate, pH 9.4) and added at a 50 µl volume to the wells at a range of loadings (1 µg-0.001 µg). The elastin peptides were air dried under UV light (366 nm) at room temperature for 18 h. Wells were then blocked for 2 h at room temperature with 5% (w/v) bovine serum albumin (BSA) made up in PBS that had been filtered through a 0.45 µm filter (Gelman Laboratories). Plates were washed three times with wash buffer (PBS, 1% (w/v) BSA, 0.1% (v/v) Tween 20). Rabbit polyclonal anti-human aortic antibodies (Elastin Products Company) were diluted to 1:2,000 in wash buffer and incubated for 1 h at room temperature. A control plate was incubated with the same dilution of normal rabbit serum. Plates were washed three times with wash buffer and incubated with a 1:2,000 dilution of goat anti-rabbit-HRP antibodies (Dako) for 30 min. After three further washes with wash buffer bound HRP-conjugated antibodies were detected by the addition of 100 µl of 3,3′, 5,5′-tetramethylbenzidine (0.1 mg/ml) (Sigma) prepared in 0.05 M phosphate-citrate buffer containing 0.006% (v/v) hydrogen peroxide. Plates were then incubated at room temperature for 10 min. The reaction was stopped by the addition of 20 µl of 1 M H_2SO_4 to each well. Plates were read at 450 nm using an ELISA plate reader (Multiskan EX, Labsystems).

2.27 Inhibition of bacterial adherence to immobilised elastin with antibodies

Human aortic elastin (Elastin Products Company) was dissolved in coating buffer (0.1M sodium bicarbonate, pH 9.4) and added at a 50-µl volume to the wells of a microtitre plate (Povair) at a concentration of 10 µg/ml (0.5 µg/well). The elastin peptides were air dried under UV light (366 nm) at room temperature for 18 h. Wells were then blocked for 2 h at room temperature with 5% (w/v) bovine serum albumin (BSA) made up in PBS that had been filtered through a 0.45 µm filter (Gelman Laboratories). A culture of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4$^+$) was grown to early

exponential phase (OD_{600nm} of 0.5) in 20 ml of TSB containing 10 μg/ml of Cm at 37°C at 200 rpm. Cells were then washed in PBS and resuspended to an OD_{600nm} of 1 (1×10^8 CFU/ml) in PBS containing a 1:100 dilution of normal rabbit serum and incubated at room temperature for 15 min. Cells were then diluted with an equal volume of one quarter dilutions of anti-rFnBPA$_{37-605}$, anti-ClfA$_{220-559}$ or anti-rEbpS$_{1-267}$ antibodies (10μg/ml-0.15ng/ml) and incubated at room temperature for 1 h. Cells were incubated with SYTO-13 at a final concentration of 2.5 μM at room temperature for 15 min in the dark. Elastin coated wells of the micotitre plates were washed three times with PBS and 100 μl of stained cells were added to the wells and incubated in the dark at room temperature for 1½ h. After incubation, wells were washed three times with PBS and the amount of cells bound was determined using a LS-50B spectrometer with excitation at 488nm and emission at 509 nm. Figures were expressed as a percentage of the fluorescence of bound cells that were not treated with antibody.

2.28 Effect of soluble fibronectin on the adherence of *S. aureus* to immobilised fibronectin and elastin

Human aortic elastin (Elastin Products Company) was dissolved in coating buffer (0.1 M sodium bicarbonate, pH 9.4) and added at a 50-μl volume to the wells of a microtitre plate (Povair) at a concentration of 10 μg/ml (0.5 μg/well). The elastin peptides were air dried under UV light (366 nm) at room temperature for 18 h. Human fibronectin (Calbiochem) was coated onto the wells of microtitre plates (Povair) (100 μl volume) by dissolving in coating buffer at a concentration of 5 μg/ml (0.5 μg/well) and incubating at 4°C for 18 h. Wells were then blocked for 2 h at room temperature with 5% (w/v) bovine serum albumin (BSA) made up in PBS that had been filtered through a 0.45-μm filter (Gelman Laboratories). A culture of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4$^+$) was grown to early exponential phase (OD_{600nm} of 0.5) in 20 ml of TSB containing 10 μg/ml of Cm at 37°C at 200 rpm. Cells were then washed in PBS and resuspended in PBS containing 100 μg/ml of human fibronectin (Calbiochem) and incubated at room temperature for 1 h. Cells were incubated with SYTO-13 at a final concentration of 2.5 μM at room temperature for 15 min in the dark. Elastin and

fibronectin coated wells of the micotitre plates were washed three times with PBS and 100 µl of stained cells were added to the wells and incubated in the dark at room temperature for 1½ h. After incubation, wells were washed three times with PBS and the amount of cells bound was determined using a LS-50B spectrometer with excitation at 488 nm and emission at 509 nm. Figures were expressed as a percentage of the fluorescence of bound cells that were not treated with inhibitor.

2.29 Inhibition of bacterial adherence to immobilised elastin with recombinant proteins

Human aortic elastin peptides were coated onto the wells of a microtitre plate (Povair) and blocked with BSA as described in section 2.24. Wells were washed three times with PBS and 50µl volumes of doubling dilutions of $rFnBPA_{37-605}$, $rFnBPA_{745-878}$, $rFnBPB_{37-540}$ or recombinant protein A (Sigma) were added to the wells (60 µM-0.2 µM) and incubated static at room temperature for 30 min. A culture of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4^{+}) was grown to early exponential phase (OD_{600nm} of 0.5) in 20 ml of TSB containing 10 µg/ml of Cm at 37°C at 200 rpm. Cells were then washed in PBS and resuspended to an OD_{600nm} of 1 (1×10^{8} CFU/ml) in PBS. Cells were incubated with SYTO-13 at a final concentration of 2.5 µM at room temperature for 15 min in the dark. 50-µl volumes of stained cells were added to each well and the plates were incubated with slow shaking at room temperature for 1 h. After incubation, wells were washed three times with PBS and the amount of cells bound was determined using a LS-50B spectrometer with excitation at 488 nm and emission at 509 nm. Figures were expressed as a percentage of the fluorescence of bound cells that were not treated with inhibitor.

Chapter 3

EbpS is associated with the cytoplasmic membrane of *Staphylococcus aureus*

3.1 Introduction

The elastin-binding protein of *Staphylococcus aureus* was originally reported to be a surface-associated 25 kDa protein encoded by a gene of 606 bp (Park *et al.*, 1996). Subsequent investigations by Dr. F. Roche prior to the current study showed that there was a DNA sequencing error in the original study by Park *et al.* and that EbpS is encoded by a 1,461 bp gene and has a predicted molecular mass of 53.08 kDa (Roche, 2000). At the onset of the current project the EbpS protein had not been unambiguously identified in *S. aureus*. In order to identify the EbpS protein in *S. aureus* it was necessary to generate an *ebpS* null mutant by allelic replacement (Roche, 2000) and to obtain specific anti-EbpS antibodies that did not cross-react with other *S. aureus* envelope-associated proteins. To identify the EbpS protein in *S. aureus* by Western immunoblotting, antibodies were raised against two recombinant proteins comprising either the N-terminal or C-terminal domains of EbpS.

The 25 kDa EbpS truncate described by Park *et al.* (1996) was released from the staphylococcal cell surface by treatment with the muralytic enzyme lysostaphin. This suggested that EbpS was in some way associated with the cell wall peptidoglycan since lysostaphin is an enzyme that specifically degrades the cell wall polymer and releases any cell-wall-associated proteins (Schneewind *et al.*, 1992), but this was not conclusively demonstrated. The predicted primary sequence of the EbpS protein revealed the absence of a N-terminal secretory signal sequence or C-terminal sorting signals associated with cell-wall-associated proteins that are covalently anchored to peptidoglycan (Roche, 2000). The elastin-binding domain of EbpS was reported to span a 21-residue region at the N terminus between residues 14 and 34 (Park *et al.*, 1999). The protein possesses three centrally located hydrophobic domains, designated H1 (residues 205–224), H2 (residues 265–280) and H3 (residues 315–342), each of which are theoretically large enough to span the cytoplasmic membrane, a characteristic usually associated with integral membrane proteins and not those that are cell-wall-associated. At the extreme C terminus of EbpS is a 50-residue domain that shares homology with the LysM motif of cell wall hydrolases of other bacterial species (Roche, 2000). This is a domain that is

generally regarded to promote binding to cell wall peptidoglycan although this has not been formally demonstrated (Joris *et al.*, 1992).

Thus another objective of this project was to determine the cellular localisation of EbpS and to determine the mechanism of secretion and anchoring to the cell envelope in the absence of a secretory signal sequence or a cell wall anchoring motif. This was achieved using Western immunoblotting analysis of purifed *S. aureus* envelope fractions treated with agents that solubilise biological membranes and release non-covalently cell-wall-associated proteins, such as lithium chloride and sodium dodecyl sulphate. To reveal the cellular location of EbpS, Western immunoblotting of proteins from purified cellular fractions was performed. Fractionation was performed on *S. aureus* and *Lactococcus lactis* expressing EbpS by the stabilised protoplast method whereby cell-wall-associated proteins were released from the cell surface by enzymatic digestion of the cell wall peptidoglycan while maintaining an osmotically stable protoplast. In addition, purified membrane fractions of *S. aureus* were prepared by mechanically smashing the cells and separating fractions by differential centrifugation.

3.2 Results

3.2.1 Production of polyclonal antibodies to the N and C termini of EbpS

In previous studies polyclonal antibodies were produced to the N-terminal 202-residues of what is now known to be a 486-residue EbpS protein (Park *et al.*, 1996). These antibodies reacted very weakly with EbpS and were heavily contaminated with antibodies against other surface proteins of *S. aureus* (data not shown). In order to unambiguously identify EbpS in *S. aureus*, polyclonal antibodies were produced to the N-terminal residues 1–267 and to the C-terminal residues 343–486 of the protein (Figure 3.1). The relevant regions of the *ebpS* gene were PCR-amplified and cloned into the 6×histidine expression vector pQE-30 and expressed in *E. coli* strain M15. The recombinant proteins, $rEbpS_{1-267}$ and $rEbpS_{343-486}$, expressed from plasmid pQE-30 possessed six histidine residues at their N termini. The six histidine residues bind with high affinity to nickel which facilitates the purification of recombinant proteins from *E. coli* lysates by nickel-chelate affinity chromatography. $rEbpS_{1-267}$ was purified under denaturing conditions because it formed inclusion bodies in the *E. coli* cytoplasm (F. Roche, personal communication). However, $rEbpS_{343-486}$ was soluble and was purified by standard methods. Purified proteins, $rEbpS_{1-267}$ and $rEbpS_{343-486}$ (5μg), were run on an SDS-PAGE gel and stained with Coomassie blue protein stain (Figure 3.2). The $rEbpS_{1-267}$ protein migrated with an apparent molecular mass of approximately 57 kDa and there was some evidence of a slightly lower molecular weight protein of around 50 kDa that may have been a proteolytic degradation product of $rEbpS_{1-267}$ or a contaminating *E. coli* protein (Figure 3.2, lane A). The $rEbpS_{343-486}$ protein migrated with an apparent molecular mass of approximately 25 kDa with a large amount of what appeared to be proteolytic degradation products ranging in size between 10 and 25 kDa (Figure 3.2 lane B). Purified $rEbpS_{1-267}$ and $rEbpS_{343-486}$ proteins were used to immunize young rabbits whose preimmune sera lacked significant amounts of anti-*S. aureus* antibodies and the immunoglobulin fractions were purified. The anti-$rEbpS_{343-486}$ antibodies required further purification by affinity chromatography using $rEbpS_{343-486}$ coupled to sepharose because the purified postimmune immunoglobulin fraction was

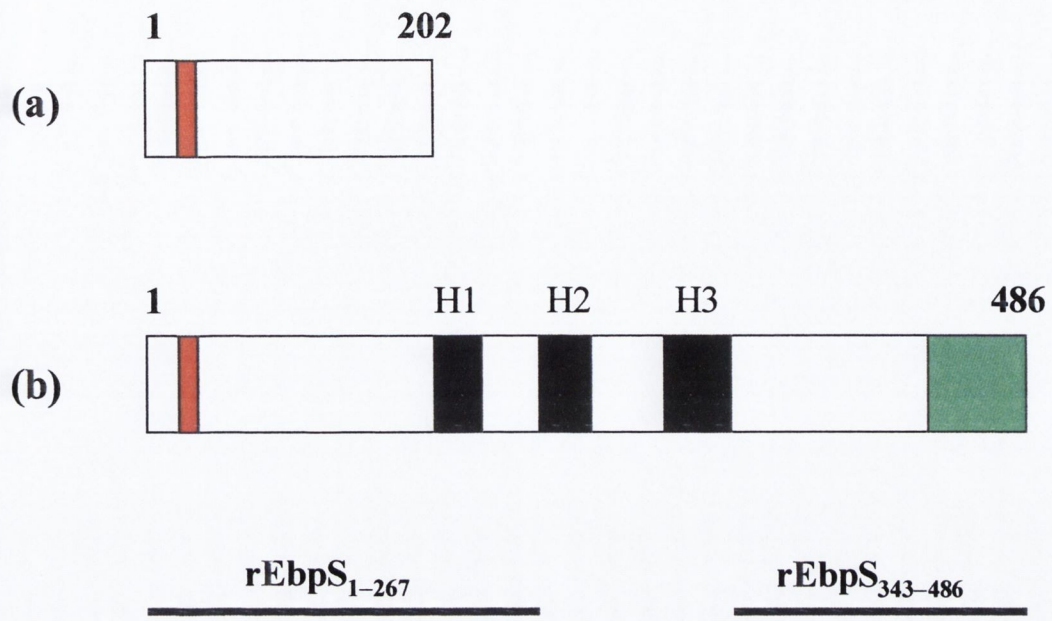


Figure 3.1 (a) Primary structure of the 202-residue EbpS protein encoded by a 606 bp gene from strain Cowan reported by Park *et al.* (1996) with the elastin binding domain between residues 14 and 34 ( ). (b) Primary structure of the full length 486-residue elastin-binding protein of strain 8325-4 (Roche, 2000) with the elastin binding domain ( ), three hydrophobic domains ( ) H1 (residues 205–224), H2 (residues 265–280) and H3 (residues 315–342) and the LysM motif ( ). Regions of EbpS to which antibodies were raised (residues 1–267) and (residues 343–486) are indicated.

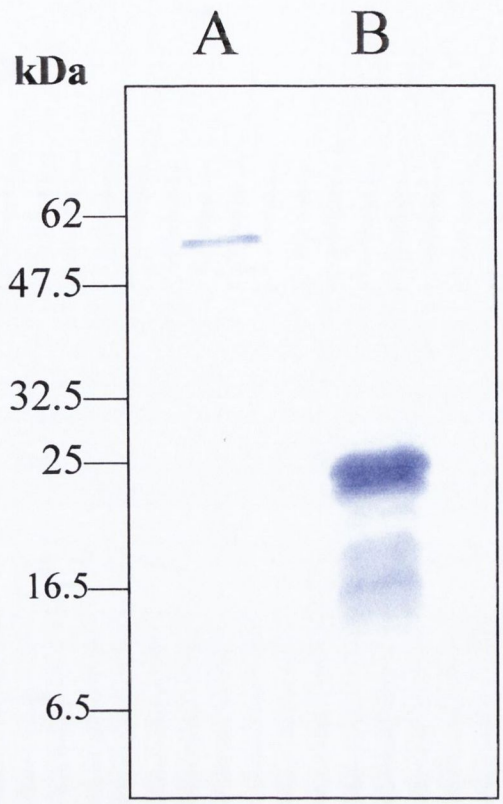


Figure 3.2 5 μg of purified $rEbpS_{1-267}$ (A) and $rEbpS_{343-486}$ (B) were resolved by SDS-PAGE and stained with Coomassie blue. The relevant regions of the *ebpS* gene were PCR-amplified and expressed as N-terminal His-tag fusions. Recombinant proteins were expressed in *E. coli* strain M15 and purified by nickel affinity chromotography. Size standards are as indicated.

heavily contaminated with antibodies to other cell envelope proteins of *S. aureus*. The anti-rEbpS$_{1-267}$ did not require any further treatment.

Expression of EbpS in strain Newman *spa* and an isogenic strain in which *ebpS* had been interrupted by allelic replacement with an erythromycin resistance cassette (Roche, 2000) was assessed in whole cell lysates. The *spa* mutants were used because protein A binds to the immunoglobulin Fc region and would interfere with Western immunoblotting analysis (Schneewind *et al.*, 1993). Cultures were grown to stationary phase washed, resuspended to the same optical density and digested with the muralytic enzyme lysostaphin before boiling in final sample buffer. Western immunoblotting analysis was performed using the polyclonal antibodies recognising either the N-terminal or C-terminal regions of EbpS. The anti-rEbpS$_{343-486}$ antibodies reacted with an 83 kDa protein in strain Newman *spa* (Figure 3.3, lane 3). This band was not present in the lysate of the isogenic *ebpS* mutant (Figure 3.3, lane 4). With the anti-rEbpS$_{1-267}$ polyclonal antibodies, both an 83 kDa protein and a smaller protein of ~70 kDa were detected in the lysate of Newman *spa* (Figure 3.3, lane 1) and not in the isogenic *ebpS* mutant (Fig.3.3, lane 2). This is likely to be a trucated form of EbpS lacking the C terminus of the protein because it was not detected using the anti-rEbpS$_{343-486}$ antibodies.

Taken together these data suggest that these antibodies recognise what is likely to be the full-length undegraded form of EbpS that has an apparent molecular mass of 83 kDa. This is considerably larger than the predicted molecular mass of 53.08 kDa.

3.2.2 EbpS is associated with the bacterial cell surface by a novel mechanism for a *S. aureus* extracellular matrix binding protein

Digestion of the *S. aureus* cell wall peptidoglycan with the muralytic enzyme lysostaphin, in the presence of an osmotic stabiliser, solubilises covalently or non-covalently cell-wall-associated proteins. This method is known to solubilise the *S. aureus* surface-associated proteins ClfA, ClfB, Cna, FnBPA and FnBPB, which are covalently anchored to the cell wall peptidoglycan via their C-terminal LPXTG motif (Navarre *et al.*, 1998). EbpS does not possess an LPXTG motif and sorting signals required for sortase-mediated covalent linkage to the cell wall (Roche, 2000). This suggests that EbpS may be associated with the cell envelope by a novel mechanism for a

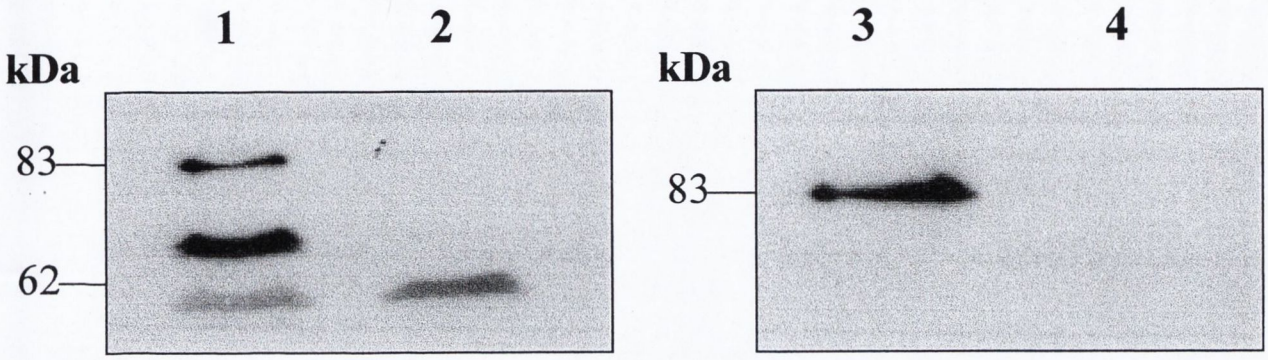


Figure 3.3 Western immunoblotting analysis of whole cell lysates of cells from *S. aureus* stationary phase cultures. Cultures were grown to stationary phase (18 h), washed and resuspended to an OD_{600nm} of 100 and digested with lysostaphin before boiling in an equal volume of final sample buffer. 10 µl of each lysate was loaded into relevant wells. Lysates of strains Newman *spa* (lane 1) and Newman *spa ebpS* (lane 2) detected with anti-$rEbpS_{1-267}$ antibodies. Lysates of strains Newman *spa* (lane 3) and Newman *spa ebpS* (lane 4) detected with anti-$rEbpS_{343-486}$ antibodies.

staphylococcal extracellular matrix (ECM) binding protein. Previously it has been reported that wall-associated proteins of Gram-positive organisms that were not covalently linked to the cell wall peptidoglycan could be solubilised by treatment of whole cells with lithium chloride (LiCl) or sodium dodecyl sulphate (SDS) (Smith and Foster, 1995; Sugai *et al.*, 1990). LiCl is a compound that has been used extensively to solublise non-covalently attached cell surface proteins of *S.aureus* (McGavin *et al.* 1993b; Liang *et al.*, 1995; Jonsson *et al.*, 1995) and is also known to solubilise biological membranes (Frasch and Gotschlich, 1974). SDS is a potent denaturing agent which is know to solubilise biological membranes but has also been used to release non-covalently attached proteins from the surface of *S. aureus* cells (Sugai *et al.*, 1990). Recent evidence has indicated that the treatment of whole cells of *Staphylococcus epidermidis* with LiCl resulted in the release of DNA, cytoplasmic proteins and a decrease in cell viability (Hussain *et al.*, 1999). Therefore treatment of whole staphylococcal cells in this way may lead to the erroneous identification of a cytoplasmic protein as wall-associated. To avoid this problem, cell envelopes comprising the cell wall and the cytoplasmic membrane were prepared from *S. aureus* strain Newman *spa* by mechanically smashing the cells using a FastPrep™ cell disrupter and purifying the envelope fraction by differential centrifugation. The envelope fraction was extensively washed to remove any cytoplasmic contamination. The cell envelopes were then treated with either 3M LiCl or 2% (w/v) SDS to solubilise any membrane-associated proteins or proteins that were non-covalently associated with the cell wall. The insoluble material was then collected by centrifugation leaving solubilised proteins in the supernatant. After removal of the supernatant, the insoluble fractions were extensively washed and digested with lysostaphin to release any proteins that remained anchored to the cell wall after LiCl or SDS treatment. Both the supernatants and the solubilised cell wall fractions were analysed by Western immunoblotting with antibodies recognizing the C terminus of EbpS and region A of ClfA (residues 220-559). ClfA, a fibrinogen-binding protein of *S. aureus,* is a cell wall associated protein with an apparent molecular mass of approximately 175 kDa which is covalently anchored to the cell wall peptidoglycan via an LPXTG motif and can only be released by the muralytic enzyme lysostaphin (McDevitt *et al.*, 1994). After LiCl treatment ClfA, detected as two proteolytic

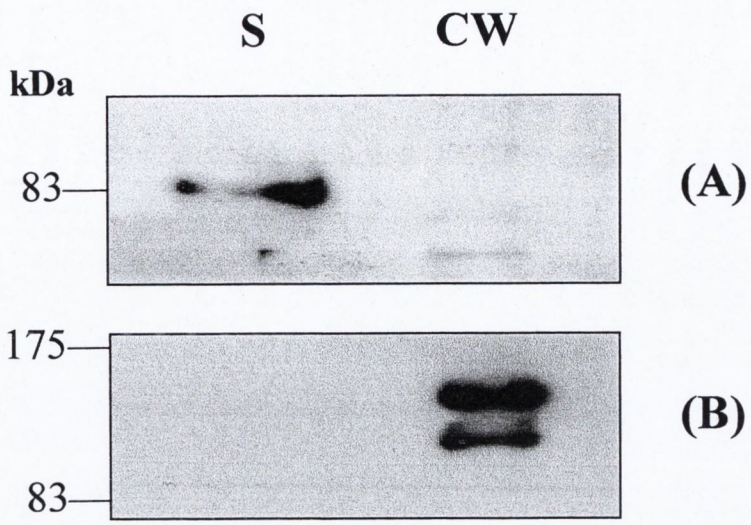


Figure 3.4 Western immunoblottting analysis of proteins from cell envelopes of *S. aureus* strain Newman *spa* solubilised (S) or retained in the insoluble cell wall fraction (CW) after treatment with 3 M LiCl. Equivalent volumes of each fraction were analysed. Panel A, fractions probed with anti-rEbpS$_{343-486}$ antibodies. Panel B, fractions probed with anti-ClfA$_{220-559}$ antibodies.

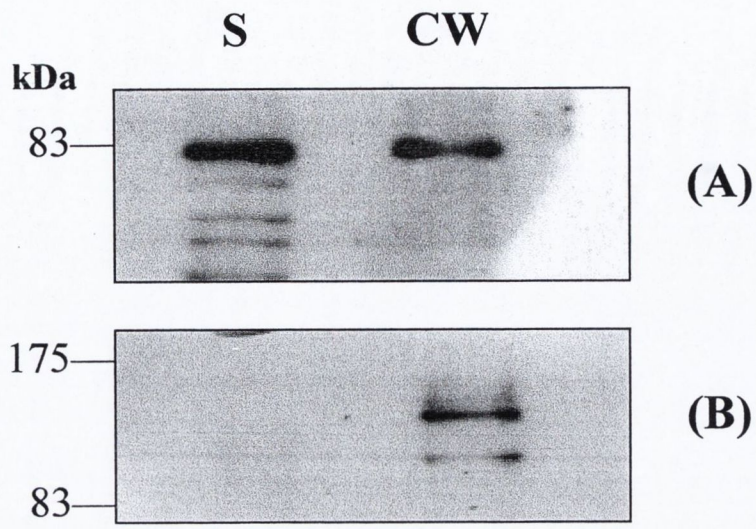


Figure 3.5 Western immunoblottting analysis of proteins from cell envelopes of *S. aureus* strain Newman *spa* solubilised (S) or retained in the insoluble cell wall fraction (CW) after treatment with 2% (w/v) SDS. Equivalent volumes of each fraction were analysed. Panel A, fractions probed with anti-rEbpS$_{343-486}$ antibodies. Panel B, fractions probed with anti-ClfA$_{220-559}$ antibodies.

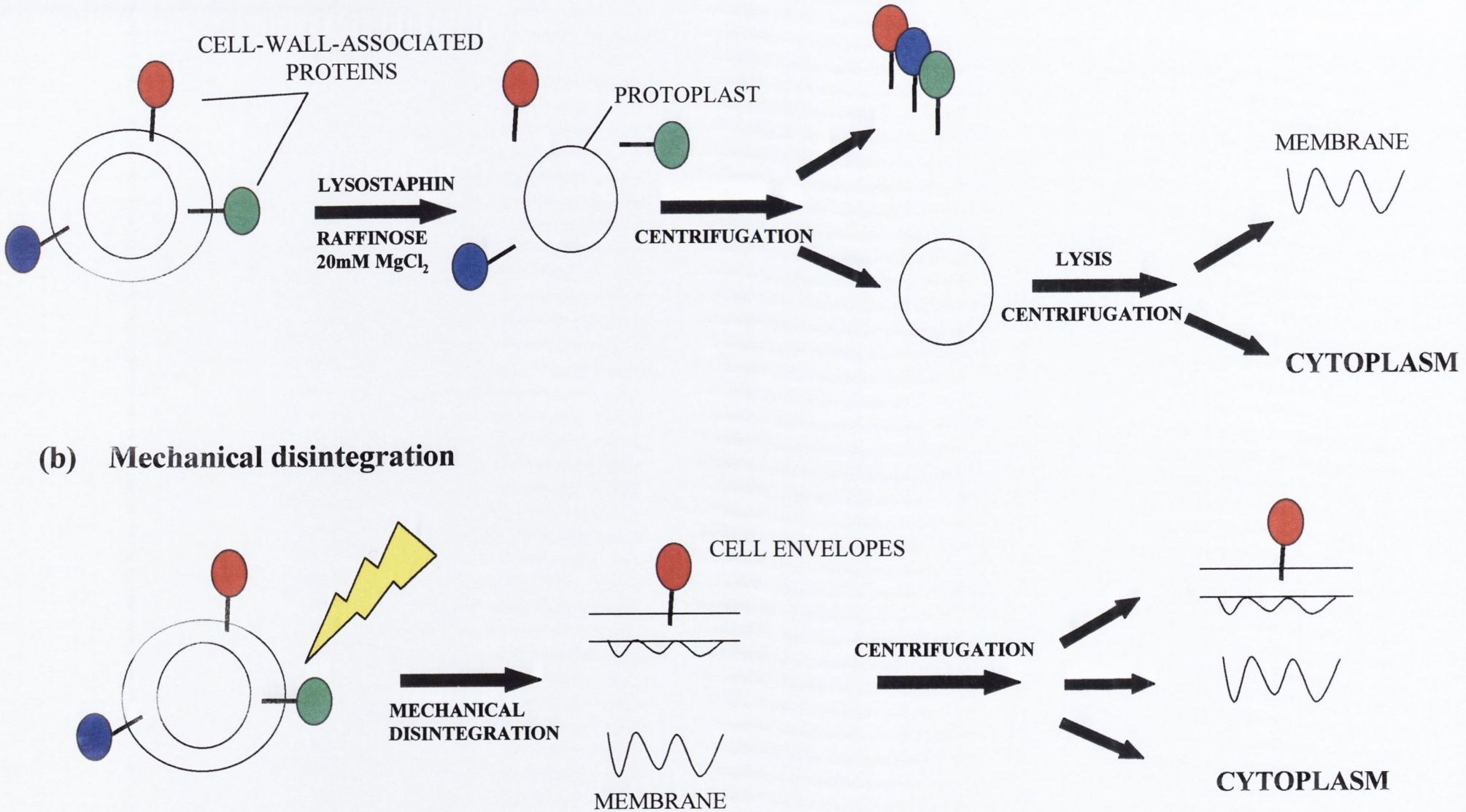
degradation products of approximately 140 kDa and 110kDa, remained exclusively in the cell wall fraction and was not released into the supernatant fraction. This is consistent with the covalent anchoring of this protein to the cell wall peptidoglycan by sortase. Conversely, EbpS was completely released into the supernatant after LiCl treatment (Figure 3.4) with none remaining in the insoluble cell wall fraction. Similarly, when cell envelopes were boiled in SDS, ClfA remained attached to the cell wall whereas EbpS and breakdown products were partially released into the supernatant (Figure 3.5). The release of EbpS from the cell envelope of *S. aureus* by LiCl or SDS treatment indicated that the protein is associated with the cell envelope by means of a distinct mechanism from that of the typical covalently bound protein ClfA. This novel interaction is either by non-covalent interactions with the cell wall or with the cytoplasmic membrane.

3.2.3 Cell fractionation

3.2.3.1 EbpS is present in the protoplast fraction of *S. aureus* and *Lactococcus lactis*

To investigate whether EbpS is associated with the cell wall or with the protoplast, fractionation studies were performed with *S. aureus* cells and with *Lactococcus lactis* cells expressing EbpS. Cultures of *S. aureus* strain Newman *spa* were grown to stationary phase. Cells were washed and fractionated into cell wall and protoplast fractions by digestion with the muralytic enzyme lysostaphin in the presence raffinose and 20mM $MgCl_2$ to maintain stable protoplasts (Figure 3.6). Fractions were separated by centrifugation, boiled in final sample buffer and equal volumes were analysed by Western immunoblotting with anti-$ClfA_{220-559}$ and anti-$rEbpS_{343-486}$ antibodies. As expected, ClfA was detected as two proteolytic degradation products predominantly in the solubilised cell wall fraction (Figure 3.7A). A small amount of higher molecular weight ClfA protein was also detected in the whole protoplast fraction. This could be due to association of the unsorted protein with the membrane via the C-terminal membrane anchor or it may cytoplasmically located ClfA that retains both its secretory signal sequence and its C terminal cell wall sorting motifs. Conversely, EbpS was detected only in the protoplast fraction and not in the cell wall fraction (Figure 3.7B).

Figure 3.6 Methods for fractionating whole *S. aureus* cells. (a) Stabilised protoplast method. Cells were resuspended in a Tris buffer containing the osmotic stabiliser raffinose (30% w/v), 20 mM $MgCl_2$ and a cocktail of protease inhibitors. Lysostaphin (0.2 mg/ml) digested the cell wall peptidoglycan and solubilised all cell-wall-associated proteins. Whole protoplasts (cytoplasmic membrane and cytoplasm) were pelleted by centrifugation and the soluble cell wall proteins were separated. The protoplasts were resuspended in a buffer lacking raffinose and lysed by osmotic stress, pipetting and vortexing. The insoluble membrane was then separated by centrifugation leaving cytoplasmic proteins in the soluble fraction. (b) Mechanical disintegration of cells. Cells were resuspended in a Tris buffer containing DNase (80 μg/ml) and a cocktail of protease inhibitors. The resuspension was then placed in a blue cap FastRNA tube and shaken in a FP120 Fastprep™ cell disrupter ( ) (see Materials and Methods). This treatment lysed the cells, which could then be separated into cell envelopes (comprising cell walls and associated cytoplasmic membrane), pure cytoplasmic membrane and cytoplasmic fractions.


(a) Stabilised protoplasts
CELL-WALL-ASSOCIATED PROTEINS
LYSOSTAPHIN
RAFFINOSE
20mM $MgCl_2$
PROTOPLAST
CENTRIFUGATION
MEMBRANE
LYSIS
CENTRIFUGATION
CYTOPLASM
(b) Mechanical disintegration
MECHANICAL DISINTEGRATION
CELL ENVELOPES
MEMBRANE
CENTRIFUGATION
CYTOPLASM

L. lactis is a gram-positive food grade organism that has been used for the heterologous expression of proteins from other Gram-positive bacterial species (Holmes *et al.*, 1998). ClfA and EbpS were expressed in *L. lactis* strain MG1363 from the expression vector pKS80 (Figure 3.8). The *L. lactis* expression vector pKS80 constitutively expresses any gene that has been cloned in-frame into the *BclI* restriction site downstream from the strong lactococcal bacteriophage C2 promoter, LPS2 (Hartford *et al.*, 2001b). ClfA expressed in *L. lactis* (provided by L. O' Brien) was sorted to the lactococcal cell wall peptidoglycan and promoted bacterial adherence to immobilised fibrinogen (O' Brien, 2001). The *ebpS* gene was cloned into the *BclI* restriction site of plasmid pKS80. The *BclI* restriction site (T$^{\vee}$GATCA) provides the bases 'TG' of the 'ATG' start translation codon (underlined). This dictates that the second codon of the cloned gene must be 'ATC' which codes for an isoleucine residue and that the third codon must begin with the base 'A'. To facilitate expression of EbpS from the plasmid pKS80 the second residue (serine) of the wild-type EbpS protein was substituted with residues isoleucine and leucine. This changed the N terminus of EbpS expressed *in L. lactis* from MSNNFK to MILNNFK.

L. lactis cultures expressing ClfA or EbpS were grown overnight at 30°C without shaking. The cellular localisation of both proteins in *L. lactis* was assessed in the same manner as for *S. aureus* except that the cell wall was solubilised using the muralytic enzymes mutanolysin and lysozyme instead of lysostaphin. In addition, culture supernatants were treated with TCA to precipitate any extracellular proteins. Western immunoblotting analysis was performed on the supernatant, cell wall and protoplast fractions. ClfA was detected both in the cell wall and in the protoplast fraction. Both the intact 175 kDa and degradation products were observed in each fraction. This indicates that the ClfA protein was secreted by the *L. lactis* Sec-secretion apparatus and became associated with the cell wall while some remained associated with the protoplast in a membrane-bound or cytoplasmic form (Figure 3.9A). Conversely, EbpS and breakdown products were only detected in the protoplast fraction and not in the cell wall (Figure 3.9B). In previous studies heterologously expressed proteins were proteolytically degraded during protoplast formation in a time dependent manner (Hartford *et al.*, 2001b). It is likely that membrane associated proteases that are unaffected by protease

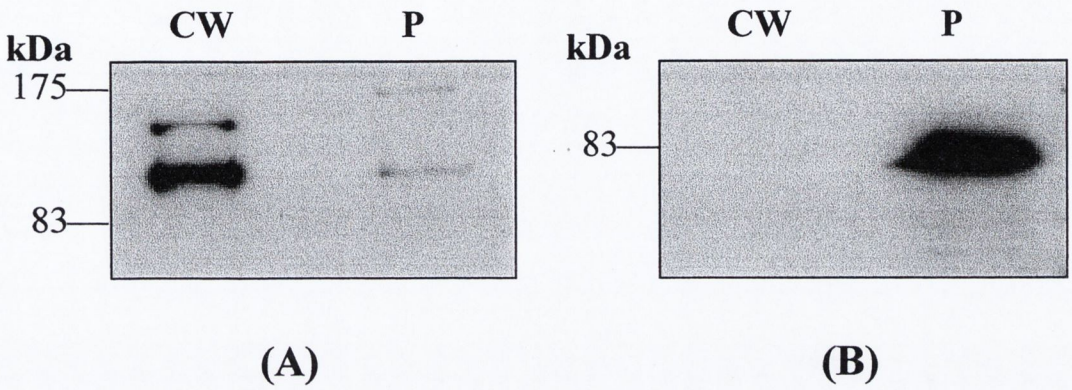


Figure 3.7 Western immunoblotting analysis of the cell wall lysostaphin digest (CW) and the protoplast (P) fractions of *S. aureus* strain Newman *spa*. Equal volumes of each fraction were analysed. (A), fractions probed with anti-$ClfA_{220-559}$ antibodies.(B), fractions probed with anti-$rEbpS_{343-486}$ antibodies.

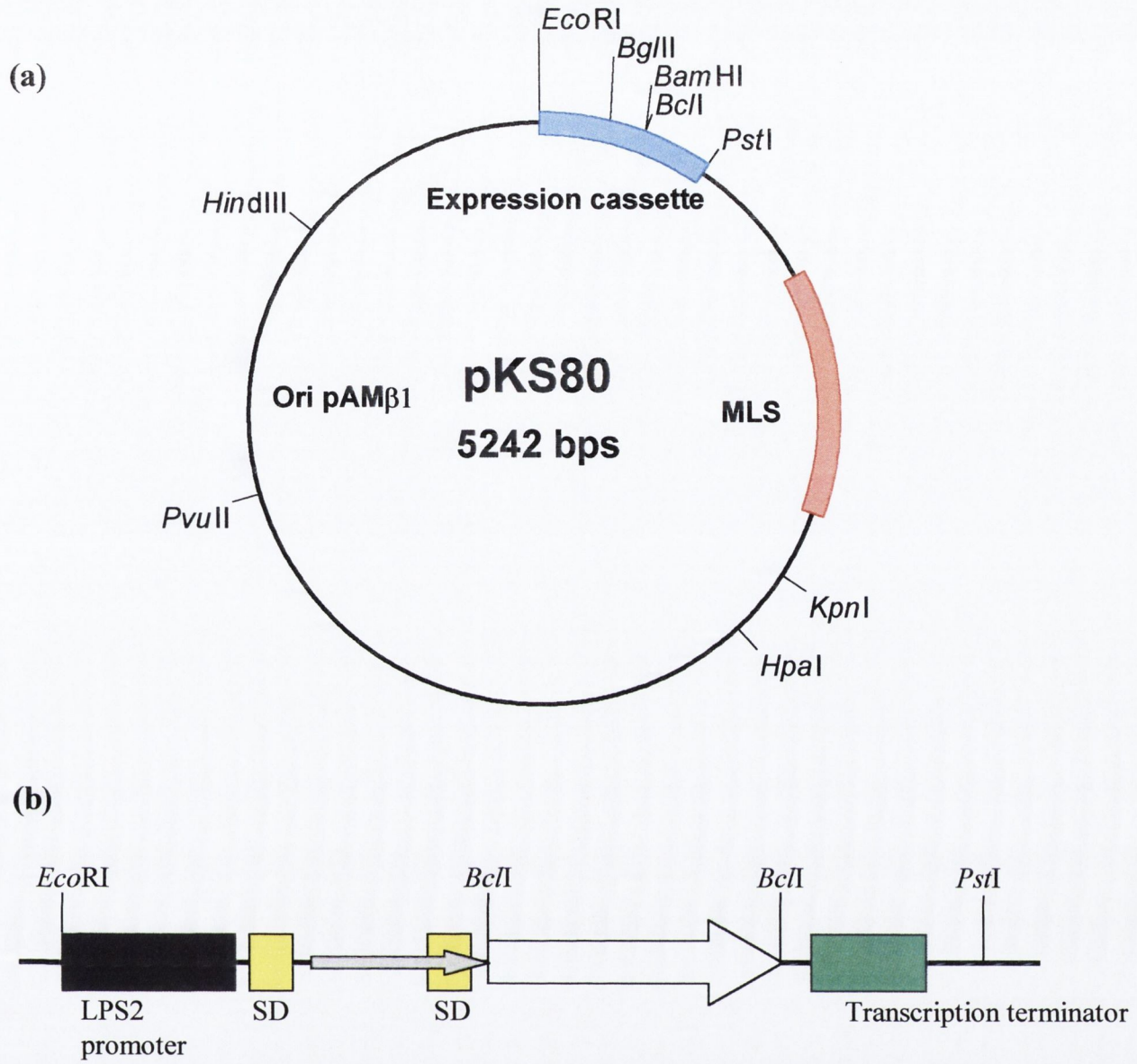


Figure 3.8 Map of the *Lactococcus lactis* expression vector pKS80. (a) Positions of the origin of replication, the expression cassette and the macrolide, lincosamide and streptogramin B (MLS) antibiotic resistance marker are indicated. (b) Constitutive expression of a cloned gene (⇨) is achieved by cloning into the *Bcl*I restriction site downstream of a short open reading frame (⇨) which has its own Shine–Dalgarno sequence (SD, ▭). Using appropriate primers the cloning step places the cloned open reading frame in frame with an ATG start translation codon overlapping the *Bcl*I restriction site. In the case of EbpS this replaces the second residue, serine, with the residues isoleucine and leucine. The ribosome is then recruited to a second SD sequence resulting in expression of the cloned gene.

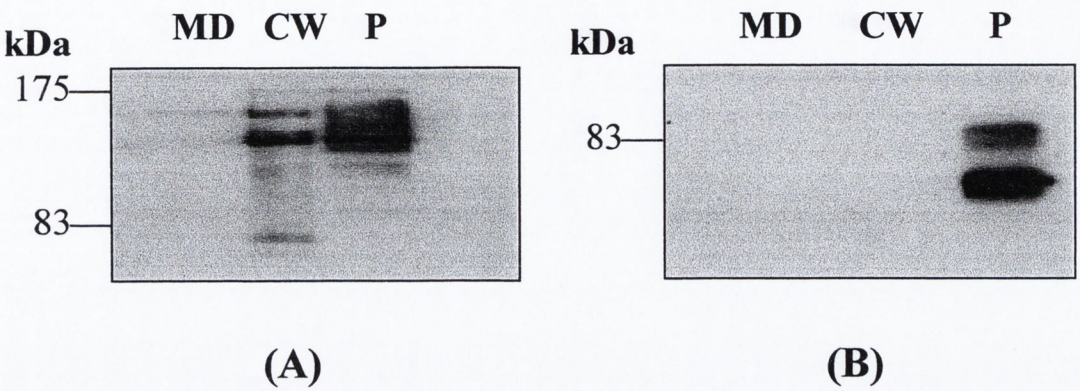


Figure 3.9 Western immunoblotting analysis extracellular proteins TCA precipitated from culture media (MD), cell wall lysozyme and mutanolysin digests (CW) and protoplast fractions (P) of *Lactococcus lactis* cultures expressing ClfA (A) and EbpS (B). An equal volume of each fraction was analysed. (A), fractions probed with anti-$ClfA_{220-559}$ antibodies.(B), fractions probed with anti-$rEbpS_{343-486}$ antibodies.

inhibitors become activated or released during protoplast formation resulting in protein degradation.

3.2.3.2 EbpS is associated with the cytoplasmic membrane of stabilised protoplasts

To determine the exact cellular location of the EbpS protein, *S. aureus* Newman *spa* cells that had been grown to stationary phase were sedimented and resuspended in a buffer containing the osmotic stabiliser raffinose. Lysostaphin was used to solubilise the cell wall peptidoglycan in order to release cell-wall-associated proteins. The stabilised protoplasts were sedimented, lysed by repeated pipetting and separated into an insoluble cytoplasmic membrane and cytoplasmic fractions by centrifugation (Figure 3.6). An extracellular protein fraction was prepared by TCA precipitation of the growth media after cells had been removed by centrifugation. Each fraction was boiled in final sample buffer and an equal volume was analysed by Western immunoblotting using anti-rEbpS$_{1-267}$ and anti-rEbpS$_{343-486}$ antibodies. The ClfA protein was used as a marker for cell-wall-associated proteins. The F_1F_0 ATPase was used a marker for the cytoplasmic membrane (Kubak and Yotis, 1981). The presence of ClfA and ATPase in each fraction was monitored by Western immunoblotting using specific antibodies. Membrane and cytoplasmic fractions were also tested for isocitrate dehydrogenase activity, a cytoplasmic protein marker (Jonquières *et al.*, 1999).

Figure 3.10B clearly demonstrates that the 83 kDa EbpS protein of strain Newman *spa* is exclusively associated with the cytoplasmic membrane fraction. It was not detected in the culture supernatant, the cell wall or the cytoplasmic fraction. When probed with anti-rEbpS$_{1-267}$ antibodies, an immunoreactive protein of 83 kDa was revealed, along with a smaller protein detected in the membrane fraction, but not in other fractions (Figure 3.10A). It is assumed that the smaller protein is a truncate lacking residues from the C-terminus because it was absent from (i) the corresponding fraction of an *ebpS* null mutant and (ii) the same material probed with anti-rEbpS$_{343-486}$ antibodies. A high molecular weight extracellular protein was also detected in the TCA precipitated growth media fraction using the anti-rEbpS$_{1-267}$ antibodies. It was assumed that this was a cross-reactive protein because it was also detected in the growth media fraction of a culture of the isogenic *ebpS* null mutant. In contrast, ClfA was associated exclusively

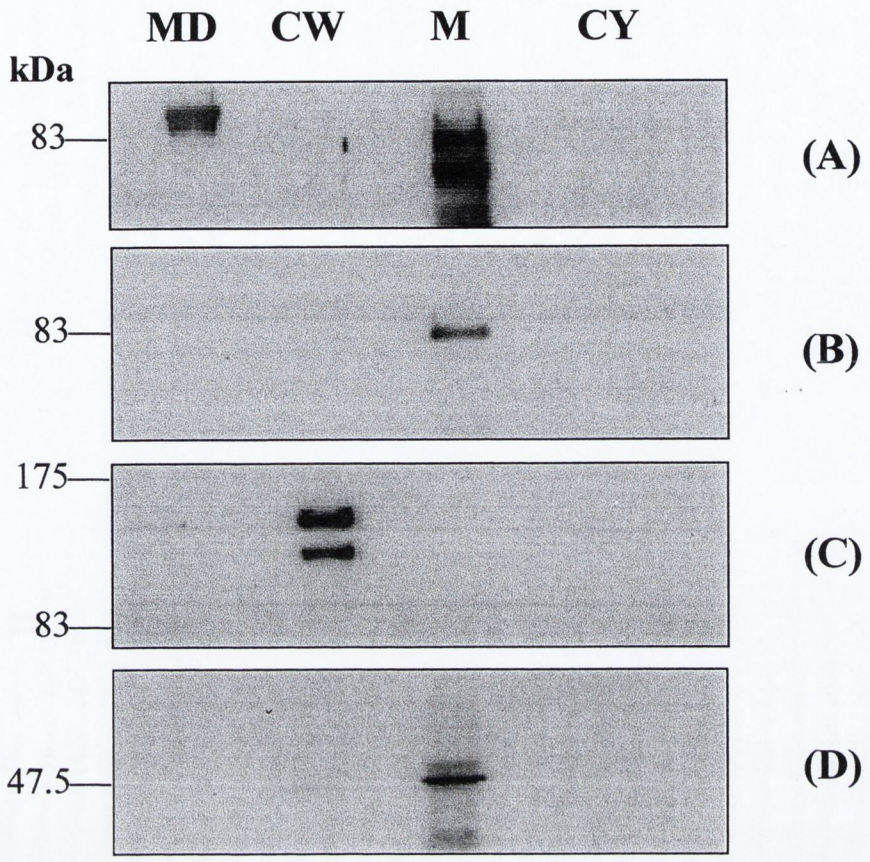


Figure 3.10 Western immunoblotting analysis of media (MD), cell wall (CW), cytoplasmic membrane (M) and cytoplasmic (CY) fractions of cells from a stationary phase culture of *S. aureus* strain Newman *spa* prepared by stabilised protoplast formation. Equal volumes of each fraction were analysed and probed with (A) anti-rEbpS$_{1-267}$ antibodies, (B) anti-rEbpS$_{343-486}$ antibodies, (C) anti-ClfA$_{220-559}$ antibodies and (D) anti-ATPase antibodies.

with the cell wall fraction (Figure 3.10C). This shows that the protoplast membrane fraction was not contaminated significantly with peptidoglycan. The anti-ATPase antibodies only reacted with a protein of 47.4 kDa in the membrane fraction (Figure 3.10D), indicating that the membrane did not contaminate the other fractions. Of the total cellular isocitrate dehydrogenase acvtivity, 83.8% was associated with the cytoplasm fraction while the cytoplasmic membrane fraction had 16.2% (n=3). This indicates that there was little contamination of the membrane fraction by cytoplasmic proteins. Similar results were observed with cells from a late exponential phase culture of the same strain indicating that EbpS is associated with the membrane throughout the growth cycle (data not shown).

3.2.3.3 EbpS is associated with cytoplasmic membranes prepared by mechanical disintegration of cells

A purified cytoplasmic membrane fraction of *S. aureus* Newman *spa* was prepared after mechanical disintegration of cells and differential centrifugation (Figure 3.6). Western immunoblotting showed that this fraction lacked the cell-wall-associated protein ClfA but did contain the 83 kDa EbpS protein (Figure 3.11). This confirms that EbpS is firmly associated with the cytoplasmic membrane and establishes that it and did not become associated with the protoplast membrane after solubilisation of the cell wall.

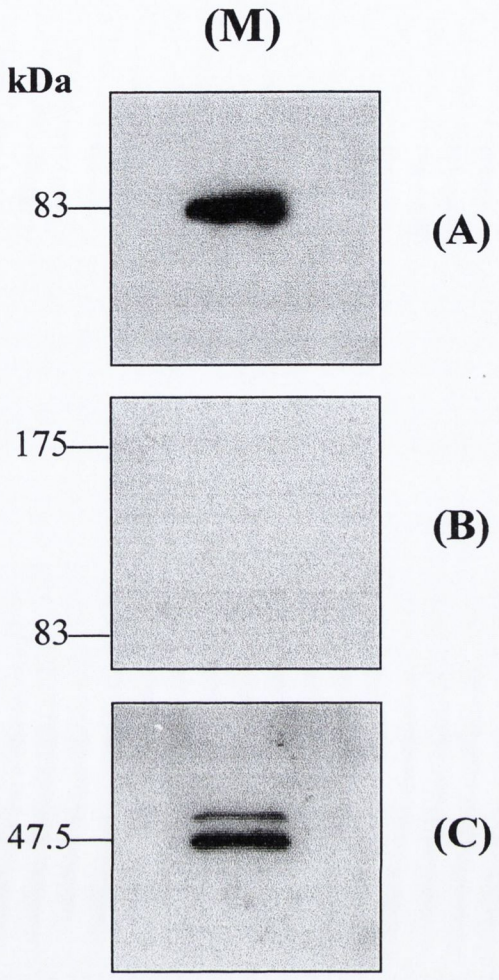


Figure 3.11 Western immunoblotting analysis of a purified cytoplasmic membrane fraction prepared by mechanical disintegration of cells of *S. aureus* strain Newman *spa*. Panel A, EbpS detected with anti-rEbpS$_{343-486}$ antibodies. Panel B, ClfA detected with anti-ClfA$_{220-559}$ antibodies (a cell wall protein). Panel C, ATPase used as a cytoplasmic membrane marker.

3.3 Discussion

Previous analysis of the elastin-binding protein of *S. aureus* strain Cowan reported it to be a surface-associated protein of 202-residues with an apparent molecular mass of 25 kDa (Park *et al.*, 1996). It is now known that this 25 kDa protein was in fact a truncated derivative of the full length protein comprising an N-terminal moiety. The native protein is 486 residues in length encoded by a 1461 bp open reading frame (Roche, 2000). It is most likely that the 25 kDa protein was formed by cleavage of EbpS by bacterial proteases during prolonged incubation in the absence of the osmotic stabiliser raffinose. Bacterial cytoplasmic and membrane-associated proteases might have been released as a result of protoplast lysis. Although EbpS was reported to be released by the muralytic enzyme lysostaphin, it was not formally shown to be cell-wall-associated. EbpS must be located on the cell surface to perform its ligand binding function but the protein does not possess any of the structural features found in the well-characterised staphylococcal surface proteins. These include a N-terminal cleavable hydrophobic signal sequence required for Sec-dependent secretion across the cytoplasmic membrane and an LPXTG motif and membrane-spanning domain required for processing and covalent anchoring to peptidoglycan by the enzyme sortase (Schneewind *et al.*, 1993).

Polyclonal antibodies were raised against an N-terminal domain (residues 1–267) and a C-terminal domain (residues 343–486) of EbpS. By careful analysis of whole cell lysates of *S. aureus* strain Newman *spa* it was shown that these antibodies reacted with a protein which migrated in SDS-PAGE with an apparent molecular mass of 83 kDa. The specificity of these antibodies was confirmed by the fact that the 83 kDa protein could not be detected in the lysate of Newman *spa* in which *ebpS* had been interrupted with an erythromycin resistance cassette. The EbpS protein appeared larger by SDS-PAGE than its predicted molecular mass of 53.08 kDa. This may be due to the presence of the three large hydrophobic domains. Other surface proteins of *S. aureus* including the fibronectin-binding proteins (McGavin *et al.*, 1997) and ClfA (Hartford *et al.*, 1997) migrate by SDS-PAGE at almost twice their predicted molecular mass. Detection by the C-terminal specific antibodies of the 83 kDa protein confirms that EbpS is the product of the newly

sequenced *ebpS* gene. This reinforces the conclusion that there was a mistake made in the original characterisation of the *ebpS* locus and gene product.

To determine the nature of the interaction that binds EbpS to the bacterial cell surface, purified cell envelopes were treated with either 3M LiCl or 2% (w/v) SDS. The staphylococcal envelope prepared by mechanical disruption of cells comprises the cell wall and cytoplasmic membrane. Both LiCl and SDS are known to solubilise biological membranes and to release non-covalently-associated cell wall proteins (Smith and Foster, 1995; Sugai *et al.*, 1990). Consequently, any proteins released from the cell envelopes by either of these compounds were either associated with the cytoplasmic membrane or non-covalently attached to the cell wall. Both compounds solubilised the 83 kDa form of EbpS whereas ClfA, a typical covalently cell-wall-anchored MSCRAMM, could only be solubilised from cell envelopes by digestion with the muralytic enzyme lysostaphin. This evidence indicates that EbpS is associated with the cell surface by a completely distinct mechanism from that of other well-characterised matrix binding proteins of *S. aureus*.

To examine further the cellular localisation of EbpS, the protein was expressed in *L. lactis*. Culture supernatants, cell wall and protoplast (membrane and cytoplasm) fractions were prepared from *L. lactis* strains expressing EbpS or ClfA. ClfA was detected in the cell wall fraction by Western immunoblotting whereas EbpS could only be detected in the protoplast fraction. Cell wall and protoplast fractions from *S. aureus* strain Newman *spa* were also subjected to Western immunoblotting analysis. In agreement with the *L. lactis* data, ClfA was detected exclusively in the cell wall fraction whereas EbpS was completely retained in the protoplast fraction. To determine the exact cellular localisation of EbpS, a complete fractionation of the *S. aureus* cells was performed. When stabilised protoplasts of *S. aureus* were formed and separated into membrane and cytoplasmic fractions, the EbpS protein remained firmly associated with the cytoplasmic membrane. This was the case in cells from both exponential and stationary phase cultures indicating that this strong membrane association occurs throughout the growth cycle. It could be argued that EbpS was originally in the cell wall fraction but became associated with the membrane fraction during protoplast formation. To address this possibility, cytoplasmic membranes were prepared by mechanical disintegration of cells and differential centrifugation. By this method the cell wall

peptidoglycan lattice is not broken down. Therefore, cell wall proteins, be they covalently or non-covalently attached, remain associated with the cell wall fraction. Western immunoblotting analysis of the pure membrane fraction confirmed that EbpS was associated with this fraction whereas ClfA was completely absent. These data validated EbpS as a true membrane-associated protein.

The majority of matrix-binding proteins of *S. aureus* are covalently attached to the cell wall peptidoglycan via an LPXTG motif, but some are non-covalently attached (Foster and Höök, 1998). The MHCII analogous protein (Map) (Jonsson *et al.*, 1995), also called extracellular adhesive protein (Eap) (Palma *et al.*, 1999), and coagulase (Bóden and Flock, 1989) are loosely associated with the cell wall and are found in large amounts in the growth media (coagulase) or can be released from cells by treatment with LiCl (Map). An amidase of *Streptococcus pneumoniae*, LytA, is loosely attached by 20-amino acid repeats to the choline residues of teichoic and lipoteichoic acid (Höltje and Tomasz, 1975). The internalin B protein, InlB, of *Listeria monocytogenes* is displayed at the cell surface by direct association with lipoteichoic acid (Jonquières *et al.*, 1999). Moreover, ActA, the actin-polymerising protein of *L. monocytogenes*, is anchored to the cytoplasmic membrane by a stretch of 20 hydrophobic residues at the C-terminus (Kocks *et al.* 1992). EbpS is the only reported membrane-associated staphylococcal matrix-binding protein. The next stage of the project was to determine if EbpS is an integral membrane protein and, if so, its topological orientation.

Chapter 4

Topology of EbpS in the cytoplasmic membrane

4.1 Introduction

4.1.1 General

Work in the previous chapter showed that EbpS is associated with the cytoplasmic membrane of *S. aureus*, so it was decided to determine the nature of this association. EbpS could be an integral membrane protein whereby it traverses the membrane via hydrophobic stretches of amino acids once (monotopic), twice (bitopic) or several times (polytopic). It could be peripherally associated with the membrane whereby it is anchored to the extracellular face of the membrane, directly or in the form of a lipoprotein, but does not traverse it. Or it could be anchored by an alternative mechanism such as via teichoic or lipoteichoic acid (Höltje and Tomasz, 1975; Kocks *et al.*, 1992). To be an integral plasma membrane protein two criteria must be satisfied (i) the protein must possess one or more stretches of between 15 and 30 mostly hydrophobic residues that can traverse the lipid bilayer by adopting a α-helical conformation (ii) the overall charge of translocated and nontranslocated hydrophilic loops must obey the 'positive inside' rule (von Heijne, 1992; von Heijne, 1994). The first rule dictates the transmembrane regions of the protein whereas the second determines the topology of the protein in the membrane indicating the regions that are located on the extracellular face of the membrane and those which are cytoplasmically located. Positively charged regions of a protein will be unable to traverse the negatively charged phospholipid bilayer and will remain cytoplasmically located. The model for EbpS topology must also be consistent with the N-terminal ligand-binding domain being surface-exposed.

With the knowledge that EbpS is associated with the cytoplasmic membrane and that the protein possesses three stretches of hydrophobic residues designated H1 (residues 205-224), H2 (residues 265-280) and H3 (residues 315-342), each of which is theoretically large enough to span the cytoplasmic membrane (Roche, 2000) it was decided to determine whether EbpS was an integral membrane protein and, if so, its topological orientation.

4.1.2 Methods for studying integral membrane protein topology

There are many ways to study the topology of integral membrane proteins. Due to the technical difficulties involved in forming crystals of integral membrane proteins for structural studies, the focus is mainly on genetic and biochemical techniques. These techniques are based on one of the two following principles (i) the cytoplasmic membrane is a physical barrier that is selectively permeable (ii) fusions to the hydrophilic loops of an integral membrane protein with reporter molecules whose activity depends on their cellular location will result in active or inactive fusion proteins depending on the proteins topology.

4.1.2.1 Techniques based on membrane permeability

The topology of an integral membrane protein can be determined by the introduction of specific tags into the hydrophilic loops of the protein and assessing the interaction of membrane-impenetrable reagents with these tags (Figure 4.1). Examples of specific tags used in this type of study include reactive cysteine residues, protease cleavage sites and N-glycoslation sites.

Cysteine scanning. Cysteine residues are readily modified by sulfhydryl reagents, some that are membrane permeable and some not. If a cysteine residue is introduced into a hydrophilic loop of an integral membrane protein by site-directed mutagenesis, it will be labeled with a membrane-permeable sulfhydryl reagent regardless of whether the cysteine is exposed at the cell surface or is cytoplasmically located. Membrane permeable reagents may be labeled with biotin (Loo and Clarke, 1995), a fluorescent group (Zhou *et al.*, 1995) or a radiolabel (Kimura *et al.*, 1997) and are easily detectable. If protein labeling with these reagents is blocked by pretreatment with a membrane impermeable sulfhydryl reagent then the cysteine residue and therefore the hydrophilic loop must be surface-exposed. Conversely, if the membrane-impermeable reagent does not inhibit labeling then the hydrophilic loop must be cytoplasmically located. This approach has been used successfully for a number of bacterial integral membrane proteins (Kimura *et al.*, 1997; Long *et al.*, 1998).

The advantages of this system are that cysteine residues can easily be introduced by site-directed mutagenesis without disturbing the topology of the protein and the

mutant form of the protein can be expressed in the native host organism. A disadvantage is that any resident cysteine residues must be replaced, usually with a serine residue, before the introduction of experimental cysteine residues. In addition, certain introduced cysteine residues may be inaccessible to reactive agents due to structural constraints of the hydrophilic loop (Chen *et al.*, 1998).

Protease cleavage sites. Exogenous proteases are unable to traverse the cytoplasmic membrane of whole cells, sphaeroplasts or protoplasts. A specific protease cleavage site is engineered into the hydrophilic loop of an integral membrane protein and susceptibility of the cleavage site to protease digestion is then determined. Surface-exposed loops are digested whereas internal loops are not, thus indicating the topology of the protein in the cytoplasmic membrane. This technique has been used successfully with specific proteases such as factor Xa (Sahin-Toth *et al.*, 1995) and endoproteinase LysC (Miller *et al.*, 1993). Similarly, non-specific proteases can be used in this type of study. Expressing the protein of interest with a specific tag engineered into the hydrophilic loops and then monitoring release of the tag when cells are treated with proteases can indicate the topology of the protein. If the tag is released by protease treatment then the loop must be surface-exposed. Examples of specific tags used in this manner are the maltose-binding protein (Miller *et al.*, 1993), a six histidine tag (McKenna *et al.*, 1992) and the commercially available FLAG® epitope (Pan *et al.*, 1998).

N-glycosylation sites. Glycosylation is a posttranslational modification of proteins. In the endoplasmic recticulum (ER) of eukaryotic cells the enzyme oligosaccharide transferase adds oligosaccharides to the N residue of the consensus sequence N-X-T/S (Welply *et al.*, 1983). The enzyme, oligosaccharide transferase, is only present in the lumen of the ER and does not traverse the ER membrane (Welply *et al.* 1983). By the introduction of glycosylation consensus sequences into the hydrophilic loops of an integral membrane protein and expressing the protein in an in *vivo* expression system or *in vitro* cell free system and determining its glycosylation state, the topology of the protein can be determined (Melchers *et al.*, 1996; van Geest, *et al.*, 1999; Weeks *et al.*, 2000). The addition of an oligosaccharide molecule to the protein increases its molecular mass by 2.5 kDa. By engineering a number of glycosylation sites at the region of interest an increase in size of the protein can be detected by SDS-PAGE if that region

of the protein has traversed the microsome (membranes derived from ER) and become glycosylated.

This method can be used without disrupting the overall topology of the protein by simply engineering the glycosylation motif into the protein. However the technique has limitations. There is an optimum distance for the positioning of a functional glycosylation site between hydrophobic domains. Incorrect positioning may result in dysfunctional glycosylation and false negative results (Nilsson and von Heijne, 1993). Another potential difficulty is that the protein may lose activity due to the posttranslational modification (Turk *et al.*, 1996).

4.1.2.2 Gene fusions

A second method for determining the topology of an integral membrane protein is the gene fusion approach. The hydrophilic loops of the protein of interest are fused to a compartment-specific enzyme that lacks a secretion signal sequence. A compartment-specific enzyme is one that is only active when located on a specific side of the cytoplasmic membrane. The hybrid protein can be a sandwich fusion whereby the enzymatic reporter is inserted into the region of interest or a C-terminal deletion whereby the reporter protein replaces the C terminus of the protein of interest (Figure 4.1). With both approaches, if the fusion position traverses the cytoplasmic membrane the integral membrane protein will act as the reporter molecule's secretion signal sequence and determine its cellular location and thus its enzymatic activity. Reporter molecules used in this way are the bacterial alkaline phosphatase (PhoA), β-galactosidase (LacZ), β-lactamase and chloramphenicol acetyltransferase.

Bacterial alkaline phosphatase (PhoA). The alkaline phosphatase of *E. coli* (PhoA) is a metalloenzyme that can generate inorganic phosphate from phosphorylated derivatives (Fan *et al.*, 1966). This protein is only active in the periplasm because factors in the cytoplasm prevent disulphide bond formation and the protein cannot adopt its proper conformation (Derman *et al.*, 1993). The enzyme is composed of two identical subunits. When transported to the bacterial periplasm, each subunit forms two intramolecular disulphide bonds and undergoes dimerisation to form the active enzyme (Akiyama and Ito, 1993). The enzyme may be fused to a hydrophilic loop of an integral

membrane protein and expressed in *E. coli*. The activity of the reporter molecule can be measured qualitatively with plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) or quantitatively by liquid assay. A hybrid protein expressing high alkaline phosphatase activity indicates that the enzyme is fused to a region of the integral membrane protein that traversed the cytoplasmic membrane and is located on the extracellular face of the membrane. This method has been used to determine the membrane topology of integral membrane proteins of both Gram-positive and Gram-negative organisms (Green and Cutting, 2000; Fulkerson and Mobley, 2001).

β-lactamase. The enzyme β-lactamase, encoded by the *blaZ* gene, can be used as an alternative to PhoA. β-lactamase is a monomeric enzyme that confers resistance to the β-lactam antibiotics by cleavage of the β-lactam ring (Ghuysen, 1991). The targets of the β–lactam antibiotics are the periplasmically located penicillin-binding proteins which are involved in the synthesis of cell wall peptidoglycan. β–lactamase only confers resistance to these antibiotics when it is located on the periplasmic side of the cytoplasmic membrane. Thus, only cells expressing fusion proteins in which β–lactamase is fused to a periplasmically-located loop of a membrane protein can grow on plates containing high concentrations of ampicillin whereas cells expressing fusions with cytoplasmically located β–lactamase cannot grow in the presence of the antibiotic (Broome-Smith and Spratt, 1986; Hirata *et al.*, 1998).

β-galactosidase. β–galactosidase (LacZ) is a large, tetrameric cytoplasmically-located enzyme. As a reporter this enzyme is only active when it is fused downstream of a cytoplasmically-located hydrophilic loop of an integral membrane protein. Fusions downstream of a periplasmically located hydrophilic loop result in inactive LacZ due to improper folding because of the inability of the large enzyme to traverse the cytoplasmic membrane (Silhavy *et al.*, 1977). The activity of the reporter molecule can be measured qualitatively with plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or quantitatively by liquid assay using o-nitrophenyl-β-D-galactopyranoside (ONPG).

Chloramphenicol acetyltransferase. The enzyme chloramphenicol acetyltransferase encoded by the *cat* gene confers resistance to the antibiotic chloramphenicol. The enzyme acetylates the antibiotic using acetyl-CoA as an acetyl donor (Thibault *et al.*, 1980). Cells expressing fusion proteins in which chloramphenicol acetyltransferase is fused to a cytoplasmically-located loop of an integral membrane protein show resistance to chloramphenicol because acetyl-CoA is only present in the cytoplasm. In contrast cells expressing fusions to the periplasmically-located loops of an integral membrane protein cannot grow in the presence of the antibiotic (Zelazny and Bibi, 1996).

There are a number of advantages and some disadvantages to the gene fusion approach of studying integral membrane protein topology. The activities of each fusion can be easily determined both qualitatively and quantitatively and the expression of each fusion protein can be monitored by Western immunoblotting using commercially available antibodies. Previously it has been shown that the topology of integral membrane proteins determined using PhoA fusions is in agreement with the structure determined by crystallographic studies, thus validating the gene fusion approach as a reliable method for the study of integral membrane protein topology (Chepuri and Gennis, 1990; Iwata *et al.*, 1995). A common strategy is to employ the complementary activities of PhoA and LacZ to study the topology of integral membrane proteins. The same hydrophilic loop of an integral membrane protein can be fused to either PhoA or LacZ and the activities determined. In this type of study a fusion with high PhoA activity should have low LacZ activity and vice versa thus reinforcing the validity of the proposed topological model (Fulkerson and Mobley, 2001). Although the fusion approach has been used extensively to study integral membrane protein topology there have been reports of conflicting results particularly with the use of LacZ as a reporter molecule (Froshauer *et al.*, 1988; Danielsen *et al.*, 1995).

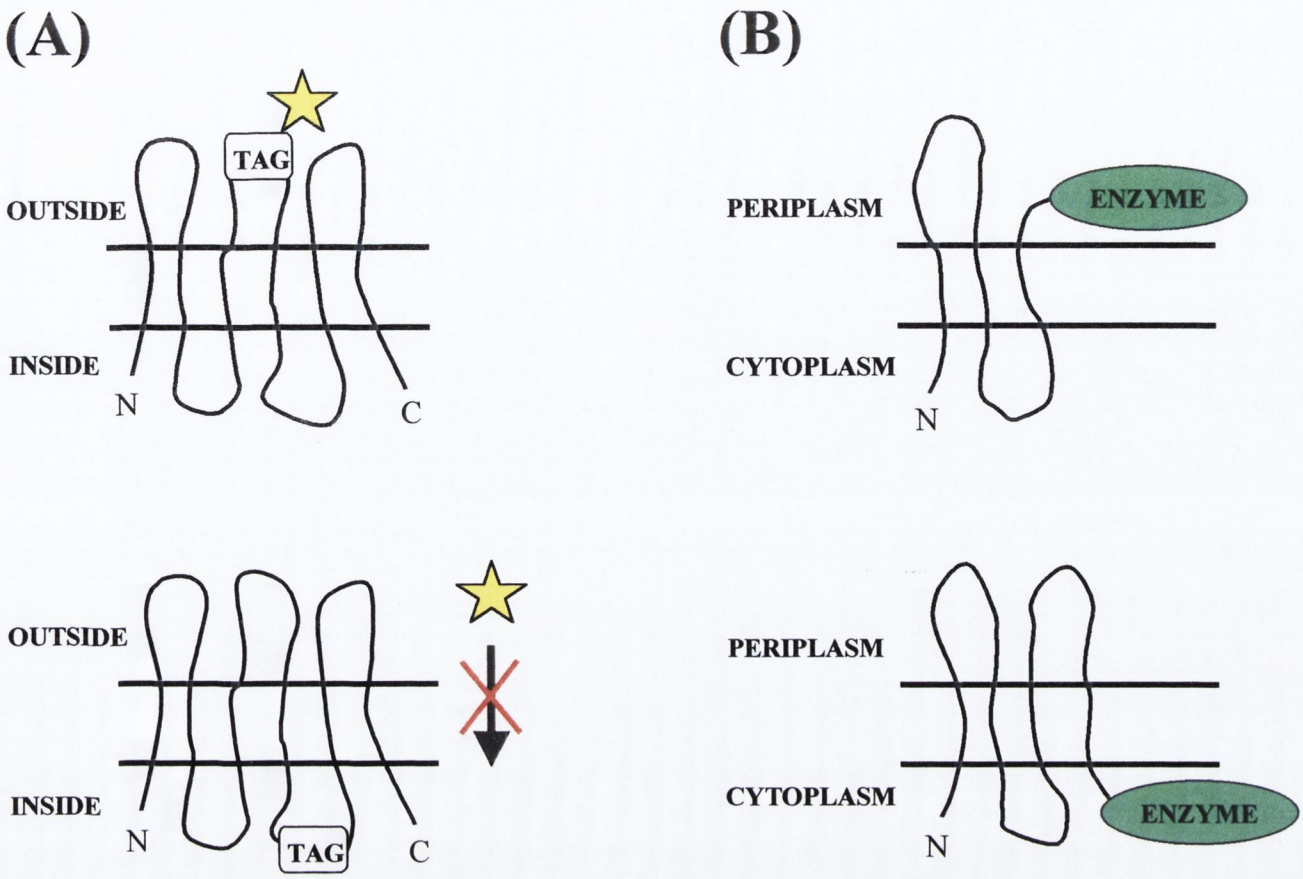


Figure 4.1 Schematic diagram of the approaches for determining the topology of integral membrane proteins. (A) Methods based on membrane impermeability. A specific 'tag' is introduced into the hydrophobic loops of the integral membrane protein. The ability of a membrane-impermeable factor (☆) to react with the tag is assessed, thus determining the topology of the integral membrane protein. (B) The gene fusion approach. A reporter enzyme is fused to the hydrophilic loops of the integral membrane protein by replacing the C terminus of the protein of interest. The activity of the reporter protein indicates the cellular location of the hydrophilic loop to which it is fused.

4.1.3 Rationale

To determine the nature of the interaction between EbpS and the cytoplasmic membrane a number of experiments were performed. Firstly, the primary amino acid sequence of EbpS was analysed by several computer programs used for the prediction of the topology of integral membrane proteins. With these data and knowledge of the hydropathy profile of EbpS, a topological analysis of EbpS was performed using EbpS-PhoA and EbpS-LacZ fusions expressed in *E. coli*. It was necessary to express the native EbpS protein in *E. coli* to ensure that the protein was stable and that it became associated with the cytoplasmic membrane of this organism. EbpS was expressed in *E. coli* from the expression vector pKK233-2. The plasmid pKK233-*ebpS* was used as a template for the construction of EbpS-PhoA and EbpS-LacZ fusions.

To ensure that the topology of EbpS determined in the cytoplasmic membrane of *E. coli* was representative of the topology of the protein in the membrane of *S. aureus*, antibody absorption experiments were performed using stabilised protoplasts of *S. aureus* and anti-rEbpS$_{1-267}$ or anti-rEbpS$_{343-486}$ antibodies. Cross-linking studies were performed using the homobifunctional cross-linking reagent dithiobis(succinimidyl proprionate) (DSP) to determine whether EbpS was present in the cytoplasmic membrane as a monomer or as a multimer.

4.2 Results

4.2.1 *In silico* topological analysis of EbpS in the cytoplasmic membrane

In previous studies, analysis of the EbpS primary amino acid sequence using the algorithim of Kyte and Doolittle (1982) to identify regions of high average hydrophobicity has shown that EbpS possesses three large hydrophobic domains, H1, H2 and H3 (Roche, 2000). For a hydrophobic region of a protein to traverse the prokaryotic cytoplasmic membrane lipid bilayer it must be between 15 and 30 residues in length (von Heijne, 1994). Regions H1 (residues 205-224), H2 (residues 265-280) and H3 (residues 315-342) of EbpS were predicted as 20, 16 and 28 residues in length, respectively (see Figure 5.8). This suggested that each domain could span the cytoplasmic membrane of *S. aureus*. With the knowledge that EbpS was membrane-associated and that the protein possessed three large hydrophobic domains each capable of spanning the cytoplasmic membrane it became apparent that EbpS may be an integral membrane protein.

There are many publically available programs on the WorldWideWeb for predicting the membrane topology of integral membrane proteins. Although these programs are powerful tools each comes with a warning that the results generated are purely speculative and should be used with caution. The TMHMM program for predicting transmembrane α-helices in both prokaryotic and eukaryotic proteins (Sonnhammer *et al.*, 1998) (www.cbs.dtu.dk./services/ TMHMM-2.0) uses the hydropathy profile of a protein and knowledge of membrane insertion mechanisms to predict transmembrane α-helices. This program identifies the hydrophobic regions of a protein and gives the probability of each domain forming a transmembrane α-helix. THHMM predicted that only the hydrophobic domains H1 (probability 0.35) and H3 (probability 1.0) were transmembrane α-helices whereas the central hydrophobic domain, H2, was unlikely to form a transmembrane α-helix (probability <0.02).

The DAS program for predicting transmembrane domains in prokaryotic membrane proteins is based on the dense alignment surface method whereby transmembrane α-helices in a protein are identified by comparing putative transmembrane domains of the query sequence to the sequence of known transmembrane

domains of non-homologous membrane proteins (Cserzo *et al.*, 1997) (www.sbc.su.se/~miklos/DAS). This program assigns a 'DAS score' to each putative transmembrane α-helix where a DAS score above 1.7 identifies a transmembrane α-helix. The hydrophobic domains of EbpS H1, H2 and H3 where assigned DAS scores of 3.3, 3.0 and 5.4 respectively. Therefore, according to this program all three hydrophobic domains were predicted as transmembrane α-helices.

Finally, the TMpred program for predicting transmembrane α-helices of prokaryotic and eukaryotic proteins was used. This program predicts transmembrane α-helices using multiple sequence alignments (Persson and Argos, 1994) (www.ch.embnet.org/software/ TMPRED_form.html) whereby putative transmembrane α-helices are assigned a positive 'TMpred value'. This program also predicted the three hydrophobic domains of EbpS H1, H2 and H3 as transmembrane α-helices with values of 1506, 734 and 2546, respectively.

Although the results from different programs varied slightly and this type of analysis must be treated with caution, a number of conclusions were made. Each of the three programs predicted EbpS as an integral membrane protein indicating that the protein met the parameters laid down by each program. Secondly, if EbpS is an integral membrane protein it would possess at least two transmembrane α-helices.

4.2.2 Expression of EbpS in *E. coli* and association with the inner membrane

In previous studies it has been shown that the topology of integral membrane proteins from Gram-positive organism could be studied in the Gram-negative organism *E. coli*. These include the tetracycline efflux protein (TetK) of *S. aureus* (Hirata *et al.*, 1998) and the Pro-σ^K complex involved in spore formation in *Bacillus subtilis* (Green and Cutting, 2000). It was decided to study the topology of EbpS by expressing EbpS-PhoA and EbpS-LacZ fusions in *E. coli* (see section 4.1.2.2). Before the production of these types of fusions it was necessary to express the native EbpS protein in *E. coli* to ensure that the full length 83 kDa protein was stably expressed and became associated with the cytoplasmic membrane of this organism.

Previously, the full length EbpS protein was expressed in *E. coli* strain M15 with an N-terminal six-histidine tag and purified by nickel-chelate affinity chromatography

(Roche, 2000). This protein formed insoluble inclusion bodies in the *E. coli* cytoplasm and had to be purified under denaturing conditions (Roche, 2000). To express the native EbpS protein in *E. coli*, the *ebpS orf* was cloned and expressed from the 'ATG' expression vector pKK233-2. The plasmid pKK233-2 is specifically designed for the regulated expression of heterologous proteins in *E. coli*. It provides the strong IPTG regulatable *trc* promoter upstream of the *lacZ* ribosome-binding site followed by an ATG start translation codon contained within an unique *Nco*I restriction site (CCATGG) and the downstream 5S rRNA gene transcription termination sequences (Amann and Brosius, 1985) (Figure 4.2). Digestion with the enzyme *Nco*I exposes the ATG codon for the cloning and expression of the gene of interest. The *ebpS orf* was PCR-amplified from plasmid pKS5.2 with a *Nco*I restriction site at the 5′ end, incorporating the ATG start translation codon, and a 3′ *Hin*dIII restriction site. Plasmid pKS5.2 is a derivative of plasmid pBluescript KS(+) with the *ebpS* gene from *S. aureus* strain 8325-4 contained on a 5.2 kb *Pst*I genomic fragment (Roche, 2000). The resulting PCR product was cloned into the unique *Nco*I/*Hin*dIII restriction sites of plasmid pKK233-2 such that the entire *ebpS orf* was joined to the ribosome binding site and ATG codon of the vector. The nature of the *Nco*I restiction site (CCATGG) demanded that the first base of the second codon must be guanine. To accommodate this the forward primer designed to clone *ebpS* into the *Nco*I site changed the N terminus of the resulting EbpS protein from MSNNFKD to MANNFKD, a substitution that should not affect the localisation or topology of EbpS because the amino acids serine and alanine have similar properties.

The plasmid pKK233-*ebpS* was transformed into *E. coli* strain XL1-blue. Cultures were grown to mid exponential phase (OD_{600nm} 0.4) and IPTG was added to final concentrations of 0.1mM, 0.5mM and 1mM to induce expression while one culture was left uninduced. Cultures grew for a further 2 hours before the cell density was corrected to the same OD_{600nm}. Each sample was boiled in final sample buffer to produce whole cell lysates. Equal volumes of each sample were analysed by Western immunoblotting with anti-r$EbpS_{343-486}$ antibodies. Each culture expressed EbpS as an 83 kDa protein including the uninduced culture (Figure 4.3). This indicated that the *E. coli* strain XL1-blue did not produce enough LacI repressor protein to inhibit EbpS expression from the *trc* promoter even in the absence of IPTG. There was a slight increase in the amount of

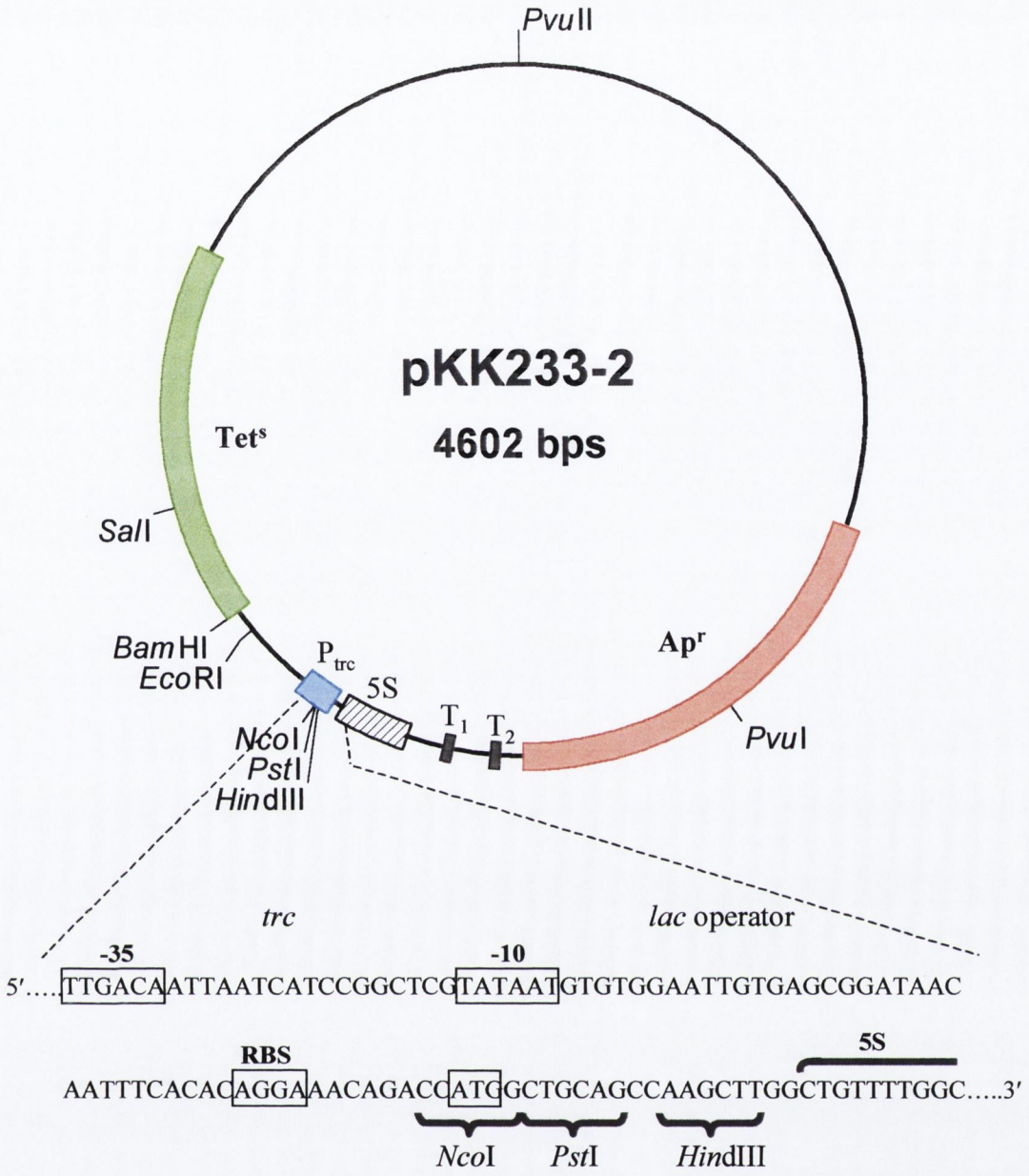


Figure 4.2 Map of plasmid pKK233-2 and relevant regulatory elements. A fragment containing the 3′ end of the 5S rRNA gene (hatched) and the transcription terminators T_1 and T_2 (▮) is indicated. The enlarged region contains the relevant regulatory elements. The boxed areas denote the –35 and –10 consensus sequences of the *trc* promoter (▮) as well as the *lacZ* ribosome binding site (RBS) and the ATG start codon.

EbpS expressed as the concentration of IPTG increased but there was also some proteolytic degradation of EbpS to low molecular weight forms of the protein ranging in size from 25 kDa to 50 kDa. The specificity of the reaction was confirmed by the fact that the immunoreactive proteins were not detected in the lysate of *E. coli* strain XL1-blue containing the empty vector pKK233-2 that had been induced with 1mM IPTG (Figure 4.3, lane 5).

To determine if the protein was in the cytoplasmic membrane and not in an inclusion body, inner and outer membrane fractions were separated on a sucrose gradient by isopycnic centrifugation. Previously it has been shown that the inner and outer membranes of Gram-negative organisms have different densities due to their different lipid contents (Johnston and Gotlisch, 1974; Witholt *et al.*, 1976). This difference in density allows the inner and outer membranes of *E. coli* to be separated on a sucrose gradient by isopynic centrifugation (Witholt *et al.*, 1976). The *E. coli* strain expressing EbpS was grown without induction by IPTG. Sphaeroplasts were formed by digestion of the cell wall peptidoglycan with the muralytic enzyme lysozyme in the presence of the osmotic stabiliser sucrose. The sphaeroplasts were then lysed in a French press and the insoluble membrane fraction was pelleted by centrifugation. The membrane fraction was loaded onto a sucrose gradient ranging in concentration from 35% (w/w) to 55% (w/w) sucrose and the inner and outer membranes were separated by ultracentrifugation. Equal volumes of the inner and outer membranes were then analysed by Western immunoblotting with anti-r$EbpS_{343-486}$ antibodies. EbpS was detected predominantly in the inner membrane as proteolytic degradation products of approximately 47 kDa and 38 kDa (Figure 4.4, lane 2) with some EbpS breakdown products also detected in the outer membrane fraction (Figure 4.4, lane 1). Immunoreactive proteins were smaller than the 83 kDa EbpS protein detected in lysates, presumably due to proteolytic degradation during the prolonged isolation procedure. Importantly these data revealed that a proportion of EbpS expressed in *E. coli* became associated with the cytoplasmic membrane.

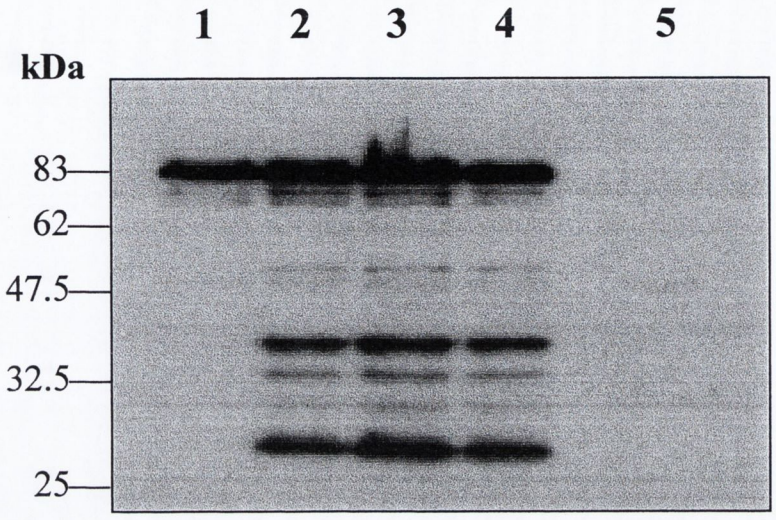


Figure 4.3 Western immunoblotting analysis of whole cell lysates of *E. coli* strain XL1-blue expressing EbpS from plasmid pKK233-2. Expression of EbpS was induced with 0.1 mM (lane 2), 0.5 mM (lane 3) or 1 mM (lane 4) IPTG. One culture was left uninduced (lane 1). Cultures were adjusted to the same OD_{600nm} and equal volumes were analysed by Western immunoblotting with anti-rEbpS$_{343-486}$ antibodies. A lysate of *E. coli* strain XL1-blue containing the empty vector was also analysed (lane 5).

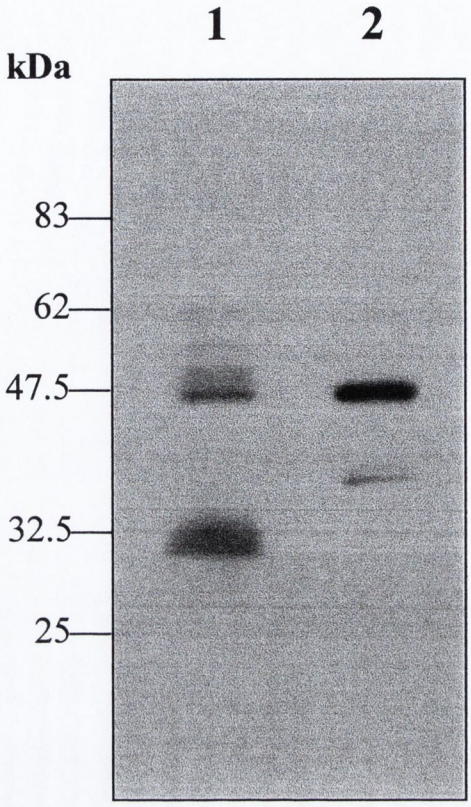


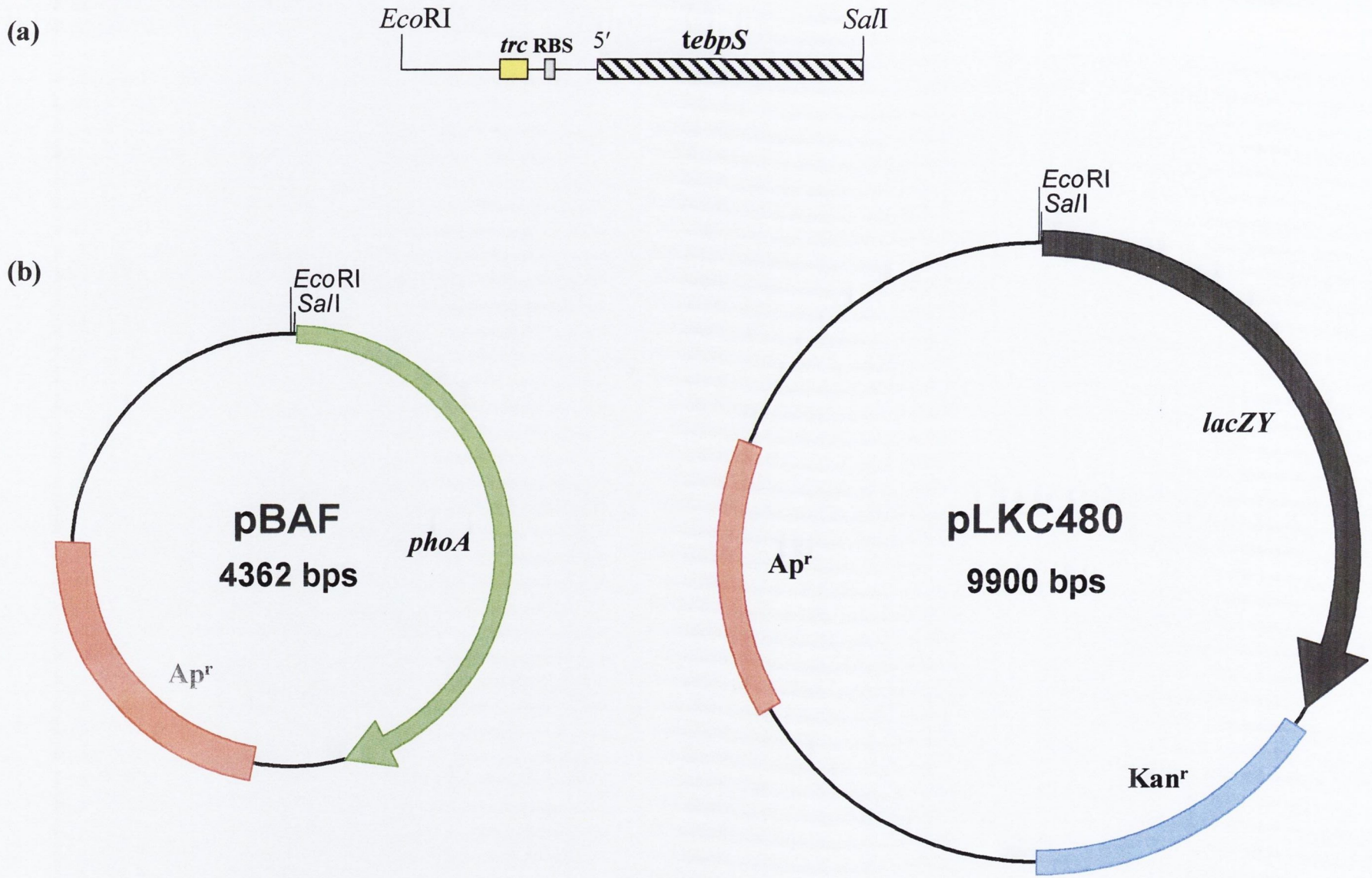
Figure 4.4 Western immunoblotting analysis of the outer membrane fraction (lane 1) and inner membrane fraction (lane 2) of *E. coli* strain XL1-blue expressing EbpS. Equal volumes of each fraction were analysed and proteins were detected with anti-rEbpS$_{343-486}$ antibodies.

4.2.3 Analysis of EbpS-PhoA and EbpS-LacZ fusion proteins

To determine the topology of EbpS in the cytoplasmic membrane a number of EbpS-PhoA and EbpS-LacZ fusions were constructed. The alkaline phosphatase of *E. coli* (PhoA) is only active in the periplasm where it can form intramolecular disulphide bonds and undergo dimerisation thus forming the active enzyme complex. Factors in the cytoplasm prevent disulphide bond formation and the protein cannot adopt its proper conformation (Derman *et al.*, 1993). The β-galactosidase of *E. coli* (LacZ) is a large tetrameric enzyme that is only active in the cytoplasm. If LacZ is attached to an export signal and attempts to traverse the cytoplasmic membrane it becomes trapped in the membrane and improperly folded which renders the enzyme inactive (Silhavy *et al.*, 1977). Confident that EbpS entered the cytoplasmic membrane of *E.coli,* a series of fusions were constructed between EbpS and alkaline phosphatase (PhoA) or β-galactosidase (LacZ) based on the protein's hydropathy profile. If the PhoA fusion is located distal to a transmembrane domain that spans the membrane from the cytoplasmic side to the periplasmic side this domain will act as a membrane translocation signal and the PhoA domain is placed in the periplasm where it becomes enzymatically active (Derman *et al.*, 1993). However if the fusion is linked to a transmembrane domain that spans the membrane from the periplasmic side to the cytoplasmic side the PhoA domain is located in the cytoplasm and cannot become enzymatically active. Conversely an EbpS-LacZ fusion will only be enzymatically active if it occurs distal to a transmembrane domain that spans the membrane in the outside-inside direction. The large tetrameric LacZ protein is only active in the cytoplasm because it is too large to be secreted into the periplasmic space.

Using pKK233-*ebpS* as the template, truncated derivatives of the *ebpS* gene lacking varying lengths of 3′ DNA were amplified by PCR along with 263 bp of sequence upstream of the ATG start codon to include the pKK233-2 IPTG–inducible promoter and ribosome binding site. The PCR products were amplified incorporating an *Eco*RI restriction site at the 5′ end and a *Sal*I restriction site at the 3′ end and cloned into the *phoA* vector pBAF (Fulkerson and Mobley, 2001) and the *lacZ* vector pLKC480 (Tiedeman and Smith, 1988) to produce in-frame *ebpS-phoA* and *ebpS-lacZ* fusions, respectively (Figure 4.5). The plasmid pBAF is a derivative of pBluescript II SK(+)

Figure 4.5 Production of pBAF*ebpS-phoA* and pLKC480*ebpS-lacZ* fusions. (a) 5′ truncates of the *ebpS* gene (t*ebpS*, ▨) were PCR-amplified from plasmid pKK233-2*ebpS* with 263 bp of upstream sequence incorporating the *trc* promoter (□) and the ribosome binding site (RBS, □) of pKK233-2 as *Eco*RI-*Sal*I fragments. (b) these fragments were cloned into the *Eco*RI-*Sal*I restriction sites upstream of the promoterless *phoA* gene and *lacZ* gene of plasmids pBAF and pLKC480, respectively, to create in-frame fusions. Expression of each fusion was driven from the imported *trc* promoter.


(a)
EcoRI
trc RBS
5′
tebpS
SalI
(b)
EcoRI
SalI
pBAF
4362 bps
phoA
Apr
EcoRI
SalI
lacZY
pLKC480
9900 bps
Apr
Kanr

containing the nucleotide sequence encoding the mature PhoA polypeptide lacking its N-terminal secretion signal sequence. Plasmid pLKC480 is a derivative of the *lacZY* fusion plasmids described by Minton (1984), containing the transposon Tn*5* kanamycin resistance gene. Both plasmids have been engineered to create active PhoA (pBAF) or LacZ (pLKC480) fusions in any desired reading frame. This feature of these plasmids allowed the same *ebpS* 5′ PCR-product to be cloned into *Eco*RI-*Sal*I restriction sites upstream of the *phoA* or *lacZ* gene to create in-frame *ebpS-phoA* and *ebpS-lacZ* fusions. This facilitated the fusion of EbpS N-terminal truncates to PhoA and LacZ at the same position allowing the complementing activities of PhoA and LacZ to be examined at the same fusion site. Fusion positions were chosen based on the hydropathy profile of EbpS and named according to the residue of EbpS after which PhoA of LacZ was fused. EbpS has three centrally located hydrophobic domains designated H1 (residues 205-224), H2 (residues 265-280) and H3 (residues 315-342) each of which is theoretically large enough to span the cytoplasmic membrane. Three fusions were made in the putative loop regions of EbpS between hydrophobic domains H1 and H2 (A234, T247 and S256) with another three between H2 and H3 (S285, A296 and H306) and two distal to domain H3 (D369 and D402) (Figure 4.6). Eight fusions were created to avoid ambiguous results. Both sets of fusions were expressed from the imported pKK233-2 IPTG-inducible *trc* promoter. The pBAF*ebpS-phoA* fusions were expressed in the *phoA E. coli* strain CC118 that has been used extensively in this type of study (Manoil and Beckwith, 1985). The pLKC480*ebpS-lacZ* fusions were expressed in the *lacZ E. coli* strain TG1 (Sambrook *et al.*, 1989). Expression of the pLKC480*ebpS-lacZ* fusions was controlled by LacI expressed by the co-resident plasmid pDIA17 to avoid problems of toxicity that occurred even when small amounts of the EbpS-LacZ proteins were expressed in the leaky $lacI^q$ host TG1. Plasmid pDIA17 is a derivative of pACYC184 with the *lacI* gene cloned into the *Bam*HI site allowing expression from the *tet* promoter. The pACYC184 replicon is compatible with that of pLKC480 so the plasmids coexist stably with antibiotic selection.

The alkaline phosphatase activities of pBAF*ebpS-phoA* fusions were initially tested on Luria agar plates incorporating IPTG to induce expression and the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP). The β-galactosidase activities of pLKC480*ebpS-lacZ* fusions were tested on Luria agar plates incorporating IPTG and 5-

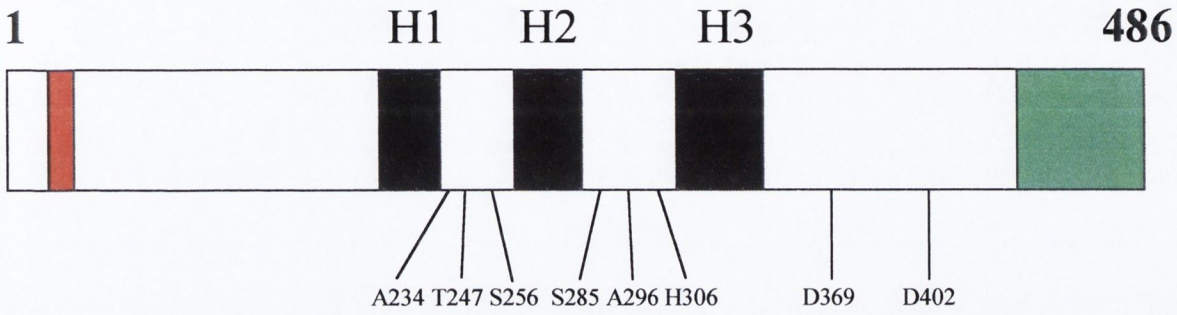


Figure 4.6 Positions of EbpS-PhoA or EbpS-LacZ fusions. The primary structure of the 486-residue elastin-binding protein of strain 8325-4 (Roche, 2000) is depicted with the elastin binding domain (▭) three hydrophobic domains (▬), H1 (residues 205–224), H2 (residues 265–280) and H3 (residues 315–342) and the LysM motif (▭). Positions of fusions were named according to the residue of EbpS after which PhoA or LacZ was fused (single letter amino acid code).

bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). High enzymatic activity in the presence of the relevant chromogenic substrate would result in blue coloured colonies. Only the EbpS-PhoA C-terminal fusions at positions D369 and D402 showed detectable PhoA activity indicated by blue coloured colonies whereas fusions at the other six positions showed no detectable activity (Table 4.1). Conversely, EbpS-LacZ fusions at positions A234, T247, S256, S285, A296 and H306 showed high levels of β-galactosidase activity whereas the D369 and D402 fusions showed no detectable activity (Table 4.1).

To quantify the levels of alkaline phosphatase or β-galactosidase activity expressed by EbpS-PhoA or EbpS-LacZ fusions, respectively, enzyme assays were performed with the relevant chromogenic substrate. Initial experiments performed with cultures grown at 37°C produce erratic and irreproducible results. To address these problems cultures of strains harbouring *ebpS-phoA* or *ebpS-lacZ* fusions were grown at 30°C in the presence of 0.4% (w/v) glucose. Both these measures are known to optimise expression of stable heterologous proteins in *E. coli* (Hanning and Makrides, 1998). Cultures were grown to mid exponential phase and then induced with IPTG at a final concentration of 3mM (*phoA* fusions) or 2mM (*lacZ* fusions) and then grown for a further 4 hours. Alkaline phosphatase activities of pBAF*ebpS-phoA* were assessed by the method of Brickman and Beckwith (1975) using BCIP as the alkaline phosphatase chromogenic substrate. β-galactosidase activities were assayed by the method of Miller (1972) using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a chromogenic substrate. Similar to the results on agar, only the C-terminal fusions at positions D369 and D402 showed detectable PhoA activity with values of 71.01 ± 6.99 and 71.25 ± 9.92 phosphatase units, respectively (Figure 4.7). This indicated that these fusions promoted exposure of PhoA in the periplasm. In contrast those occurring between hydrophobic domains H1-H2 and H2-H3 showed little detectable PhoA activity with values ranging between 2.27 and 4.53 phosphatase units above background levels, indicating that PhoA at these fusion positions were cytoplasmically located. This was in direct contrast to the LacZ fusions at the same residues, where the proximal fusions between H1-H2 and H2-H3 gave high levels of β-galactosidase ranging between 229 and 886 Miller units whereas the C-terminal fusions, D369 and D402, were almost devoid of activity (Figure 4.7).

Position of Fusion	Colour of Colonies Expressing EbpS-PhoA Fusion on BCIP Indicator Plates	Colour of Colonies Expressing EbpS-LacZ Fusion on X-Gal Indicator Plates
A234	White	Blue
T247	White	Blue
S256	White	Blue
S285	White	Blue
A296	White	Blue
H306	White	Blue
D369	Blue	White
D402	Blue	White

Table 4.1 Alkaline phosphatase and β-galactosidase activities of EbpS-PhoA and EbpS-LacZ fusions as determined on L-agar incorporating 40 μg/ml BCIP (PhoA fusions) or 40 μg/ml X-Gal (LacZ fusions). The position of each fusion is indicated. High enzymatic activity is indicated by blue coloured colonies.

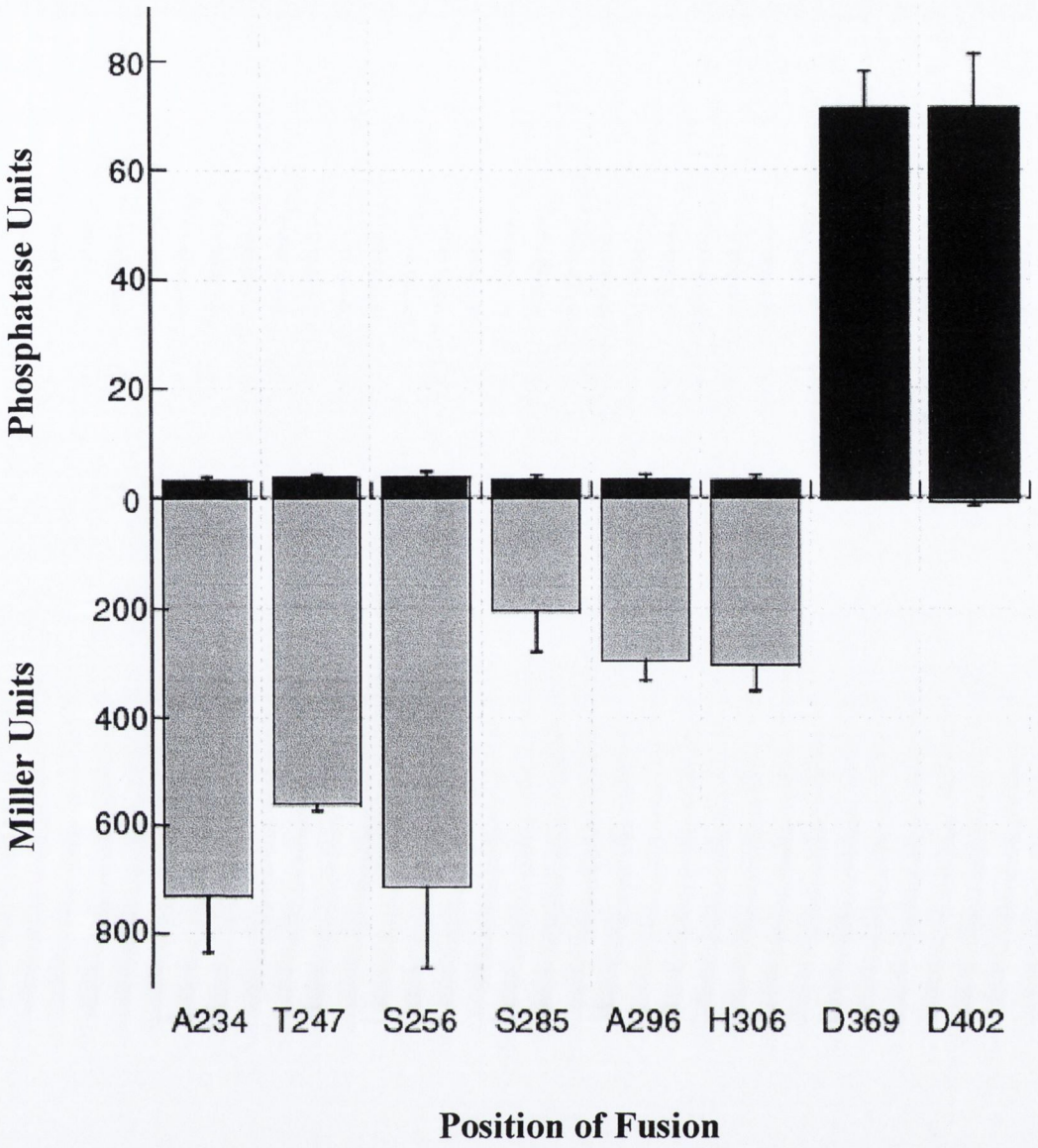


Figure 4.7 Enzymatic activity expressed by EbpS-PhoA and EbpS-LacZ fusions. Alkaline phosphatase (AP) activities of EbpS-PhoA fusions expressed in *E.coli* CC118 (mean specific activities of three independent experiments). Background levels of AP activity from CC118 (pBAF) were subtracted. β-galactosidase activities of EbpS-LacZ fusions expressed in *E.coli* TG1 (pDIA17) (mean specific activities of three independent experiments). Background enzyme activity from TG1(pDIA17/pLKC480) was subtracted.

To ensure that each EbpS-PhoA and EbpS-LacZ fusion protein was expressed, Western immunoblotting analysis was performed on envelope fractions, containing inner and outer membranes, of each strain. Membranes of *E. coli* CC118 and TG1 expressing EbpS-PhoA and EbpS-LacZ fusions respectively were prepared by mechanically disrupting cells. Cultures were grown and induced with IPTG and then corrected to the same OD_{600nm} before they were lysed in a FastPrep™ cell disrupter in the presence of the cell wall peptidoglycan-degrading enzyme lysozyme. Membranes of each strain were then pelleted by centrifugation and resuspended before equal volumes were analysed by Western immunoblotting. Western immunoblotting analysis with polyclonal anti-PhoA antibodies indicated that each of the EbpS-PhoA fusions expressed an immunoreactive protein of between 100-150 kDa that was not present in membranes of strain CC118(pBAF) (Figure 4.8). The size of the proteins increased in proportion to the length of EbpS sequence incorporated. This indicated that differing PhoA activities could be attributed to protein localisation and not to proteolytic degradation or lack of expression. Each of the EbpS-LacZ fusions, detected with polyclonal antibodies against β-galactosidase, expressed a single major immunoreactive protein apart from the inactive D369 and D402 fusions (Figure 4.8). In the last two samples, several smaller immunoreactive bands were sometimes detected, which indicates that extensive degradation was occurring. Degradation of β-galactosidase as the protein attempts to traverse the cyoplasmic membrane has been reported previously (Fulkerson and Mobley, 2001). After induction, cells bearing these two mutants grew very slowly, which is consistent with synthesis of a toxic protein.

The EbpS-PhoA and EbpS-LacZ fusion data allows two models to be proposed for the membrane topography of EbpS (Figure 4.9). Since the ligand-binding domain of EbpS is located at the N terminus between residues 14 and 34 (Park *et al.*, 1999) this region of the protein must be located on the extracellular face of the cytoplasmic membrane for the protein to perform its ligand-binding function. The low PhoA activities and high LacZ activities of fusions at positions between hydrophobic domains H1-H2 and H2-H3 indicates that this region of EbpS is located in the cytoplasm, suggesting that the domain H1 acts as a transmembrane α-helix. Similarly, the high PhoA and low LacZ activities of the fusions at positions D369 and D402 indicate that the region of EbpS

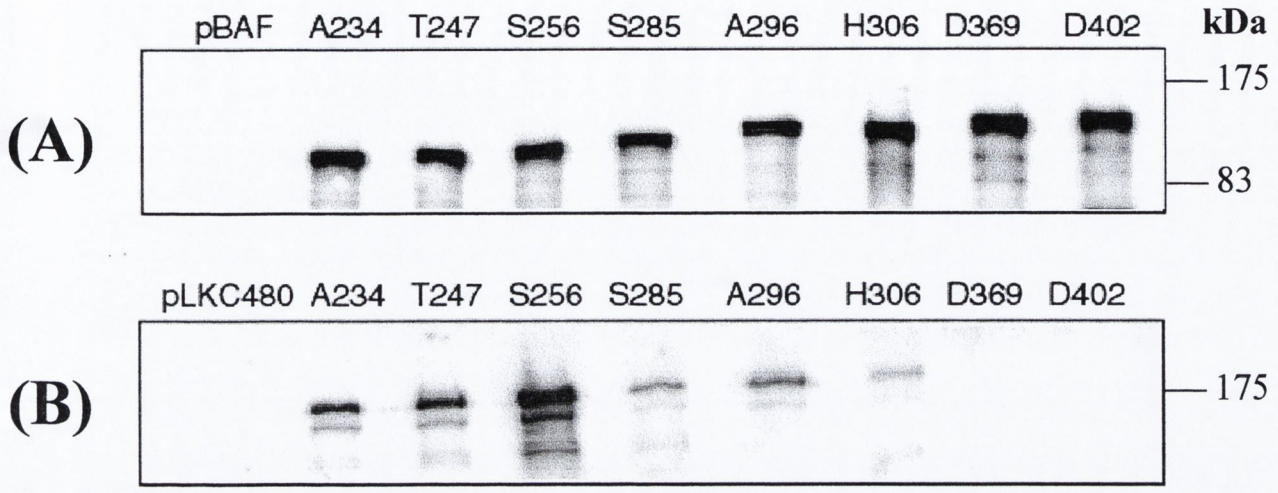


Figure 4.8 Western immunoblotting analysis of EbpS-PhoA and EbpS-LacZ fusions. (A) EbpS-PhoA fusion proteins in the membrane fraction of *E.coli* CC118 (pBAF*ebpS-phoA*) detected with polyclonal anti-PhoA antibodies. (B) EbpS-LacZ fusions proteins in envelope fraction of *E.coli* TG1 (pDIA17, pLKC480*ebpS-lacZ*) detected with anti-β-galactosidase antibodies. The positions of fusions are indicated.

distal to the hydrophobic domain H3 is located in the periplasm on the extracellular face of the cytoplasmic membrane and that domain H3 is also a transmembrane domain. In both models, the N-terminal and the C-terminal domains are located on the outer face of the cytoplasmic membrane while the central part of the protein, containing the hydrophobic domain H2, is on the cytoplasmic face. The middle hydrophobic domain H2 might be membrane-associated but probably does not traverse the membrane.

The positive-inside rule states 'that positively charged amino acids residues (arginine and lysine) are much more abundant in the cytoplasmic hydrophilic loops as compared to the periplasmic loops of bacterial integral membrane proteins' with an average of 5% of positively charged residues present in membrane-translocated hydrophilic loops and an average of 15% positively charged residues in untranslocated loops (von Heijne, 1992). In previous studies it has been shown that the deletion or introduction of positively charged residues into the loops flanking hydrophobic transmembrane domains can alter the topology of an integral membrane protein in *E. coli* (Andersson *et al.*, 1992). This is due to the inability of highly positively charged regions of membrane proteins to traverse the negatively charged lipid bilayer. Previous studies have shown that the cellular location of hydrophilic regions greater than 70 residues in length is determined independently of the distribution of charged residues (von Heijne and Gavel, 1988). With regard to EbpS, both the N terminus and C terminus, which are located on the extracellular face of the cytoplasmic membrane, are greater than 70 residues in length thus their cellular location is determined independently of their net charge. However, 10/40 (25%) of the hydrophilic residues located in the cytoplasm between domains H1 and H2 of EbpS and 6/34 (18%) of the residues between domains H2 and H3 are positively charged. There is a high concentration of positively charged residues located adjacent to the transmembrane helices H1 and H3 on their cytoplasmic side which may act as 'stop translocation' signals due to the inability of a concentrated region of positive charged residues to traverse the negatively charged phospholipid bilayer (Figure 4.10). This indicates that the proposed topology of EbpS in the cytoplasmic membrane satisfies the positive-inside rule.

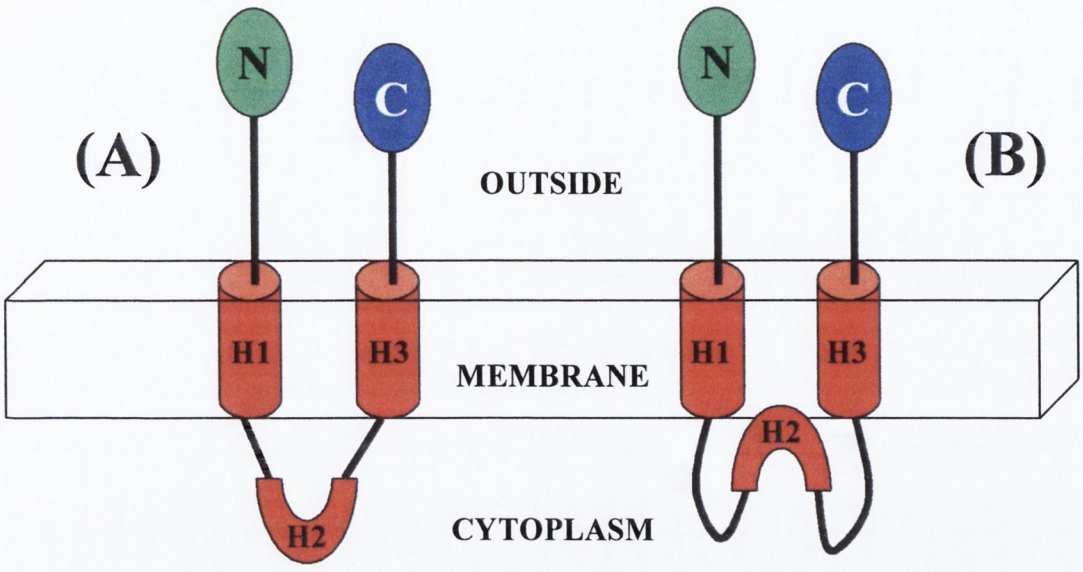


Figure 4.9 Schematic diagram of the deduced topology of EbpS in the cytoplasmic membrane. Both the N terminus and C terminus are located on the extracellular face of the cytoplasmic membrane. The hydrophobic domains H1 and H3 are transmembrane α-helices. The central hydrophobic domain, H2, is located on the cytoplasmic side of the cytoplasmic membrane. This domain may be unassociated with the cytoplasmic membrane (A) or associated with the inner face of the cytoplasmic membrane (B).

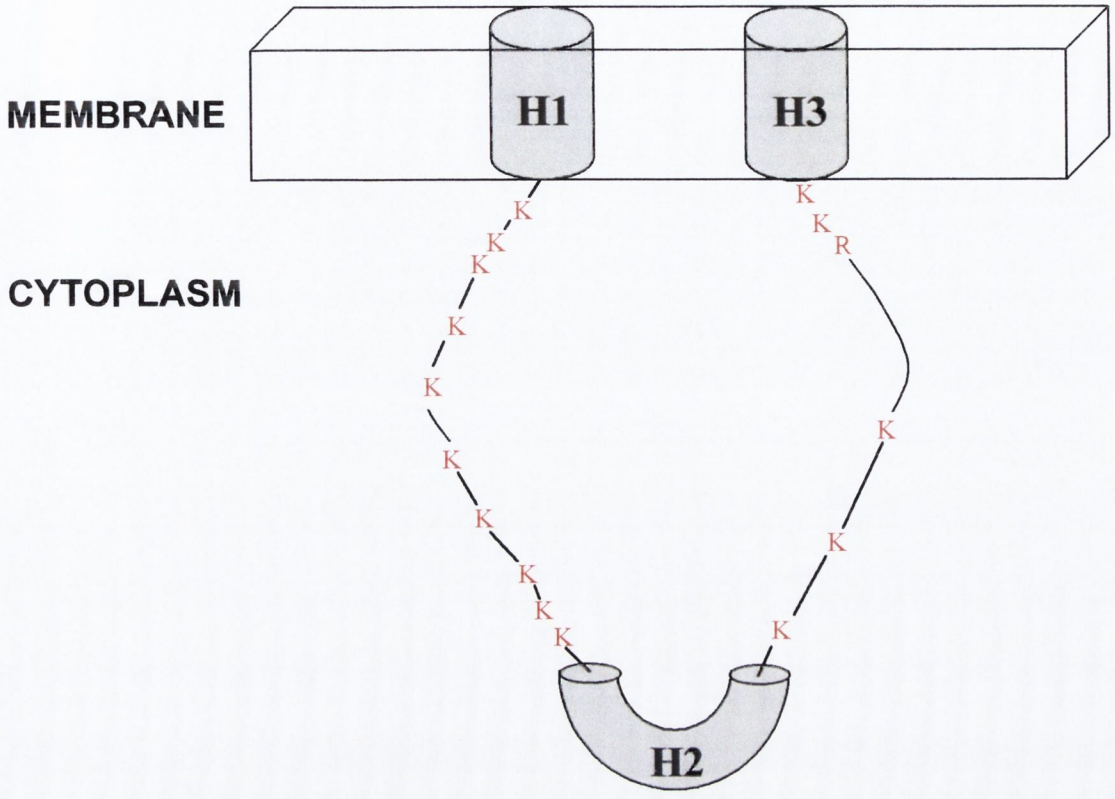


Figure 4.10 Distribution of positively charged lysine (K) and arginine (R) residues in the cytoplasmically located hydrophilic loop of EbpS between hydrophobic domains H1 and H3.

4.2.4 Topology of EbpS in the cytoplasmic membrane of *S. aureus*

Previously it had been reported that the N terminus of EbpS was detected on the surface of intact *S. aureus* cells by whole cell immunoblotting, but that the C terminus was undetectable (Roche, 2000). EbpS was only detected by this method when overexpressed (8-fold compared to wild-type) from the multicopy shuttle vector pCU1 (Roche, 2000). Since PhoA/LacZ analysis had shown that both the N terminus and C terminus of EbpS were located on the extracellular face of the cytoplasmic membrane, perhaps the N terminus of the protein was projected away from the cell wall peptidoglycan thus allowing this region to be detected with antibodies while the C terminus remained buried in the peptidoglycan lattice and inaccessible to antibodies. To address this problem, stabilised protoplasts of *S. aureus* were used in antibody absorption experiments.

If a region of an integral membrane protein to which specific antibodies have been raised is exposed on the surface of stabilised protoplasts then the antibodies can bind to their antigen. The protoplasts and associated antibodies can then be pelleted leaving any remaining antibodies in the supernatant. Thus a fixed concentration of antibodies incubated with increasing amounts of protoplasts will result in less and less antibodies in the supernatant due to absorption. Conversely, if the region of the protein to which antibodies have been raised is cytoplasmically located then the antibodies cannot gain access to their antigen across the membrane lipid bilayer. Thus the concentration of antibody in the supernatant after pelleting will remain constant irrespective of the amount of protoplasts added (Figure 4.11). After the antibodies have been incubated with the protoplasts and the protoplasts have been pelleted the supernatants containing any unabsorbed antibodies, can be quantified by their ability to bind to the immobilised purified protein antigen by dot immunoblotting. If the antigen was exposed on the surface of the protoplasts, the antibodies will be completely absorbed leaving no antibodies in the supernatant. This supernatant will then be unable to recognize recombinant antigen immobilised on a membrane by dot immunoblotting. Conversely, if the antigen is not surface-exposed then the antibodies will not be absorbed and the immobilised recombinant antigen will react in dot immunoblotting (Figure 4.11).

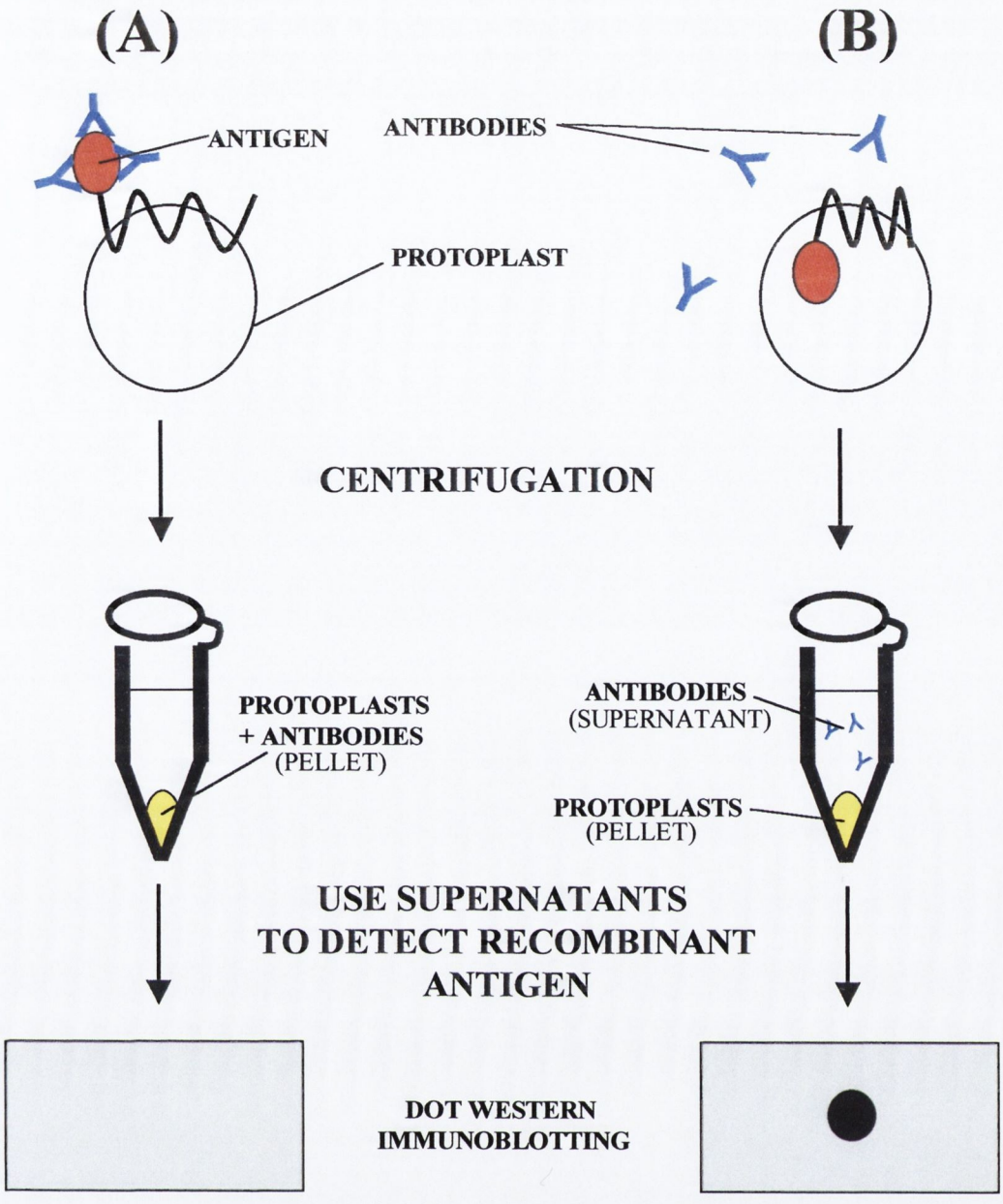


Figure 4.11 Schematic representation of the strategy used to determine protein topology by antibody absorption. (A) If the region of the protein to which antibodies have been raised (●) is located on the extracellular face of the membrane then the antibodies can bind to their antigen. The protoplasts and associated antibody can then be pelleted by centrifugation and the supernatant, devoid of antibodies, will be unable to recognize the immobilised purified antigen by dot immunoblotting. (B) If the antigen is cytoplasmically located the antibodies cannot bind to their antigen. The protoplasts can then be pelleted, leaving the antibodies in the supernatant. This supernatant will react strongly with the immobilised purified recombinant antigen by dot immunoblotting.

Stabilised protoplasts of cells from late exponential phase cultures (OD_{600nm} 1.5) of *S. aureus* strains Newman *spa ebpS* and the same strain in which the *ebpS* mutation had been complemented by expression of EbpS from the multicopy plasmid pCU-*ebpS*$^+$ were prepared by lysostaphin digestion of the cell wall peptidoglycan in the presence of 30% (w/v) raffinose and 20mM $MgCl_2$. The plasmid pCU-*ebpS*$^+$ contains the *ebpS* gene cloned with 300 bp of upstream sequence and 200 bp of downstream sequence and has been shown to express 8-fold more EbpS than wild-type and to complement the elastin binding phenotype (Roche, 2000). Cells from late exponential phase cultures were used because they produce stabler protoplasts than cells from stationary phase cultures (data not shown). Anti-rEbpS$_{1-267}$ and anti-rEbpS$_{343-486}$ antibodies were incubated with increasing amounts of stabilised protoplasts of each strain after the protoplasts had been incubated with anti-ClfA$_{220-559}$ antibodies to saturate an undefined membrane-associated immunoglobulin binding protein. After incubation the stabilised protoplasts were pelleted by centrifugation and antibodies remaining in the supernatants measured by incubating with polyvinylidene diflouride (PVDF) membranes spotted with 1μg amounts of rEbpS$_{1-267}$ (anti-rEbpS$_{1-267}$ antibodies) or rEbpS$_{343-486}$ (anti-rEbpS$_{343-486}$ antibodies). Increasing concentrations of protoplasts of strain Newman *spa ebpS* (pCU- *ebpS*$^+$) resulted in absorption of the anti-rEbpS$_{1-267}$ antibodies onto the protoplast surface as shown by a lower titre in the reaction with immobilised rEbpS$_{1-267}$ (Figure 4.12, panel A). The absorption was specfic to EbpS since it did not occur with protoplasts of the *ebpS* null mutant (Figure 4.12, panel B). Anti-rEbpS$_{343-486}$ antibodies also were absorbed by the protoplasts in an EbpS-dependent manner (Figure 4.12, panels C and D). To ensure that protoplasts were not lysing and therefore allowing antibodies access to the cytoplasmic face of the membrane, samples were examined by phase contrast microscopy at every stage of the procedure.

These data validate the PhoA/LacZ experiments in *E. coli* indicating that both the N and C termini of EbpS are located on the extracellular face of the cytoplasmic membrane of *S. aureus*. Previous experiments whereby EbpS was detected on the surface of whole *S. aureus* cells suggested that only the N terminus was surface exposed (Roche, 2000). Now a model for membrane-associated EbpS can be proposed whereby both the N and C termini of EbpS are located on the extracellular face of the membrane with the N

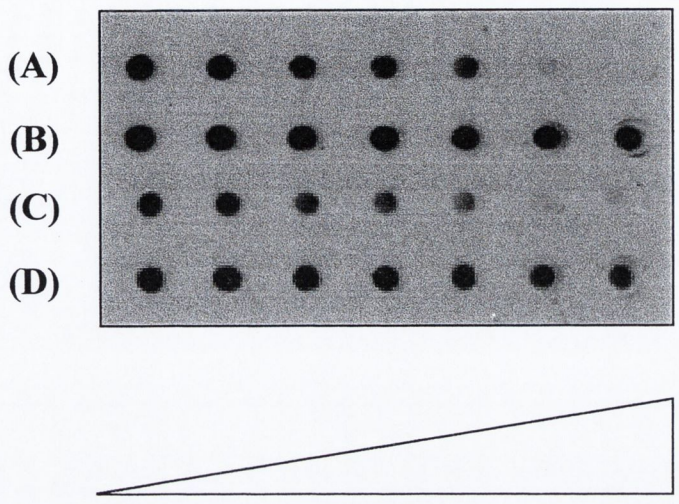


Figure 4.12 Western dot immunoblotting with immobilised recombinant protein detected with antibodies absorbed against staphylococcal protoplasts. Panel A, $rEbpS_{1-267}$ detected with anti-$rEbpS_{1-267}$ antibodies that had been absorbed against increasing concentrations of protoplasts of *S. aureus* strain Newman *spa ebpS* (pCU-*ebpS*$^{+}$). Panel B, $rEbpS_{1-267}$ detected with anti-$rEbpS_{1-267}$ antibodies that had been absorbed against increasing concentrations of protoplasts of *S. aureus* strain Newman *spa ebpS*. Panel C, $rEbpS_{343-486}$ detected with anti-$rEbpS_{343-486}$ antibodies that had been absorbed against increasing concentrations of protoplasts of *S. aureus* strain Newman *spa ebpS* (pCU-*ebpS*$^{+}$). Panel D, $rEbpS_{343-486}$ detected with anti-$rEbpS_{343-486}$ antibodies that had been absorbed against increasing concentrations of protoplasts of *S. aureus* strain Newman *spa ebpS*.

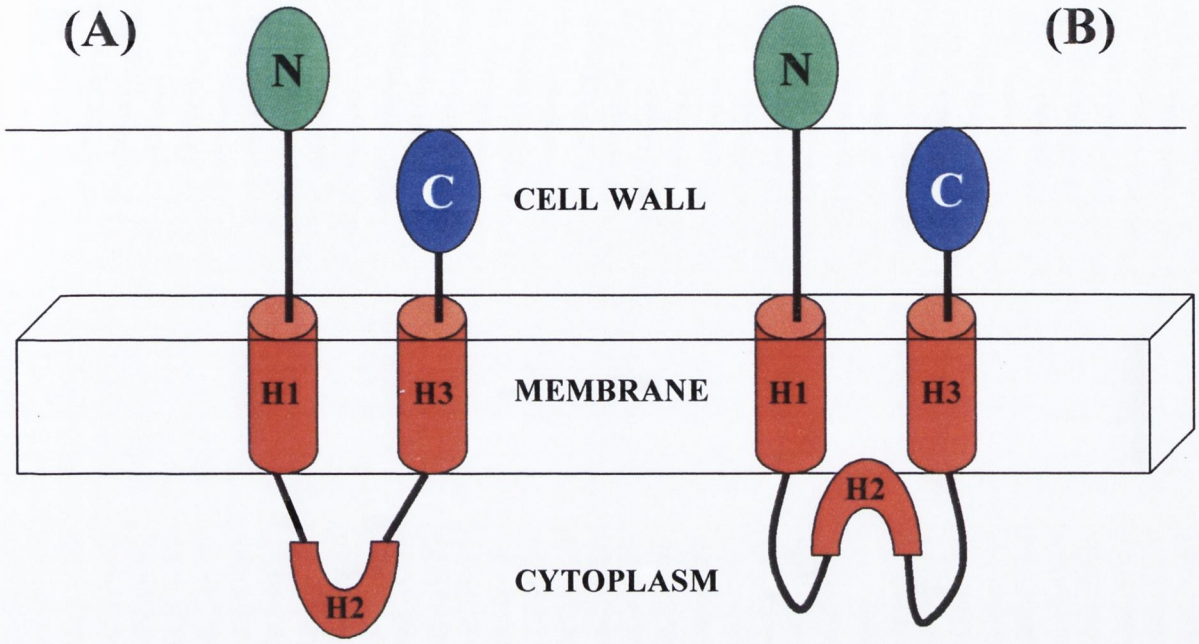


Figure 4.13 Schematic diagram of the deduced topology of EbpS in the cytoplasmic membrane with both the N terminus and C terminus are located on the extracellular face of the cytoplasmic membrane. The hydrophobic domain H2 may be unassociated with the cytoplasmic membrane (A) or associated with the inner face of the cytoplasmic membrane (B). The N terminus protrudes through the cell wall and is displayed on the cell surface. The C terminus is not displayed on the cell surface

terminus projected away from the cell wall peptidoglycan and the C terminus buried in the peptidoglycan lattice (Figure 4.13).

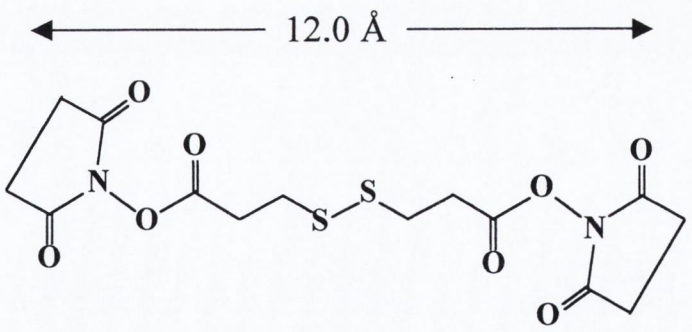
4.2.5 Cross-linking studies

To obtain some indication as to whether EbpS is present in the *S. aureus* cytoplasmic membrane as a monomer or as a multimeric complex, cross-linking studies were performed. The basis of this type of study is that if a protein is part of a homomeric or heteromeric protein complex, then the components of this complex can be joined covalently using a homobifunctional chemical cross-linking. This results in an increase in the apparent molecular weight of the protein of interest as determined by SDS-PAGE analysis. If the protein of interest is a monomer then treatment with a chemical cross-linking agent will not result in an increase in molecular weight.

Dithiobis(succinimidyl proprionate) (DSP) is a membrane-soluble, homobifuctional and thiol-cleavable cross-linking reagent. It possesses an ester group at each end of the molecule that reacts with amino groups of amino acids (Figure 4.14a) (Joshi and Burrows, 1990). The cytoplasmic membrane of *S. aureus* strain Newman *spa* was prepared by mechanical disruption of cells in a FastPrep™ cell disrupter and differential centrifugation. The purified cytoplasmic membrane was treated with DSP at a ratio of protein to DSP of 50:1 (weight) and analysed by SDS-PAGE and Western immunoblotting under non-reducing conditions with anti-rEbpS$_{343\text{-}486}$ antibodies. In the absence of DSP the 83 kDa EbpS monomer was detected (Figure 4.14b, lane 1). In the presence of DSP the 83 kDa EbpS monomer was observed along with a single high molecular weight band of approximately 250 kDa (Figure 4.14b, lane 2). Attempts to increase the intensity of this high molecular weight protein by increasing the ratio of DSP to protein resulted in indistinguishable protein smears on Western immunoblots (data not shown).

These data indicated that EbpS may be present in the cytoplasmic membrane as a multimeric complex. Although this experiment gives no indication as to whether this a heteromeric or homomeric complex it is interesting to note that that the molecular mass of the high molecular weight band observed in the presence of DSP was three times the

(a)



Dithiobis(succinimidyl propionate)

(b)

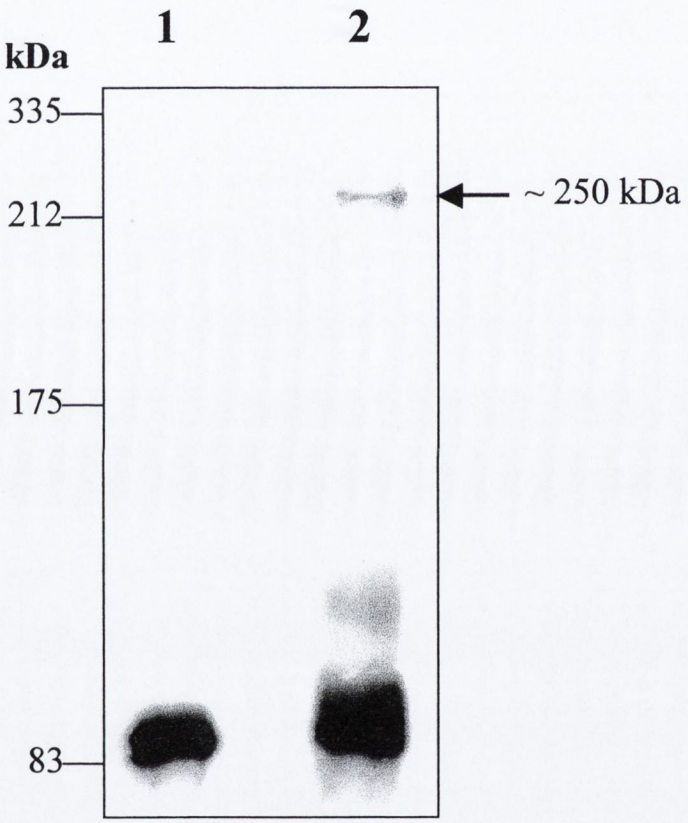


Figure 4.14 EbpS cross-linking studies. (a) Chemical structure of the homobifunctional cross-linking reagent dithiobis(succinimidyl propionate) (DSP). (b) Western immunoblotting analysis of untreated purified cytoplasmic membrane from *S. aureus* strain Newman *spa* (lane 1) and cytoplasmic membrane from *S. aureus* strain Newman *spa* treated with DSP at a ratio of DSP to protein concentration of 1:50 (weight) (lane 2). Proteins were detected with anti-rEbpS$_{343-486}$antibodies.

mass of the monomeric form of EbpS suggesting that the protein may be present in the cytoplasmic membrane as a homotrimeric complex.

4.3 Discussion

Previously it has been shown that the elastin-binding protein of *S. aureus* (EbpS) is a 486-residue protein with three centrally located regions composed largely of residues with hydrophobic side chains. Each of these hydrophobic domains, designated H1 (residues 205-224), H2 (residues 265-280) and H3 (residues 315-342) is theoretically large enough to span the cytoplasmic membrane (Roche, 2000). For a protein to be an integral membrane protein it must possess at least one hydrophobic transmembrane α-helix and it must satisfy the positive-inside rule whereby the net positive charge of untranslocated hydrophilic loops is greater than that of translocated loops (von Heijne, 1992). With the knowledge that EbpS is associated in some way with the cytoplasmic membrane and that it possesses three large hydrophobic domains it was decided to investigate whether EbpS is an integral membrane protein and, if so, its topological orientation.

In silico topological analysis of the EbpS primary amino acid sequence was performed. A number of publically available programs were used to predict if EbpS is an integral membrane protein and, if so, its topological orientation in the membrane. The TMHMM program (Sonnhammer *et al.*, 1998) predicted that EbpS is an integral membrane protein with two transmembrane α-helices produced by regions H1 and H3 whereas the central hydrophobic region, H2, was not predicted to be a transmembrane α-helix. Furthermore, the DAS (Cserzo *et al.*, 1997) and the TMpred (Persson and Argos, 1994) programs predicted EbpS to be an integral membrane protein although these programs calculated that all three hydrophobic domains form transmembrane α-helices. Although the results from this type of analysis were purely speculative it has been used in previous studies to predict topological models for integral membrane proteins (von Heijne, 1992). These data indicated that the primary amino acid sequence of EbpS satisfied the strict parameters laid down by each program for the prediction of transmembrane α-helices in an integral membrane protein and endorsed the notion that EbpS may be an integral membrane protein.

In previous studies the topology of integral membrane proteins from *S. aureus* and *B. subtilis* has been determined using gene fusions expressed in the Gram-negative organism *E. coli* (Hirata *et al.*, 1998; Green and Cutting, 2000). Expression of EbpS with a N-terminal six-histidine tag in *E. coli* established that the protein formed inclusion bodies in the *E. coli* cytoplasm (Roche, 2000). This would hinder any topological analysis of EbpS when the protein was expressed in this organism. To ensure that this did not occur with the native form of the protein, EbpS was expressed in *E. coli* from the 'ATG' expression vector pKK233-2. This vector permitted the IPTG-regulated expression of EbpS in *E. coli* in its native form. EbpS was expressed from this vector in whole cell lysates of *E. coli* strain XL1-blue as an 83 kDa protein with evidence of some proteolytic degradation as determined by Western immunoblotting. The protein was expressed in this strain even in the absence of IPTG indicating that strain XL1-blue did not produce enough LacI repressor protein to prevent EbpS expression. To establish if EbpS expressed in *E. coli* was associated with the cytoplasmic membrane or formed inclusion bodies, cells were fractionated into inner and outer membranes using isopynic centrifugation on a sucrose gradient (Withholt *et al.*, 1976). This technique separated the inner and outer membranes due to their different densities, which focuses each membrane at a different level on a linear sucrose gradient (Johnston and Gotlisch, 1974). Each purified membrane fraction was analysed by Western immunoblotting with anti-rEbpS$_{343-486}$ antibodies and EbpS was detected predominantly in the inner membrane fraction as proteolytic degradation products of approximately 47 kDa and 38 kDa. The specificity of the reaction was ensured by the fact that this protein was not detected in whole cell lysates of strain XL1-blue containing the empty vector. The extensive proteolytic degradation of EbpS could be attributed to the long isolation procedure performed in the absence of protease inhibitors. These data indicated that a proportion of EbpS is associated with the cytoplasmic membrane of *E. coli*, although a fraction of the protein could remain in the cytoplasm in the form of inclusion bodies.

Next it was decided to determine if the protein is an integral membrane protein using alkaline phosphatase (PhoA) and β-galactosidase (LacZ) fusions to C-terminal regions of EbpS. Previously it had been shown that the topology of the integral membrane protein, cytochrome c oxidase from *Paracoccus denitificans*, as determined

using PhoA fusions agreed with the topology of the protein resolved by crystallographic studies (Iwata *et al.*, 1995) indicating that this type of gene fusion analysis is an effective and reliable method for the study of integral membrane protein topology. Using plasmid pKK233-*ebpS* as a template for PCR, 5′ regions of the *ebpS* gene were amplified along with the upstream *trc* promoter region and ribosome-binding site of pKK233-2. The resulting PCR products were cloned in-frame into the PhoA and LacZ expression vectors pBAF and pLKC480, respectively, to produce EbpS-PhoA and EbpS-LacZ fusions. These vectors had been engineered in such a way that the same PCR product could be cloned in-frame into each vector thus allowing the analysis of PhoA and LacZ fused to the same position of EbpS. Three fusions were made in the putative loop regions of EbpS between hydrophobic domains H1 and H2 (A234, T247 and S256) with another three between H2 and H3 (S285, A296 and H306) and two distal to domain H3 (D369 and D402) with each reporter enzyme. Analysis of the PhoA and LacZ activities of each fusion both qualitatively on agar containing the relevant chromogenic substrates and quantitatively in liquid assays revealed that the region of EbpS distal to region H3 is located on the extracellular face of the cytoplasmic membrane as indicated by the high PhoA activities of EbpS-PhoA fusions at positions D369 and D402 and the low LacZ activities of the corresponding EbpS-LacZ fusions. All six fusion positions between regions H1 and H3 demonstrated high LacZ activity and low PhoA activity thus indicating that this region of EbpS is located in the cytoplasm. Expression of EbpS-LacZ fusions was controlled by LacI expressed from the co-resident plasmid pDIA17. In the absence of this plasmid large amounts of toxic fusion proteins were produced even without IPTG induction resulting in cell lysis.

Western immunoblotting analysis of total membrane fractions of strains expressing EbpS-PhoA and EbpS-LacZ with anti-PhoA and anti-LacZ antibodies confirmed that the different activities of each fusion could be attributed to the cellular localisation of the reporter molecule and not due to lack of expression. Although no protein was detected in the membranes of the strains expressing EbpS-LacZ fusions at positions D369 and D402, proteolytic degradation products were sometimes observed and cultures grew slower than cultures of the same strain containing the empty vector pLKC480 thus indicating the production of toxic proteins. As the large LacZ protein

attempts to traverse the cytoplasmic membrane it becomes improperly folded which may make it more susceptible to proteolytic cleavage (Silhavy *et al.*, 1977). This type of degradation of improperly folded membrane-bound LacZ has been reported previously (Fulkerson and Mobley, 2001).

Since the N terminus of EbpS must be on the outside of the cytoplasmic membrane to perform its ligand-binding function (Park *et al.*, 1999) these data revealed that EbpS is an integral membrane protein with both the N terminus and C terminus of the protein located on the extracellular face of the cytoplasmic membrane and with two transmembrane α-helices, H1 and H3. The hydrophobic domain H2 is either cytoplasmically located or interacts with the inner face of the cytoplasmic membrane but probably does not traverse it. This proposed topology for EbpS was supported by analysis of the distribution of positively charged residues within the protein. According to the positive-inside rule the net positive charge of cytoplasmically located hydrophilic loops must be greater than that of periplasmically located loops. This rule only applies to stretches of hydrophilic residues less than 70 residues in length since the topological orientation of larger regions is determined independently of charge distribution. This implied that the cellular location of both the N terminus of EbpS proximal to transmembrane α-helix H1 and the C terminus of EbpS distal to transmembrane helix H3, which are both located on the extracellular face of the membrane, is determined independently of the net charge of these regions since they are greater than 70 residues in length. The high concentration of positively charge residues discovered in the hydrophilic loops between domains H1 and H2 (25%) and between H2 and H3 (18%) was in agreement with the cytoplasmic location of these regions of EbpS and validated the topological model.

The topological model for the membrane topology of EbpS was further supported by antibody absorption experiments. Previously it had been shown that the N terminus but not the C terminus of EbpS could be detected on the surface of *S. aureus* cells using specific antibodies (Roche, 2000). Since both the C terminus and the N terminus of EbpS were located on the extracelluar surface of the cytoplasmic membrane this suggested that the N terminus of the protein was displayed on the cell surface and was accessible to antibodies whereas the C terminus was not surface-exposed but remained buried in the

cell wall peptidoglycan. Anti-rEbpS$_{1-267}$ and anti-rEbpS$_{343-486}$ antibodies were absorbed onto the surface of stabilised protoplasts of *S. aureus* in an EbpS-dependent manner. This indicated that both the N terminus and C terminus of EbpS were exposed on the extracellular face of the *S. aureus* cytoplasmic membrane and were accessible to antibodies in the absence of the cell wall peptidoglycan. These data reinforced the EbpS topological model in the cytoplasmic membrane and permitted a further model to be proposed whereby both the N terminus and C terminus were located on the extracellular face of the cytoplasmic membrane with the N terminus displayed on the cell surface but with the C terminus hidden in the peptidoglycan lattice.

To gain some insight into whether EbpS is present in the cytoplasmic membrane as a monomer or as a mutimeric complex, purified cytoplasmic membrane from *S. aureus* was treated with the homobifunctional cross-linking reagent dithiobis(succinimidyl proprionate) (DSP). Purified membrane was used rather than whole cells to avoid the possible cross-linking of EbpS to cell wall or cytoplasmic proteins with the membrane soluble cross-linking reagent DSP. Analysis of DSP-treated membranes by Western immunoblotting with anti-rEbpS$_{343-486}$ antibodies revealed that some of the EbpS molecules were associated with a 250 kDa band while the majority of the protein remained in the 83 kDa monomeric form. Attempts to increase the proportion of the high molecular weight form of the protein by increasing the DSP concentration resulted in indistinguishable protein smears on Western immunoblots, a problem frequently encounter with this type of analysis (M. Meehan, personal communication). These data revealed that at least a fraction of EbpS was present in the cytoplasmic membrane as part of a multimeric complex. It could be argued that the high molecular weight form of EbpS was just the protein randomly cross-linked to other membrane-associated proteins. If this were the case then EbpS would be detected as a number of different high molecular bands because the protein would be cross-linked to proteins of differing molecular weights. Conversely, EbpS was detected as a single high molecular weight band after treatment with DSP suggesting that the protein was consistently cross-linked to the same member(s) of a multimeric complex. It is tempting to hypothesise that this multimeric form of EbpS is a trimer since its molecular weight is approximately three times that of the 83 kDa monomer, but this would require further investigation.

The secretion of extracellular and integral membrane proteins has been studied in various Gram-negative organisms, particularly *E. coli*. Studies with this organism have revealed that secretion of the vast majority of membrane associated or integral membrane proteins occurs by the Type II Sec-dependent secretion pathway (Fekkes and Dreissen, 1999). The Sec pathway consists of membrane-associated and cytoplasmic proteins involved in the ATP-dependent translocation of polypeptides across the cytoplasmic membrane (Pugsley, 1993; Dreissen *et al.*, 1998). Proteins secreted by this mechanism require a hydrophobic N-terminal signal sequence that is cleaved off during secretion by a peptidase component of the Sec apparatus (Izard and Kendall, 1994). Integral membrane proteins are delivered to the cytoplasmic membrane by the *E. coli* homologue of the signal recognition particle (SRP) apparatus of eukaryotic cells (DeGier *et al.*, 1998). The cytoplasmic component of this apparatus, Ffh, binds to the nascent polypeptide chain and delivers it to the cytoplasmic membrane via an interaction with the membrane-associated component of the apparatus, FtsY. Whether subsequent insertion of the integral membrane protein into the cytoplasmic membrane absolutely requires the Sec translocon or can occur in its absence is still a matter of great debate.

Previously, it was reported that the only posttranslational modification of EbpS was removal of the initial methionine residue (Park *et al.*, 1991). This indicated that the protein did not possess an N-terminal Sec-dependent signal sequence required for the conventional translocation of the N terminus across the cytoplasmic membrane. This form of posttranslational processing has been reported previously for a cell-wall-associated cell wall hydrolase, CwlC, of *B. subtilis* (Smith and Foster, 1995). Similarly, a large number of integral cytoplasmic membrane proteins of *E. coli* have been reported with their N termini on the extracellular face of the cytoplasmic membrane even though they do not possess a Sec-dependent signal sequence (Dabley *et al.*, 1995). Included in this group is ProW, a constituent of the *E. coli* ProU osmoregulatory system. This protein has seven transmembrane helices with the first 100 residues of its N terminus located on the extracellular face of the cytoplasmic membrane even though it does not possess a Sec-dependent signal sequence (Whitley *et al.*, 1994). Studies with this protein have shown that secretion of the N terminus across the inner membrane absolutely required the presence of the first downstream transmembrane α-helix and the SRP-targeting pathway

but could occur independently of the Sec apparatus (Cristóbal, *et al.*, 1999). Although most of these studies were performed in the Gram-negative organism *E. coli* they may offer some insight into the mechanism by which the N terminus of EbpS traverses the cytoplasmic membrane of *S. aureus* in the absence of a N-terminal secretion signal sequence.

Chapter 5

Functional analysis of EbpS

5.1 Introduction

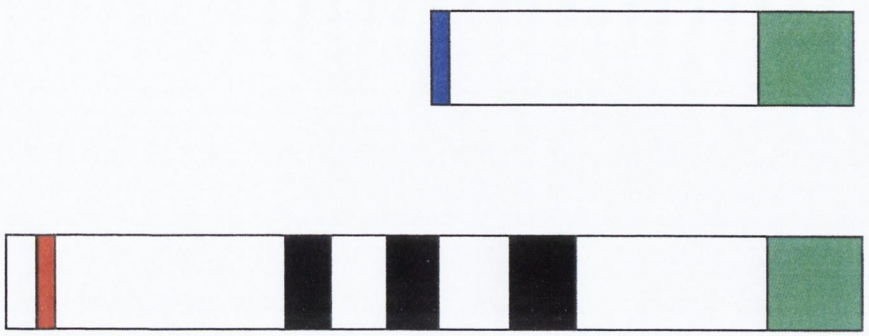
Previous studies have show that the elastin-binding protein of *Staphylococcus aureus* (EbpS) is the dominant bacterial factor promoting binding to soluble elastin peptides (Park *et al.*, 1996; Roche, 2000) and that the ligand-binding site is located between residues 14 and 34 at the N terminus of the protein (Park *et al.*, 1999). It was suggested that EbpS might be a member of the MSCRAMM class of staphylococcal surface proteins that promote bacterial attachment to and colonisation of mammalian tissue (Park *et al.*, 1996). These include the collagen-binding protein, Cna, the fibrinogen-binding proteins, ClfA and ClfB, and the fibronectin-binding proteins, FnBPA and FnBPB (Foster and Höök, 1998). The majority of staphylococcal MSCRAMMs are covalently attached to the uncross-linked pentaglycine bridge of nascent cell wall peptidoglycan by the enzyme sortase (Narvarre *et al.*, 1998). This occurs at the C terminus where the proteins contain conserved features required for cell wall sorting. These include a positively charged tail at the extreme C terminus, a membrane-spanning domain and a LPXTG motif (Schneewind *et al.*, 1993). However, EbpS is an integral membrane protein and although it has been demonstrated that the protein can bind to soluble elastin peptides and tropoelastin, it has not been shown to promote bacterial attachment to immobilised elastin. To determine whether EbpS can promote binding of *S. aureus* to immobilised elastin, the capacity of *S. aureus* wild-type and isogenic *ebpS* mutants to bind to elastin peptides immobilised on the wells of a microtitre plate was compared.

Due to the membrane location and topology of EbpS it is possible that the protein might have a function(s) other than binding to elastin. Protein searches with the BLAST service provided by the National Institutes of Health were used to identify regions of homology between EbpS and other proteins. Searches of the Brookhaven Protein Data Bank, Genbank™, EMBL and SWISSPROT revealed that the N-terminal 205 residues of EbpS share no significant homology with other proteins in the database (Roche, 2000). Using this type of analysis it was found that EbpS possesses a putative LysM domain in the 48 residues at the extreme C terminus of the protein (Roche, 2000). The LysM

domain, so-called because it was originally identified in bacterial lysins (lysin motif), is a widespread module that has been proposed to function as a peptidoglycan-binding motif, although this has not been formally demonstrated (Joris *et al.*, 1992; Ponting *et al.*, 1999). This module has been identified in both prokaryotic and eukaryotic proteins although it is predominantly found in cell wall degrading enzymes of bacterial species and is presumed to anchor these enzymes to their substrates. The domain can be found in these enzymes as a single module or repeated a number of times at either the N terminus or C terminus of the protein. The LysM module has also been identified in proteins involved in bacterial pathogenesis. These include protein A, the immunoglobulin binding protein of *S. aureus* (Uhlén *et al.*, 1984) and intimin, the outer membrane protein of enterohaemorrhagic and enteropathogenic *E. coli* involved in attachment to mammalian cells (Jerse *et al.*, 1990). Structural studies of the LysM module of the membrane-bound lytic murein transglycosylase D (MtlD) of *E. coli* have shown that the domain has a βααβ structure with the two α-helices packing onto the same side of an anti-parallel β sheet (Bateman and Bycroft, 2000). These studies have also identified a putative peptidoglycan-binding site. Sequence alignments of the putative LysM module of EbpS with those of other proteins revealed that this domain shares the greatest homology with the sequence of the single putative LysM motif of the YpbE protein of *Bacillus subtilis* (Figure 5.1). YpbE is a putative membrane-associated protein of unknown function. The LysM modules of EbpS and YpbE are 52% identical at the level of primary amino acid sequence (Roche, 2000).

To determine if EbpS has another function(s) apart from binding to soluble elastin, the growth of *S. aureus* wild-type strain 8325-4 and its isogenic *ebpS* mutant were compared by a number of methods. These included competition experiments where the strains were grown in the same culture to determine if one had an advantage over the other, standard growth curves and determination of growth yields by dry weight. Several truncated derivatives of EbpS were expressed in *S. aureus* to determine if regions other than the elastin-binding domain are involved in the function of the protein.

(A)



(B)

YpbE	H	T	V	Q	K	K	E	T	L	Y	R	I	S	M	K	–	–	Y	Y	K	S	R	T	G
EbpS	H	T	V	N	G	Q	E	N	L	Y	R	I	A	I	Q	–	–	Y	Y	G	S	G	S	P
Consensus	H	–	V	–	–	G	D	T	L	–	–	I	A	–	–	–	–	Y	–	–	–	–	–	–

YpbE	E	–	–	E	K	I	R	A	Y	N	H	L	N	G	N	D	V	Y	T	G	Q	V	L	D
EbpS	E	N	V	E	K	I	R	R	A	N	G	L	S	G	N	N	I	R	N	G	Q	Q	I	V
Consensus	–	–	–	–	–	–	–	N	–	–	–	L	–	–	–	–	I	–	–	G	Q	–	L	–

Figure 5.1 (A) Schematic representation of the proteins YbpE from *B. subtilis* and EbpS from *S. aureus*. The elastin binding domain ( ), secretion signal sequence ( ), hydrophobic domains ( ) and LysM modules ( ) are indicated. (B) Multiple sequence alignments of the LysM modules of YpbE of *B. subtilis* and EbpS with the consensus LysM sequence defined by Joris *et al.* (1992) (Roche, 2000). Conserved residues are denoted in red (100% identity) and green (67-83% identity).

5.2 Results

5.2.1 EbpS does not promote bacterial adherence to immobilised elastin peptides

It was reported previously that soluble ^{125}I-labeled α-elastin peptides (oxalic acid-solubilised form of insoluble elastin) from bovine *ligamentum nuchae* and ^{125}I-labeled human recombinant tropoelastin (hrTE), the monomeric precusor form of elastin, bound specifically to *S. aureus* cells (Park *et al.*, 1991). Subsequent studies demonstrated that EbpS was responsible for this specific interaction. *S. aureus* strain Newman *ebpS* showed a 72% reduction in soluble ^{125}I-hrTE binding compared to the parental strain. Binding was reduced to background levels by incubation with excess cold elastin peptides, reaching the same level as the *ebpS* mutant incubated with cold peptides. The complementing plasmid pCU-*ebpS*$^{+}$ restored elastin binding to the same level as the parental strain (Park *et al.*, 1996; Roche, 2000). These data indicated that EbpS is the dominant factor of *S. aureus* Newman for binding soluble elastin, but also suggested that the bacteria express a second elastin-binding moiety. In addition, the recombinant N-terminal truncate of EbpS (rEbpS$_{1-267}$) was shown to bind in a dose-dependent manner to immobilised κ-elastin peptides (sodium hydroxide-solubilised form of insoluble elastin) from bovine *ligamentum nuchae* in an ELISA-based assay (Roche, 2000). Taken together these data suggest that EbpS may also promote the adherence of *S. aureus* cells to immobilised elastin *in vitro* and may even promote bacterial colonisation of elastin rich tissue *in vivo*.

ELISA-based assays have been used to study the adherence of *S. aureus* to immobilised mammalian extracellular matrix proteins such as fibronectin, collagen and fibrinogen (Wolz *et al.*, 1996). A technique was developed whereby elastin peptides dissolved in 0.1M sodium bicarbonate were coated onto the wells of a 96-well microtitre plate by incubating in UV light at room temperature for 18 hours (Hinek *et al.*, 1999; Roche, 2000). *S. aureus* strains were treated with the fluorescent nucleic acid probe SYTO-13. Labeled cells emit a green fluorescent signal after excitation at 488 nm that can be detected with a fluorescence spectrometer. Using this technique it was found that a number of *S. aureus* strains adhered to immobilised bovine *ligamentum nuchae* κ-elastin

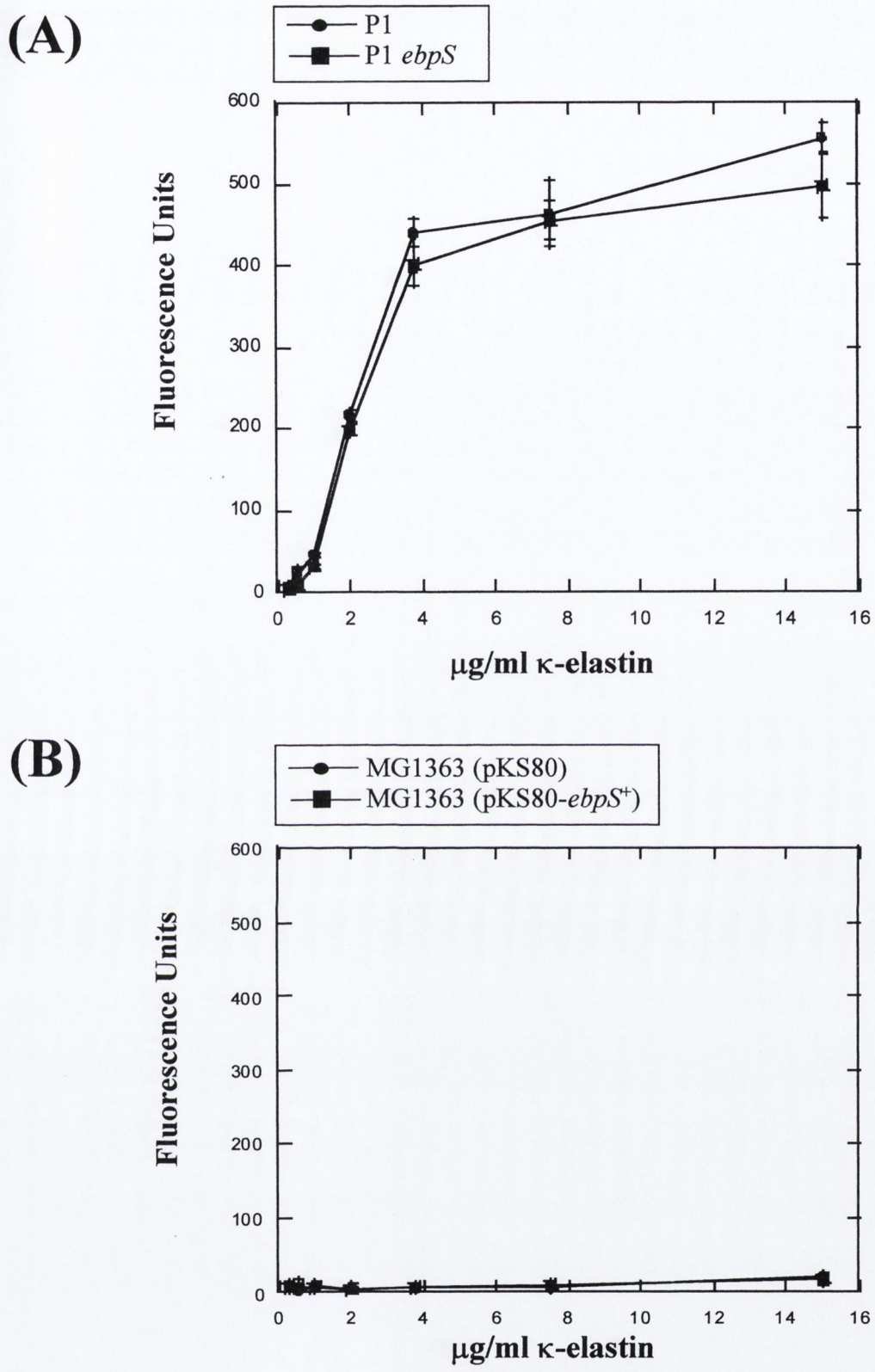


Figure 5.2 (A) Adherence of *S. aureus* strains P1 and P1 *ebpS* to immobilised κ-elastin peptides from bovine *ligamentum nuchae*. (B) Adherence of *L. lactis* strains MG1363 (pKS80) and MG1363 (pKS80-*ebpS*$^{+}$) to immobilised κ-elastin peptides.

and that binding was growth phase-dependent, occurring only with cells from early exponential phase cultures (OD_{600nm} = 0.5) (Roche, 2000).

Of the *S. aureus* strains tested, P1 and Cowan demonstrated strong adherence to immobilised elastin (Roche, 2000). To investigate whether EbpS was responsible for this interaction, the ability of *S. aureus* strains P1 and P1 *ebpS* to adhere to immobilised bovine *ligamentum nuchae* κ-elastin was tested. ELISA plates were coated with doubling dilutions of κ-elastin and cultures of each strain were grown to early exponential phase. There was no significant difference in the elastin-binding activity of the two strains, indicating that EbpS is not the surface factor responsible for the promotion of bacterial adherence to immobilised elastin peptides (Figure 5.2A). Also, EbpS expressed in *L. lactis* strain MG1363 from plasmid pKS80 (see section 3.2.3.1) did not promote adherence of this strain to immobilised κ-elastin (Figure 5.2B). These data revealed that EbpS is not an elastin adhesin and that the adherence of *S. aureus* to immobilised elastin peptides is promoted by another surface factor.

5.2.2 *S. aureus* wild-type dominates when grown in mixed culture with an isogenic *ebpS* mutant

Although EbpS binds soluble elastin it does not promote adherence of bacteria to immobilised elastin. Due to the membrane location of EbpS, the possibility that the protein might have another function(s) apart from elastin binding was investigated. Integral membrane proteins of Gram-positive organisms have a wide variety of functions in metabolism (Breidt *et al.*, 1987), transport (Morrissey *et al.*, 2000), antibiotic resistance (Guffanti *et al.*, 1998) and sensing environmental signals (Lina *et al.*, 1998).

In previous studies, the fitness of a strain compared to another has been tested by growing both strains in the same culture. The fitter strain will dominate the culture while the less fit strain will diminish (Zambrano *et al.*, 1993). If EbpS has a function other than soluble elastin binding then this could be manifested as an *ebpS* mutant being less fit compared to the parental strain. To test this hypothesis *S. aureus* strain 8325-4 and its isogenic *ebpS* mutant were grown together in the same culture. These experiments were performed in a chemically defined media (CDM) (Hussain *et al.*, 1991). This media is less rich than trypticase soy broth (TSB) and has been used to study the growth dynamics

of *S. aureus* under various conditions (Watson *et al.*, 1998a; Clements *et al.*, 1999a). *S. aureus* strains 8325-4 and 8325-4 *ebpS* were grown separately for 18 hours in 3 ml of CDM. Each culture was diluted to an OD_{600nm} of 0.03 in the same test tube in 3 ml of fresh CDM. Viable counts were determined at 2 hours (early exponential phase), 8 hours (early stationary phase) and 24 hours (stationary phase) by diluting samples of the culture in sterile PBS and plating on trypticase soy agar. After colonies were counted the plates were replica plated onto trypticase soy agar plates containing 10μg/ml of erythromycin. This identified the colonies that were deficient in EbpS expression since the *ebpS* gene was interrupted with the *ermC* erythromycin resistance cassette (Roche, 2000). The *ebpS* mutant formed 45.5% (± 2.1) of the population after 2 hours of growth, 13.6% (± 3.6) after 8 hours and 11.3% (± 2.6) after 24 hours. To ensure that the decrease in CFU of the 8325-4 *ebpS* strain was not due to the presence of an antibiotic resistance on the chromosome of the strain, the same experiments were performed with 8325-4 and 8325-4 *fnbB*. Strain 8325-4 *fnbB* is a derivative of 8325-4 that is defective in expression of the cell-wall-associated fibronectin-binding protein B (FnBPB) because the *fnbB* gene has been interrupted by *ermC* (Greene *et al.*, 1995). The *fnbB* mutant formed 48.6% (± 2.9) of the population after 2 hours of growth, 47.4% (± 5.8) after 8 hours and 48.2% (± 3.5) after 24 hours.

These data indicted that an *ebpS* mutant is less able to compete with the parental strain and that this defect is not due to the presence of *ermC*.

5.2.3 Growth curves

To determine if the *ebpS* mutant grew slower than the parental strain, growth curves of each strain were compared. 20 ml of CDM was inoculated to an OD_{600nm} of 0.03 with an overnight culture of either 8325-4 or 8325-4 *ebpS*. The growth of each culture was observed by measuring the optical densities over a 24-hour period (Figure 5.3). An initial lag was observed in the growth curves of some cultures but this was not associated with cultures of a particular strain. Strain 8325-4 grew with a mean generation time of 34.45 min (± 5.67) during the exponential phase of growth. Strain 8325-4 *ebpS* had a mean generation time of 37.50 min (± 3.61) during the same phase of growth. This difference in the rate of division was not statistically significant as determined by the

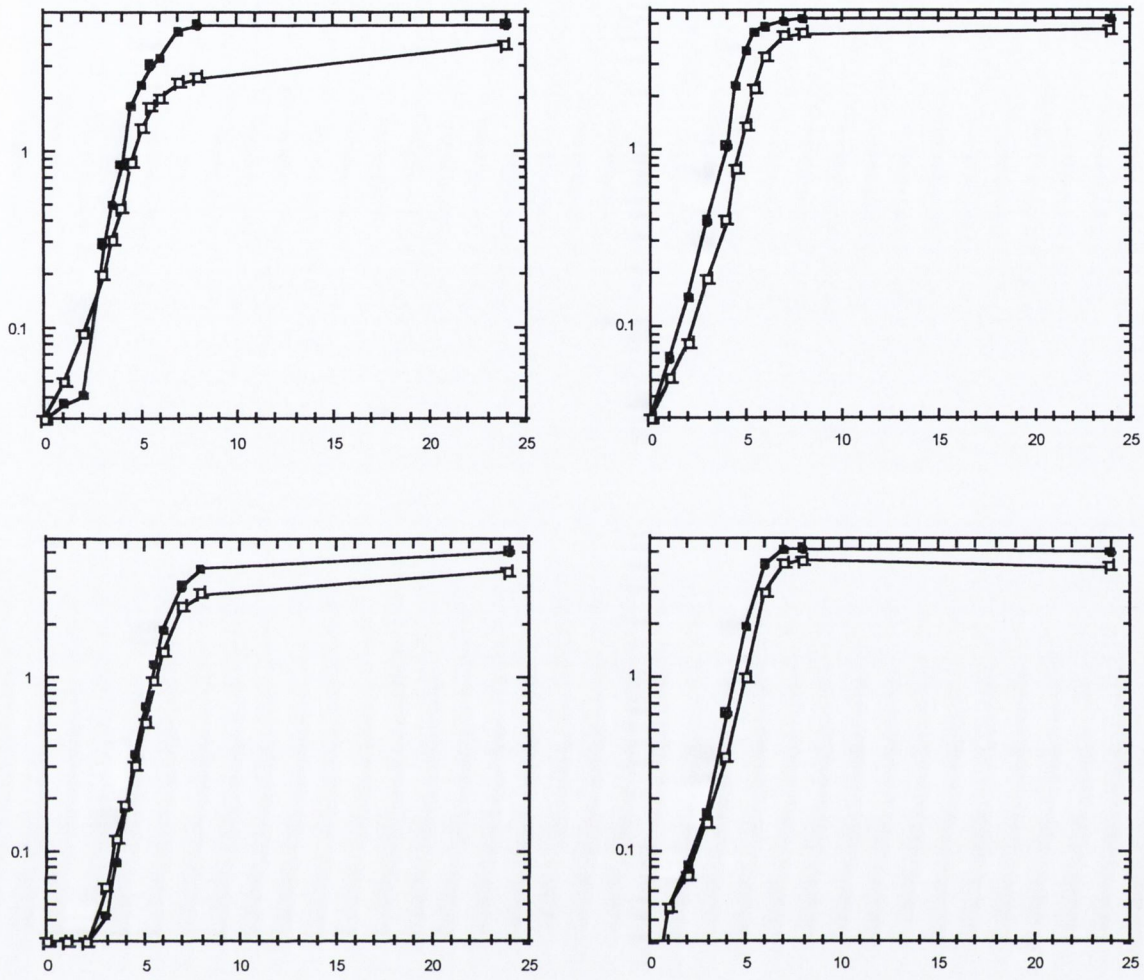


Figure 5.3 Typical growth curves of *S. aureus* strains 8325-4 (—■—) and 8325-4 *ebpS* (—□—) with hours of growth on the x-axis and optical density of the culture at 600 nm on the y-axis.

Student pairwise t test (Table 5.1). After 24 hours of growth the *ebpS* mutant consistently reached a lower optical density than the parental strain. The parental strain reached an OD_{600nm} of 5.06 (± 0.06) whereas the isogenic *ebpS* mutant reached an OD_{600nm} of 4.0 (± 0.12) (Table 5.1).

To ensure the difference in growth of the cultures was due to the lack of EbpS expression, the *ebpS* mutation was complemented by expressing EbpS from the single-copy integrating vector pCL84 (Figure 5.4) (Lee *et al.*, 1991). Plasmid pCL84 is capable of autonomous replication in *E. coli* but does not possess an origin of replication for *S. aureus*. This plasmid establishes itself in *S. aureus* by integrating into the chromosome, which is achieved using the integration system of bacteriophage L54a. L54a integrates into the *S. aureus* chromosome at an *attB* site located in the chromosome. Plasmid pCL84 contains the viral attachment sequence *attP*. Integration into the chromosome is promoted by the viral integrase supplied *in trans* in *S. aureus* strain CYL316. The plasmid integrates into the *attB* site that is located near the 3′ end of the glycerol ester hydrolase gene (*geh*). Integrated plasmids are stably inherited in the absence of selection (Lee *et al.*, 1991). The *ebpS* gene was cloned into plasmid pCL84 along with 300 bp of upstream sequence and 200 bp of downstream sequence to create plasmid pCL84-*ebpS*$^{+}$. This plasmid was introduced into the chromosome of strain CYL316 and transduced into strain 8325-4 *ebpS*. As a control, the empty vector was introduced into the same strain. Expression of EbpS as an 83 kDa protein from the integrated plasmid in strain 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) was confirmed by Western immunoblotting. Cells from stationary phase cultures of strains 8325-4, 8325-4 *ebpS*, 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) and 8325-4 *ebpS* (pCL84) grown in CDM were corrected to the same OD_{600nm}. Stabilised protoplasts were prepared and boiled in final sample buffer. Equal volumes of each sample were analysed by SDS-PAGE and Western immunoblotting with anti-r$EbpS_{343-486}$ antibodies (Figure 5.5).

Growth curves of strains 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) and 8325-4 *ebpS* (pCL84) were prepared in the same manner as described (Figure 5.6). There was no statistically significant difference between the exponential phase growth rates of the two strains ($p=0.2629$). Strain 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) grew with a mean generation time of 34.18 min (± 2.60) during the exponential phase of growth. Strain 8325-4 *ebpS* (pCL84)

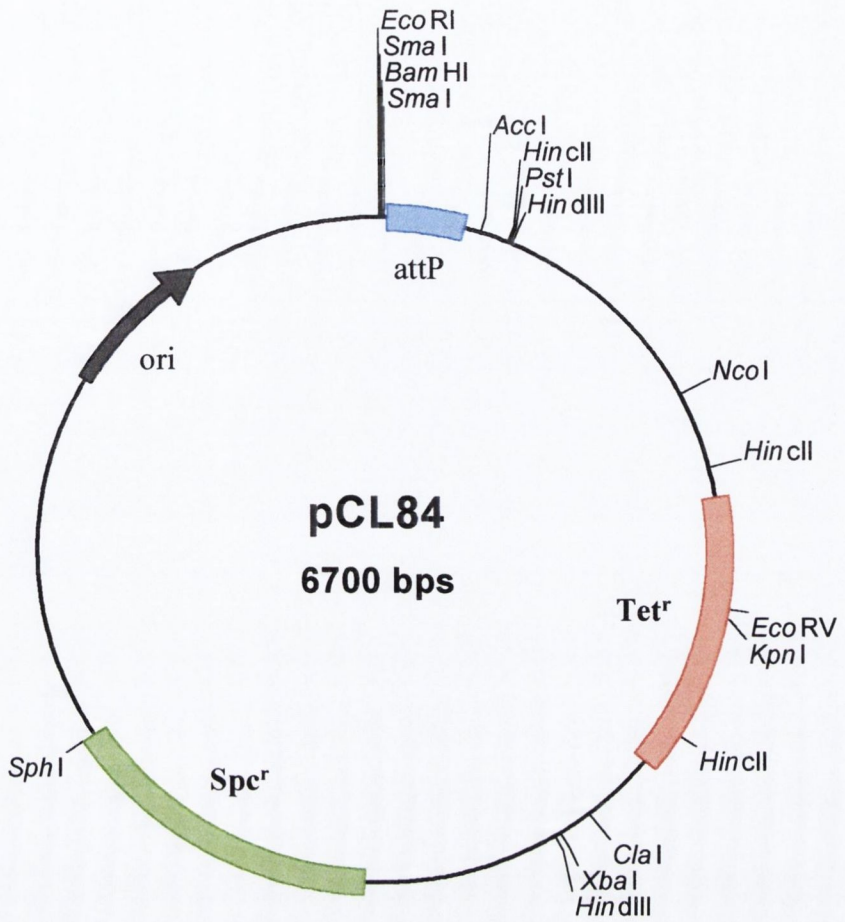


Figure 5.4 Map of the *S. aureus* integrating vector pCL84. The *E. coli* origin of replication (ori) and the *E. coli* spectinomycin resistance determinant (Spc^r) are indicated. The *attP* site promotes integration of the plasmid into the *attB* site in the glycerol ester hydrolase gene (*geh*) of *S. aureus* where it confers tetracycline resistance (Tet^r).

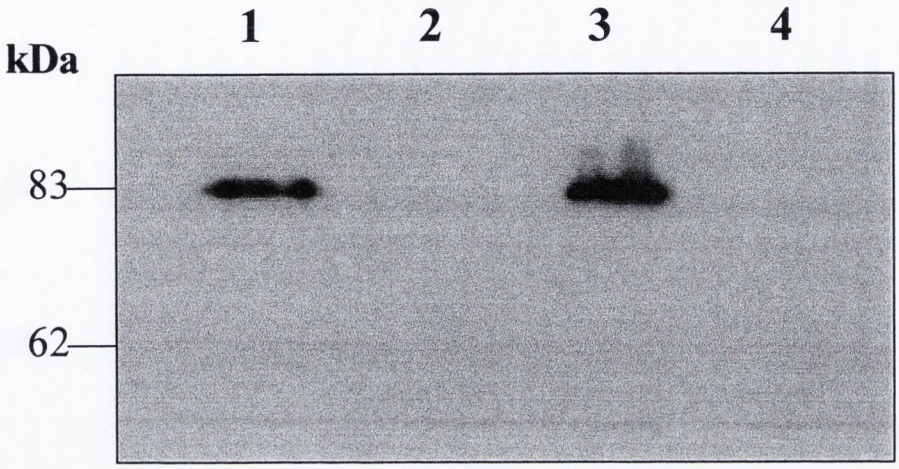


Figure 5.5 Western immunoblotting analysis of the stabilised protoplast fraction of *S. aureus* strains 8325-4 (lane 1), 8325-4 *ebpS* (lane 2), 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) (lane 3) and 8325-4 *ebpS* (pCL84) (lane 4) with anti-rEbpS$_{343-486}$ antibodies.

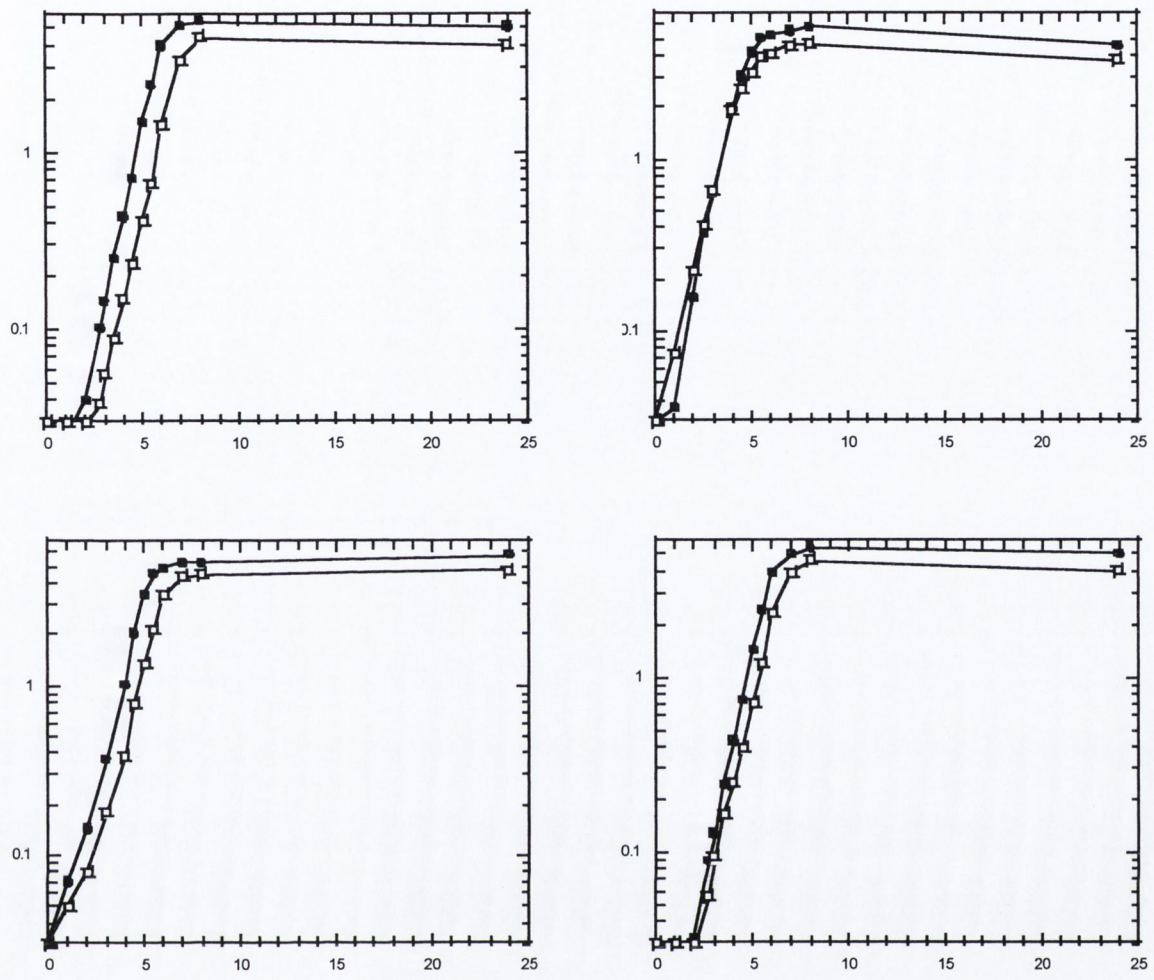


Figure 5.6 Typical growth curves of *S. aureus* strains 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) (—■—) and 8325-4 *ebpS* (pCL84) (—□—) with hours of growth on the x-axis and optical density of the culture at 600 nm on the y-axis.

Strain	OD_{600nm}	Mean generation time (min)
8325-4	5.06 ± 0.06	34.45 ± 5.67
8325-4 *ebpS*	4.00 ± 0.12 (p=0.0090)	37.50 ± 3.61 (p=0.2947)
8325-4 *ebpS* (pCL84-*ebpS*$^+$)	5.07 ± 0.04	34.18 ± 2.60
8325-4 *ebpS* (pCL84)	4.01 ± 0.01 (p=0.0001)	38.00 ± 1.96 (p=0.2629)

Data were measurements from four independent cultures of each strain.

Pairwise Student's t test performed between $EbpS^+$ and $EbpS^-$ strains.

Table 5.1 Optical densities and mean generation times of *S. aureus* strains 8325-4, 8325-4 *ebpS*, 8325-4 *ebpS* (pCL84-*ebpS*$^+$) and 8325-4 *ebpS* (pCL84) grown for 24 hours in chemically defined media (CDM).

had a mean generation time of 38.0 min (± 1.96) during the same phase of growth (Table 5.1). After 24 hours of growth, strain 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) reached an OD_{600nm} of 5.07 (± 0.04) whereas strain 8325-4 *ebpS* (pCL84) reached an OD_{600nm} of 4.01 (± 0.01) (Table 5.1). Once again an initial lag was observed in the growth of some cultures but this occurred randomly between EbpS expressing and EbpS non-expressing strains and was not considered to be significant.

This revealed that strains of *S. aureus* 8325-4 expressing EbpS could grow to a higher culture density than strains lacking EbpS and that the difference in culture density is independent of the rate of cell division during the exponential growth phase.

5.2.4 Growth yields by dry weight analysis

To ensure that the differences observed between the optical densities of the EbpS expressing and non-expressing strains were not due to cell aggregation, the dry weight yield of cultures of each strain were determined. *S. aureus* strains 8325-4, 8325-4 *ebpS*, 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) and 8325-4 *ebpS* (pCL84) were grown for 18 hours in 20 ml of CDM. The cells from each culture were harvested, washed and pelleted in a pre-weighed glass tube. The cells were then baked for 24 hours at 105°C and the tubes were weighed to measure the weight of the dried cells (Table 5.2). The *ebpS* mutant exhibited a 16% reduction in dry weight compared to the parental strain, with strains 8325-4 and 8325-4 *ebpS* having dry weights of 1.79 g/L and 1.5 g/L, respectively. This indicated that the difference in optical density observed between these two strains was not due to differences in cell numbers and not to cell aggregation, assuming that cells were of the same size and cell wall thickness. When EbpS was expressed from the integrated plasmid pCL84 in strain 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) the dry weight was restored to 1.80 g/L. This restoration of yield was specific to EbpS since it did not occur with strain 8325-4 *ebpS* (pCL84). These differences were shown to be statistically significant with confidence intervals of 99% by the pairwise Student's t test. These data indicated that the differences observed in the culture optical densities could be attributed to differences in cell numbers and that the phenotype was specific to EbpS.

Interestingly, when the same experiments were performed with cultures grown in trypticase soy broth (TSB), a rich media routinely used for the growth of *S. aureus*

Strain	OD_{600nm}	Dry Weight (g/L)
8325-4	5.83 ± 0.10	1.79 ± 0.02
8325-4 *ebpS*	4.65 ± 0.14 *	1.50 ± 0.04 *
8325-4 *ebpS* (pCL84-*ebpS*$^+$)	5.98 ± 0.08	1.80 ± 0.02
8325-4 *ebpS* (pCL84)	4.75 ± 0.10 *	1.50 ± 0.05 *

Data were duplicate measurements from three independent cultures of each strain.

Pairwise Student's t test performed between $EbpS^+$ and $EbpS^-$ strains.

* indicates p=<0.0001

Table 5.2 Optical densities and dry weight yields of *S. aureus* strains 8325-4, 8325-4 *ebpS*, 8325-4 *ebpS* (pCL84-*ebpS*$^+$) and 8325-4 *ebpS* (pCL84) grown for 18 hours in chemically defined media (CDM).

Strain	OD_{600nm}	Dry Weight (g/L)
8325-4	9.82 ± 0.27	2.75 ± 0.09
8325-4 *ebpS*	10.12 ± 0.19 p=0.0509	2.75 ± 0.05 p=0.9508
8325-4 *ebpS* (pCL84-*ebpS*$^+$)	9.93 ± 0.31	2.77 ± 0.07
8325-4 *ebpS* (pCL84)	10.01 ± 0.09 p=0.5549	2.69 ± 0.05 p=0.0551

Data were duplicate measurements from three independent cultures of each strain.
Pairwise Student's t test performed between $EbpS^+$ and $EbpS^-$ strains.

Table 5.3 Optical densities and dry weight yields of *S. aureus* strains 8325-4, 8325-4 *ebpS*, 8325-4 *ebpS* (pCL84-*ebpS*$^+$) and 8325-4 *ebpS* (pCL84) grown for 18 hours in trypticase soy broth (TSB).

cultures, there was no statistically significant difference observed in the dry weight yields of all four strains (Table 5.3).

5.2.5 Effects of truncated derivatives of EbpS on bacterial dry weight yield

To determine the region of EbpS that was responsible for the growth yield phenotype, a number of truncated derivatives of EbpS were expressed from plasmid pCL84 in *S. aureus* strain 8325-4 *ebpS*. Different regions of the *ebpS* gene were deleted by PCR. Plasmid pKS5.2 is a derivative of plasmid pBluescript KS(+) with the *ebpS* gene from *S. aureus* strain 8325-4 contained on a 5.2 kb *Pst*I genomic fragment (Roche, 2000). PCR was performed from plasmid pKS5.2 using a forward primer complementary to sequence 300 bp upstream of the *ebpS* gene and a reverse primer, incorporating a *Bam*HI restriction site, complementary to sequence within the *ebpS* gene located 5′ to the sequence to be deleted. Secondly, PCR was performed using a reverse primer complementary to a sequence 200 bp downstream from the *ebpS* gene and an internal forward primer, incorporating a *Bam*HI restriction site that was complementary to sequence located 3′ to the region to be deleted. This resulted in two PCR-products that could be ligated together in plasmid pCL84. The sequences to be deleted were replaced by a G and S residue encoded by the *Bam*HI restriction site introduced by the two internal primers (Figure 5.7). Derivatives of pCL84 containing truncated forms of the *ebpS* gene were introduced into the chromosome of *S. aureus* strain CYL316 and transduced into strain 8325-4 *ebpS*. Four truncates of EbpS were expressed (Figure 5.8). The first truncate lacked the elastin-binding domain of EbpS located between residues 14 and 34 ($EbpS_{\Delta 14-34}$). The second lacked a region at the C terminus of EbpS located C-terminal to the hydrophobic region H3, which contained a stretch of SKD repeats ($EbpS_{\Delta 365-389}$). The third region to be deleted was a Q- and N-rich region also located C-terminal to domain H3 located between residues 402 and 431 ($EbpS_{\Delta 402-431}$). The fourth region to be deleted was the LysM motif located between residues 440 and 479 ($EbpS_{\Delta 440-479}$). Expression of each EbpS derivative in strain 8325-4 *ebpS* was confirmed by Western immunoblotting with stabilised protoplasts. The $EbpS_{\Delta 14-34}$ protein was detected in the protoplast fraction of strain 8325-4 *ebpS* ($EbpS_{\Delta 14-34}$) with anti-$rEbpS_{343-486}$ antibodies as a protein with a molecular mass of approximately 75 kDa (Figure 5.9A, lane3). Similarly, the

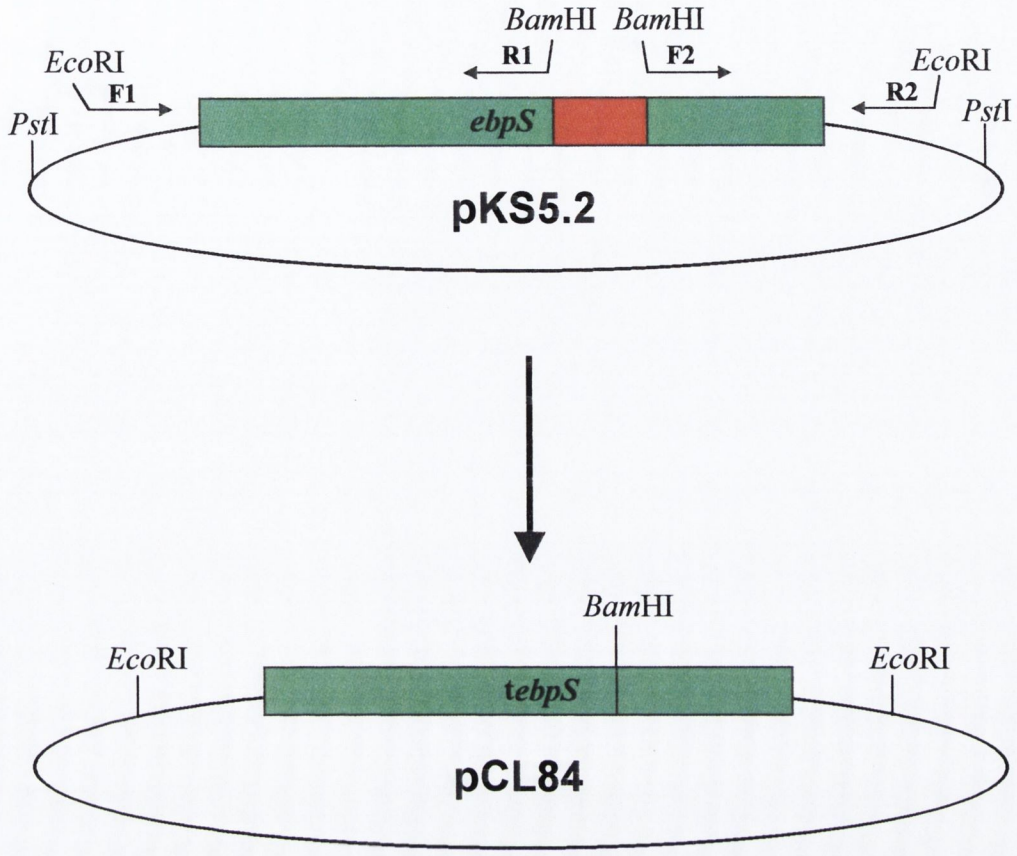


Figure 5.7 Schematic representation of the strategy used to produce truncated derivatives of the *ebpS* gene on the integrating plasmid pCL84. PCR was performed from plasmid pKS5.2 using a forward primer (F1), incorporating an *Eco*RI site, complementary to sequence 300 bp upstream of the *ebpS* gene and a reverse primer (R1), incorporating a *Bam*HI restriction site, 5′ to the sequence to be deleted (■). Secondly, PCR was performed using a reverse primer (R2) complementary to sequence 200 bp downstream of the *ebpS* gene and an internal forward primer (F2), incorporating a *Bam*HI restriction site, complementary to sequence 3′ to the sequence to be deleted. This resulted in two PCR-products that could be ligated together in the *Eco*RI restriction site of plasmid pCL84, thus replacing the sequence to be deleted with a G and S residue encoded by the *Bam*HI restriction site introduced by the two internal primers.

MSNNFKDDFE KNRQSIDTNS HQDHTEDVEK DQSELEHQDT IENTEQQFPP

RNAQRRKRRR DLATNHNKQV HNESQTSEDN VQNEAGTIDD RQVESSHSTE

SQEPSHQDST PQHEEEYYNK NAFAMDKSHP EPIEDNDKHD TIKNAENNTE

HSTVSDKSEA EQSQQPKPYF TTGANQSETS KNEHDNDSVK QDQDEPKEHH

NGKKAAAIGA GTAGVAGAAG AMAASKAKKH SNDAQNKSNS GKANNSTEDK

ASQDKSKDHH NGKKGAAIGA GTAGLAGGAA SKSASAASKP HASNNASQNH

DEHDNHDRDK ERKKGGMAKV LLPLIAAVLI IGALAIFGGM ALNNHNNGTK

ENKIANTNKN NADESKDKDT SKDASKDKSK STDSDKSKED QDKATKDESD

ND QNNANQAN NQAQNNQNQQ QANQNQQQQQ Q RQGGGQRHT

VNGQENLYRI AIQYYGSGSP ENVEKIRRAN GLSGNNIRNG QQIVIP

Figure 5.8 Primary amino acid sequence of the elastin-binding protein (EbpS) from strain 8325-4. The hydrophobic domains H1, H2 and H3 are underlined. Regions deleted from EbpS are indicated in colour. EbpS truncated derivatives $EbpS_{\Delta 14-34}$ (red), $EbpS_{\Delta 365-389}$ (blue), $EbpS_{\Delta 402-431}$ (orange) and $EbpS_{\Delta 440-479}$ (green) expressed in strain 8325-4 *ebpS* from the integrated plasmid pCL84 are indicated.

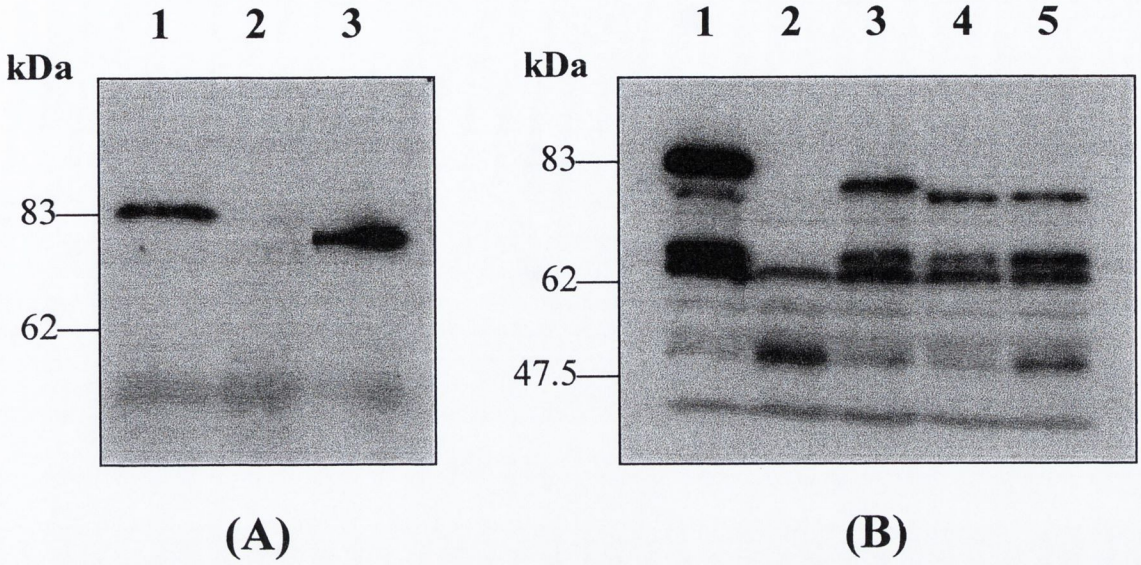


Figure 5.9 Western immunoblotting analysis of the protoplast fraction of *S. aureus* strains expressing truncated derivatives of EbpS from the integrated plasmid pCL84. Cultures were corrected to the same OD_{600nm} before protoplast formation and equal volumes of each protoplast fraction were analysed. (A) Protoplast fraction of strains 8325-4 (lane 1), 8325-4 *ebpS* (lane 2) and 8325-4 *ebpS* ($EbpS_{\Delta 14-34}$) detected with anti-$rEbpS_{343-486}$ antibodies. (B) Protoplast fraction of strains 8325-4 (lane 1), 8325-4 *ebpS* (lane 2), 8325-4 *ebpS* ($EbpS_{\Delta 365-389}$) (lane 3), 8325-4 *ebpS* ($EbpS_{\Delta 402-431}$) (lane 4) and 8325-4 *ebpS* ($EbpS_{\Delta 440-479}$) (lane 5) detected with with anti-$rEbpS_{1-267}$ antibodies.

$EbpS_{\Delta 365-389}$, $EbpS_{\Delta 402-431}$ and $EbpS_{\Delta 440-479}$ proteins were detected as truncates in the protoplast fraction of strains 8325-4 *ebpS* ($EbpS_{\Delta 365-389}$), 8325-4 *ebpS* ($EbpS_{\Delta 402-431}$) and 8325-4 *ebpS* ($EbpS_{\Delta 440-479}$), respectively, with anti-$rEbpS_{1-267}$ antibodies (Figure 5.9B, lanes 3,4 and 5). There was evidence of proteolytic degradation of the native EbpS protein to a truncated derivative of approximately 65 kDa. The other protein bands observed were proteins that cross-reacted with the anti-$rEbpS_{1-267}$ antibodies since they were present in the protoplast fraction of strain 8325-4 *ebpS* (Figure 5.9B, lane 2). The proteins $EbpS_{\Delta 365-389}$, $EbpS_{\Delta 402-431}$ and $EbpS_{\Delta 440-479}$ were present at lower levels than the native EbpS protein.

The dry weight yield and optical density of each strain after 18 hours growth in CDM was measured and compared to that of strains 8325-4 *ebpS* (pCL84-*ebpS*$^+$) and 8325-4 *ebpS* (Table 5.4). Strains 8325-4 *ebpS* (pCL84-*ebpS*$^+$) and 8325-4 *ebpS* achieved dry weights of 1.55 g/L and 1.29 g/L, respectively. Strains of 8325-4 *ebpS* expressing either the $EbpS_{\Delta 14-34}$ or $EbpS_{\Delta 365-389}$ derivative produced yields of 1.50 g/L and 1.53 g/L, respectively. This indicated that these EbpS truncates could fully complement the growth defect of strain 8325-4 *ebpS* even though the $EbpS_{\Delta 365-389}$ was expressed at a lower level than the native form of the protein. Conversely, strains 8325-4 *ebpS* ($EbpS_{\Delta 402-431}$) and 8325-4 *ebpS* ($EbpS_{\Delta 440-479}$) achieved yields of 1.45 g/L and 1.24 g/L, respectively. The proteins $EbpS_{\Delta 402-431}$ and $EbpS_{\Delta 440-479}$ were also expressed at lower levels than the native EbpS protein as determined by Western immunoblotting (Figure 5.9). These data revealed that while the $EbpS_{\Delta 402-431}$ truncate could partially complement the 8325-4 *ebpS* growth defect, the $EbpS_{\Delta 440-479}$ truncate could not. These differences were shown to be statistically significant by the pairwise Student's t test (Table 5.4). This indicated that $EbpS_{\Delta 440-479}$ protein expressed in strain 8325-4 *ebpS* ($EbpS_{\Delta 440-479}$) could not complement the growth defect of strain 8325-4 *ebpS*. The dry weight yield data was mirrored by the optical density of each strain (Table 5.4).

Strain	OD_{600nm}	Dry Weight (g/L)
8325-4 *ebpS* (pCL84-*ebpS*)	5.09 ± 0.05	1.55 ± 0.02
8325-4 *ebpS*	4.19 ± 0.06 p=<0.0001	1.29 ± 0.03 p=<0.0001
8325-4 *ebpS* ($EbpS_{\Delta 14-34}$)	4.89 ± 0.05 p=0.0011	1.50 ± 0.01 p=0.0025
8325-4 *ebpS* ($EbpS_{\Delta 365-389}$)	5.14 ± 0.11 p=0.2834	1.53 ± 0.01 p=0.0915
8325-4 *ebpS* ($EbpS_{\Delta 402-431}$)	4.70 ± 0.08 p=<0.0001	1.45 ± 0.05 p=0.0054
8325-4 *ebpS* ($EbpS_{\Delta 440-479}$)	4.01 ± 0.14 p=<0.0001	1.24 ± 0.05 p=<0.0001

Data were duplicate measurements from three independent cultures of each strain. The Student's pairwise t test was performed between values of strain 8325-4 *ebpS* (pCL84-*ebpS*) and other strains.

Table 5.4 Optical densities and dry weight yields of *S. aureus* strains 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$), 8325-4 *ebpS*, 8325-4 *ebpS* ($EbpS_{\Delta 14-34}$), 8325-4 *ebpS* ($EbpS_{\Delta 365-389}$), 8325-4 *ebpS* ($EbpS_{\Delta 402-431}$) and 8325-4 *ebpS* ($EbpS_{\Delta 440-479}$) grown for 18 hours in chemically defined media (CDM).

5.3 Discussion

Previously it has been shown that the elastin-binding protein (EbpS) is the dominant factor promoting binding of soluble elastin and tropoelastin to *S. aureus* (Park *et al.*, 1991; Roche, 2000). It was hypothesised that EbpS promotes bacterial adherence to immobilised elastin *in vitro* and could therefore promote bacterial colonisation of elastin-rich tissue *in vivo*. EbpS would perform a similar function to that of other mammalian extracellular matrix-binding proteins expressed on the surface of *S. aureus*, including the fibrinogen-binding proteins, ClfA and ClfB, the fibronectin-binding proteins, FnBPA and FnBPB and the collagen-binding protein, Cna. The ability of different *S. aureus* laboratory strains to adhere to immobilised elastin peptides derived from bovine neck ligament was compared. Strains P1 and Cowan adhered strongly in a growth phase-dependent manner. Cells from exponential phase cultures adhered strongly whereas cells from stationary phase cultures did not (Roche, 2000). Growth phase specific adherence to immobilised elastin peptides was also observed with 10 clinical *S. aureus* strains isolated from patients with native-valve endocarditis (Roche, 2000).

To determine if EbpS is responsible for growth phase-dependent adherence to immobilised elastin, the ability of *S. aureus* strain P1 and its isogenic *ebpS* mutant to adhere to this material was tested. There was no significant difference in the ability of the mutant to bind compared to the parental strain. Similarly, EbpS expressed in the heterologous Gram-positive host *L. lactis* could not promote adherence of this organism to immobilised elastin peptides. This indicated that EbpS is not the surface factor that promotes bacterial adherence to immobilised elastin peptides *in vitro* and therefore is unlikely to promote bacterial colonisation of elastin-rich tissue *in vivo*. Furthermore, EbpS is present in the cytoplasmic membrane throughout the *S. aureus* growth cycle (Roche, 2000) whereas only exponential phase bacteria bound to immobilised elastin. These data seem to eliminate EbpS as the MSCRAMM responsible for the adherence of *S. aureus* to immobilised elastin peptides. Previous studies have shown that the elastin-binding domain of EbpS, situated at the N terminus of the protein, is located on the cell surface (Park *et al.*, 1991; Roche, 2000). Perhaps the ligand-binding domain of the

protein is located in clefts in the cell wall peptidoglycan that enable the protein to bind soluble elastin but not to the immobilised ligand. Bacterial adherence to immobilised elastin must therefore be promoted by some other factor.

The inability of EbpS to promote bacterial attachment to immobilised elastin peptides combined with its location in the membrane prompted investigation as to whether the protein has another function. Integral membrane proteins of Gram-positive organisms may be involved in housekeeping functions, antibiotic resistance, resistance to antimicrobial peptides and signal transduction. Growth experiments were performed to determine if EbpS has a function that would be manifested as a growth defect in mutant cells. A chemically defined media (CDM) (Hussain *et al.*, 1991) used previously to study the response of *S. aureus* to changes in growth conditions and stress (Clements *et al.*, 1999a) as well as the characterisation of proteins involved in the *S. aureus* starvation survival response (Watson *et al.*, 1998a) was employed. CDM is composed of all 18 amino acids, carbohydrates, vitamins, minerals and trace elements. The advantage of using CDM was that production of the media was consistent and the components of the media could be altered to determine their affect on growth.

To compare the ability of a *S. aureus ebpS* mutant to grow in competition with the parental strain, mixed culture experiments were performed. Strains 8325-4 and 8325-4 *ebpS* were grown in the same culture and the proportion of wild-type to *ebpS* mutant colony forming units was monitored throughout the growth cycle. After 8 hours of growth (early stationary phase) approximately 10% of the colony forming units in the culture were the *ebpS* mutant and a similar figure was observed with stationary phase cultures (24 hours). The growth rate of a strain might be compromised by the presence of an antibiotic resistance determinant in the chromosome. To ensure that the reduction in growth observed with the *ebpS* mutant was not due to the presence of the *ermC* erythromycin resistance cassette used to inactivate the *ebpS* gene by allelic replacement, the same mixed culture experiments were performed using strains 8325-4 and 8325-4 *fnbB*. In strain 8325-4 *fnbB* the gene for the cell-wall-associated fibronectin-binding protein, FnBPB, is interrupted with the same *ermC* cassette used to inactivate the *ebpS* gene. When grown in mixed culture with the parental strain, the *fnbB* mutant accounted for approximately 50% of the culture throughout the growth cycle. This indicated that the

growth defect observed in the *ebpS* mutant was due to loss of EbpS expression and not due to the presence of an antibiotic resistance marker. While these experiments indicated that the *ebpS* mutant was at a growth disadvantage compared to the wild-type strain, they did not determine whether this was due to direct competition between the strains for factors such as nutrients or whether the *ebpS* mutant simply grew slower than the parental strain.

Growth curves of cultures of strains 8325-4 and 8325-4 *ebpS* revealed that there was no significant difference between the division times of each strain during exponential growth. However, cultures of strain 8325-4 *ebpS* did not reach the same optical density as cultures of the parental strain. This growth defect could be complemented by expression of EbpS from the integrating plasmid pCL84. This indicated that the growth defect was due to a lack of EbpS expression. It was essential to use a method for complementation that did not require antibiotic selection since the presence of an antibiotic in the growth media could affect the growth rate of the organism. These data implied that the difference in the number of colony forming units observed in the mixed culture experiments was due to a difference in culture growth dynamics between the *ebpS* mutant and the parental strain.

The growth of each of the above strains was monitored using a spectrophotometer by measuring the optical density of the cultures at 600 nm. Optical density is not a direct indicator of the number of colony forming units in a culture. While the optical density of a culture may be low the viable count may be higher due to bacterial aggregation. To ensure that the difference in the optical densities of EbpS-expressing and EbpS-non-expressing strains was due to differences in the number of bacteria present and not to bacterial aggregation, the dry weight yields of cultures of strains 8325-4, 8325-4 *ebpS*, 8325-4 *ebpS* (pCL84-*ebpS*$^{+}$) and 8325-4 *ebpS* (pCL84) were measured. Again, a difference in dry weight yield of approximately 16% was observed between EbpS-expressing and EbpS-non-expressing strains, suggesting that the difference observed in the optical densities of cultures could be attributed to differences in cell numbers and not aggregation, assuming that the cells from *ebpS*$^{+}$ and *ebpS*$^{-}$ cultures were the same size and had the same cell wall thickness.

Interestingly, the lower dry weight yields of the *ebpS* mutant was not observed when cells were grown in trypicase soy broth (TSB), a rich media routinely used for the growth of *S. aureus*. This suggests that the growth defect of the *ebpS* mutant could be due to the availability of nutrients since media composition is the major difference between CDM and TSB. This is purely speculation and there may be many other factors that contribute to the phenotype. TSB is a complex media composed of digested casein, digested soybean, dextrose, sodium chloride and dipotassium phosphate and it is impossible to directly compare the constituents of CDM and TSB.

It is interesting to note that when strains 8325-4 and 8325-4 *ebpS* were grown in the same culture there was a reduction in *ebpS* mutant cells with this strain accounting for only 1/10 of the colony forming units after 8 hours of growth whereas the difference in the dry weight yield of the two strains, grown independently for 24 hours, was only 16%. This could be accounted for by the fact that growth of the two strains together would exaggerate any difference in the culture growth dynamics of the strains. Cultures of the *ebpS* mutant did not reach the same optical density as cultures of the parental strain. This meant that cells of the *ebpS* mutant were slowly dividing as they entered stationary phase, while cells of the parental strain were still dividing at the exponential rate. The wild-type cells could proliferate faster than the *ebpS* mutant cells leaving the latter at a disadvantage when competing for nutrients and space in the culture. This could explain the amplification of the growth defect observed in mixed culture experiments.

To determine if the growth defect phenotype was the result of a function of EbpS other than soluble elastin binding, truncated derivatives of the protein were expressed from the integrated plasmid pCL84 in strain 8325-4 *ebpS* and their ability to complement the growth defect was determined using culture dry weight yields. Four truncates of EbpS were expressed. The EbpS protein lacking the elastin-binding domain ($EbpS_{\Delta 14-34}$) could fully complement the growth defect of strain 8325-4 *ebpS*. This revealed that the EbpS growth defect is a function of EbpS that is completely separate from its elastin-binding function. Similarly, the EbpS protein lacking the serine-lysine-aspartic acid repeat region at the C terminus could fully complement the growth defect of the same strain even though the protein was expressed at a lower level than the native EbpS protein. This indicated that this region of EbpS played no role in the growth phenotype. Conversely,

the EbpS derivative lacking the glutamine-asparagine rich region at the C terminus of the protein could only partially complement the EbpS growth defect whereas the EbpS protein with the LysM module deleted could not complement. These proteins were expressed at a lower level than the native EbpS protein, which could account for the inability of these proteins to complement the growth defect. However, both these proteins were expressed at approximately the same level as $EbpS_{\Delta 365-389}$, yet there is a distinct difference in the ability of the two proteins to complement the growth defect. This suggests that residues at the extreme C terminus of the protein are required to complement the growth defect of strain 8325-4 *ebpS*. The expression of the LysM module appears to be essential for the second function of EbpS. Perhaps the glutamine-asparagine rich region is not directly involved in this function but is required for the correct conformation of the LysM module in the cell wall.

Entry into stationary phase has been intensively studied in the Gram-positive organism *B. subtilis* and the Gram-negative organism *E. coli*. In *B. subtilis*, starvation, as in most bacterial species, is the signal for entry into stationary phase. In this organism survival is mediated by the formation of environmentally resistant spores. The process of sporulation is controlled by greater than 125 genes and the expression of these genes is controlled by four DNA-binding proteins and five RNA polymerase σ subunits (Haldenwang, 1995; Stragier and Losick, 1996). Furthermore, this system is regulated by a quorum sensing mechanism that involves two signaling peptides, ComX and CSF (Magnuson *et al.*, 1994; Solomon *et al.*, 1996).

In the non-sporulating bacteria, such as *E. coli*, entry into stationary phase is marked by changes in global gene expression and increased resistance to environmental stresses such as high temperature, osmotic stress and oxidative reagents (Zambrano and Kolter, 1996; Ishihama, 1997). The bacterial cells adopt a smaller and more spherical morphology with the membranes becoming less fluid and the nucleoid becoming more condensed (Loewen and Hengge-Aronis, 1994). These physiological changes are controlled by the expression of stationary phase specific genes whose expression is regulated by the stationary phase RNA polymerase σ factor σ^S (Hengge-Aronis, 1996). Expression of σ^S is maximal at the onset of stationary phase (Jishage *et al.*, 1996) and is controlled by a diverse number of factors including the concentration of the stringent

response molecule ppGpp (Lange *et al.*, 1995), the concentration of cAMP (Loewen and Hengge-Aronis, 1994), the concentration of UDP-glucose (Bohringer *et al.*, 1995) and DNA superhelicity (Kusano *et al.*, 1996). Binding of the σ^S subunit to the promoters of stationary phase-specific genes results in the expression of these genes and entry into stationary phase.

In *S. aureus* the availability of glucose is the major stimulus for the entry of cells into the starvation survival state (Watson *et al.*, 1998a). This is accompanied by a reduction on cell size and increased resistance to acid shock and oxidative stress (Watson *et al.*, 1998a). The alternative RNA polymerase σ factor σ^B (SigB) is maximally expressed during stationary phase and is involved in the recovery of cells from environmental stresses but not starvation survival (Chan *et al.*, 1998). Other phenotypic changes observed in a *sigB* deletion mutants of different *S. aureus* strains include increased alpha hemolysin production (Cheung *et al.*, 1999), decreased ability to form biofilm (Rachid *et al.*, 2000), reduction in yellow pigmentation (Kullik *et al.*, 1998) and restored methicillin sensitivity in methicillin-resistant strains (Wu *et al.*, 1996). Presently there is very little understanding of the molecular mechanisms involved in entry of *S. aureus* cultures into stationary phase.

It is possible that EbpS is in some way involved in the entry of cells into stationary phase as demonstrated by the growth defect of the EbpS-deficient mutant. The protein might be involved in signal transduction similar to the membrane associated histidine protein kinase, ComP, of *B. subtilis* that is involved in sporulation (Lazazzera, 2000). This could be tested using a bacterial two-hybrid system used to detect protein-protein interactions. If EbpS is involved in a signaling pathway then it must interact with other membrane proteins or cytoplasmic proteins. Commerically available bacterial two-hybrid systems such as Bacteriomatch™ from Stratagene, which uses ampicillin resistance and β–galactosidase activity as reporters, could be used to test for the interaction of regions of EbpS with other proteins of *S. aureus*. Regions such as the LysM module or the cytoplasmically located loop could be used as bait. Unfortunately, EbpS does not share any homology to known protein kinases at the primary amino acid level (Roche, 2000). Alternatively, EbpS might be involved in the uptake of essential nutrients or the efflux of toxic metabolites. It is interesting to note that the open reading frames

flanking the *ebpS* locus on the *S. aureus* chromosome share a similar structural organisation to the *recQ-cmk* region of the *B. subtilis* chromosome (Roche, 2000). In *B. subtilis* this region is thought to be part of a supraoperon mostly specifying enzymes involved in amino acid synthesis (Xie *et al.*, 1999). This suggests that EbpS might have a role to play in amino acid biosynthesis or transport. This could be examined using phenotypic microarrays. Biolog Inc. produce phenotypic microarrrays that test the ability of an organism to utilize different carbohydrate and nitrogen sources, and to grow in the absence of a number of amino acids. The performance of an *ebpS* mutant and the parental strain in these microarrays could be compared to identify a role for EbpS in bacterial metabolism or transport of nutrients.

EbpS could be involved in bacterial resistance to bacteriocidal or bacteriostatic agents such as antibiotics, antimicrobial peptides or detergents. This could be tested by comparing the minimum inhibitory concentrations (MIC) of an *ebpS* mutant and the parental strain grown on agar or in broth cultures in the presence of such agents.

Regarding the soluble elastin-binding activity of EbpS, there is no clear reason why *S. aureus* should bind soluble elastin. Elastin peptides generated by elastases are chemotactic for neutrophils as a signal of tissue damage (Varga *et al.* 1989) so it is possible that EbpS is a part of a signal transduction system providing early warning of an impending neutrophil influx or it may simply mop up the elastin peptides before a chemotatic signal is activated. This could be tested in a neutrophil chemotaxis chamber by comparing the number of neutrophils attracted to *S. aureus* wild-type cells and *ebpS* mutant cells in the presence of elastin. Whatever the role of EbpS, the importance of the protein is demonstrated by the fact that the *ebpS* gene was detected by Southern blotting in every strain of 25 distinct clinical isolates (Smeltzer *et al.*, 1997).

While many questions remain regarding the function of EbpS, it is clear that the properties and the functions of the protein bear little resemblance to the hypothesis and extrapolations originally suggested. EbpS was reported as a cell-wall-associated protein that could probably promote bacterial colonisation of elastin-rich tissue. It is now known that EbpS is an integral membrane protein of known membrane topology that does not function as an elastin adhesin but has another function distinct from soluble elastin binding. Since the original interest in EbpS stemmed from its possible role as an elastin

adhesin it was decided to identify and characterise the factor(s) on the surface of *S. aureus* responsible for the adherence of the bacterium to immobilised elastin peptides, which is the focus of the next chapter.

Chapter 6

Studies on the elastin binding ability of *Staphylococcus aureus*

6.1 Introduction

The initial step in the infection process is the colonisation of host tissue. *S. aureus* is a human pathogen that possessses a number of proteins on its cell surface that promote bacterial adherence to proteins of the mammalian extracellular matrix (ECM). Collectively, these proteins are called microbial surface components recognising adhesive matrix molecules or MSCRAMMs. Binding to the ECM allows bacteria to adhere to and to colonise host tissue. Surface proteins that interact with collagen (Switalski *et al.*, 1993), fibrinogen (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998), fibronectin (Signas *et al.*, 1989) and von Willebrand factor (Hartlieb *et al.*, 2000) have been characterised. The importance of the MSCRAMM proteins to virulence has been demonstrated using *S. aureus* gene knock out mutants defective in expression of a particular MSCRAMM and comparing the virulence of the mutant strain to the parental strain in various animal models. Using this approach the collagen-binding protein of *S. aureus*, Cna, has been implicated in osteomylitis and septic arthritis (Switalski *et al.*, 1993; Patti *et al.*, 1994b; Elasri *et al.*, 2002). Similarly, *S. aureus* derivatives defective in the expression of the fibrinogen-binding protein, ClfA, demonstrated a reduced capacity to induce experimental endocarditis (Moreillon *et al.*, 1995) and septic arthritis (Josefsson *et al.*, 2001).

Elastin, along with microfibrillar components, is a major component of the elastic fibre ECM (Mecham and Davies, 1994). Elastin plays a crucial role in maintaining the structural integrity and function of tissues in which reversible extensibility or deformability is required (Sandberg *et al.*, 1981). Thus elastin and elastic fibres are present in abundance in tissues that require elasticity such as the lung, skin, and major blood vessels. Physiologic elastin, like interstitial collagen and other matrices, is an insoluble polymeric fibre. Elastin is comprised of cross-linked tropoelastin monomers that are first secreted as soluble precursors from cells (Mecham and Davies, 1994). Lysine residues in the cross-linking domain of secreted tropoelastin are rapidly cross-linked both inter- and intramolecularly by the enzyme lysyl oxidase (Kagan and Trackman, 1991).

S. aureus is known to infect a number of elastin rich tissues such as lung and heart valves to cause diseases such as pneumonia and native-valve endocarditis (Tenover and Gaynes, 2000). Studies to identify an elastin adhesin on the surface of *S. aureus* identified the elastin-binding protein (EbpS) as the factor that promotes the binding of soluble elastin peptides and recombinant tropoelastin, the monomeric form of elastin, to *S. aureus* (Park *et al.*, 1996). It was suggested that EbpS might be the surface factor responsible for the colonisation of elastin-rich tissue and a virulence determinant in diseases of such tissue (Park *et al.*, 1996). If EbpS is to function as an elastin adhesin it must promote bacterial adherence to immobilised elastin, a property that is essential if the protein is to promote bacterial colonisation of elastin-rich tissue. To study the interactions between *S. aureus* and immobilised elastin an adherence assay was developed whereby commercially available elastin peptides are coated onto the wells of a microtitre plate (Roche, 2000). Originally, this method was developed to study the effects of elastin binding on the elastin receptors on the surface of mammalian cells and involves drying elastin peptides onto the wells of a microtitre plate using UV light (Hinek *et al.*, 1999). Previously, to examine the binding of *S. aureus* cells to immobilised matrix proteins such as fibronectin and fibrinogen, bound cells were detected by crysal violet staining of their cell wall peptidoglycan (Ní Eidhin *et al.*, 1998). Unfortunately, is was discovered that crystal violet stains immobilised elastin peptides, thus rendering this system for the detection of cells bound to this material useless (Roche, 2000). A method for the detection of cells bound to elastin was developed using the fluorescent nucleic acid probe SYTO-13. SYTO-13 penetrates Gram-positive cells and binds to nucleic acid. Labeled cells emit a fluorescent signal after excitation at 488 nm that can be detected with a fluorescence spectrometer. This probe has been used previously for detecting *S. aureus* cells by flow cytometry (Mason *et al.*, 1998). The optimum conditions for the staining of *S. aureus* cells with SYTO-13 was determined and the method was validated by studies of the binding of *S. aureus* strain Newman and its isogenic *clfA* derivative to immobilised fibrinogen (Roche, 2000).

Using this method it was found that a number of laboratory *S. aureus* strains adhered to immobilised κ-elastin from bovine neck ligament and that adherence was growth phase dependent because it only occurred with cells from early exponential phase

cultures (Roche, 2000). It was also shown that this binding was not dependent on EbpS but depended of the expression of the fibronectin-binding proteins, FnBPA and FnBPB, because elastin binding did not occur in a strain of *S. aureus* in which both the *fnb* genes were disrupted by allelic replacement (Roche, 2000).

In this chapter the role of the fibronectin-binding proteins in bacterial adherence to immobilised elastin peptides was assessed by the complementation of FnBP expression from multicopy plasmids and expression of the proteins in the Gram-positive heterologous host *L. lactis*. The region of the fibronectin-binding proteins responsible for elastin binding was determined by inhibition of adherence with antibodies and purified recombinant truncated derivatives of the proteins. In addition, the purity of commercially available elastin peptides was assessed by Western immunoblotting and the relevance of using elastin peptides dried onto the wells of microtitre plates as representative of elastin tissue was assessed in an ELISA-based assay.

6.2 Results

6.2.1 Adherence of *S. aureus* to immobilised elastin peptides

Previously it has been shown that cells from cultures in the exponential phase of growth of several *S. aureus* laboratory strains could adhere to immobilised elastin peptides (Roche, 2000). It has also been established that EbpS is not responsible for this interaction (see section 5.2.1). In the original experiments the wells of microtitre plates were coated with a very high concentration of elastin peptides (1 mg/ml = 50 μg/well) to test for bacterial cell binding (Roche, 2000). To assess the ability of *S. aureus* laboratory and clinical strains to adhere in a growth phase-dependent manner to lower concentrations of immobilised elastin peptides, 10 μg/ml (0.5 μg/well) of κ-elastin from bovine *ligamentum nuchae* was coated onto the wells of a microtitre plate. Cultures of the laboratory strains Newman, P1, 8325-4, Cowan, COL and RN4220, and the clinical isolates MSSA and EMRSA-16 were grown to early exponential phase (OD_{600nm} = 0.5) and stationary phase (OD_{600nm} = 12) and their ability to bind to immobilised elastin peptides was assessed. Of the laboratory strains tested only strains P1 and Cowan promoted strong adherence to immobilised elastin peptides in the early exponential phase of growth and the ability to bind was lost in stationary phase cultures (Figure 6.1). This was in agreement with data from the original study. Of the clinical strains tested only strain MSSA adhered strongly. MSSA is a virulent strain isolated from community-acquired infections in the U.K. (Enright *et al.*, 2000). Similar to the laboratory strains, the adherence of MSSA to immobilised elastin was growth phase-dependent and occurred only with cells from exponential phase cultures (Figure 6.1).

6.2.2 Adherence of *S. aureus* to immobilised elastin is dependent on fibronectin-binding proteins

Previous studies with *S. aureus* strain P1 revealed that when the genes for both the fibronectin-binding proteins, *fnbA* and *fnbB*, were inactivated by allelic replacement the ability to adhere to immobilised elastin was reduced to background levels (Roche, 2000). The fibronectin-binding proteins, FnBPA and FnBPB, are two cell-wall-associated

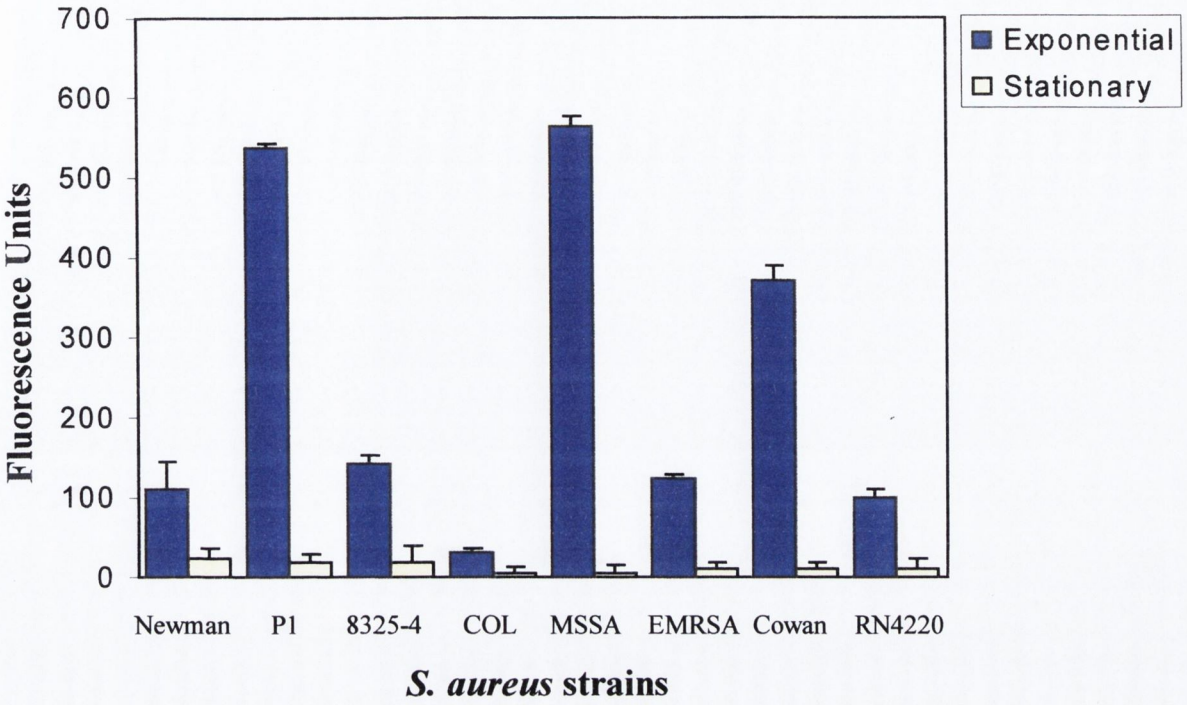


Figure 6.1 Adherence of cells from cultures of *S. aureus* strains Newman, P1, 8325-4, MSSA, EMRSA-16 (EMRSA), Cowan and RN4220 grown to early exponential phase (OD_{600nm} = 0.5) and stationary phase (OD_{600nm} = 12) to immobilised κ-elastin from bovine *ligamentum nuchae* (10 μg/ml).

proteins that belong to the MSCRAMM family of *S. aureus* proteins that promote bacterial binding to immobilised fibronectin (Greene *et al.*, 1995). They also mediate bacterial invasion of epithelial and endothelial *in vitro* (Lammers *et al.*, 1999; Sinha *et al.*, 1999). They are encoded by two closely linked genes, *fnbA* and *fnbB*, that are transcribed separately (Jöhsson *et al.*, 1991; Greene *et al.*, 1995). The N-terminal domains of FnBPA and FnBPB are only 45% identical but the C-terminal domains are 95% identical (See Introduction, section 1.2.1.1.2). To further investigate the role of the fibronectin-binding proteins in elastin binding, the ability of the fibronectin-binding proteins A and B expressed in strain P1 *fnbA fnbB* from plasmids pFNBA4 and pFNBB4 to complement the defective elastin-binding phenotype was assessed. Plasmids pFNBA4 and pFNBB4 are *S. aureus* plasmids that express the FnBPA and FnBPB proteins of strain 8325-4, respectively (Greene *et al.*, 1995). Plasmids pFNBA4 and pFNBB4 were transduced into strain P1 *fnbA fnbB* to produce strains P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$), respectively. Transcription of the *fnb* genes is restricted to the early exponential phase of growth (Greene *et al.*, 1995; Wolz *et al.*, 2000) and the fibronectin-binding ability is reduced as cultures enter stationary phase. This is thought to be a result of the degradation of the proteins by *S. aureus* proteases (McGavin *et al.*, 1997) and masking of the proteins by capsular polysaccharide (Pöhlmann-Dietze *et al.*, 2000). The expression of the fibronectin-binding proteins by these strains was assessed. Cultures of strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) were grown to early exponential phase (OD_{600nm} = 0.5). Cells from each culture were washed and resuspended to the same optical density. The cell-wall-associated proteins were solubilised by digestion of the cell wall peptidoglycan with lysostaphin in the presence of the osmotic stabiliser, raffinose, to maintain stable protoplasts. Equal volumes of the solubilised fractions were analysed by SDS-PAGE and the FnBP proteins were detected by ligand affinity blotting using biotinylated human fibronectin (Figure 6.2). In strain P1 the fibronectin-binding proteins were detected as high molecular mass bands of 175 kDa with some evidence of proteolytic degradation of the proteins to lower molecular mass forms (Figure 6.2, lane 1). These bands were not detected in the solubilized cell wall fraction of strain P1 *fnbA fnbB* (Figure 6.2, lane 2). FnBPA expressed from plasmid pFNBA4 was detected as a high molecular mass band of

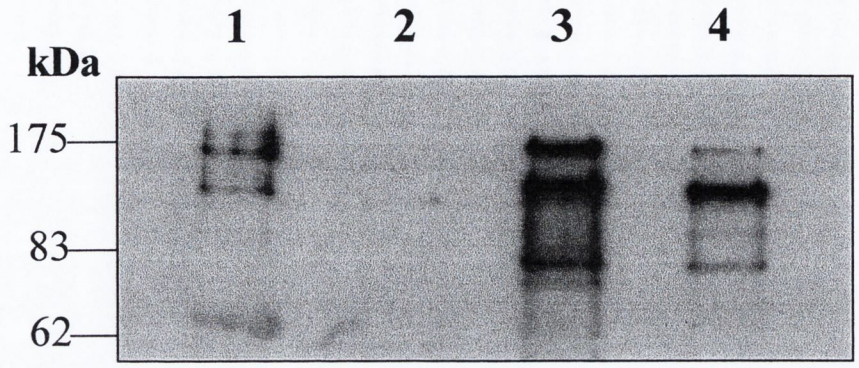


Figure 6.2 Ligand affinity blot of the lysostaphin released cell wall fraction of cells from early exponential phase cultures of *S. aureus* strains P1 (lane 1), P1 *fnbA fnbB* (lane 2), P1 *fnbA fnbB* (pFNBA4$^+$) (lane 3) and P1 *fnbA fnbB* (pFNBB4$^+$) (lane 4). The FnBP proteins were detected using biotinylated human fibronectin (50 μg/ml).

175 kDa with evidence of extensive proteolytic degradation to lower molecular mass forms (Figure 6.2, lane 3). Similar results were observed with FnBPB expressed from plasmid pFNBB4 (Figure 6.2, lane 4). The ability of the FnBP proteins expressed in strain P1 *fnbA fnbB* from plasmids pFNBA4 and pFNBB4 to complement binding to immobilised human fibronectin was also tested. As expected, both FnBPA and FnBPB expressed from plasmids pFNBA4 and pFNBB4, respectively, restored the fibronectin-binding phenotype to strain P1 *fnbA fnbB* (Figure 6.3).

Microtitre plates were coated with solutions of κ-elastin from bovine *ligamentum nuchae* ranging from 15 μg/ml-0.06 μg/ml. Cultures of strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) were grown to early exponential phase and their ability to bind to immobilised elastin peptides was tested (Figure 6.4). Cells of strain P1 adhered in a dose-dependent manner to the immobilised ligand whereas no binding was observed with strain P1 *fnbA fnbB*. FnBPA expressed from plasmid pFNBA4 promoted dose-dependent adherence to elastin but not to the same level as the wild-type strain. Similarly, FnBPB expressed from plasmid pFNBB4 in strain P1 *fnbA fnbB* (pFNBB4$^+$) complemented the elastin-binding phenotype better than FnBPA but not to the same level as the wild-type strain.

Similar results were observed with strain 8325-4. As described in section 6.2.1, strain 8325-4 showed weak adherence to immobilised κ-elastin. This low level adherence was dose-dependent. No binding was observed with strain 8325-4 *fnbA fnbB* (Figure 6.5). Expression of FnBPA or FnBPB from the multicopy plasmids pFNBA4 or pFNBB4 significantly increased the elastin binding capacity of strain 8325-4 *fnbA fnbB*. FnBPB promoted better binding than FnBPA. These data strongly suggest that FnBPA and FnBPB are the surface proteins responsible for bacterial adherence to immobilised elastin peptides.

6.2.3 *S. aureus* aggregation in the presence of elastin peptides is dependent on fibronectin-binding proteins

The majority of *S. aureus* MSCRAMMs promote binding to a soluble ligand as well as bacterial adherence to an immobilised ligand (Foster and Höök, 1998). The fibrinogen-binding protein of *S. aureus*, ClfA, promotes adherence to immobilised

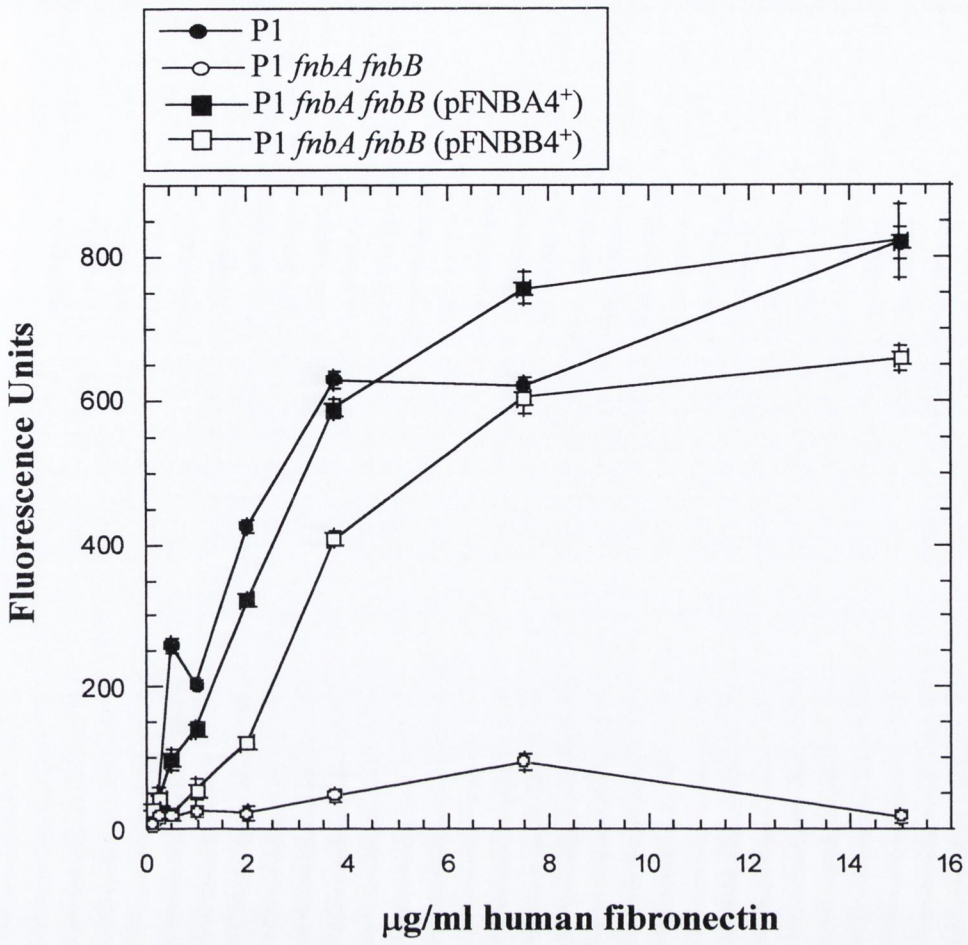


Figure 6.3 Adherence of cells from early exponential phase cultures of *S. aureus* strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) to immobilised human fibronectin.

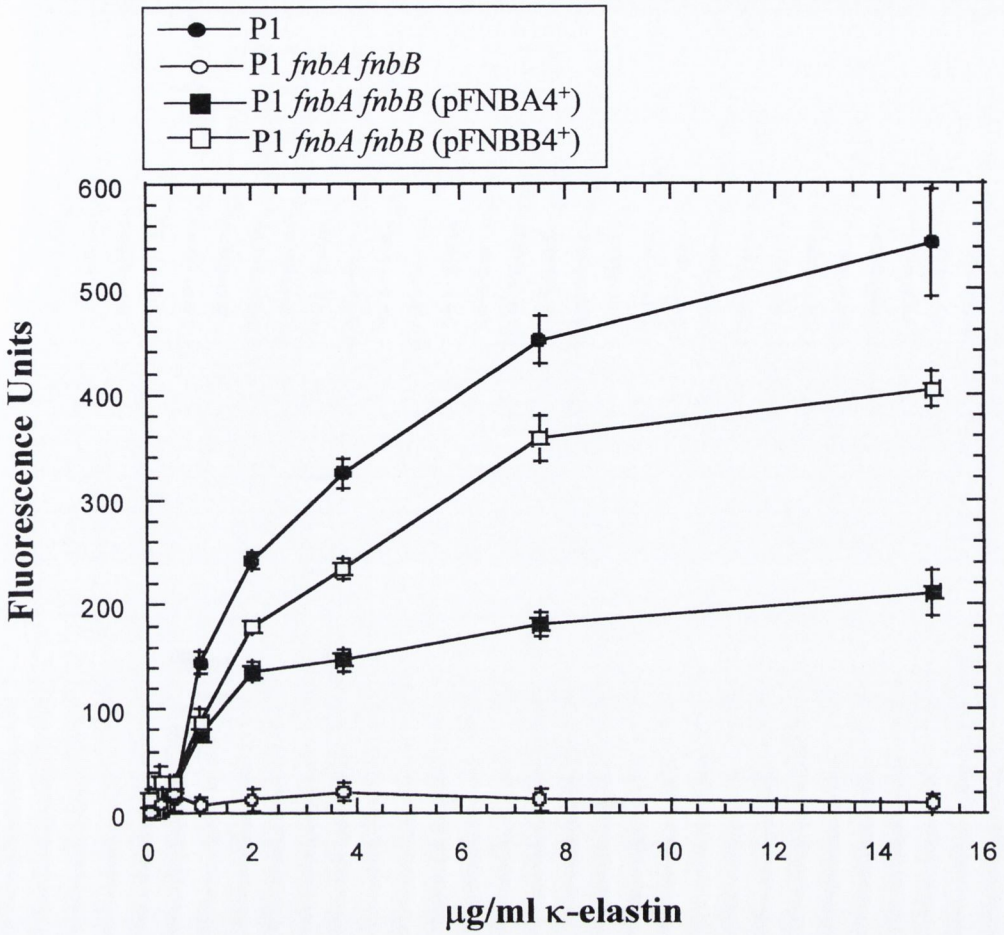


Figure 6.4 Adherence of cells from early exponential phase cultures of *S. aureus* strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) to immobilised κ-elastin peptides from bovine *ligamentum nuchae*.

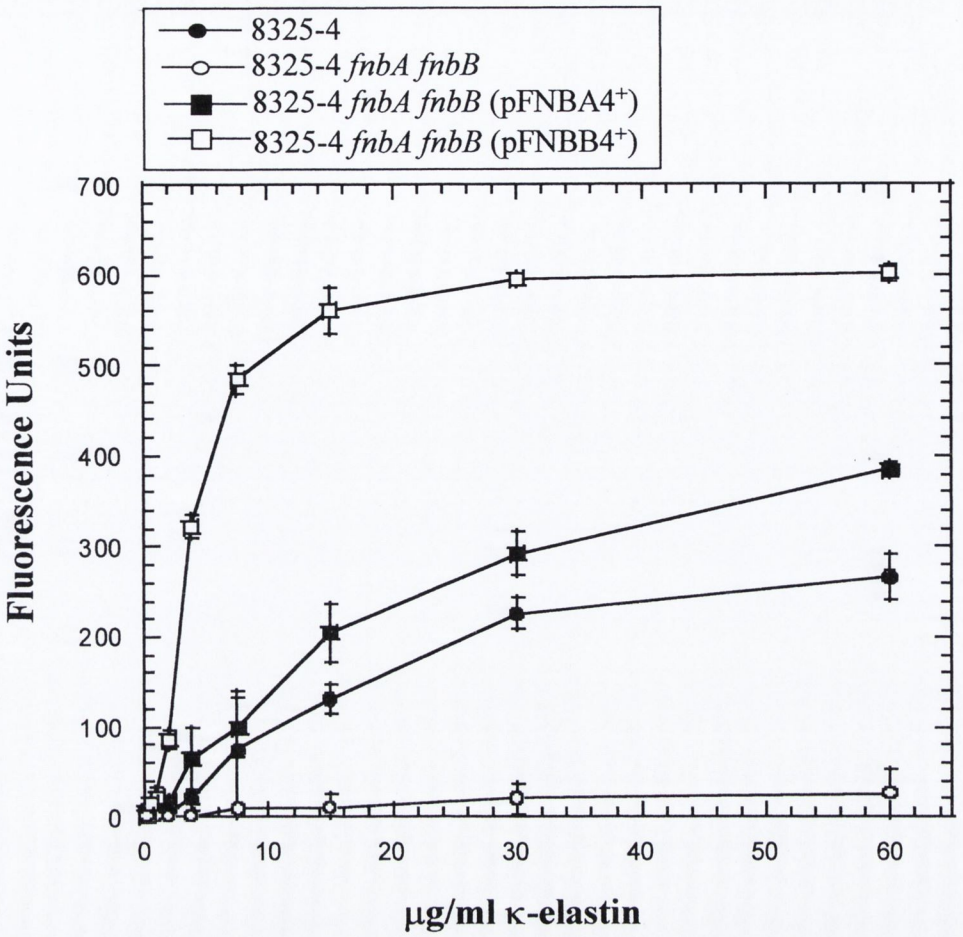


Figure 6.5 Adherence of cells from early exponential phase cultures of *S. aureus* strains 8325-4, 8325-4 *fnbA fnbB*, 8325-4 *fnbA fnbB* (pFNBA4$^+$) and 8325-4 *fnbA fnbB* (pFNBB4$^+$) to immobilised κ-elastin peptides from bovine *ligamentum nuchae*.

fibrinogen and fibrin as well the formation of macroscopic cell clumps when cells are suspended in soluble fibrinogen (McDevitt *et al.*, 1994).

To determine if the fibronectin-binding proteins could promote bacterial binding to soluble elastin peptides, strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) were grown in the presence of the ligand. Overnight cultures of each strain were inoculated into TSB containing 50 μg/ml of κ-elastin from bovine *ligamentum nuchae*. Cultures were incubated with shaking at 37°C and observed for the formation of macroscopic cell clumps. After 1 to 2 hours of growth it was noticed that strains P1, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) formed visible cell aggregates. Aggregation was quantified by measuring the reduction in absorbance of the cultures at 600 nm in comparison to the absorbance of cultures of the same strains grown without elastin peptides (Figure 6.6). Cell aggregation was not observed in the culture of strain P1 *fnbA fnbB* grown in the presence of elastin peptides indicating that the phenotype was specific to cells expressing fibronectin-binding proteins. The aggregation phenomenon was shown to be specific for elastin peptides. It did not occur when cultures were grown in the presence of the same concentration of bovine serum albumin (Figure 6.6). The cells aggregates observed in early exponential phase cultures of strains P1, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) disintegrated as the cultures entered late exponential phase (data not shown).

6.2.4 Expression of the fibronectin-binding proteins in *L. lactis*

Previously it has been shown that the interactions between staphylococcal cell-wall-associated proteins and their ligands can be studied by expression of the protein in the Gram-positive heterologous host *L. lactis*. Staphylococcal surface proteins that have been studied in *L. lactis* include ClfA of *S. aureus* (Que *et al.*, 2000), FnBPA of *S. aureus* (Massey *et al.*, 2001) and SdrG of *S. epidermidis* (Hartford *et al.*, 2001b).

To determine if the fibronectin-binding proteins could promote the adherence of *L. lactis* to immobilised elastin, FnBPA and FnBPB from *S. aureus* strain P1 were expressed from plasmid pKS80 in *L. lactis* strain MG1363. DNA fragments containing the *fnbA* or the *fnbB* gene were PCR-amplified from chromosomal DNA of strain P1. The resulting PCR products were cloned into the *L. lactis* expression vector pKS80 (see

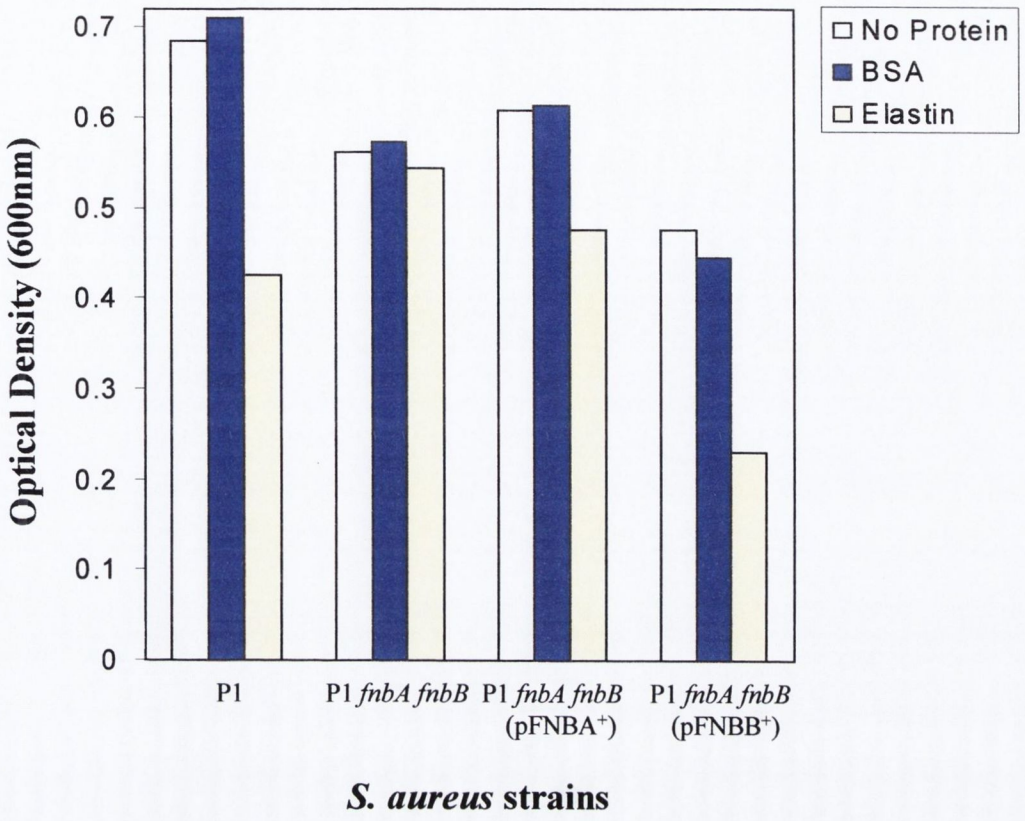


Figure 6.6 Aggregation of *S. aureus* strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) grown in TSB or TSB containing 50 μg/ml of κ-elastin from bovine *ligamentum nuchae* or bovine serum albumin (BSA). This figure is representative of the results from three independent experiments performed in duplicate.

Figure 3.8) and introduced into *L. lactis* strain MG1363 to produce strains MG1363 (*fnbA*$^+$) and MG1363 (*fnbB*$^+$). The fibronectin-binding ability of strains MG1363 (*fnbA*$^+$) and MG1363 (*fnbB*$^+$) was compared to *S. aureus* strain P1. Human fibronectin was coated onto the wells of a microtitre plate and incubated with cells from an early exponential phase culture of strain P1 and late exponential phase cultures of *L. lactis* strains MG1363, MG1363 (*fnbA*$^+$) and MG1363 (*fnbB*$^+$) (OD_{600nm} of 2). Both FnBPA and FnBPB promoted the adherence of *L. lactis* to immobilised fibronectin in a dose-dependent manner, to a level similar to that of *S. aureus* strain P1 (Figure 6.7). Adherence was specific to FnBP expression because it did not occur with *L. lactis* strain MG1363 containing the empty vector, pKS80. Conversely, FnBPA and FnBPB expressed in *L. lactis* did not promote the adherence of this organism to immobilised κ-elastin from bovine *ligamentum nuchae* (Figure 6.8). Adherence was not promoted even at higher concentrations of immobilised elastin or at any phase of the growth cycle (data not shown).

To determine the levels of the FnBP proteins expressed on the surface of *L. lactis*, Western immunoblotting and ligand affinity blotting of cell wall extracts was performed and compared with *S. aureus* P1. Cells from each culture were washed and resuspended to the same optical density. Cultures of *S. aureus* strain P1 *fnbA fnbB* and P1 *fnbA fnbB* (pFNBA4$^+$) were grown to early exponential phase and the cell wall proteins were solubilised by digestion of the cell wall peptidoglycan in the presence of the osmotic stabiliser raffinose, to maintain stable protoplasts. Similarly, cell-wall-associated proteins from cultures of *L. lactis* strains MG1363, MG1363 (*fnbA*$^+$) and MG1363 (*fnbB*$^+$) grown to late exponential phase were solubilised by digestion of the cell wall peptidoglycan with the muralytic enzymes lysozyme and mutanolysin, in the presence of raffinose. Equivalent volumes of each sample were analysed by Western immunoblotting with anti-$rFnBPA_{37-605}$ antibodies that recognise regions A and B of FnBPA and are weakly cross-reactive with region A of FnBPB (E. Wann, personal communication). FnBPA was detected in the soluble cell wall fraction of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4$^+$) as a number of bands ranging in size from greater than 175 kDa to below 60 kDa (Figure 6.9A., lane 2). A cross-reactive protein band of approximately 50 kDa was detected in the solublised cell wall fraction of *S. aureus* strain P1 *fnbA fnbB* (Figure 6.9A, lane 1).

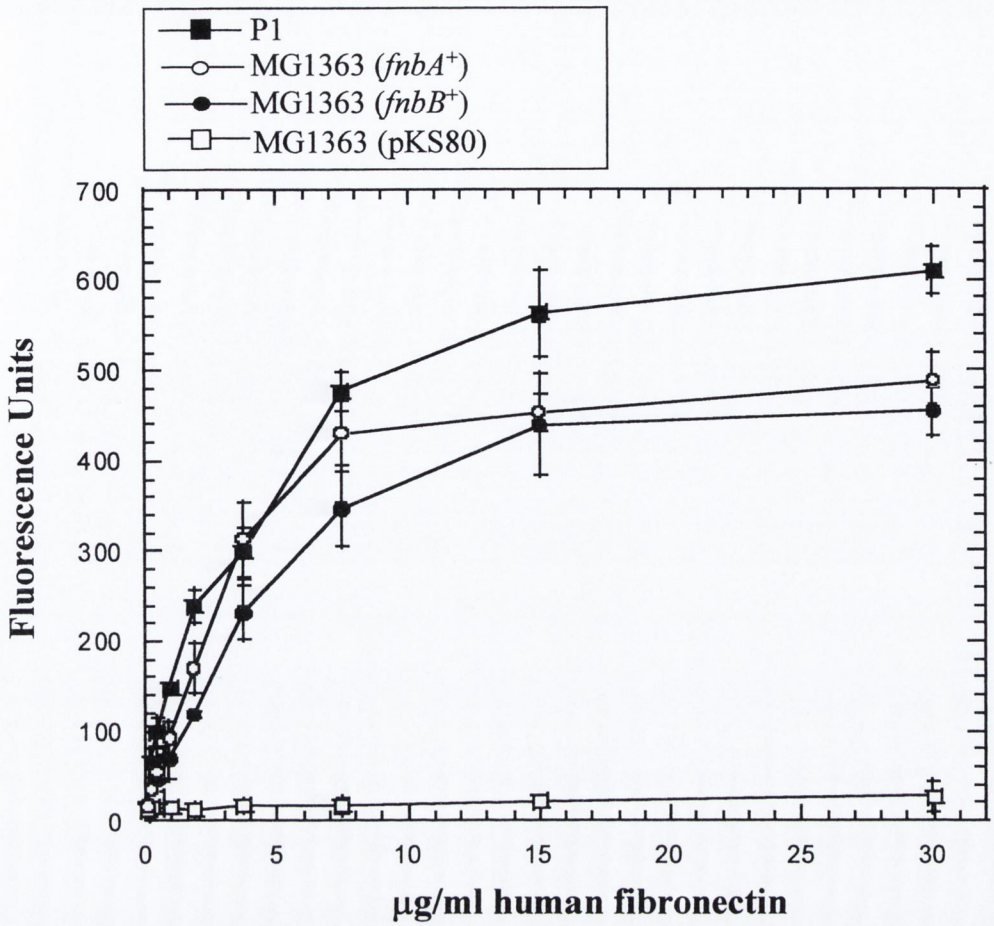


Figure 6.7 Adherence of cells from an early exponential phase culture of *S. aureus* strain P1 and late exponential phase cultures of *L. lactis* strains MG1363 (*fnbA*$^+$), MG1363 (*fnbB*$^+$) and MG1363 (pKS80) to immobilised human fibronectin.

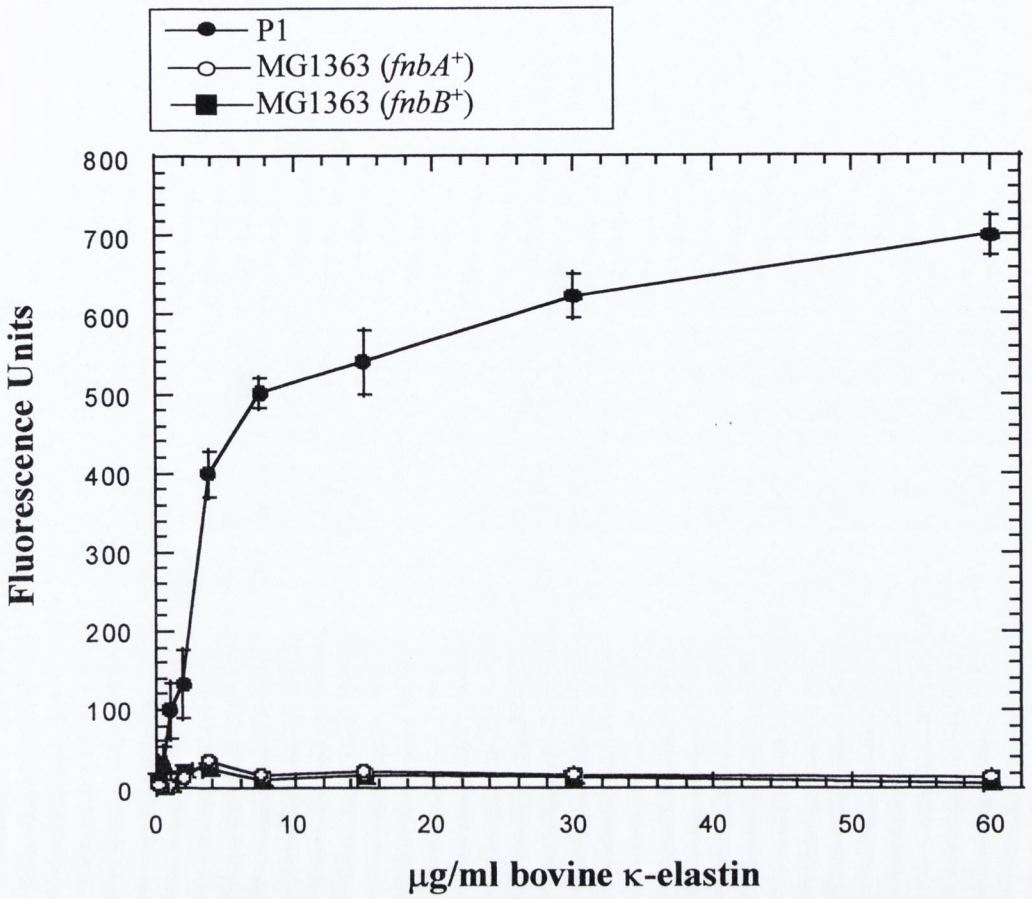


Figure 6.8 Adherence of cells from an early exponential phase culture of *S. aureus* strain P1 and late exponential phase cultures of *L. lactis* strains MG1363 (*fnbA*$^+$), MG1363 (*fnbB*$^+$) and MG1363 (pKS80) to immobilised κ-elastin from bovine *ligamentum nuchae*.

FnBPA in the soluble cell wall fraction of *L. lactis* strain MG1363 (*fnbA*$^+$) was weakly expressed as a number of proteolytic degradation products ranging in size from 70 kDa to 30 kDa (Figure 6.9A, lane 3). FnBPB expressed in *L. lactis* strain MG1363 (*fnbB*$^+$) was weakly expressed as a single band of approximately 130 kDa (Figure 6.9A, lane 4). Similar results were observed with ligand affinity blot analysis of the same samples using biotinylated human fibronectin (Figure 6.9B). FnBPA was weakly detected as a number of proteolytic degradation products ranging in size from 70 kDa to 30 kDa (Figure 6.9B, lane 3). A fibronectin-binding protein of approximately 120 kDa, that was not FnBPA or FnBPB, was observed in the solubilised cell wall digest of *S. aureus* strain P1 *fnbA fnbB*, and *L. lactis* strains MG1363 (*fnbB*$^+$) and MG1363 (pKS80) (Figure 6.9B, lanes 1,4 and 5, respectively).

To ensure that the muralytic enzymes lysozyme and mutanolysin efficiently digested the cell wall peptidoglycan of *L. lactis* and solubilised cell wall proteins, the solubilised cell wall fraction of strains MG1363 (*fnbA*$^+$) and MG1363 (*fnbB*$^+$) were compared to the solubilised cell wall fraction of *L. lactis* strain MG1363 (*sdrC*$^+$) by silver nitrate staining of SDS-PAGE gels. Strain MG1363 (*sdrC*$^+$) expresses the *S. aureus* cell-wall-associated protein SdrC from plasmid pKS80 (O'Brien, 2001). Previous studies have shown that SdrC is expressed and sorted to the cell wall of this organism where it can be released in its full length form by digestion of the cell wall with lysozyme and mutanolysin (O'Brien, 2001). No high molecular weight protein bands were detected in the cell wall fractions of strains MG1363 (*fnbA*$^+$) and MG1363 (*fnbB*$^+$) (Figure 6.10, lanes 2 and 3). Conversely, SdrC was detected as two high molecular weight proteins bands of approximately 120 kDa and 150 kDa (Figure 6.10, lane 4). This indicated that the cell wall proteins of *L. lactis* strains were solubilised by digestion of the cell wall peptidoglycan with lysozyme and mutanolysin.

These data suggest that the fibronectin-binding proteins are poorly expressed on the surface of *L. lactis*. The breakdown in *L. lactis* is likely due to proteolysis that occurs on the cell surface or during solubilisation. Small amounts of truncated fibronectin-binding proteins on the surface of the organism could promote bacterial binding to their high affinity ligand, fibronectin, but there was insufficient protein to promote adherence to elastin. This might also indicate that the elastin-binding activity of the fibronectin-

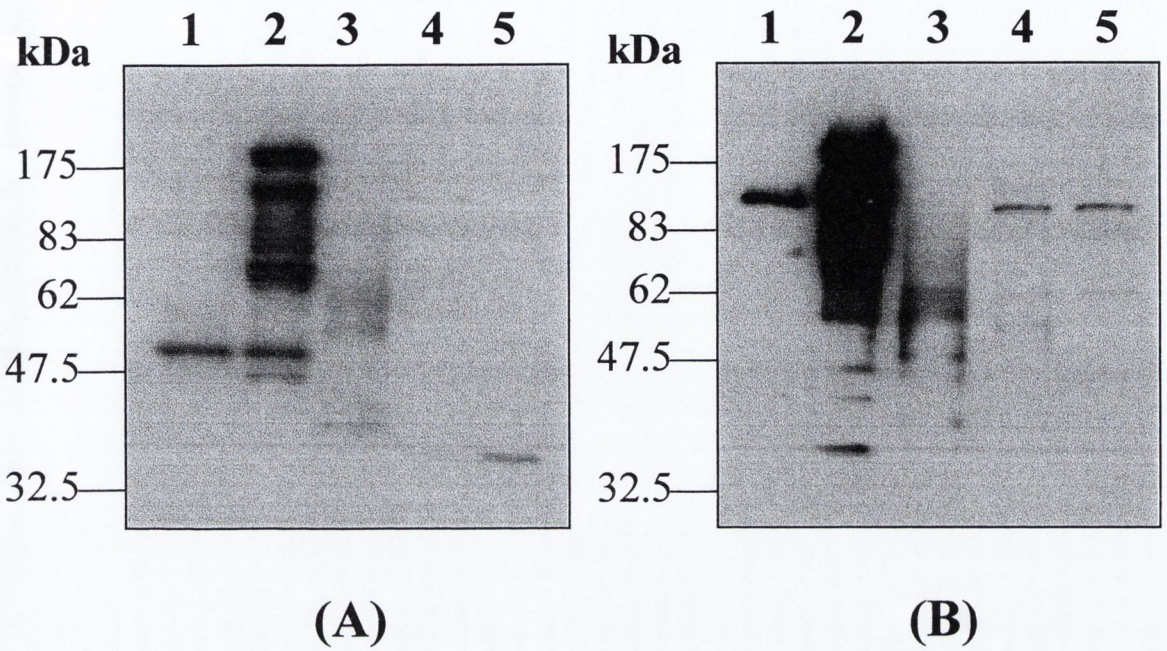


Figure 6.9 Western immunoblot (A) and Western ligand affinity blot (B) of lysostaphin cell wall digests of *S. aureus* strains P1 *fnbA fnbB* (lanes 1) and P1 *fnbA fnbB* (pFNBA4$^+$) (lanes 2) and lysozyme/mutanolysin cell wall digests of *L. lactis* strains MG1363 (*fnbA*$^+$) (lanes 3), MG1363 (*fnbB*$^+$) (lanes 4) and MG1363 (pKS80) (lanes 5) probed with anti-rFnBPA$_{37-605}$ antibodies (A) or biotinylated human fibronectin (B).

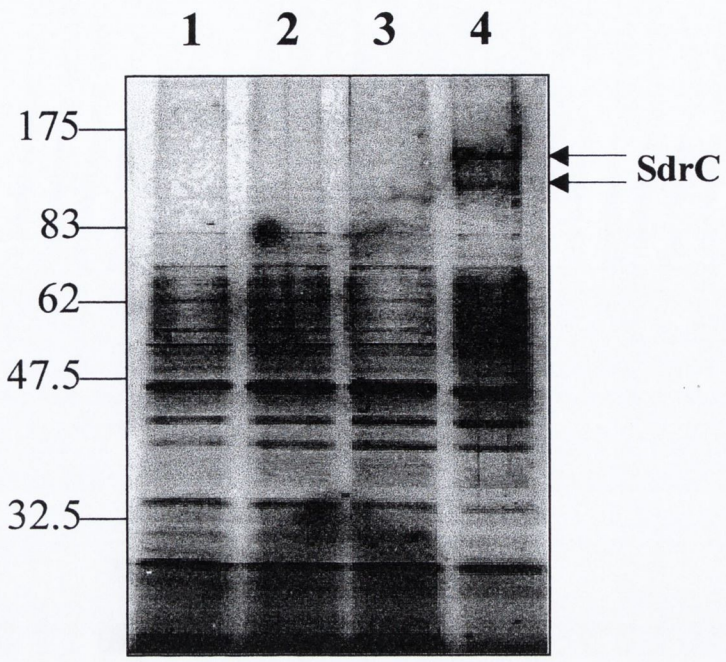


Figure 6.10 Silver nitrate stained SDS-PAGE gel of lysozyme/mutanolysin solubilised cell-wall-associated proteins of *L. lactis* strains MG1363 (pKS80) (lane 1), MG1363 (*fnbA*$^+$) (lane 2), MG1363 (*fnbB*$^+$) (lane 3) and MG1363 (*SdrC*$^+$) (lane 4).

binding proteins is located in a region of the proteins that is distinct from the regions involved in fibronectin binding.

6.2.5 *S. aureus* adheres to human aortic elastin and human lung elastin in an FnBP-dependent manner

As demonstrated in section 6.2.2 *S. aureus* adheres to immobilised elastin peptides purified from bovine neck ligament in an FnBP-dependent manner. If the interaction between the fibronectin-binding proteins and elastin is to be an important factor in human diseases of elastin-rich tissue, such as endocarditis and pneumonia, then these proteins must promote bacterial adherence to immobilised human elastin. There is a great deal of sequence homology between all mammalian elastins (Boyd *et al.*, 1991).

κ-elastin from bovine *ligamentum nuchae*, human aortic elastin that had been prepared by successive treatments with hot oxalic acid (Partridge, 1962) and human lung elastin that had been prepared by the method of Starcher and Galione (1976) and digested with human sputum elastase, were coated onto the wells of microtitre plates. A culture of *S. aureus* strain P1 was grown to early exponential phase (OD_{600nm} = 0.5) and the ability of cells to bind to immobilised elastin peptides was tested. The cells bound in a dose-dependent manner to all three forms of immobilised elastin and most avidly to human aortic elastin (Figure 6.11).

Bacterial adherence to immobilised human aortic and human lung elastin was shown to be FnBP-dependent since it did not occur with strain P1 *fnbA fnbB*. The phenotype could be complemented by expression of FnBPA or FnBPB from plasmids pFNBA4 and pFNBB4, respectively (Figures 6.12 and 6.13).

6.2.6 Bovine and human elastin preparations are not contaminated with fibronectin or fibrinogen

Previously it has been shown that the D repeats at the C terminus of the fibronectin-binding proteins contain the fibronectin binding domains (McGavin *et al.*, 1993a; Patti e*t al.*, 1995) and that the N-terminal A region of FnBPA can interact with the γ-chain of fibrinogen (Wann *et al.*, 2000). Since the commercially available elastin peptides used in adherence assays were purified from animal and human sources, it was

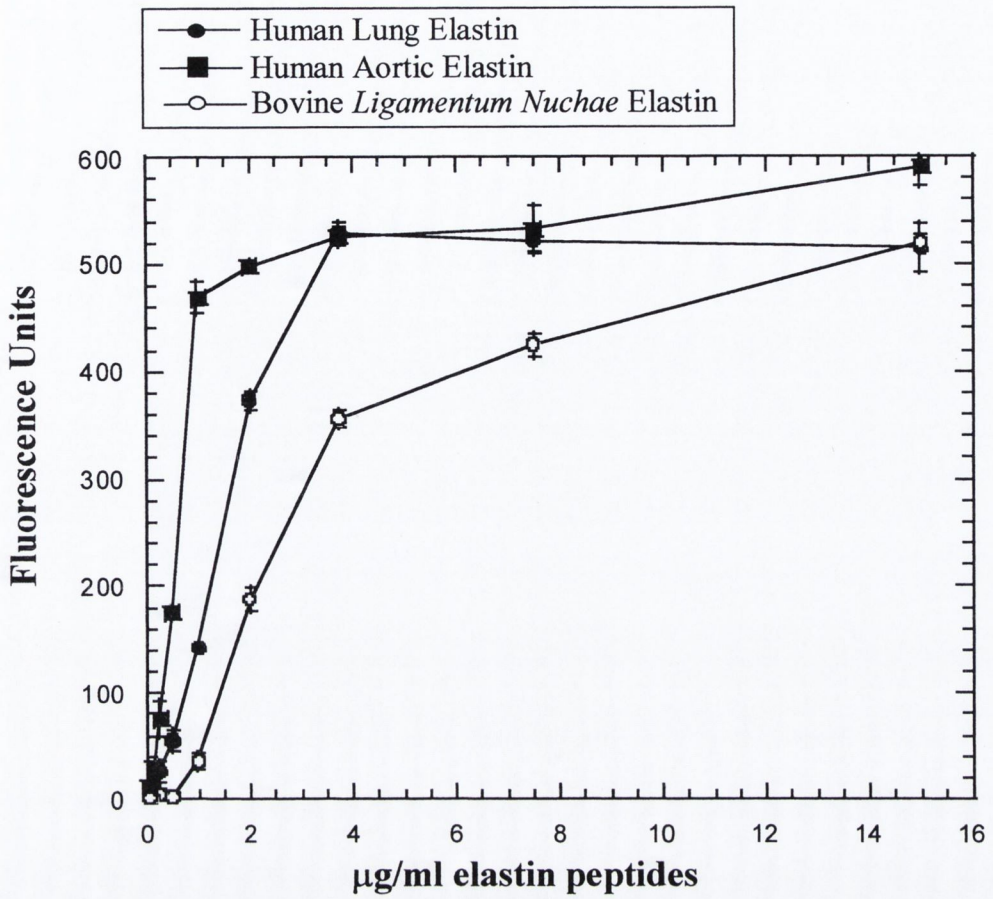


Figure 6.11 Adherence of cells from an early exponential phase culture of *S. aureus* strain P1 to immobilised elastin from human lung, human aorta and bovine *ligamentum nuchae*.

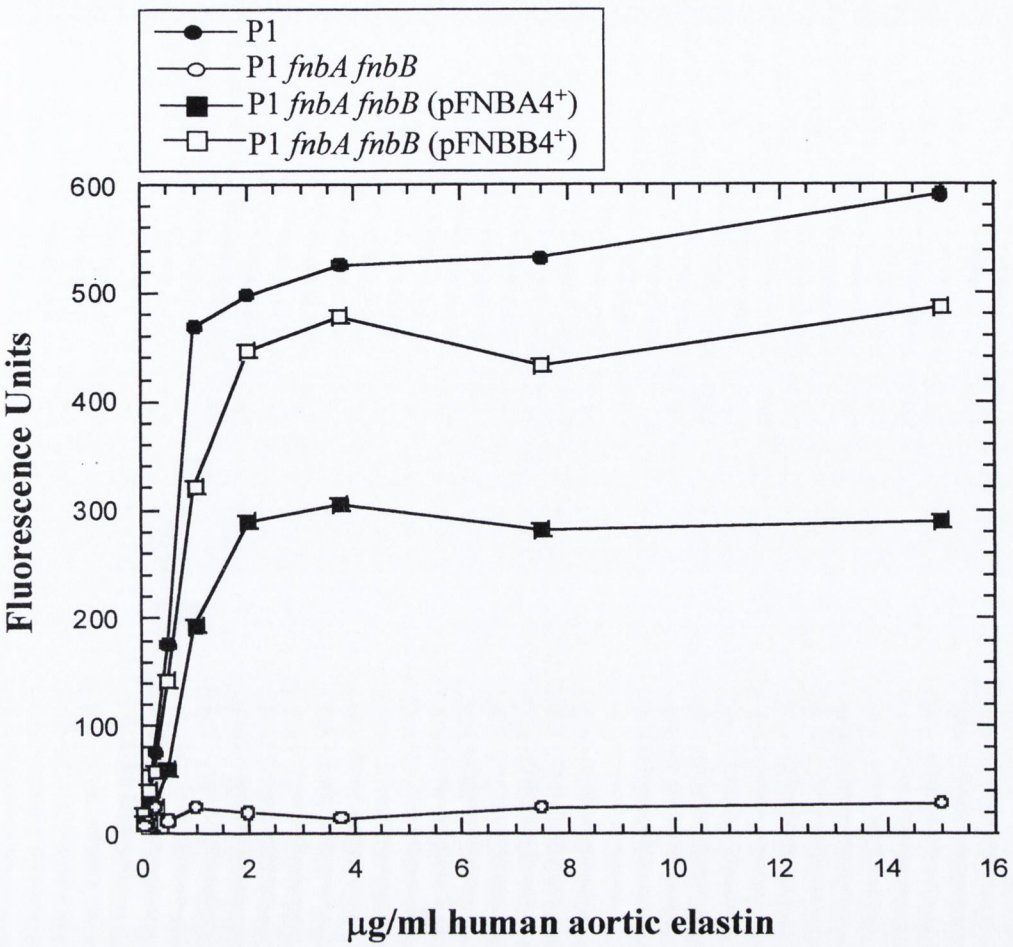


Figure 6.12 Adherence of cells from early exponential phase cultures of *S. aureus* strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) to immobilised human aortic elastin peptides.

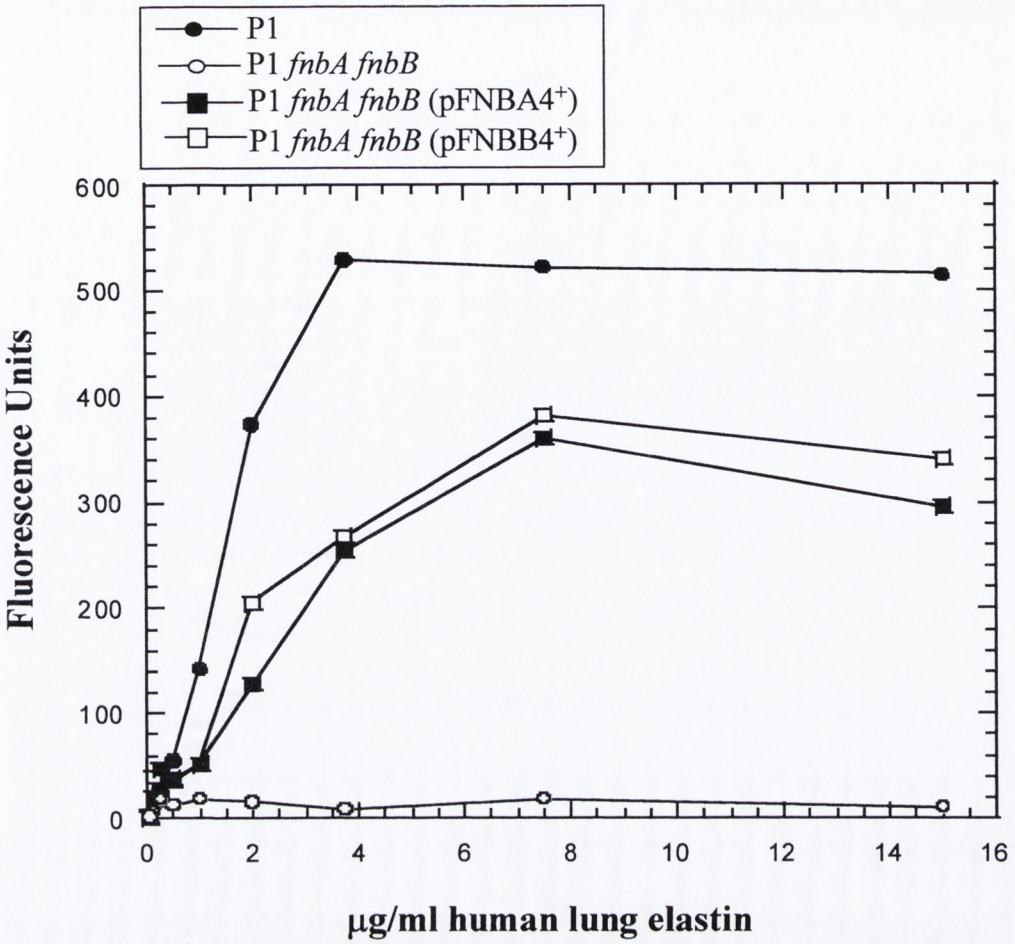


Figure 6.13 Adherence of cells from early exponential phase cultures of *S. aureus* strains P1, P1 *fnbA fnbB*, P1 *fnbA fnbB* (pFNBA4$^+$) and P1 *fnbA fnbB* (pFNBB4$^+$) to immobilised human lung elastin peptides.

suggested that they could be contaminated with fibronectin or fibrinogen. However, experts in the elastin field, who routinely use elastin preparations from the same commercial source (Elastin Products Company), have never found fibronectin contamination as determined by Western immunoblotting using anti-fibronectin antibodies (R. Mecham and A. Hinek, personal communications). Similarly, N-terminal sequencing of protein bands of an α-elastin preparation from bovine *ligamentum nuchae* run on a SDS-PAGE gel revealed that there was no detectable contamination with other proteins (Park *et al.*, 1991).

To determine if there was contamination of elastin preparations with fibronectin or fibrinogen, Western immunoblotting analysis was performed using polyclonal anti-human fibronectin antibodies and polyclonal anti-human fibrinogen antibodies. 2 μg of human fibronectin was tested as a control with 30 μg of human aortic elastin and 30 μg of human lung elastin by Western immunoblotting. Human fibronectin (2 μg) reacted strongly as a large number of protein bands ranging in size from greater than 175 kDa to 50 kDa (Figure 6.14, lane 1). Conversely, no immunoreactive protein was detected in either the human aortic elastin preparation (Figure 6.14, lane 2) or the human lung elastin preparation (Figure 6.14, lane 3). Similarly, the α, β and γ chains of human fibrinogen (2 μg) were detected with polyclonal anti-human fibrinogen antibodies (Figure 6.14, lane 4), but no immunoreactive proteins were detected in 30 μg of either the human aortic elastin preparation (Figure 6.14, lane 5) or the human lung elastin preparation (Figure 6.14, lane 6).

Similar results were observed by Western immunoblotting analysis of κ-elastin prepared from bovine *ligamentum nuchae* with anti-human fibronectin antibodies. 2 μg of human fibronectin and bovine fibronectin, and 30 μg of bovine κ-elastin were run on an SDS-PAGE gel and detected with anti-human fibronectin antibodies. Again, the human fibronectin was detected as a number of high molecular weight bands (Figure 6.14, lane 7). The antibodies cross-reacted with bovine fibronectin, which was detected as several protein bands of greater than 175 kDa (Figure 6.14, lane 8). Conversely, no protein was detected in the bovine κ-elastin preparation (Figure 6.14, lane 9). Unfortunately, the anti-human fibrinogen antibodies did not cross-react with the bovine

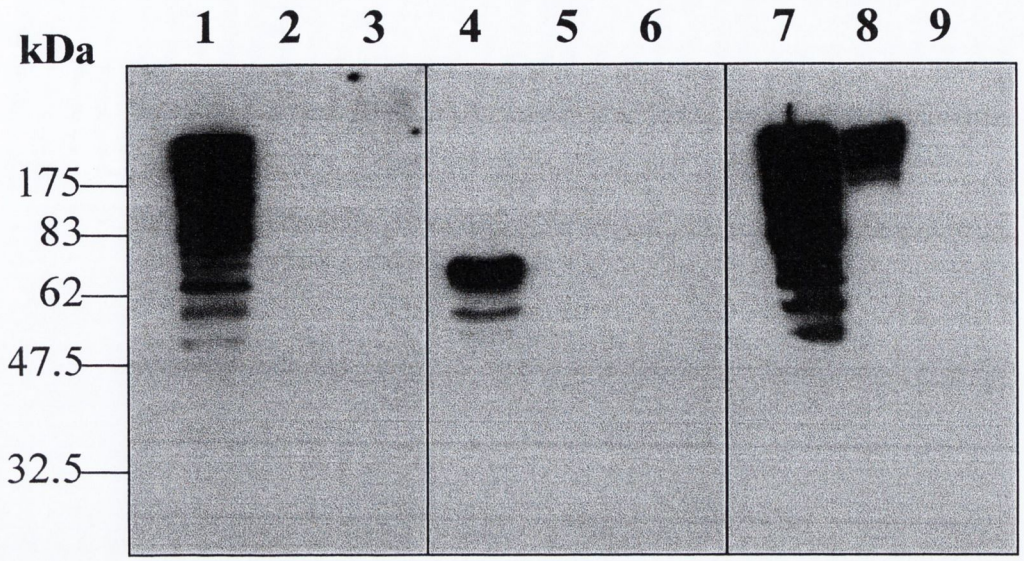


Figure 6.14 Western immunoblotting analysis of elastin preparations purified from bovine and human sources. Lane 1, 2 μg of human fibronectin; lane 2, 30 μg of human aortic elastin and lane 3, 30 μg of human lung elastin, probed with polyclonal anti-human fibronectin antibodies. Lane 4, 2 μg of human fibrinogen; lane 5, 30 μg of human aortic elastin and lane 6, 30 μg of human lung elastin, probed with polyclonal anti-human fibrinogen antibodies. Lane 7, 2 μg of human fibronectin; lane 8, 2 μg of bovine fibronectin and lane 9, 30 μg of bovine κ-elastin peptides from *ligamentum nuchae*, probed with polyclonal anti-human fibronectin antibodies.

form of the protein and could not be used to test for fibrinogen contamination of the bovine κ-elastin preparation (data not shown).

These data suggest that the commercially available elastin peptide preparations used in the bacterial adherence assays are not contaminated with fibronectin or fibrinogen and that the bacteria adhered to elastin and not to a contaminating ligand of the fibronectin-binding proteins.

6.2.7 Immobilised elastin peptides react with antibodies to soluble elastin peptides

To study the adherence of bacteria to an immobilised ligand, the protein is coated onto the wells of a microtitre plate and the binding of the bacteria is assessed. This method has been used to study the interactions between *S. aureus* and a number of mammalian extracellular matrix proteins such as fibrinogen, fibronectin and collagen (Wolz *et al.*, 1996). Ligands spontaneously bind to the wells of a microtitre plate when incubated at 4°C in sodium bicarbonate for 18 hours. However, attempts to coat wells of a microtitre plate with elastin peptides were unsuccessful (Roche, 2000). The technique used to coat elastin peptides onto the wells of a microtitre plate involves incubating the proteins in 0.1 M sodium bicarbonate for 18 hours under UV light at room temperature (Roche, 2000). This method was developed from a technique used to study elastin receptors on the surface of mammalian cells (Hinek *et al.*, 1999).

To determine if human aortic elastin dried under UV light onto the wells of a microtitre plate is representative of the soluble form of the protein, the ability of specific antibodies to interact with the immobilised form was assessed in an ELISA-based assay. Human aortic elastin (1 μg/ml – 2 ng/ml) was coated onto the wells of a microtitre plate. A fixed concentration of rabbit anti-human aortic elastin antibodies was added to each well. The antibodies were shown to bind to the immobilised material in a dose-dependent manner (Figure 6.15). This interaction was shown to be specific to the anti-human aortic elastin antibodies since it did not occur using the same dilution of normal rabbit serum (Figure 6.15).

These data indicate that elastin peptides dried onto the wells of a microtitre plate contain some of the epitopes possessed by the soluble form of the antigen used to immunize a rabbit.

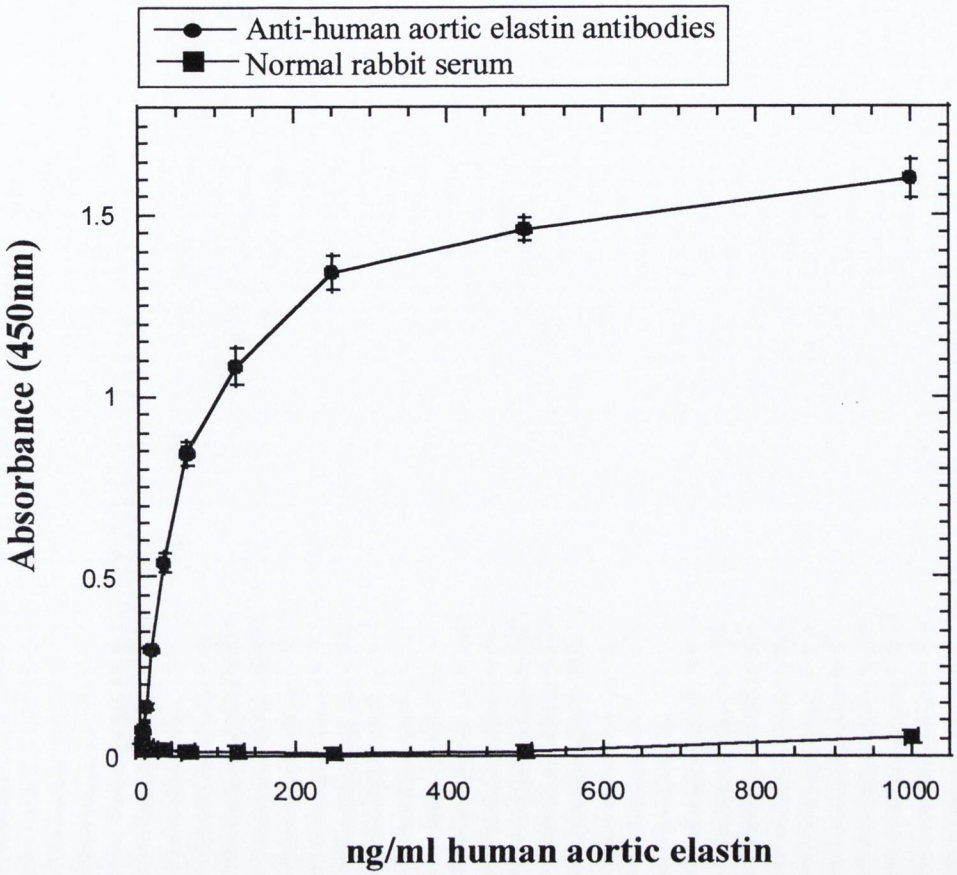


Figure 6.15 Binding of anti-human aortic elastin antibodies to increasing concentrations of immobilised human aortic elastin.

6.2.8 Inhibition of *S. aureus* elastin binding by anti-FnBP antibodies and soluble fibronectin

Previously, the regions of the fibronectin-binding proteins responsible for the interaction with fibronectin and fibrinogen have been characterised using various genetic and biochemical techniques. It has been shown that the D1 to D4 repeat regions at the C terminus of both FnBPA and FnBPB are the main fibronectin-binding regions of the proteins and that there are two other fibronectin-binding regions in FnBPA. These include the Du region at the C terminus of the protein (Joh *et al.*, 1998) and the B region of the protein (Massey *et al.*, 2001). Region A of FnBPA has been shown to bind to the γ-chain peptide of human fibrinogen (Wann *et al.*, 2000). To determine which regions of the fibronectin-binding proteins are involved in elastin binding, inhibition studies were performed using FnBP-specific antibodies and soluble fibronectin.

Strain P1 *fnbA fnbB* (pFNBA4$^+$) was grown to early exponential phase. The cells were preincubated with normal rabbit serum to saturate protein A, the cell-wall-associated immunoglobulin-binding protein. The cells were then incubated with dilutions of anti-rFnbpA$_{37-605}$ antibodies, which recognise regions A and B of FnBPA, anti-rEbpS$_{1-267}$ antibodies or anti-ClfA$_{220-559}$ antibodies. If the antibodies bound to a region of a protein that is responsible for elastin binding then this interaction would be inhibited. The ability of the cells to adhere to immobilised human aortic elastin peptides was assessed. The anti-rFnbpA$_{37-605}$ antibodies inhibited FnBPA mediated elastin binding in a dose-dependent manner, with the highest concentration of inhibiting antibody (10μg/ml) resulting in 90 ± 0.4% inhibition of bacterial binding (Figure 6.16). Conversely, the same range of concentrations of the anti-rEbpS$_{1-267}$ antibodies and anti-ClfA$_{220-559}$ antibodies did not inhibit elastin binding.

To determine if the fibronectin-binding regions of FnBPA were involved in the elastin-binding phenotype, the ability of soluble fibronectin to inhibit elastin binding was assessed. Strain P1 *fnbA fnbB* (pFNBA4$^+$) was grown to early exponential phase and preincubated with soluble human fibronectin (100 μg/ml). The ability of cells to bind to immobilised fibronectin and elastin was then tested. Pre-incubation of cells with soluble fibronectin resulted in 76 ± 2.7% inhibition of binding to immobilised fibronectin (Figure

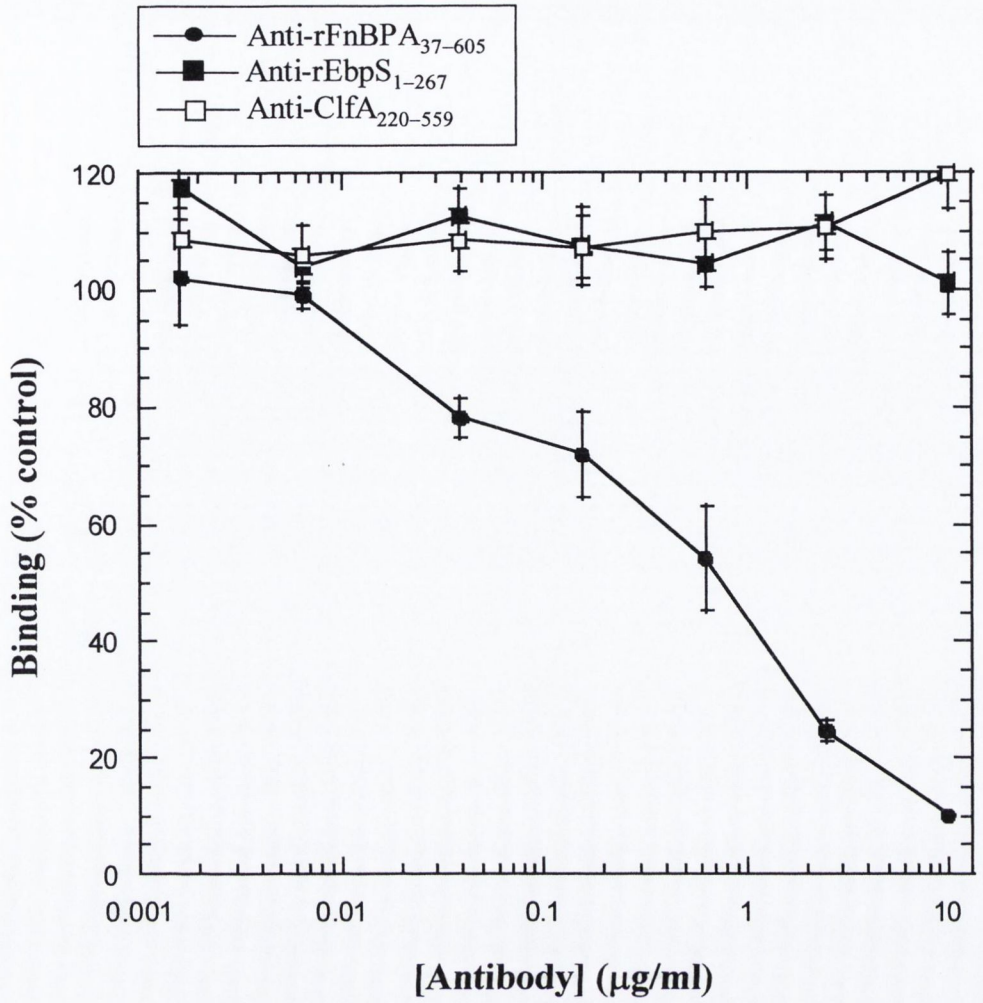


Figure 6.16 Inhibition of cells of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4$^+$) binding to immobilised human aortic elastin (10 μg/ml = 0.5 μg/well) by preincubation with anti-rFnBPA$_{37–605}$, anti-rEbpS$_{1–267}$ or anti-ClfA$_{220–559}$ antibodies. The values are expressed as percentages of a control lacking inhibitor.

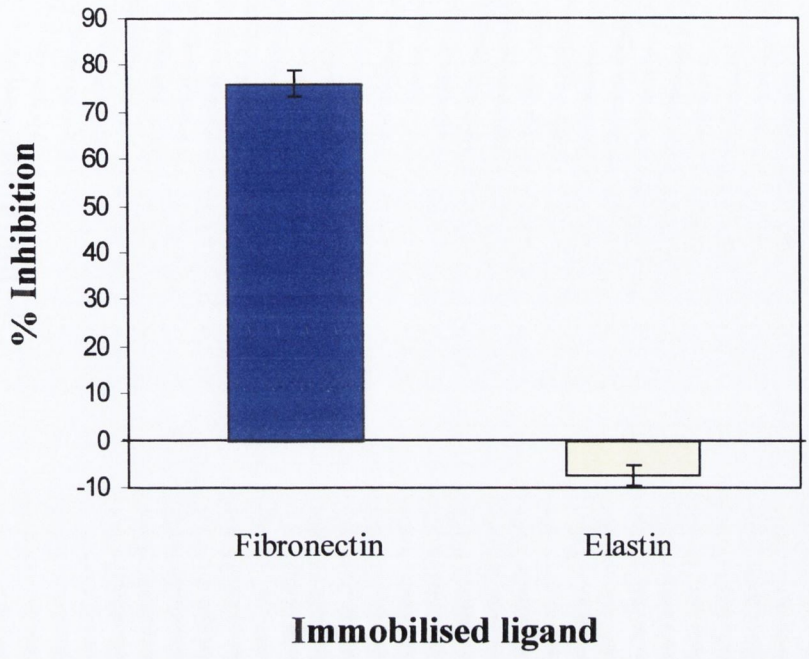


Figure 6.17 Inhibition of *S. aureus* binding to immobilised fibronectin and elastin by preincubation of cells with human fibronectin. Cells of *S. aureus* strain P1 *fnbA fnbB* (pFNBA4^{+}) were preincubated in human fibronectin (100 μg/ml) and then their ability to bind to immobilsed human fibronectin (0.5 μg/well) and human aortic elastin (0.5 μg/well) was tested. The values are expressed as percentages of a control lacking inhibitor.

6.17). Conversely, pre-incubation of cells expressing FnBPA with soluble fibronectin did not inhibit binding to immobilised human aortic elastin.

These data suggest that the region(s) of FnBPA involved in elastin binding is distinct from the regions involved in fibronectin binding and that this region(s) is located at the N terminus of the protein within regions A and B.

6.2.9 Expression and purification of fibronectin-binding proteins truncated derivatives in *E. coli*

To further study the region of the fibronectin-binding proteins involved in elastin binding, recombinant truncated derivatives of FnBPA and FnBPB were expressed in *E. coli* as N-terminal 6×histidine fusion proteins. DNA fragments corresponding to region AB of FnBPA (residues 37-605) and region D1 to D4 of FnBPA (residues 745-878) were PCR–amplified from plasmid pFNBA4. Similarly, a DNA fragment corresponding to region A of FnBPB (residues 37-540) was PCR-amplified from plasmid pFNBB4 (Figure 6.18). The resulting PCR fragments were cloned into the expression vector pQE-30 and expressed in the protease deficient *E. coli* strain, Topp 3. The recombinant proteins, rFnBPA$_{37-605}$, rFnBPB$_{37-540}$ and rFnBPA$_{745-878}$ were purified from *E. coli* lysates by nickel-chelate affinity chromatography and analysed on a Coomassie blue stained SDS-PAGE gel and by Western ligand affinity blotting using the India™ HisProbe.

Proteins rFnBPA$_{37-605}$, rFnBPB$_{37-540}$ and rFnBPA$_{745-878}$ (5μg) were run on an SDS-PAGE gel and stained with Coomassie blue (Figure 6.19A). The rFnBPA$_{37-605}$ had a predicted molecular mass of 62.7 kDa but the protein migrated with an apparent molecular mass of approximately 85 kDa (Figure 6.19A, lane 1). Similarly, the proteins rFnBPB$_{37-540}$ and rFnBPA$_{745-878}$ migrated at approximately 70 kDa and 25 kDa, respectively, slower than their predicted molecular mass (Figure 6.19A, lane 2 and 3).

The same size protein bands were detected by Western ligand affinity blotting using the commercially available India™ HisProbe. India™ HisProbe is nickel chelated to horseradish peroxidase (HRP), which has a high affinity for histidine residues. The six histidine residues at the N terminus of each of the purified recombinant proteins was detected using this probe (Figure 6.19B).

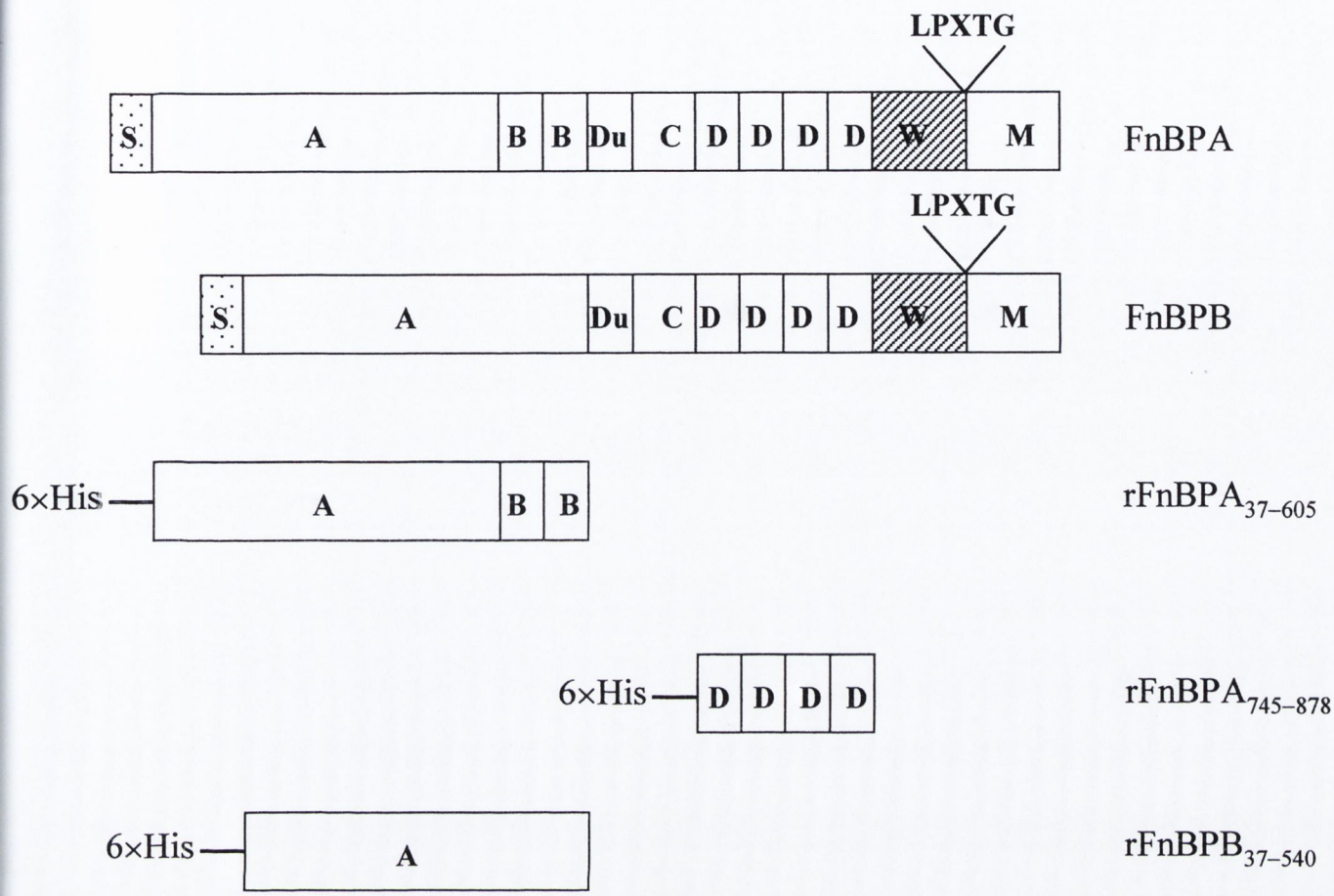


Figure 6.18 Schematic diagram of the fibronectin-binding proteins of *S. aureus*, FnBPA and FnBPB, and recombinant truncated derivatives. A secretory signal sequence (S), regions A, B, Du, C and D, cell spanning domains (W) and membrane spanning domains (M) are indicated. The LPXTG motifs, involved in anchoring the proteins to the cell wall peptidoglycan, are also indicated. The recombinant proteins $rFnBPA_{37–605}$, $rFnBPB_{37–540}$ and $rFnBPA_{745–878}$ with N-terminal six histidine tags (6×His) are shown.

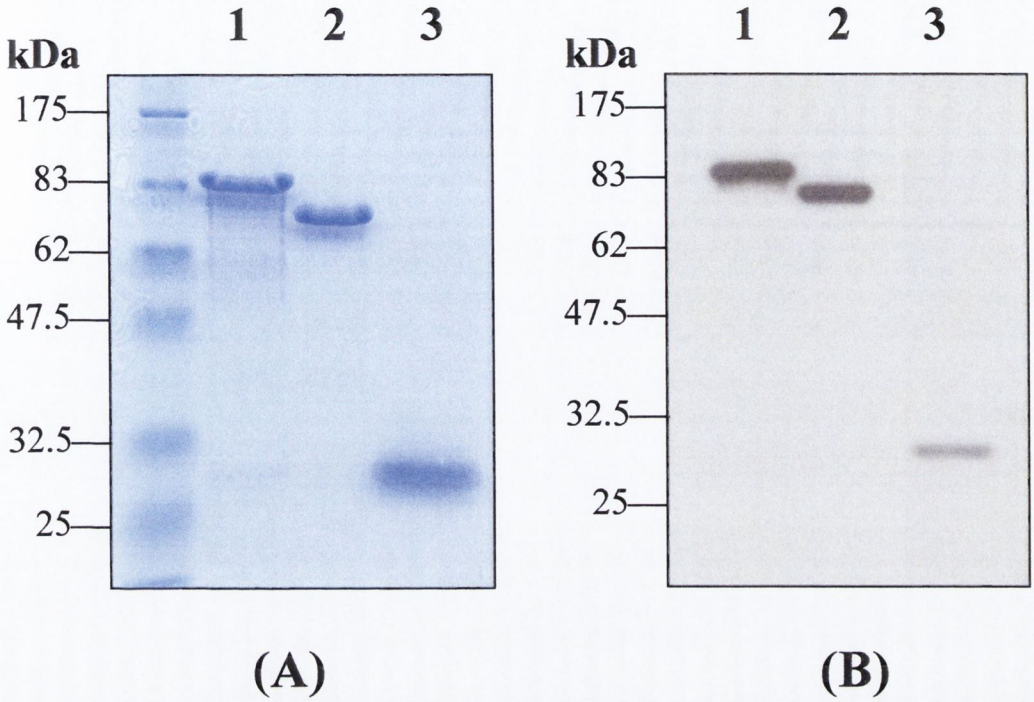


Figure 6.19 (A) 5 μg of purified rFnBPA$_{37-605}$ (lane 1), rFnBPB$_{37-540}$ (lane 2) and rFnBPA$_{745-878}$ (lane 3) resolved by SDS-PAGE and stained with Coomassie blue. (B) Western ligand affinity blotting analysis of rFnBPA$_{37-605}$ (lane 1), rFnBPB$_{37-540}$ (lane 2) and rFnBPA$_{745-878}$ (lane 3) probed with India™ HisProbe.

6.2.10 Competitive inhibition of *S. aureus* binding to immobilised elastin by recombinant FnBP derivatives

To determine the regions of FnBPA and FnBPB that promote bacterial adherence to immobilised elastin, the ability of the recombinant proteins $rFnBPA_{37-605}$, $rFnBPB_{37-540}$ and $rFnBPA_{745-878}$ to inhibit *S. aureus* cells binding to immobilised human aortic elastin was tested.

Human aortic elastin (10 μg/ml or 0.5 μg/well) was coated onto the wells of a microtitre plate. The wells were then blocked with BSA and after washing with PBS, increasing concentrations of $rFnBPA_{37-605}$, $rFnBPB_{37-540}$, $rFnBPA_{745-878}$ and protein A were added. A culture of *S. aureus* strain P1 *fnbA fnbB* ($pFNBA4^+$) was grown to early exponential phase and its ability to adhere to immobilised elastin, which had been preincubated with recombinant proteins, was tested. Pretreatment of the immobilised elastin peptides with the recombinant proteins, $rFnBPA_{37-605}$ and $rFnBPB_{37-540}$, inhibited the binding of strain P1 *fnbA fnbB* ($pFNBA4^+$) to the immobilised ligand in a dose-dependent manner, thus indicating that recombinant region AB of FnBPA and recombinant region A of FnBPB could inhibit FnBPA-mediated bacterial adherence to immobilised elastin. Conversely, protein A and $rFnBPA_{745-878}$ could not inhibit bacterial cell binding (Figure 6.20).

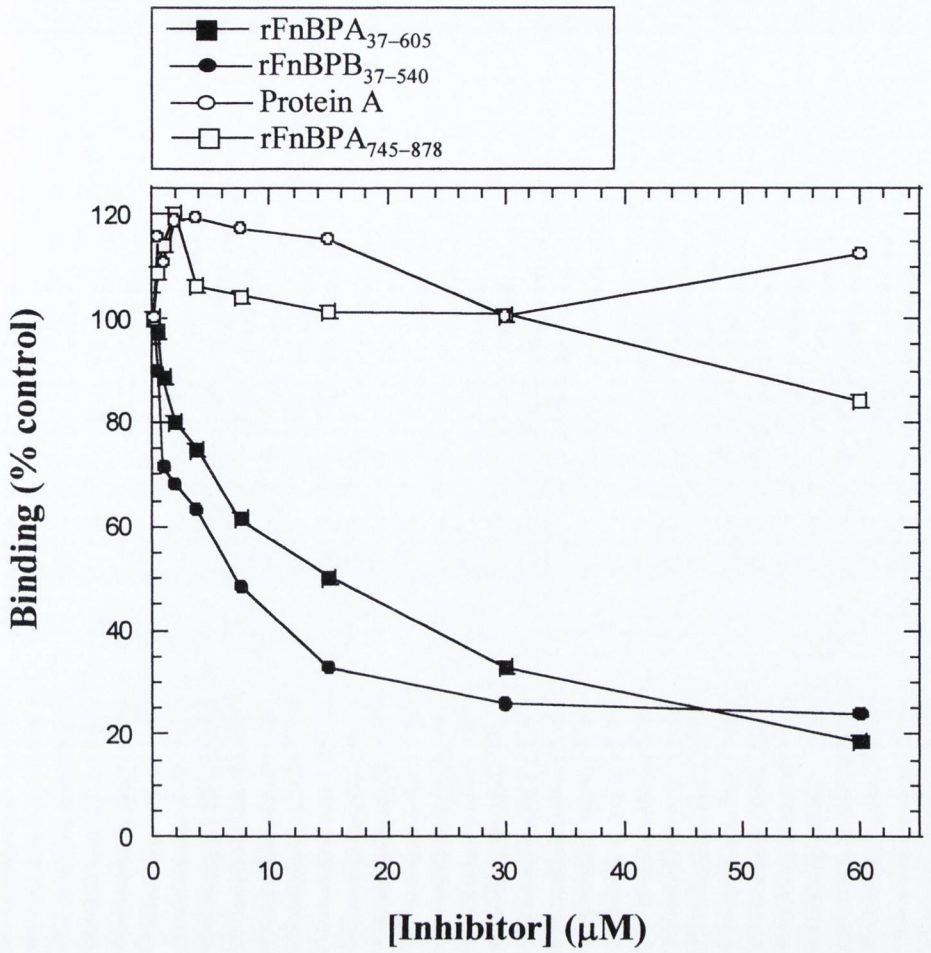


Figure 6.20 Inhibition of *S. aureus* binding to immobilised elastin peptides. 10 μg/ml (0.5 μg/well) of human aortic elastin peptides were coated onto the wells of a microtitre plate. Wells were preincubated with increasing concentrations of $rFnBPA_{37-605}$, $rFnBPB_{37-540}$, $rFnBPA_{745-878}$ and protein A. After incubation the ability of cells from an early exponential phase culture of *S. aureus* strain P1 *fnbA fnbB* ($pFNBA4^+$) to bind to the immobilised elastin was tested. The values are expressed as percentages of a control lacking inhibitor. This figure is representative of the results of three independent experiments performed in duplicate.

6.3 Discussion

An important factor in pathogenesis is the ability of an organism to colonise host tissue. *S. aureus* possesses on its cell surface a number of proteins, collectively called MSCRAMMs, that promote the binding of the organism to components of the host extracellular matrix and play an important role in bacterial virulence. *S. aureus* causes a number of diseases of elastin-rich tissue such as lung and heart valves and for this reason the interaction of the organism with elastin was studied. These studies identified the elastin-binding protein (EbpS) as the surface protein that promotes the adherence of soluble elastin to *S. aureus* cells (Park *et al.*, 1996; Roche, 2000). It was proposed that EbpS could behave like other well characterised matrix-binding proteins of *S. aureus* and promote the adherence of the organism to elastin rich tissue and facilitate bacterial colonisation. An ELISA-based assay was developed to study the adherence of *S. aureus* to immobilised or fixed elastin. This method involves drying elastin peptides onto the wells of a microtitre plate and detecting bound bacteria using a fluorescent nucleic acid probe, SYTO-13 (Roche, 2000). Using this technique it was found that a number of *S. aureus* strains bind in a growth phase-dependent manner to elastin purified from bovine neck ligament but that binding was independent of EbpS since it still occurred with a strain of *S. aureus* defective in EbpS expression (see section 5.2.1). Similarly, EbpS expressed in the heterologous Gram-positive host *L. lactis* could not promote the adherence of this organism to immobilised elastin (see section 5.2.1). Further studies with various *S. aureus* derivatives defective in the expression of different MSCRAMMs found that a *S. aureus* strain that did not express the cell-wall-associated fibronectin-binding proteins, FnBPA and FnBPB, could not adhere to immobilised elastin peptides from bovine *ligamentum nuchae* (Roche, 2000). These data strongly suggested that these proteins are responsible for the adherence of this organism to immobilised elastin peptides and might have a role to play in the colonisation of elastin-rich tissue. This theory was supported by the fact that a number of clinical *S. aureus* isolates from patients with native-valve endocarditis demonstrated, on average, a higher capacity to adhere to immobilised bovine elastin peptides then strains isolated from patients with primary septic arthritis and/or osteomylitis or nasal carriage isolates (Roche, 2000).

In the original studies the wells of the microtitre plates were coated with very high concentrations of elastin peptides to study elastin binding. To ensure that this interaction could be physiologically relevant the ability of *S. aureus* strains to adhere to lower concentrations of elastin purified from bovine neck ligament was tested. Similar to the original results, strains P1 and Cowan adhered strongly to this immobilised material, as did the clinical isolate MSSA. This adherence only occurred with cells from early exponential phase cultures and not with cells from stationary phase cultures of the same strains. This indicated that the surface factor responsible for the adherence of *S. aureus* to immobilised elastin was only expressed during the early exponential phase of growth or was occluded by some other surface factor, such as capsular polysaccharide, during the later phase of growth.

Due to the high elastin binding activity of *S. aureus* strain P1 and the availability of derivatives of the strain defective in the expression of a number of MSCRAMMs it was decided to used this strain in further experiments. It was found that the ability of P1 to adhere to immobilised bovine elastin peptides was lost when the organism did not express the fibronectin-binding proteins, FnBPA and FnBPB (Roche, 2000). To follow Koch's molecular postulates, the mutations in strain P1 *fnbA fnbB* were complemented by the expression of FnBPA or FnBPB from multicopy plasmids. Plasmids pFNBA4 and pFNBB4 express FnBPA and FnBPB, respectively, from strain 8325-4 and have been shown to complement the fibronectin-binding defect of strain 8325-4 *fnbA fnbB* (Greene *et al.*, 1995). These plasmids were shown to complement FnBP expression in strain P1 *fnbA fnbB* by ligand affinity blotting using biotinylated human fibronectin. The FnBP proteins expressed from these plasmids in strain P1 *fnbA fnbB* complemented the fibronectin-binding defect of this strain to levels similar to that of the wild-type strain, P1. The FnBP proteins expressed in strain P1 *fnbA fnbB* also complemented the elastin-binding defect of the strain. While FnBPB expressed from plasmid pFNBB4 almost complemented elastin binding to the same level as strain P1, FnBPA expressed from plasmid pFNBA4 complemented elastin binding to a lower level then the wild-type strain. This difference in the levels of elastin binding exhibited by the proteins expressed in P1 *fnbA fnbB* could be a result of different expression levels, differences in protein

stability or different affinities for the immobilised ligand. Similar results were observed when the FnBP proteins were over expressed in strain 8325-4 *fnbA fnbB*.

These data indicate that the fibronectin-binding proteins of *S. aureus* are the proteins responsible for bacterial adherence to immobilised bovine elastin peptides by cells from the early exponential phase of growth. This finding is in agreement with the temporal expression of the fibronectin-binding proteins in *S. aureus*. Transcription of the *fnb* genes is restricted to the early exponential phase of growth (Greene *et al.*, 1995; Wolz *et al.*, 2000) and the fibronectin-binding ability of the FnBP proteins is reduced as cultures enter stationary phase. This is thought to be a result of the degradation of the proteins by *S. aureus* proteases (McGavin *et al.*, 1997) and masking of the proteins by capsular polysaccharide (Pöhlmann-Dietze *et al.*, 2000). The differences in elastin-binding patterns between strains may be attributed to differences in the levels of FnBP expression. Strains P1 and Cowan express high levels of FnBPs (Sinha *et al.*, 1999) and promote strong binding to immobilised fibronectin and elastin. Strain 8325-4 has been shown to express low levels of FnBPs compared to other strains as determined by Western ligand affinity blotting (Bisognano *et al.*, 1997) and binds weakly to immobilised elastin peptides. Strain Newman expresses similar levels of FnBPs to strain Cowan but it does not bind well to immobilised fibronectin (Lindberg *et al.*, 1990; Vaudaux *et al.*, 1998) or elastin, which suggests that some other factor, such as the presence of a capsule, may be masking the proteins. The levels of FnBP expression of the clinical strains MSSA, EMRSA-16 and COL were not investigated.

Further evidence for the role of the fibronectin-binding proteins in elastin binding was supplied by an agglutination assay. When *S. aureus* cells were grown in the presence of elastin peptides from bovine neck ligament they formed macroscopic clumps in an FnBP-dependent manner as cultures entered exponential phase. This suggests that the FnBPs on the surface of the different cells bound the same elastin peptide, thus allowing the elastin peptides to act as cross-bridges between cells and forming a large macroscopic lattice. Previously it was shown that the dominant binding factor of *S. aureus* for soluble elastin was EbpS because an *ebpS* deficient strain bound 72% less soluble elastin than its parental strain (Roche, 2000). These experiments were performed with cells from stationary phase cultures. Therefore only small amounts of the FnBPs were present on the

surface of the cells. Perhaps the FnBPs could account for the remaining 28% of residual binding of soluble elastin seen in *ebpS* mutants.

The FnBPs from strain P1 were expressed in the heterologous Gram-positive host, *L. lactis*. This approach has been used to study the interactions of a number of staphylococcal MSCRAMMs with mammalian proteins such as fibrinogen, fibronectin and von Willebrand factor and to examine the processes of *S. aureus* invasion of mammalian cells (Massey *et al.*, 2001) and platelet activation (O'Brien *et al.*, 2002a). The FnBPs expressed in *L. lactis* promoted adherence of this organism to immobilised human fibronectin to levels similar to those of *S. aureus* strain P1. However, the FnBPs did not promote the adherence of *L. lactis* to immobilised elastin peptides from bovine neck ligament. Western immunoblotting with anti-FnBPA region AB antibodies and Western ligand affinity blotting with biotinylated human fibronectin suggested that the FnBPs were expressed poorly by *L. lactis*. The FnBPs are known to be susceptible to proteases (McGavin *et al.*, 1997) and perhaps *L. lactis* expresses a protease with specificity for the FnBPs that cleaves off the A regions but leaves the D repeats intact on the cell surface. This would account for the ability of *L. lactis* strains expressing FnBPA and FnBPB to adhere to immobilised fibronectin but not to immobilised elastin. Other possibilities are that the FnBPs are poorly expressed or poorly sorted to the cell wall peptidoglycan of this organism, or that degradation of the proteins occurred during solubilisation. The end result is that the expression of the FnBPs in *L. lactis* is not a good system for the study of FnBP-mediated elastin binding. However, these data did suggest that the regions of the FnBPs responsible for the elastin-binding phenotype were distinct from the regions responsible for fibronectin binding.

If the fibronectin-binding proteins are responsible for the colonisation of elastin-rich tissue in the human they must bind to elastin prepared from human tissue and not just to elastin prepared from bovine neck ligament. The ability of *S. aureus* strain P1 to adhere to immobilised elastin peptides prepared from human aorta and human lung was compared to its ability to bind to elastin peptides from bovine *ligamentum nuchae*. Strain P1 bound to all three forms of elastin in a dose-dependent manner and appeared to have greatest affinity for elastin prepared from human aorta. This suggests that the FnBPs may

have evolved to bind most avidly to human elastins. As with bovine elastin binding the adherence to human elastins was FnBP-dependent.

The identification of the FnBPs as the proteins responsible for the binding of cells to immobilised elastin peptides raised the question of the purity of the elastin preparations used in adherence assays. The elastin peptides were prepared from animal sources and could be contaminated with other ligands of the FnBPs such as fibronectin and fibrinogen. The preparation of elastin peptides from bovine neck ligament involves boiling the tissue in sodium hydroxide and hydrolysing the elastin in weak basic conditions. Finally, only high molecular weight proteins are retained after a chromatography step (Mecham and Lange, 1982). Human aortic elastin was prepared by the method of Partridge (1962) whereby elastin peptides were purified from tissue by successive treatments with hot oxalic acid. The human lung elastin peptides were prepared by the rigorous method of Starcher and Galione (1976) whereby elastin peptides were purified from finely divided tissue treated with saline-EDTA, formic acid, urea and digested with trypsin. The FnBP binding sites at the N terminus of fibronectin and the C-terminal residues of the γ-chain of fibrinogen are unlikely to tolerate such harsh treatments. Even if fragments of fibronectin of fibrinogen survived these treatments they would be lost at the chromatography step in the case of bovine elastin peptides. The lack of fibrinogen or fibronectin contamination in any of the commercially available elastin preparations was confirmed by Western immunoblotting using polyclonal antibodies to human fibronectin and human fibrinogen. This was also confirmed by the ability of *L. lactis* strains expressing FnBPs to adhere to immobilised fibronectin but not to immobilised elastin. If the FnBPs were adhering to contaminating fibronectin in the elastin preparation then the *L. lactis* strains would adhere to this material, but no binding was observed.

The interactions of *S. aureus* with mammalian extracellular matrix proteins such as fibrinogen, fibronectin and von Willebrand factor has been studied by immobilising these proteins onto inert surfaces and testing the binding ability of bacterial cells (Green *et al.*, 1995;Wolz *et al.*, 1996; Ní Eidhin *et al.*, 1998). These ligands have been shown to spontaneously coat the wells of a microtitre plate when incubated at 4°C for 18 hours. However, attempts to immobilise elastin peptides in this way failed. To coat the wells of

microtitre plates with elastin peptides the material was dried onto the plastic using UV light. This method has been used to study the affects of immobilised elastin peptides on the elastin receptors of mammalian cells (Hinek *et al.*, 1999). Drying of a protein under UV light is a very harsh treatment. To ensure that this dried protein still retained the epitopes specific to elastin the ability of anti-human aortic elastin antibodies to recognise immobilised human aortic elastin was assessed in an ELISA-based assay. These antibodies were raised in rabbits immunized with the soluble form of the protein and were shown to bind in a dose-dependent manner to the immobilised antigen. This suggests that the overall structure of immobilised elastin peptides is not grossly affected by drying under UV light and the protein still retains the major epitopes of the soluble form of the antigen. These data, in combination with the confirmation of the purity of the commercially available elastin preparations, indicate that the elastin adherence assay used in these studies is an effective and reliable method of studying the interactions of *S. aureus* with mammalian elastin. This is supported by the fact that *S. aureus* promotes cell aggregation when grown in the presence of soluble elastin peptides in an FnBP-dependent manner.

To identify the regions of the fibronectin-binding proteins responsible for the binding of bacterial cells to elastin, inhibition studies were performed using antibodies, soluble fibronectin and recombinant proteins. The regions of the fibronectin binding proteins responsible for fibronectin and fibrinogen binding have been identified. Fibrinogen binding is promoted by region A of FnBPA (Wann *et al.*, 2000) and region A of FnBPB (Prof. M. Höök, personal communocation). Fibronectin binding is primarily promoted by the D repeats of each protein (McGavin *et al.*, 1993a; Patti *et al.*, 1995) but also by the B repeats and Du region of FnBPA (Joh *et al.*, 1998; Massey *et al.*, 2001). Antibodies raised against rFnBPA$_{37-605}$ (regions A and B of FnBPA) preincubated with *S. aureus* cells expressing FnBPA from plasmid pFNBA4 inhibited cell binding to immobilised human aortic elastin peptides. Conversely, the preincubation of cells from the same culture with soluble fibronectin inhibited binding to immobilised fibronectin but not to immobilised elastin peptides. Similarly, the inability of fibronectin bound to FnBPA on the surface of *S. aureus* to inhibit elastin binding suggests that neither the D repeats, the Du region or the B repeats of FnBPA are involved in elastin binding. The

ability of recombinant truncates of FnBPA and FnBPB to inhibit the elastin-binding function of FnBPA expressed on the surface of cells of strain P1 *fnbA fnbB* (pFNBA4$^+$) was assessed. Preincubation of elastin-coated wells with recombinant region AB of FnBPA (rFnBPA$_{37-605}$) or region A of FnBPB (rFnBPB$_{37-540}$) inhibited FnBPA mediated bacterial cell adherence in a dose-dependent manner, thus indicating that the A regions are most likely responsible for elastin binding. It is interesting to note that recombinant region of A of FnBPB inhibited FnBPA-mediated bacterial adherence. This suggests that FnBPA and FnBPB bind to the same region or very close regions on the elastin molecule. EbpS promotes adherence to the N terminus of soluble tropoelastin (Park *et al.*, 1991). It will be interesting to identify the region of elastin to which the FnBPs bind and to determine if they compete with EbpS for a binding site.

This is the first report of proteins on the surface of *S. aureus* promoting adherence to immobilised elastin. This interaction may have a role to play in staphylococcal diseases of elastin-rich tissue such as heart valves and lung. The role of the fibronectin-binding proteins in infective endocarditis has been studied in animal models. Immunization with a recombinant fibronectin-binding protein resulted in partial protection in a rat endocarditis model (Schennings *et al.*, 1993). Later studies revealed that a *S. aureus* mutant defective in the expression of both the fibronectin-binding proteins could cause endocarditis in an animal model (Flock *et al.*, 1996), raising questions about the role of these MSCRAMMs in causing this disease. However, recent studies have shown that FnBPA expressed on the surface of *L. lactis* is sufficient to produce endocarditis in rats with catheter induced aortic vegetations (Que *et al.*, 2001). *S. aureus* expresses a number of surface proteins that bind to mammalian extracellular matrix proteins such as fibrinogen, fibronectin, collagen and von Willebrand factor that may act as bridging molecules for the organism to adhere to the vascular vegetation in an endocarditis infection. These surface proteins may also aid bacterial persistence at the site of infection and promote the shedding of bacteria into the blood stream resulting in secondary infections (Ing *et al.*, 1997). It has been suggested that the *S. aureus* collagen adhesin, Cna, may play a role in bacterial persistence within a vegetative lesion (Hienz *et al.*, 1996). Perhaps the fibronectin-binding proteins could perform a similar function by binding to the exposed elastin fibers of the damaged heart valve.

Recent studies have shown that the fibronectin-binding proteins are virulence factors in staphylococcal pneumonia. The proteins were found to promote bacterial adherence to human airway epithlium and strains of *S. aureus* isolated from cystic fibrosis patients and patients with nosocomial pneumonia exhibited elevated levels of FnBP expression and fibronectin binding (Mongodin *et al.*, 2002). Perhaps FnBP mediated elastin binding is involved in this phenomenon.

Chapter 7

Discussion

The elastin binding protein EbpS of *S. aureus* strain Cowan was originally reported to be a surface-associated, lysostaphin-releasable protein of 25 kDa comprising 202 residues, and classified as a staphylococcal MSCRAMM (Park *et al.*, 1991; Park *et al.*, 1996). Since then it has been demonstrated that the *ebpS orf* of strain Cowan in fact comprises 1461 bp and encodes an EbpS protein of 486 residues (Roche, 2000). The EbpS protein of 8325-4 is the same size and is 95% identical (Roche, 2000). It is now clear that an error occurred in the original DNA sequence of the Cowan *ebpS* gene.

Analysis of the cell envelope of $EbpS^+$ *S. aureus* indicated that EbpS is strongly associated with the cytoplasmic membrane. When stable protoplasts of *S. aureus* were formed by lysostaphin digestion of peptidoglycan, the EbpS protein remained firmly associated with the membrane fraction, whereas ClfA, a cell-wall-associated protein anchored to peptidoglycan by the LPDTG motif and sortase, was completely solubilised. It could be argued that EbpS was originally in the wall fraction but became associated with the membrane during protoplast formation. This is unlikely because EbpS was also detected in a membrane fraction prepared by mechanical disintegration and differential centrifugation.

Examination of the amino acid sequence and analysis by secondary structure prediction algorithms suggested three hydrophobic domains in EbpS, two of which were predicted to span the cytoplasmic membrane. The N-terminus of the protein must be located on the extracellular face of the membrane because (i) residues 14-34 comprise the ligand-binding domain, (ii) intact bacterial cells bind to elastin via the N-terminal domain and (iii) antibodies to the N-terminal recombinant protein rEbpS1-267 bound to intact *S. aureus* cells.

EbpS was shown to be an integral membrane protein with two transmembrane domains by analyzing EbpS-PhoA and EbpS-LacZ expressed in *E. coli*. Initially the wild-type EbpS protein was expressed from the ATG vector pKK233-2. Cell fractionation experiments showed that EbpS was associated with the cytoplasmic membrane of *E. coli*. Expression of alkaline phosphatase activity and β-galactosidase activity by the fusions indicated the N-terminal domain lying proximal to the hydrophobic region and the C-terminal domain located distal to the hydrophobic region are exposed on the outer face of the cytoplasmic membrane. However, only the N-terminus is surface-exposed as

indicated by whole cell immunoblots performed with anti-rEbpS$_{1-267}$ and anti-rEbpS$_{343-486}$ antibodies where a positive reaction was only obtained with the former.

To be classified as a staphylococcal MSCRAMM a protein must promote bacterial adherence to the pure ligand immobilised onto an inert surface and also bind to the soluble ligand. Data from previous studies and from this study has indicated that *S. aureus* cells adhere to immobilised elastin, but in an EbpS-independent manner. Therefore, although EbpS binds to soluble elastin, it cannot be classified as a true MSCRAMM.

Comparison of 8325-4-EbpS$^+$ and 8325-4-EbpS$^-$ strains grown in mixed culture and independently in a chemically defined medium indicated that strains not expressing EbpS had a growth defect. This growth defect was found to be independent of the elastin-binding domain and to be dependent on the presence of the LysM motif at the C terminus of the protein. This suggests that EbpS could be involved in a cellular process that is required for optimum growth, such as signal transduction, nutrient uptake or cell wall turnover. Whatever the function of EbpS, it has been eliminated as a staphylococcal MSCRAMM.

Since the original interest in EbpS stemmed from the possibility that it could promote bacterial adherence to elastin and therefore might be a virulence factor in diseases of elastin-rich tissue, focus shifted to the identification and characterisation of the protein(s) responsible for bacterial adherence to the immobilised ligand. The fibronectin-binding proteins, FnBPA and FnBPA, were identified as the surface proteins responsible for bacterial adherence to immobilised elastin peptides using *S. aureus* FnBP deficient mutants and complementation analysis. The fibronectin-binding proteins are multifunction proteins that bind to fibronectin and fibrinogen, and promote bacterial internalization by endothelial and epithelial cells. The interaction between FnBPA and elastin was found to occur in the AB region of FnBPA using antibody inhibition and recombinant proteins. The role of the fibronectin-binding proteins in bacterial pathogenesis is well documented, particularly in infective endocarditis and staphylococcal pneumonia. Perhaps the interaction between the FnBPs and elastin promotes bacterial adherence to deep tissue and persistence at the site of infection. The ability of *S. aureus* to adhere to elastin-rich tissue, such as tendon, in an FnBP-dependent

manner could be tested. The tissue would be treated with a protease, such as trypsin, that would degrade contaminating fibronectin and fibrinogen but would leave the protease-resistant elastin intact.

The human tropoelastin gene has been cloned and the protein has been expressed in *E. coli*. The ability of *S. aureus* to adhere to immobilised tropoelastin could be tested in an ELISA-based assay. Truncates of the tropoelastin molecule could then be assessed for their ability to inhibit this interaction and the region of the molecule to which the FnBPs bind could be determined. It would be interesting to ascertain if the FnBPs compete for the EbpS binding site on tropoelastin (exons 9 and 10).

Similar to the approach used to determine the residues responsible for the binding of ClfA to fibrinogen, an alanine scan site-directed mutagenesis approach could be used to determine the residues in region A of FnBPA and FnBPB responsible for binding to elastin. Recombinant mutated forms of the proteins could be expressed and purified from *E. coli* and their ability to bind to immobilised elastin tested in an ELISA-based assay. Mutated forms of the FnBPs could be expressed on the surface of *S. aureus* and their ability to promote bacterial adherence tested. Many of these questions are to be answered in the near future.

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