Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Characterisation of the Elastin Binding Protein (EbpS) of *Staphylococcus aureus*

A thesis submitted for the degree of Doctor of Philosophy

by

Fiona M. Roche

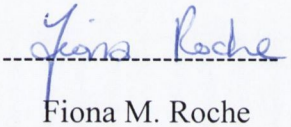
Department of Microbiology

Trinity College, Dublin

October 2000

Declaration

I hereby declare that this thesis represents my own work, except where acknowledged in the text and that it has not previously been submitted for a degree at this or any other university. I declare that this thesis may be borrowed or copied on request at the library of Trinity College Dublin.



Fiona M. Roche

Acknowledgements

At last the moment I've been waiting for....feet up, a cup of coffee and now to ponder on the "thank you's". Firstly I would like to thank my supervisor, Professor Tim Foster for his constant guidance and support over the last three years. I really appreciate it! I would also like to thank all those Fosterettes past and present for their constant advice, good humour and friendship. What a team! A special thanks to my bay partner Evelyn who would drop anything instantly to solve a problem and of course to my EbpS partner, Rob (a.k.a. Jim Corr), for his eternal optimism. Thanks to all my drinking buddies in the Moyne and to everyone in the prep room for providing clean glassware, media, technical support and great chats!

A big thanks to Robert P. Mecham and Pyong Woo Park who opened up the doors to EbpS in the first place. Thanks for allowing me to work in your lab for 10 fabulous summer days and for putting me up in the exclusive Sheridan Hotel in sunny St. Louis. I'll be back! I'm also grateful to Dr. Sharon Peacock for providing a wide range of clinical isolates and to Mark Smeltzer for his willingness to further investigate the *sar* regulation story.

I would like to express my warmest thanks to my parents and family for their interest and continuous encouragement throughout the project. Thanks to all my friends particularly Kate, Sharon and Mary for all the laughs and for always keeping my spirits high.

Finally and most importantly I would like to offer my warmest thanks to my dearest Karsten. Vielen Dank mein lieber für all Deine Hilfe. Liebe ist die Quelle der Kraft!

Summary

Staphylococcus aureus expresses an array of surface proteins that promote interaction of the bacterium with the host. Some surface proteins promote binding to components of the host extracellular matrix and can act as adhesins. Others bind proteins in plasma and may interfere with host defenses. Recently it has become apparent that some surface proteins can bind two or possibly more ligands. This thesis describes an unusual membrane associated surface protein of *S.aureus* that promotes interactions with elastin.

Park *et al.* (1991) first reported that *S.aureus* strain Cowan could bind elastin, a major structural component of elastic tissue. They subsequently reported that elastin binding was mediated by a 25 kDa cell wall-associated elastin binding protein (EbpS) encoded by a 609 bp *ebpS* gene (Park *et al.*, 1996). This thesis reports a detailed molecular analysis of EbpS. The *ebpS* gene was cloned from genomic DNA of strain 8325-4. DNA sequencing revealed an open reading frame of 1461 bp encoding a protein of 486 residues. The *ebpS* gene of strain Cowan was also sequenced and was shown to comprise 1461 bp. It was concluded that a frameshift sequence error occurred in the original publication of the *ebpS* sequence (Park *et al.*, 1996).

Analysis of the extended EbpS open reading frame indicated that EbpS had three hydrophobic domains in the middle of the primary sequence with potential to span the cytoplasmic membrane. EbpS has recently been shown to be associated with the cytoplasmic membrane (R.Downer, personal communication). The C-terminus of the protein does not bear the features of the family of surface proteins that are covalently anchored to peptidoglycan via an LPXTG motif. Instead the C-terminus has a LysM domain found in a variety of bacterial proteins that interact with peptidoglycan. It is conceivable that the LysM domain of EbpS is also located within the cell wall peptidoglycan. The ligand-binding domain is located at the N-terminus and is surface exposed.

A site-specific mutation in the *ebpS* gene was isolated by allelic replacement. The *ebpS*::*erm* mutation was constructed *in vitro* in a temperature sensitive shuttle plasmid. It was transferred into *S.aureus* and allowed to recombine with the chromosomal *ebpS* locus. The *ebpS*::*erm* mutation was subsequently transduced into

several other *S.aureus* strains. In addition, the wild type 8325-4 *ebpS* gene was cloned into the shuttle plasmid pCU1 and transferred into *ebpS* mutants to allow complementation. The 5' part of *ebpS* encoding residues 1-267 of the EbpS protein was cloned into an expression vector in *E.coli* which provided six histidine residues at the N-terminus. The recombinant protein was expressed and purified by Ni^{2+}-chelate chromatography. The purified protein was used to immunise rabbits to obtain polyclonal anti-EbpS antibodies. Antibodies recognising the C-terminal residues 343-486 were also available. Western immunoblotting analysis of proteins released from lysed protoplasts of wild type *S.aureus*, the *ebpS* mutant and the *ebpS* mutant complemented with pCU-*ebpS*$^{+}$ allowed identification of the *ebpS* gene product as a protein of 83 kDa. The 25 kDa protein described by Park *et al.* (1996) is thus an N-terminal truncate.

Western immunoblotting analysis of cells obtained from the exponential and stationary phases of growth indicated that EbpS is present at all stages of the growth cycle. A transcriptional fusion between the *ebpS* promoter and the *lacZ* gene of *E.coli* was constructed in the plasmid vector pAZ106. When this plasmid integrated into the *ebpS* locus of *S.aureus* by a single cross-over recombination event it enabled a transcriptional *ebpS-lacZ* fusion. Transcription was shown to occur in the exponential phase and to cease in stationary phase cells. The *ebpS-lacZ* fusion was transduced into strains with mutations in global regulators *sar*, *agr* and *sigB*. Only the *sar* mutation had a measurable effect on *ebpS* transcription where the level of β-galactosidase activity was reduced by three to four-fold following aerobic growth. The effect of *sar* on *ebpS* transcription was also reflected in the level of EbpS protein detected in the envelope of the *sar* mutant. A further reduction in *ebpS* expression in the *sar* mutant was observed following growth under limited oxygen conditions.

Radioiodinated tropoelastin (a soluble precursor of elastin) was used in binding assays with a wild type strain, an *ebpS* mutant and a complemented mutant. These experiments confirmed that EbpS is the predominant receptor for soluble tropoelastin. This was confirmed when binding was eliminated with excess unlabelled elastin peptides (soluble degradation products of elastin). Attempts were made to determine if EbpS is an adhesin that can promote bacterial attachment to immobilised elastin peptides. Strain Newman and 8325-4 did not adhere to immobilised elastin peptides. In contrast strain P1, Cowan and several clinical

isolates did but adherence was not dependent on EbpS. Thus EbpS can only interact with soluble elastin. The adherence of strain P1 to immobilised elastin peptides is growth phase dependent occurring only between early and mid exponential phase of growth. Preliminary experiments indicate that adherence of P1 to immobilised elastin peptides is mediated by the fibronectin binding proteins (FnBPs). The ability of the FnBPs to interact with elastin is currently being investigated by an independent method to ensure this interaction is specific.

Contents

List of Tables

List of Figures

Appendix

Single letter amino acid code

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Bases

A	adenine
T	thymine
C	cytosine
G	guanine

Antibiotics

Amp	ampicillin
Cm	chloramphenicol
Em	erythromycin
Kan	kanamycin

Key to abbreviations

aa	amino acid residues
agr	accessory gene regulator
bp	base pair(s)
BSA	bovine serum albumin
CFU	colony forming unit
ClfA	clumping factor A
ClfB	clumping factor B
Cna	collagen adhesin
CP	capsular polysaccharide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DIG	digoxygenin
dNTP	deoxy-nucleoside triphosphate
EbpS	Elastin binding protein
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ET	exfoliative toxin
Fc	crystallisable fragment of IgG
FnBP	fibronectin binding protein
h	hour
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-β-D-thio-galactoside
kb	kilobase pair
kDa	kilodalton
LB	Luria broth
MAP	MHC class II analogous protein

Key to abbreviations

nt	nucleotide(s)
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocytes
PVL	Panton Valentine leucocidin
rpm	revolutions per minute
sar	staphylococcal accessory regulator
sarH1	staphylococcal accessory regulator homolog 1
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEs	staphylococcal entertoxins
sigB	sigma factor B
SSS	scalded skin syndrome
TBS	tris-buffered saline
TNF	tumour necrosis factor
Tris	trishydroxymethylaminomethane
Ts	temperature sensitive
TSA	trypticase soy agar
TSB	trypticase soy broth
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin 1
v/v	volume per volume
w/v	weight per volume
wt	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D galactopyranoside

A few observations and much reasoning lead to error; many observations and a little reasoning to truth.

Alexis Carrel, Nobel laureate of Medicine, 1912

FOR MY PARENTS

Chapter 1

Introduction

1.1 The biology of staphylococci

The genus *Staphylococcus* consists of gram-positive, clump-forming, facultative aerobic cocci (0.5-1.5μm in diameter) that characteristically divide to form grape-like clusters. Bacterial taxonomy joins Gram-positive catalase-positive cocci such as staphylococci and micrococci in the family *Micrococcaceae*, although the two genera exhibit several different molecular features (*e.g.* cell wall composition, sensitivity to lysostaphin and GC content). Most strains of staphylococci are catalase positive and oxidase negative. They are salt-tolerant with most species capable of growing in the presence of 1.7M (10% w/v) NaCl. The temperature range for growth is 10°– 45°C. DNA-DNA hybridisation and later 16S and 23S rRNA sequence analysis has allowed the construction of a phylogenetic tree of the genus *Staphylococcus* (Kloos, 1997). There are at least 26 species of staphylococci, 13 of which are associated with humans. The ability to express coagulase has enabled the subdivision of staphylococcal species into two major groups: the coagulase positive and coagulase negative staphylococci. *Staphylococcus aureus* (*S.aureus*) is coagulase positive and is by far the most studied due to its prevalence as a human and animal pathogen. *S.aureus* can be easily identified by its ability to express extracellular coagulase, clumping factor, (a cell wall associated fibrinogen binding protein (ClfA)), and DNase.

Staphylococci are most frequently found inhabiting the skin, skin glands, and mucous membranes of mammals. Ecological studies have revealed that some species of staphylococci have habitat preferences. For example, *S.epidermidis* is found widely distributed on human skin whereas *S.aureus* and *S.auricularis* are predominantly found in the anterior nares and auditory canals, respectively (Kloos, 1997). Approximately 20% of the human population is colonised by *S.aureus*, and 30-50% may have transient colonisation (short term contamination) (Kauffman and Bradley, 1997; Kluytmans *et al.*, 1997). *S.aureus* is an opportunistic pathogen. If the natural barriers are damaged *S.aureus* can infect the host tissue and cause localised and invasive infections.

1.2 Staphylococcal diseases

1.2.1 Skin Infections

Approximately 50% of all skin infections are caused by *S.aureus* (Tenover and Gaynes, 2000). Minor skin infections include folliculitis, furnucles, carbuncles and cellulitis. Impetigo initates as a minor skin infection but can develop into a more serious form of the disease, described as bullous impetigo characterised by small blisters that continually break and become infected. Impetigo is predominantly a disease associated with children and HIV patients (Noble, 1997). Scalded skin syndrome begins with generalised erythema, followed by loosening of the epidermis associated with the formation of sterile bullae which cause the epidermis to split. The exfoliative toxins A and B, (ETA and ETB) have been implicated as virulence factors in this disease (Noble, 1997). Patients with eczema often suffer a secondary infection due to heavy colonisation and subsequent infection of the lesions by *S.aureus* (Bibel *et al.*, 1977).

1.2.2 Bacteraemia

Bacteraemia is the growth of bacteria in the bloodstream. It is accompanied by fever and malaise but can lead to septicaemia and hence more severe symptoms such as organ failure and septic shock. A persistent bactereamia is due to continuous seeding of bacteria into the bloodstream from an infected heart valve or prosthetic device. Coagulase negative staphylococci (CoNS) and *S.aureus* are the most common causes of bacteremia in the community and hospital environment (Kauffman and Bradley, 1997; Náwas *et al.*, 1998). The most important species of CoNS associated with bacteremia is *S.epidermidis*. Most patients with bacteremia have predisposing underlying conditions such as diabetes, renal failure (hemodialysis patients), being immunocompromised, or HIV infection (Lautenschlager *et al.*, 1993; Jacobson *et al.*, 1988). The risk factors associated with these conditions include trauma, altered host defenses, skin disease and intravenous drug abuse (IVDA). However, the most important risk factor for *S.aureus* and CoNS bacteremia is the use of indwelling lines or intravascular catheters. Upon insertion, catheters quickly become coated with plasma proteins. *S.aureus* colonisation of catheters is thought to occur through bacterial adherence to host proteins (Vaudaux *et al.*, 1989). Several *in vitro* studies have shown that attachment of *S.aureus* to polymeric surfaces is

promoted by immobilised fibrinogen, fibronectin, laminin, collagen, thromospondin, and vitronectin (Vaudaux *et al.*, 1995). Using an *ex vivo* arteriovenous shunt model, Vaudaux *et al.* (1995) demonstrated that fibrinogen was the major plasma protein that mediated *S.aureus* adherence to the experimental shunt. *S.epidermidis* has not been shown to bind avidly to host matrix proteins. It has been proposed that direct contamination of the catheter from the outside by *S.epidermidis* does not involve binding to blood-conditioned biomaterial, rather binding directly to plastic, forming a biofilm and growing along the catheter into the vein (Rupp, 1997). Infection predominantly occurs at the site of exit of the catheter (46%) and can remain localised or become systemic (Ing *et al.* 1997). Approximately 50% of all hospitalised patients require intravascular catheters therefore extensive research into the use of alternative materials to reduce host protein deposition on the catheter material is being carried out (Mermel *et al.*, 1993).

1.2.3 Endocarditis

S.aureus continues to be a major pathogen causing infective endocarditis (IE) with most cases being associated with community acquired *S.aureus* bacteremia. There are three types of *S.aureus* IE which include right-sided endocarditis (mostly associated with intravenous drug users), left-sided endocarditis (also known as native-valve endocarditis) and prosthetic valve endocarditis (PVE). Right-sided endocarditis is less severe but more common whereas left-sided endocarditis results in severe medical complications including heart failure (51%) (Tenover and Gaynes, 2000). *S.aureus* is a successful pathogen at inducing IE as it is equipped with many virulence factors enabling initial adherence to the vascular vegetation as well as persistence within the vegetation. Recent data suggests that *S.aureus* is able to exploit the presence of many host proteins (fibrinogen, fibronectin, collagen and von Willebrand factor) which serve as bridging ligands for *S.aureus* to adhere to and persist within the vegetation site (Ing *et al.*, 1997). Once bound, the bacteria become phagocytosed by endothelial cells and appear to follow one of two paths. A shift in phenotype to become small colony variants allows the bacteria to persist within the endothelial cell and offers protection from host defenses (e.g. polymorphonuclear leukocytes and complement) and antimicrobial agents (e.g. aminoglycosides and β-

lactams). The alternative route is invasion of the endothelium and its destruction primarily caused by expressing the cytolytic α-toxin (Ing *et al.*, 1997).

1.2.4 Central nervous system infections

Staphylococci are important causative organisms of several central nervous system infections such as cerebrospinal fluid (CSF) shunt infections, meningitis, and brain abscess. CoNS are primarily associated with CSF shunt infection (58%) followed to a lesser extent by *S.aureus* (20%). The pathogenesis of CoNS (in particular, *S.epidermidis*) in this infection is mostly due to the ability of the bacteria to adhere to and proliferate on catheter surfaces, and produce slime for protection from host defenses and antimicrobial agents. Neutrophils are unable to adhere to such surfaces therefore cannot phagocytose bacteria (Roos and Scheld, 1997). Although quite rare, *S.aureus* is the causative agent in most cases of staphylococcal meningitis. Infection usually develops from bacteremia, endocarditis or following neurosurgery.

1.2.5 The Eye

An intact cornea is quite resistent to most microbial contamination as an epithelial layer and a precorneal tear film containing lysozyme, antibodies and inflammatory cells protect it. However, predisposing factors such as tissue damage or contact lens increase vunerability to infection. *S.aureus* is accountable for many types of human ocular infections including conjunctivitis, keratitis and endophthalmitis. Keratitis commonly follows exposure to contaminated contact lenses. A recent report by Rhem *et al.* (2000) studied the role of the collagen binding adhesin (Cna) in promoting bacterial adherence to the corneal surface. *S.aureus* strains defective in *cna* were less virulent in a rabbit model of soft contact lens-associated bacterial keratitis compared to the parental and complemented strain. O'Calleghan *et al.* (1997) used purified protein and isogenic mutants to demonstrate that α-toxin and to a lesser extent β-toxin also play a role as virulence factors in this disease. The γ-toxin has been shown to contribute to experimental endophthalmitis (Supersac *et al.*, 1997).

1.2.6 Bone and joint infections

S.aureus accounts for 50 –70 % of all osteomyelitis cases and up to 60% of non-gonococcal bacterial arthritis cases (Gentry, 1997; Bremell *et al.*, 1991). Bacteria can spread to the bone hematogenously (by the bloodstream) from adjacent sites of infection (*i.e.* wound sepsis) or alternatively may gain direct access to the bone following a surgical procedure. Certain medical conditions can predispose to a staphylococcal bone infection such as immunosuppressive therapy, hemodialysis or diabetes. The development of experimental animal models has enabled the identification of virulence factors expressed by *S.aureus* which contribute to the development of the disease (Bremell *et al.*, 1991; Bremell *et al.*, 1994). The ability of the bacteria to adhere to matrix proteins rich in the bone and joint such as collagen, bone sialoprotein, osteopontin and fibronectin has been related to the ability to colonise at these sites (Hudson *et al.*, 1999). Furthermore, the expression of enterotoxins (A-D), toxic shock syndrome toxin-1 (TSST-1) and the simultaneous production of alpha and gamma toxins have been demonstrated as virulence factors in the development of *S.aureus* arthritis (Bremell and Tarkowski, 1995; Nilsson *et al.*, 1999).

1.2.7 Respiratory tract infections

S.aureus is a cause of several respiratory diseases such as sinusitis, bronchitis and pneumonia. Due to its colonisation of the nares, bacteria can transfer onto the skin and enter the bloodstream through a wound and then invade the lung or enter the respiratory tract directly through the nose. While *S.aureus* is not commonly found in community acquired pneumonia it is a common causative agent of hospital acquired pneumonia and as a secondary infection to influenza (20-30% of cases) (Tenover and Gaynes, 2000).

1.2.8 Toxic shock syndrome

Staphylococcal toxic shock syndrome (TSS) is a disease which became apparent in the 1980s because its associated with tampon use in menstruating women. Now it is clear that other sources of infection including skin lesions, abscesses, pneumonia and osteomyelitis can lead to TSS (Crass and Bergdoll, 1986). Expression of TSST-1 toxin (expressed by 90% of menstrual isolates) and staphylococcal enterotoxin A (SEA) (expressed by 88% of menstrual isolates) are

correlated with menstrual disease whereas, staphylococcal enterotoxin B (SEB) is associated with non-menstrual disease (Chesney, 1997). The superantigenic activity of these toxins results in the activation of a variety of cytokines which contribute to the severity of this illness.

1.2.9 Food poisoning

Staphylococcal food poisoning is caused by the staphylococcal enterotoxins (SEs). Outbreaks are commonly associated with contaminated protein-rich foods such as meat and dairy products. Symptoms commence about four hours following ingestion and include nausea, vomiting, diarrhea and abdominal cramping. Antibiotic treatment is futile since the illness is due to a preformed toxin. The mechanisms by which SEs cause the major symptoms of food poisoning are partially understood. Evidence suggests that following ingestion, the enterotoxins interact with gastrointestinal cells and stimulate the vomiting centre in the brain. There is also evidence of SEs interacting with mast cells stimulating histamine and leukotriene release. Another mechanism may involve the massive release of cytokines (Il-2, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFα) from macrophages or T-cells as a result of the superantigenic properties of the enterotoxins (Barg and Harris, 1997).

1.3 Virulence factors of *S.aureus*

S.aureus is the leading cause of wound and hospital-acquired infections worldwide. This is due to the ability of the organism to infect, persist and replicate in the host. The factors which contribute to staphylococcal pathogenesis are collectively termed virulence factors. These can be categorised into five groups according to their role in infection: (i) attachment to cells or extracellular matrices, (adhesins) (ii) evasion of the host defenses, (evasins) (iii) invasion of host tissue (invasins) (iv) nutrient uptake under *in vivo* growth (pabulins) and (v) damage of host tissue (cytotoxins) (Moxon and Tang, 2000). These pathogenicity factors are not always essential for growth *in vitro* but are advantageous in particular environments within the host. Several animal models have been developed to study the function of virulence factors *in vivo*. However, pathogenicity is a complex phenotype and in

most staphylococcal diseases no single factor is responsible. Therefore it has been difficult to define the role of any individual factor in contributing to disease.

Recently, alternative strategies such as *in vivo* expression technology (IVET) and signature tagged mutagenesis (STM) have been adopted to enable the identification of genes that are expressed *in vivo* or are required for *in vivo* growth. STM allows the analysis of a large number of individually tagged mutants within a pool to be introduced into a specific animal model and screened for loss of virulence (Hensel *et al.*, 1995). Mutants which are viable *in vitro* (the infecting pool) but cannot grow *in vivo* (the recovered pool) would contain an insertional mutation in a gene required for *in vivo* survival. Cloning and sequencing the DNA regions flanking the transposon can then identify the tagged gene.

IVET allows the identification of genes that are expressed *in vivo* and are essential for survival but are relatively inactive *in vitro* (Mahan *et al.*, 1993). This system does not involve mutagenesis but uses a promoter trap that relies on genetic recombination as a reporter for the activation of genes *in vivo*. Lowe *et al.* (1998) used this approach in *S.aureus* to identify genes that are induced during renal abscess infections. In brief, the strategy used involved cloning digested genomic DNA fragments to a promoterless resolvase gene. Transcriptionally active fragments cause expression of the resolvase which results in excision of a kanamycin resistance cassette flanked by direct repeats of res, the sequence at which the resolvase acts. Loss of kanamycin resistance in cells grown *in vivo* acts as a marker for genes expressed even transiently *in vivo*.

Both of these approaches have been tested in various animal models and have allowed identification of genes affecting *S.aureus* virulence (Coulter *et al.*, 1998; Mei *et al.*, 1997; Lowe *et al.*, 1998). The majority are genes encoding peptide and amino acid transporters (e.g. *putP*, a high affinity proline permease uptake system), genes involved in cell wall biosynthesis (e.g. *femA* and *femB*), and enzymes involved in cellular biosynthetic pathways. However, these approaches have not identified classical virulence factors. STM in particular, did not identify extracellular toxins, presumably due to complementation by neighbouring cells. The reasons for not detected adhesins include (i) the redundancy of surface protein adhesins and (ii) the tissues of the animal model used may not express the appropriate ligands. IVET on the other hand focuses only on *in vivo* expressed genes therefore most classical virulence factors are missed because they are expressed *in vitro*. Despite these

limitations, identification of genetic loci and pathways important for growth and persistence *in vivo* is clinically relevant and may prove to be effective drug targets for anti-staphylococcal therapies.

The availability of complete bacterial genome sequences will provide an immense fund of information for studying bacterial virulence (www.sanger.ac.uk/Projects/S_aureus; www.genome.ou.edu/staph.html; www.tigr.org/tdb/mdb/mdbinprogress.html). Bioinformatics is essential to interpret the information available and to tackle such questions as gene order within genomes, identification of virulence related genes acquired by gene transfer and detection of gene families and putative regulators. The relatively small sizes of bacterial genomes are also well suited to global expression profiling with microarray technology. This approach enables the study of genetic variation between strains and differences in expression profiles under different conditions of growth. The availability of this information will undoubtedly make a massive contribution to the classical methods of studying bacterial virulence.

1.3.1 Ligand binding proteins

S.aureus can colonise host tissue by adhering to different components of the extracellular matrix (ECM). Adherence is predominantly mediated by a class of cell wall-associated surface proteins termed microbial surface components recognising adhesive matix molecules (MSCRAMMs), which are in most cases covalently anchored to the cell wall peptidoglycan. These proteins have several common features: (i) an N-terminal signal peptide (approximately 40 residues in length) required for Sec-dependent secretion, (ii) a wall spanning domain either rich in proline and glycine residues or composed of serine and aspartate dipeptide repeats, (iii) an LPXTG motif required for covalent anchoring of the protein to the pentaglycine crossbridge in peptidoglycan, (iv) a hydrophobic membrane-spanning domain followed by (v) several positively charged residues (Figure 1.1). The LPXTG motif is important for accurate sorting of the proteins. Mazmanian *et al.* (1999) recently identified an enzyme called sortase which catalyses the cleavage between the threonine and the glycine of the LPXTG motif releasing the carboxyl of the threonine to form an amide bond with the amino of the pentaglycine cross bridge in the staphylococcal peptidoglycan (Navarre and Schneewind, 1999; Figure 1.2). Sortase, a sulfhydryl-containing enzyme, anchors surface proteins during cell wall

Figure 1.1 Schematic diagram of the structural organisation of staphylococcal MSCRAMMs. S represents signal sequences, R represents the Ser-Asp dipeptide repeats, W represents the proline and glycine rich cell wall-spanning domain, and M represents the membrane spanning region followed by the positively charged tail. Ligand binding domains are marked by asterisks. The position of the LPXTG motif is indicated in FnBPA. An arrow indicates the position of the LPXTG motif in the remaining proteins.

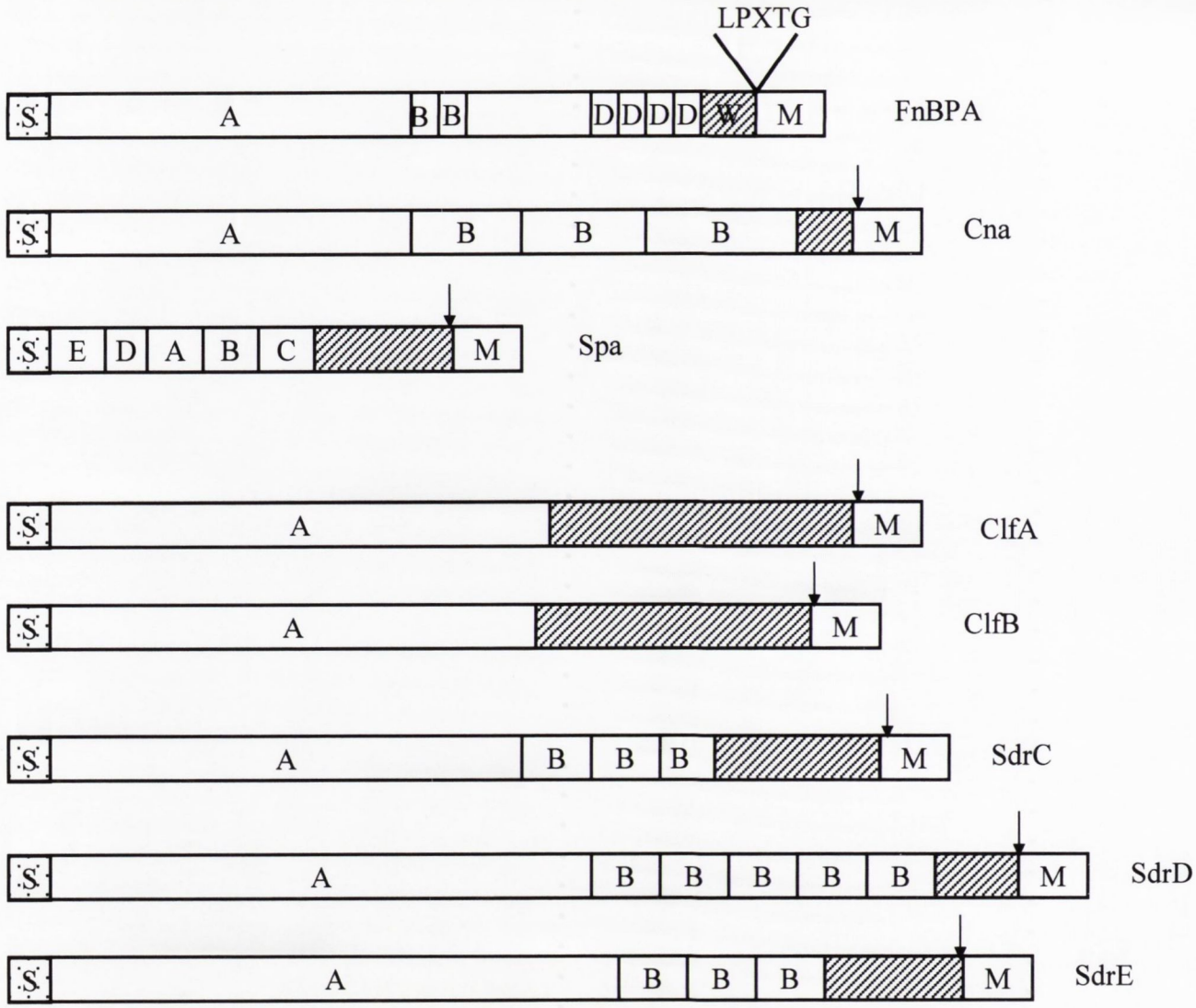
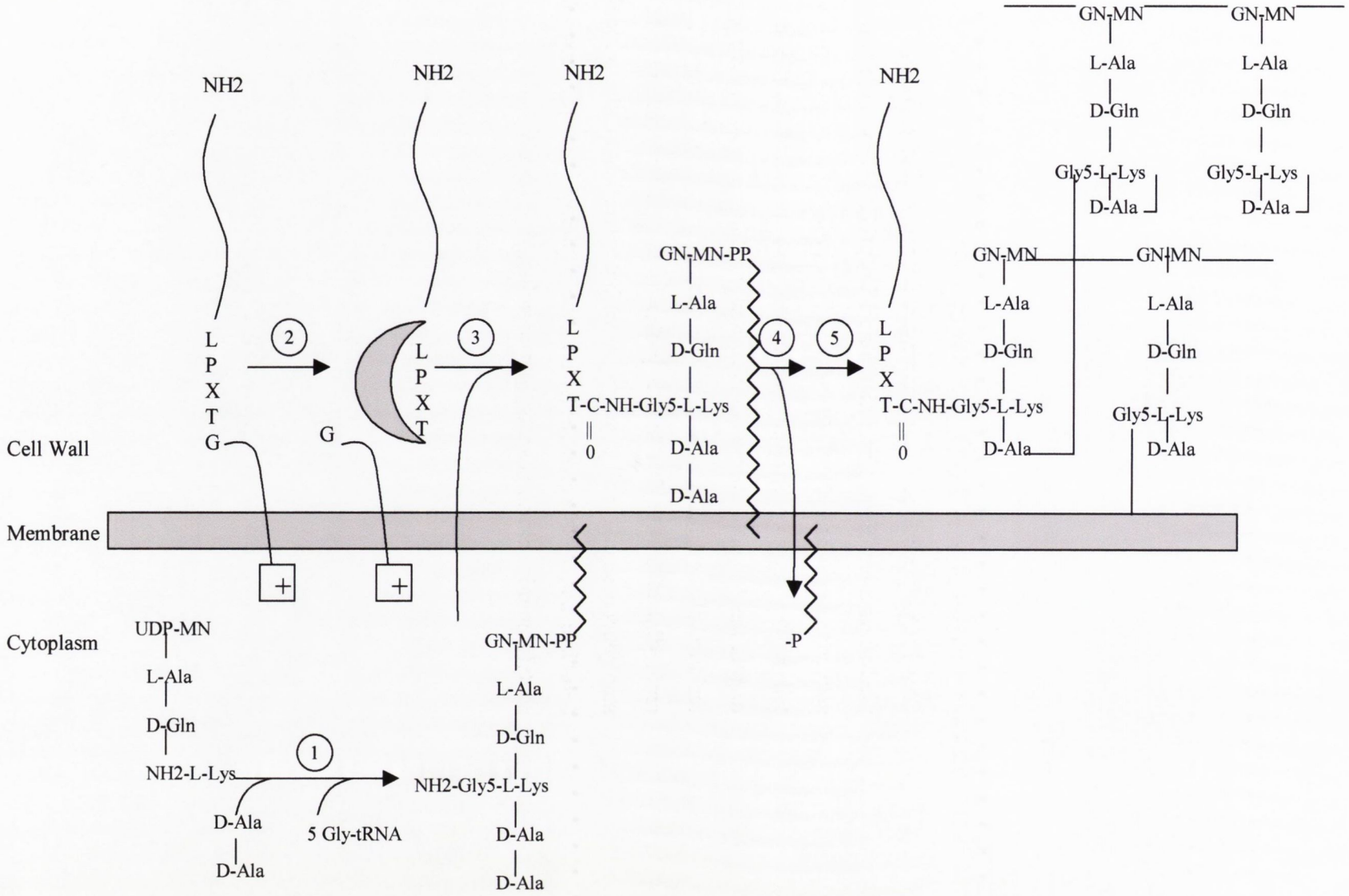

LPXTG
S
A
B
B
D
D
D
D
W
M
FnBPA
S
A
B
B
B
M
Cna
S
E
D
A
B
C
M
Spa
S
A
M
ClfA
S
A
M
ClfB
S
A
B
B
B
M
SdrC
S
A
B
B
B
B
B
M
SdrD
S
A
B
B
B
M
SdrE

Figure 1.2 The structure and sorting of LPXTG proteins of *S.aureus* to the cell wall.

Step 1. Cytoplasmic protein precursors are exported through the cell membrane by the Sec pathway. During secretion a signal peptidase cleaves the N-terminal signal sequence to generate the mature form of the protein. Step 2. A positively charged tail at the carboxy terminus stops secretion and anchors the protein to the membrane. Step 3. The protein is then cleaved between the threonine and glycine residues of the LPXTG motif by the enzyme sortase and (Step 4) subsequently amide-linked to the free amino group in the pentaglycine crossbridge of the staphylococcal cell wall through the carboxy terminal of the threonine residue. MurNAc and GlcNAc correspond to the peptidoglycan precursor molecules N-acetylmuramic acid and N-acetylglucosamine, respectively.


NH2
L
P
X
T
G
2
G
L
P
X
T
3
NH2
NH2
T-C-NH-Gly5-L-Lys
0
GN-MN-PP
L-Ala
D-Gln
D-Ala
D-Ala
4
5
NH2
T-C-NH-Gly5-L-Lys
0
GN-MN
GN-MN
L-Ala
D-Gln
Gly5-L-Lys
D-Ala
GN-MN
GN-MN
L-Ala
D-Gln
Gly5-L-Lys
D-Ala
Cell Wall
Membrane
Cytoplasm
UDP-MN
L-Ala
D-Gln
NH2-L-Lys
1
D-Ala
D-Ala
5 Gly-tRNA
GN-MN-PP
L-Ala
D-Gln
NH2-Gly5-L-Lys
D-Ala
D-Ala
-P

assembly. *S.aureus* mutants lacking sortase fail to process and display surface proteins and have been shown to cause reduced virulence in a mouse peritoneal infection (Mazmanian *et al.*, 2000). Therefore a hunt for chemical inhibitors of sortase is being explored as a new therapy for *S.aureus* infections (Schneewind and Ton-That, 1999). *S.aureus* also expresses other ligand binding proteins which are not LPXTG-anchored. These include coagulase and Efb, which are predominantly extracellular and Map which is associated with the cell wall by an unknown mechanism. Furthermore, staphylococcal autolysins (Aas and AtlE) have adhesive as well as autolytic properties (Hell *et al.*, 1998; Heilmann *et al.*, 1997). They also lack the motifs typical of Gram-positive surface proteins but are thought to associate with the cell wall by interactions with a series of putative peptidoglycan binding repeats (Navarre and Schneewind, 1999).

1.3.1.1 Protein A

Protein A (Spa) is the archetype of the LPXTG-anchored family of cell wall-associated proteins of *S.aureus*. It makes up nearly 7% of the cell wall of *S.aureus* and is present in nearly 95% of all *S.aureus* strains (Forsgren, 1970). Protein A is encoded by the *spa* gene as a 509 amino acid preprotein in strain 8325-4 with an N-terminal signal sequence (Uhlén *et al.*, 1984) (Figure 1.1). The Fc-binding region is composed of four or five repeated domains of ~58 residues in length, each of which is capable of binding to IgG. Nuclear magnetic resonance studies showed that each domain is composed of three α-helices (Starovasnik *et al.*, 1996). X-ray crystallographic analysis of the subdomain B complexed with the Fc fragment identified 11 residues on Spa which constitute the IgG binding domain and 9 residues on the Fc fragment which are important in the interaction (Gouda *et al.*, 1998). A comparison of the IgG-binding domains indicates that they arose by tandem duplication because of the gradient of sequence similarity (Uhlén *et al.*, 1984). Residues in the ligand binding domains involved in interaction with IgG have been conserved.

Protein A is actively synthesized during early to mid-exponential phase of growth but expression is repressed as the cells enter stationary phase. It is suggested that *sar* and *agr* repress *spa* expression while the recently identified *sarH1* has been proposed to be an activator of *spa* transcription (Vandenesch *et al.*, 1991; Cheung *et al.*, 1997b; Tegmark *et al.*, 2000) (Regulation is covered in detail in chapter 5).

The importance of Spa in staphylococcal infections is still unclear. The ability of *S.aureus* to bind IgG via its Fc-region may contribute to the resistance of the organsim to phagocytosis by masking binding sites for opsonins present in normal serum. Peterson *et al.* (1977) demonstrated that *S.aureus* strains expressing high levels of Spa are more resistant to phagocytosis than strains expressing low levels of Spa. Gemmel *et al.* (1991) supported this observation by demonstrating that *spa*-defective mutants are more easily phagocytosed by human PMNs than their parental strains in normal human serum indicating that Spa is anti-opsonic. When IgG-deficient serum was used, protein A-rich strains were more readily phagocytosed than were strains with less Spa on the surface suggesting that protein A is capable of activating complement in the absence of IgG (Peterson *et al.*, 1977).

A recent study has revealed that isolates taken from patients suffering with Kawasaki disease express higher levels of protein A than isolates from Toxic shock syndrome (Wann *et al.*, 1999). Kawasaki disease is an acute vasculitis of young children and is characterised by significant immune activation. TSST-1 has been implicated as a virulence factor in this disease through activation of T-cells and macrophages (Wann *et al.*, 1999). Due to the diverse immunobiological properties of protein A, including the ability to activate B cells (Ringden, 1985) and complement (Peterson *et al.*, 1977), and the high levels of expression in Kawasaki disease isolates, protein A may be associated with some of the immune activation seen in Kawasaki disease.

It was recently postulated that protein A plays a role in the pathogenesis of infective endocarditis. Protein A-mediated adherence of *S.aureus* to von Willebrand factor (Herrmann *et al.*, 1997; Hartlieb *et al.*, 2000) and the interaction between protein A and the gC1qR/p33 receptor expressed on the surface of platelets and endothelial cells (Nguyen *et al.*, 2000) may promote colonisation of the damaged valve surface. Peacock *et al.* (1999) showed that there was no difference in the ability of strain 8325-4 and its isogenic protein A defective mutant to bind non-activated endothelial cells *in vitro*. However, cell surface expression of the gC1qR/p33 receptor by endothelial cells is low unless cells are activated (Nguyen *et al.*, 2000).

Although the functions of protein A are quite diverse *in vitro*, its precise function *in vivo* is not clearly defined. Patel *et al.* (1987) demonstrated that protein A-deficient mutants were less virulent in both subcutaneous abscess and peritonitis

infections compared to the parental strain. However apart from this observation limited studies have been carried out to assess the role of protein A *in vivo*.

1.3.1.2 Sbi : a second IgG-binding protein

The IgG-binding capacity of *S.aureus* was solely attributed to protein A until the recent identification of a second IgG binding protein, Sbi. The Sbi protein is 436 amino acids in length bearing one functional IgG-binding domain (Zhang *et al.*, 1998). It possesses a cleavable N-terminal signal sequence but lacks the conserved C-terminal LPXTG motif suggesting the protein is not covalently associated with the cell wall. The IgG binding domain is composed of approximately 52 residues showing significant homology to the IgG-binding repeats of protein A. However, the significance of Sbi in staphylococcal pathogenesis remains to be elucidated.

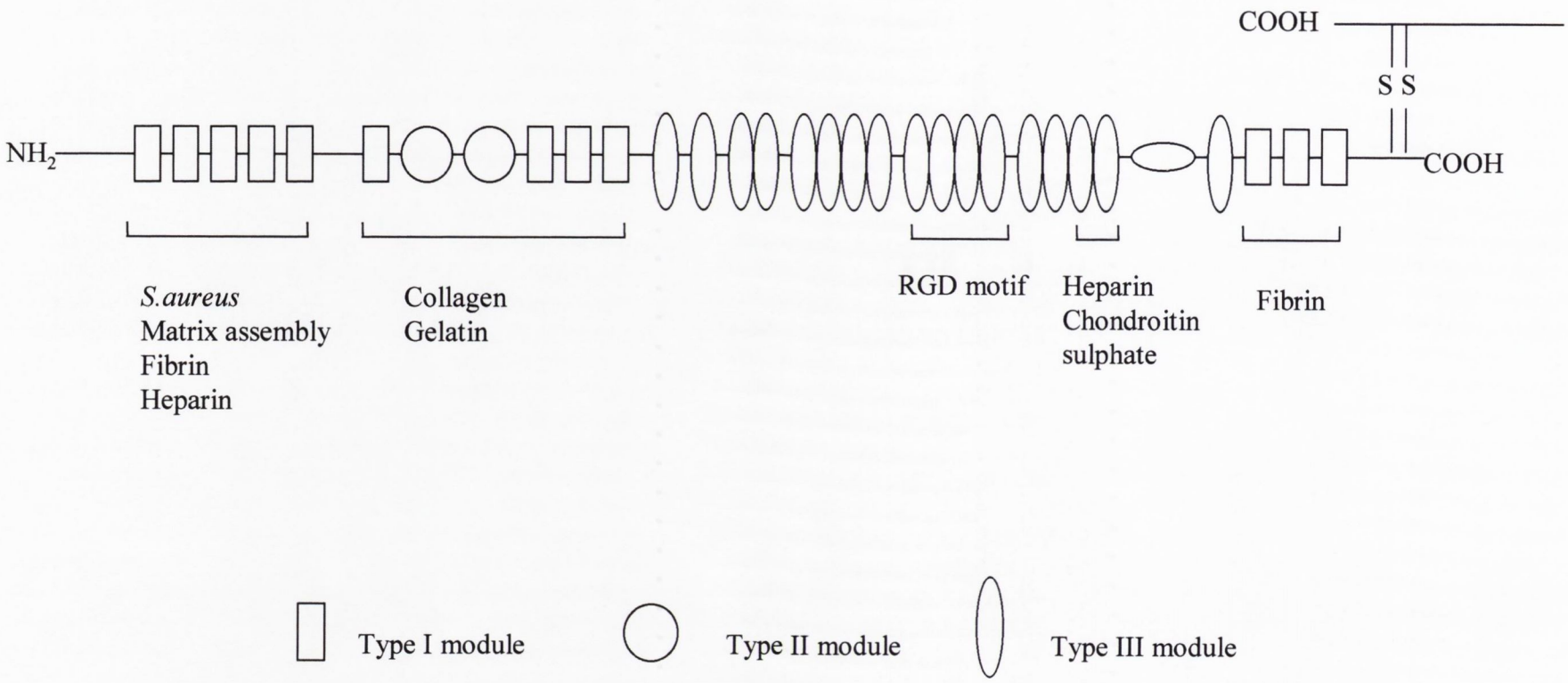
1.3.1.3 Fibronectin binding proteins

1.3.1.3.1 Fibronectin

Fibronectin is a high molecular weight extracellular glycoprotein found in both soluble (human plasma) and insoluble (extracellular matrix, ECM) forms. It plays important roles in many physiological processes such as mediating cell adhesion and migration, affecting cell morphology and differentiation, and wound healing (Hynes, 1993). Each fibronectin monomer is between 235 and 270 kDa in size which before being secreted dimerises by the formation of two disulphide bonds near the C-terminus. Like many proteins of the extracellular matrix, fibronectin is composed of modular protein units (Figure 1.3). There are three distinct modules (type I, type II, and type III) that are clustered into functional domains which bind ligands such as heparin, fibrin, collagen, glycosaminoglycans and thrombospondin. The ability of fibronectin to interact with a diverse range of ECM proteins has been associated with its ability to assemble a fibrillar matrix. In addition, fibronectin also mediates adhesion of various cells through the RGD integrin receptor located in the centre of the molecule.

Fibronectin is a human plasma protein that contributes to the coating of implanted medical devices and promotes bacterial attachment. Although fibrinogen was demonstrated to be the major plasma protein that mediated bacterial adherence to *ex vivo* catheters, the binding sites in fibronectin are more resistant to proteolytic

Figure 1.3 The domain organisation of the fibronectin monomer. Showing positions of the type I, II and III modules (based on Potts and Campbell, 1994) and their interactions with other host proteins and *S.aureus* fibronectin binding proteins.


COOH
S S
NH_2
COOH
S.aureus
Matrix assembly
Fibrin
Heparin
Collagen
Gelatin
RGD motif
Heparin
Chondroitin
sulphate
Fibrin
Type I module
Type II module
Type III module

degradation than those in fibrinogen (Vaudaux *et al.*, 1995; Vaudaux *et al.*, 1993). Therefore fibrinogen is proposed to be the dominant protein mediating bacterial adherence on newly implanted devices but is readily degraded so that in older devices fibronectin becomes the dominant factor. *S.aureus* is a major cause of infections associated with indwelling catheters and cardiovascular and orthopaedic devices (Vaudaux *et al.*, 1989). Adhesion of bacteria to fibronectin-coated catheters is thought to be a crucial step in the initiation of infection.

1.3.1.3.2 Fibronectin-binding MSCRAMMs

Kuusela (1978) was the first to demonstrate the interaction between *S.aureus* and fibronectin which was later shown to occur with a high affinity (K_d = 1.8 nM) (Bozzini *et al.*, 1992). The interaction between *S.aureus* and fibronectin is mediated by two cell wall-associated proteins, FnBPA and FnBPB, which belong to the family of MSCRAMMs (reviewed by Foster and Höök, 1998). The genes *fnbA* and *fnbB*, from *S.aureus* 8325-4, are tandemly arrayed but are transcribed separately (Signäs *et al.*, 1989; Jönsson *et al.*, 1991). Studies with isogenic mutants and recombinant proteins showed that both FnBPs promote bacterial attachment to immobilised fibronectin *in vitro* and to *ex vivo* coverslips removed from subcutaneous tissue cages in guinea pigs and they mediate bacterial adherence to plasma clots (Greene *et al.*, 1995; Vaudaux *et al.*, 1993; Vaudaux *et al.*, 1995). This suggests the importance of FnBPs as virulence factors in staphylococcal disease. The significance of the FnBPs as virulence factors is controversial. Kuypers *et al.* (1989) reported reduced adherence of a fibronectin binding defective mutant to traumatised rat heart valves, yet Flock *et al.* (1996) published contradictory results using a rat endocarditis model. These discrepancies may be explained by the use of different strains. Strain 8325-4 used by Flock *et al.* (1996) is known to express low levels of FnBPs *in vitro*.

The expression of the *fnb* genes is restricted to the early exponential phase of growth (Greene *et al.*, 1995). Wolz *et al.* (2000) demonstrated that *fnbA* expression is negatively regulated by *agr* and positively regulated by *sar*. However, an additional mechanism independent of *agr*, is proposed to restrict *fnb* transcription to the early exponential phase of growth (Wolz *et al.*, 1996; Wolz *et al.*, 2000). In addition to transcriptional regulation, proteolysis was proposed to reduce the level of FnBP on the surface of cells in stationary phase. McGavin *et al.* (1997) demonstrated that diminished fibronectin binding function was a result of FnBP proteolysis by V8

serine protease (Ssp). However inactivation of the *ssp* gene in several strains suggested that proteolysis of FnBP by V8 protease is not a common mechanism (Peacock *et al.*, 2000). Proteases other than Ssp were implicated as well as masking by capsular polysaccharide of ligand binding activity (Pöhlmann-Dietze *et al.*, 2000).

Several groups have studied the prevalence and functional expression of the *fnb* genes among *S.aureus* strains showing that FnBP production is widespread. Smeltzer *et al.* (1997) detected *fnb* genes in all 25 *S.aureus* strains tested. Peacock *et al.* (2000) carried out a larger study on a panel of 163 clinical isolates representing both nasal carriage and strains from invasive diseases and showed that 162 out of the 163 possessed the *fnb*A gene and 96% of these displayed fibronectin binding.

Analysis of the amino acid sequences of FnBPA and FnBPB revealed that the proteins are quite distinct in the N-terminal region A (45% identical) whereas the C-terminus bearing the D repeats and the wall and membrane spanning domains are 95% identical (FnBPA is shown in Figure 1.1). The major fibronectin-binding domain is located within the D-repeats. The FnBP proteins possess 3-5 consecutive D repeats of 38-40 residues, each of which can bind to fibronectin with low affinity. However, in tandem these domains promote high affinity binding. McGavin *et al.* (1993) localised the fibronectin binding domain to individual residues within the C-terminal 20 amino acids of each D motif. The conserved residues of this core sequence contain GG(X3,4)(I/V)DF, which is also present in other fibronectin-binding proteins (e.g. *Streptococcus dysgalactiae* FnBA, FnBB) suggesting recognition of a common sequence within the fibronectin molecule. The ligand-binding domain of FnBPA lacks discernable secondary structure but when bound to fibronectin type I modules it undergoes a structural change demonstrated by CD spectroscopy and the generation of novel epitopes (ligand induced binding sites) (House-Pompeo *et al.*, 1996). A similar structural conformation occurs with the ligand-binding site on FnbA of *Streptococcus dysgalactiae* in the presence of fibronectin because monoclonal antibodies only recognised the bound form of the ligand binding region (Speziale *et al.*, 1996). The ligand induced binding site in the FnBPs has been proposed as a host defense mechanism. Antibodies generated against the unfolded ligand binding domains cannot interfere with the fibronectin-FnBP interaction (House-Pompeo *et al.*, 1996). This hypothesis is supported by studies with sera from patients with staphylococcal infections, which could bind the repeat

domains of the FnBPs but were unable to inhibit the FnBP-fibronectin interaction (Casolini *et al.*, 1998).

Recently, the N-terminal A region of FnBPA was shown to have fibrinogen binding activity (Wann *et al.*, 2000). This region is different from the A region of FnBPB but has about 25% amino acid sequence identity to the A regions of ClfA, ClfB and SdrG, all of which bind fibrinogen.

The major bacterial binding site on fibronectin occurs in the N-terminal 28 kDa domain which contains the five type I modules (Mosher *et al.*, 1980). Deletion of any single module resulted in loss of binding to *S.aureus* cells indicating that all five type I modules are required for activity (Sottile *et al.*, 1991). Bozzini *et al.* (1992) identified a second lower affinity *S.aureus* binding site in the C-terminal type III heparin binding domain (type III_{14}) which is only exposed in immobilised fibronectin. However, Sakata *et al.* (1994) postulated that this domain extends to the neighbouring fibrin binding domain located in the type I repeats.

S.aureus has been considered to be exclusively an extracellular pathogen. However, recent studies have revealed that *S.aureus* can invade non-professional phagocytic cells such as human endothelial cells, human and bovine epithelial cells and human fibroblasts (Peacock *et al.*, 1999; Dziewanowska *et al.*, 1999; Sinha *et al.*, 1999). Invasion is dependent on FnBP expression. Isogenic mutants defective in expression of FnBPs showed marked reduction in adhesion to and uptake by these cells compared to wild type strains (Peacock *et al.*, 1999; Sinha *et al.*, 1999). Moreover, complementation with multicopy plasmids overexpressing FnBPs restored binding and conferred invasiveness to non-invasive strains (Peacock *et al.*, 1999; Sinha *et al.*, 1999). As mentioned previously, *S.aureus* FnBPs interact with the N-terminus of fibronectin whereas the $\alpha_5\beta_1$ integrin on mammalian cells interacts with the RGD in fibronectin. Fibronectin therefore behaves as a bridging molecule bringing the bacterial cell in close contact with the mammalian cells. Anti-$\alpha_5\beta_1$ antibodies reduced internalisation of *S.aureus* cells by 50% suggesting an important role for the $\alpha_5\beta_1$ integrin (Sinha *et al.*, 1999). The mechanism of internalisation involves stimulation of a signal transduction event by the host cell tyrosine kinases which stimulate actin rearrangements necessary to engulf bacteria into a phagocyte vesicle (Sinha *et al.*, 1999; Dziewanowska *et al.*, 1999).

1.3.1.4 Fibrinogen binding proteins

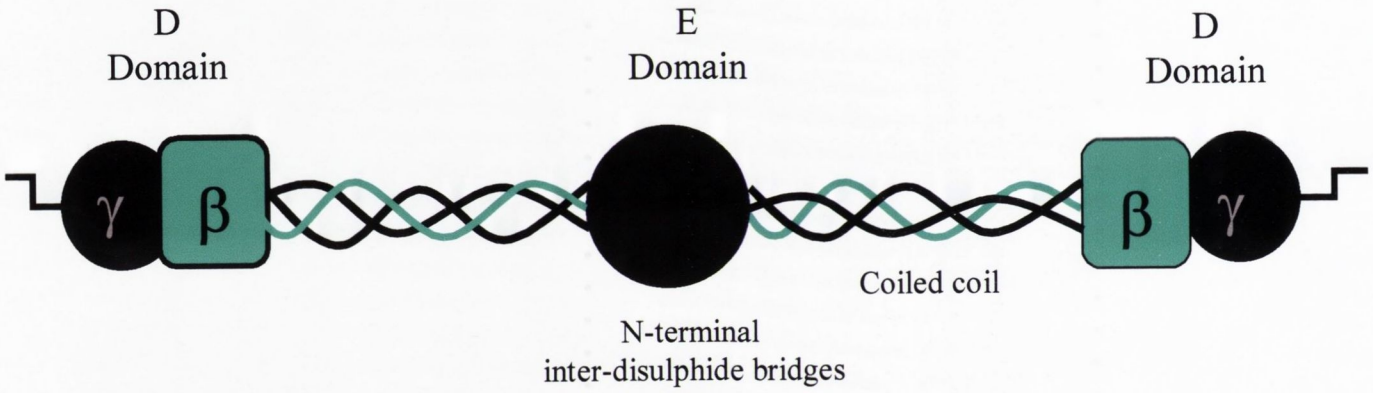
1.3.1.4.1 Fibrinogen

Fibrinogen is a soluble plasma protein of 340 kDa that is converted by thrombin to form insoluble fibrin, the primary ingredient of blood clots. The molecule consists of three polypeptide chains (α, β, and γ) that are assembled by their N-termini through complex interchain disulphide bonding (Figure 1.4). This central region, designated the E domain, is connected to the C-terminal D regions by coiled coil regions. The C-termini of the β and γ polypeptides are located at the extremity of the molecule but a short segment of the γ chain extends away from the D region. Ca^{2+} binding sites located in the E and D domains are important in fibrin formation. Following fibrin polymerisation, the clot is stabilised by the plasma transglutaminase, factor XIII, which covalently crosslinks the C-terminal proturberances of the α and γ chains. Fibronectin is also incorporated into blood clots by interacting with fibrinogen and fibrin. In addition to its role in clot formation, fibrinogen is also essential for platelet aggregation. Fibrinogen binds to a specific platelet integrin αIIb/β3 via the C-terminus of the γ chain and also to the RGD motif on the α chain (Hawiger *et al.*, 1982). The divalent nature of the fibrinogen molecule enables aggregation of platelets to occur. Fibrinogen also interacts with the other integrins expressed on the surface of endothelial cells and leukocytes.

1.3.1.4.2 Fibrinogen-binding MSCRAMMs

Staphylococcus aureus forms macroscopic clumps when mixed with soluble plasma. The clumping reaction is due to the binding of soluble fibrinogen to the surface protein receptors, clumping factor A (ClfA) and clumping factor B (ClfB) (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998). The structural organisation of ClfA and ClfB is consistent with the family of LPXTG-anchored wall-associated proteins from Gram-positive bacteria including an N-terminal signal sequence, a C-terminal wall-spanning domain, an LPXTG motif, a hydrophobic membrane domain and a positively charged C-terminus (Figure 1.1). The fibrinogen-binding sites are located in the A domains (McDevitt and Foster, 1995; Ní Eidhin *et al.*, 1998). In the case of ClfA, the ligand binding site has been further mapped to residues 220-559 of region A (McDevitt and Foster, 1995). A distinctive feature of these proteins is a dipeptide

Figure 1.4 Schematic diagram of the fibrinogen structure. The α, β, and γ chains are joined at the N-terminus by disulphide bridges (E domain). The region connecting the N- and C-termini is a coiled coil region, whereas the carboxy terminal domains (D domains) are globular in structure. The C-terminus of the α chain is in an anti – parallel conformation and is sensitive to proteolysis (not shown in diagram). This figure is modified from Ruggeri, 1994.


D
Domain
E
Domain
D
Domain
γ
β
β
γ
Coiled coil
N-terminal
inter-disulphide bridges

repeat region comprising alternating serine and aspartate residues (region R). The size of this domain in ClfA varies in different isolates from 193 to 440 amino acids (McDevitt and Foster, 1995). Its function is to act as a 'stalk' to project the N-terminal ligand binding domains away from the cell surface thus promoting interactions with fibrinogen (Hartford *et al.*, 1997). Despite their structural and functional similarity, ClfA and ClfB are expressed at different stages in the growth cycle. ClfA is expressed on the cell surface throughout the growth cycle whereas ClfB is preferentially expressed during early exponential phase and cannot be detected in later stages of growth (Ní Eidhin *et al.*, 1998).

One of the *S.aureus* binding sites on fibrinogen has been localised to the C-terminal 15 amino acids of the fibrinogen γ chain that project from the γ module (Strong *et al.*, 1982). McDevitt *et al.* (1997) demonstrated that this interaction is mediated by region A of ClfA. Furthermore, O'Connell *et al.* (1998) showed that the interaction between recombinant region A and a synthetic peptide corresponding to the binding site on the fibrinogen γ chain is inhibited by Ca^{2+}(IC_{50} 3-5mM). The likely Ca^{2+}-binding site in region A was identified as an EF-hand-like motif characteristic of eukaryotic Ca^{2+} binding proteins. Site-specific mutations within this domain reduced inhibition by Ca^{2+} (O'Connell *et al.*, 1998). The γ chain of fibrinogen is also the primary binding site for the platelet integrin αIIbβ3 and analogous to ClfA, the ligand binding activity of αIIbβ3 is regulated by Ca^{2+}ions. The recombinant region A of ClfA is a potent inhibitor of platelet aggregation (McDevitt *et al.*, 1997). Since platelet aggregation stimulates secretion of antimicrobial peptides, the ability of *S.aureus* to block this event may be a mechanism of combatting the host defenses.

Ní Eidhin *et al.* (1998) demonstrated that ClfB recognises the α and β chains of fibrinogen. The ability of *S.aureus* to interact with different parts of the host ligand might promote a more stable attachment to resist conditions such as blood flow.

The ability of *S.aureus* to adhere to fibrinogen is of importance in initiation of infection (Vaudaux *et al.*, 1989; Herrmann *et al.*, 1993; Moreillon *et al.*, 1995). Since ClfA and ClfB have both been shown to promote bacterial clumping in the presence of soluble fibrinogen and to adhere to immobilised fibrinogen under *in vitro* and *ex vivo* conditions it is probable that they are virulence factors in wound and foreign body infections. In support of this hypothesis, Moreillon *et al.* (1995) demonstrated

that ClfA is a virulence factor in a rat endocarditis model. The significance of ClfB *in vivo* remains to be elucidated.

1.3.1.4.3 Non-covalently anchored fibrinogen binding proteins

In addition to the MSCRAMMs, *S.aureus* also secretes proteins that bind fibrinogen. Coagulase is a predominantly extracellular protein which stimulates the conversion of fibrinogen to fibrin to cause the formation of clots in mammalian plasma (Bóden and Flock, 1989). The protein lacks cell wall or membrane anchoring domains and is predominantly a secreted protein (Phonimdaeng *et al.*, 1990). The expression of coagulase a feature of *Staphylococcus aureus* is often used to distinguish it from other staphylococcal species (coagulase negative staphylococci). The fibrinogen binding site on coagulase is localised in the C-terminal repeated region of the protein (McDevitt *et al.*, 1992). Although coagulase is an extracellular protein, a small fraction of the protein remains firmly attached on the bacterial cell surface. Cell surface coagulase can promote binding of soluble fibrinogen, especially in an *agr* mutant where it is overexpressed, but it does not mediate bacterial adherence to immobilised fibrinogen (McDevitt *et al.*, 1992; Wolz *et al.*, 1996).

Bóden and Flock, (1994) identified another fibrinogen binding protein secreted by *S.aureus*. The 19 kDa protein was previously called Fib but has been renamed the extracellular fibrinogen binding protein (Efb). The N-terminus of the secreted protein contains two 22 amino acid repeats with homology to the repeats at the C-terminus of coagulase (Bóden and Flock, 1994). The *efb* gene is unique to *S.aureus* and is highly conserved (Bóden-Wäsfelt and Flock, 1995). Inactivation of the *efb* gene caused reduced virulence in a rat model of wound infection suggesting a role for Efb in staphylococcal virulence. Vaccination of mice with a combination of coagulase and Efb was partially protective against experimental mastitis suggesting that Efb is also a protective antigen in this disease (Palma *et al.*, 1996; Mamo *et al.*, 1994).

1.3.1.5 Collagen binding proteins

1.3.1.5.1 Collagen

Collagen is the most abundant protein of vertebrates and is present in virtually every tissue. Its principle role is to provide tensile strength to connective tissues such as cartilage, teeth, tendon, ligament, bone, and the fibrous matrices of skin and blood

vessels. A large number of genetically distinct collagen types have been identified. Collagen types I-III are known as fibrillar collagens, which play a structural role in stabilising scaffolds in extracellular matrices (Olsen and Ninomiya, 1994). Type I collagen is found in tendons, ligaments, skin and bone. Type II collagen is an important component of cartilage and commonest form used in *S.aureus* adherence studies. Type III collagen has thinner fibres and is found in skin and vascular tissues. Type IV collagen is the major collagenous component of basement membranes where it forms a network structure for other extracellular matrix components to interact.

However, all collagen proteins contain domains with a triple helical conformation. These domains are composed of three polypeptide chains known as α-chains which contain the repetitive sequence motif $(GXY)_n$, where X is often proline and Y is often hydroxyproline. The polypeptide chains wind around each other to form a right handed superhelical structure which is responsible for the tensile strength of collagen fibers.

1.3.1.5.2 The Collagen-binding MSCRAMM

The adherence of *S.aureus* to collagen is mediated by the collagen binding MSCRAMM, Cna (Figure 1.1). The *cna* gene has been cloned and sequenced and encodes a protein that is structurally similar to other surface proteins isolated from Gram-positive bacteria (Patti *et al.*, 1992). The N-terminal A domain, which consists of approximately 500 residues of unique sequence, contains the ligand binding site. By analysing the collagen binding activity of recombinant proteins corresponding to different segments of the MSCRAMM, Patti *et al.* (1993) firstly localised the binding domain to a 168 amino acid segment corresponding to residues 151-318. Critical residues (209-233) involved in collagen binding were then identified by testing the ability of overlapping synthetic peptides to neutralise the inhibitory activity of a polyclonal antibody generated against Cna (Patti *et al.*, 1995). The crystal structure of the collagen binding domain was resolved and found to fold like a "jelly roll" in two β-sheets connected by two short α-helices (Symersky *et al.*, 1997). A trench was found on one of the β-sheets and docking studies using synthetic collagen probes suggest that this trench can accommodate a collagen triple helix. Site-directed

mutagenesis of residues within the trench resulted in decreased collagen binding activity further demonstrating the importance of the trench in collagen binding.

The B domain of Cna consists of between one and four copies of a 187 amino acid region, depending on the *S.aureus* strain from which the Cna was isolated (Gillaspy *et al.*, 1997). The function of the B repeats remains unclear. Studies have shown that they are not involved in binding collagen, and do not affect the collagen binding activity of the A domain (Rich *et al.*, 1998; Hartford *et al.*, 1999). It was also proposed that they may serve as a stalk projecting the A domain away from the cell surface. However this hypothesis is unlikely since Snodgrass *et al.* (1999) demonstrated that the presence of multiple B domains do not extend the A domain outward to avoid the masking effect of capsule production on collagen binding activity. Another possibility is that the B domains may contain binding sites for other ligands apart from collagen, although this remains to be elucidated.

Like most MSCRAMMs, *cna* expression is highest during exponential phase and diminishes to almost undetectable levels as cells enter the post exponential phase of growth (Gillaspy *et al.*, 1998). Blevins *et al.* (1999) demonstrated that SarA is the primary regulatory element controlling expression of *cna*. Inactivation of the *sar* locus results in increased transcription of *cna*. This *sar*-mediated transcription occurs via an *agr*-independent mechanism since complementation of the *sar* open reading frame in a *sar agr* double mutant restored *cna* transcription to wild type levels. Electrophoretic mobility shift assays demonstrated the ability of SarA to directly interact with *cna* upstream sequence further supporting the above hypothesis.

Unlike most MSCRAMMs, Cna is not expressed by the majority of *S.aureus* strains and is present on a discrete DNA fragment that extends 200 bp upstream and 100 bp downstream of the *cna* gene (Gillaspy *et al.*, 1997; Smeltzer *et al.*, 1997). The ability of *S.aureus* to bind collagen appears to be directly proportional to the presence of the *cna* gene and variations in the collagen binding capactiy of Cna+ strains is due to differences in *cna* transcription levels (Gillaspy *et al.*, 1998). Analysis of *S.aureus* strains isolated from patients with osteomyelitis and septic arthritis revealed that the majority of strains contained the *cna* gene and promoted adherence to collagen and cartilage *in vitro*. On the contrary, only one third of strains isolated from soft tissue infections contained the *cna* gene suggesting a role for Cna as a virulence factor in infections of collagen rich tissue. The biological importance

of the collagen binding adhesin in the pathogenesis of *S.aureus* infections was further investigated by comparing the virulence of a parental strain against its isogenic *cna*-defective mutant in animal infection models of endocarditis, septic arthritis and keratitis (Hienz *et al.*, 1996; Patti *et al.*, 1994; Rhem *et al.*, 2000). In all models tested the *cna* mutant was significantly less virulent than the wild type strain. Nilsson *et al.* (1998) further showed that vaccination of mice with a recombinant fragment of the collagen adhesin provides protection against sepsis-induced death following challenge with *S.aureus* strain Phillips. This protective effect is antibody mediated since protection was also provided using passive immunisation with anti-*cna* antibodies. This data reveals that Cna is expressed *in vivo* and can be recognised by immunoglobulins therefore represents a candidate for development of immunopreventive and therapeutic strategies to fight staphylococcal disease.

1.3.1.6 The Sdr proteins

The R domain of ClfA and ClfB consists of numerous repeats of the dipeptide Ser-Asp which are encoded by an 18-base pair DNA repeat. Southern hybridisation identified three additional genes specifying new members of the SD-repeat family (Sdr) named SdrC, SdrD and SdrE (Josefsson *et al.*, 1998). The structural organisation of the these proteins is similar to ClfA and ClfB except for the presence of B-repeats located between region A and region R (Figure 1.1). The B repeats are 110-113 residues in length and each contains a high affinity Ca^{2+}-binding site that resembles an EF-hand motif (Josefsson *et al.*, 1998). The A domains of the Sdr family (including ClfA and ClfB) are 20-30% identical and have a conserved motif, TYTFTDYVD, which overlaps the C-terminal end of the EF-hand in ClfA.

Tung *et al.* (2000) recently described a bone sialoprotein-binding protein (Bbp) which displays 76% identity in region A with the corresponding region in SdrE. Other members of the Sdr family also occur in *S.epidermidis*. Nilson *et al.* (1998) identified a fibrinogen binding protein named Fbe which contains the characteristic SD repeats of Sdr proteins. The ligand binding domain resides in the A region and has 24-41% identity to the A domains of the *S.aureus* Sdr proteins and has a TYTFTDYVD motif. Other recently identified members of this family include SdrF, SdrG and SdrH (McCrea *et al.*, 2000). SdrF and SdrG have similar sequence and structural organisation to other Sdr proteins. In contrast SdrH has a shorter A region

at its N terminus, a unique 277-residue C region and lacks a LPXTG motif. The function of these proteins has not yet been reported.

The *sdr* genes of *S.aureus* are tandemly arrayed and probably arose by gene duplication. Some strains only contain two *sdr* genes. Strain 8325-4 lacks *sdrE* while strain eMRSA-16 lacks *sdrD*. Due to their structural similarity to other MSCRAMMs, it is most likely that these proteins can interact with host extracellular matrix molecules although ligands for the proteins have not yet been reported.

1.3.1.7 MAP

Treatment of *S.aureus* cells with 1M LiCl results in the release of a 72 kDa protein capable of binding to several extracellular matrix proteins, including fibronectin, fibrinogen, vitronectin, bone sialoprotein and thrombospondin (Homonylo-McGavin *et al.*, 1993). The protein contains an N-terminal signal sequence but lacks features associated with wall-anchored proteins, such as an LPXTG motif and a hydrophobic domain. The protein has six repeated domains of 110 residues in length which show significant sequence similarity to the peptide-binding groove of the β-chain of the major histocompatibility complex class II (MHCII) molecules (Jönsson *et al.*, 1995). Hence, the 72 kDa protein is designated Map (MHCII analogous protein). The significance of Map *in vivo* has not yet been examined but due to its broad specificity it is likely to act as a multifunctional surface adhesin and contribute to staphylococcal colonisation of a variety of tissues. Palma *et al.* (1999) studied a similar protein, Eap, which acts both as an adhesin and ligand. The secreted protein can bind to several plasma proteins and to the surface of *S.aureus* (perhaps via an MSCRAMM). Moreover, adherence of *S.aureus* to mammalian cells is increased in the presence of Eap, which presumably acts as a bridging molecule between the plasma proteins coating mammalian cells and the *S.aureus* surface.

1.3.1.8 Autolysins

Bacterial autolysins are cell wall-associated proteins that function to promote cell separation during growth by specific cleavage of cell wall peptidoglycan. Recent data suggests that staphylococcal autolysins may have an additional adhesive function. The *S.saprophyticus* autolysin, Aas was shown to bind fibronectin and sheep erythrocytes while an autolysin of *S.epidermidis*, AtlE, promotes bacterial

attachment to polystyrene. It can also bind vitronectin in ligand blots and participates in the first phase of biofilm formation (Hell *et al.*, 1998; Heilmann *et al.*, 1997). The overall structural organisation and amino acid sequence of these proteins is similar with a corresponding similarity to Atl, an autolysin of *S.aureus*. All contain an N-terminal signal sequence, an alanine amidase and a glucosaminidase. It has been proposed that the part interconnecting the enzymatically active domains is responsible for the binding activity of Aas and AtlE. Autolysins may contribute to virulence by releasing immunologically active cell wall components or toxins (Diaz *et al.*, 1992).

1.3.2 Exotoxins

A list of the toxins secreted by *S.aureus* are shown in Table 1.1. *S.aureus* exotoxins can be categorised into two groups according to their mechanism of action: (i) membrane-active toxins and (ii) toxins with superantigenic activity (Bohach and Foster, 2000). The most potent and best characterised membrane-active toxin is α-toxin (Bhakdi *et al.*, 1994). Several cell types including erythrocytes, monocytes, epithelial and endothelial cells, and platelets are damaged by alpha toxin. The release of proinflammatory cytokines and procoagulatory compounds from monocytes and platelets, respectively, is proposed to be responsible for the symptoms of septic shock during severe *S.aureus* infections. Several animal infection models have shown that α-toxin is associated with virulence (Callaghan *et al.*, 1997; Foster *et al.*, 1990).

The *S.aureus* superantigens include the pyrogenic and exfoliative toxin family (Bohach and Foster, 2000). These toxins share a number of biological properties which include induction of fever, T-cell proliferation, immunosuppression and induction of cytokines. The pyrogenic toxin family are most associated with toxin shock syndrome and the exfoliative toxin family are known to induce other diseases such as food poisoning and scalded skin syndrome.

1.3.3 Extracellular enzymes

S.aureus expresses several extracellular enzymes but their significance in the pathogenesis of *S.aureus* infections remains unclear. Table 1.2 lists some of the extracellular enzymes produced by *S.aureus* and records their possible roles in

virulence. In addition to its fibrinogen binding activity, coagulase also binds to prothrombin to form a complex named staphylothrombin. This in turn converts fibrinogen to fibrin, the main component of blood clots. One possible function of coagulase *in vivo* could be to induce fibrin clots around infection foci to protect bacteria from the host defense. Sawai *et al.* (1997) demonstrated that loss of coagulase activity led to reduced virulence in a mouse model of blood-borne staphylococcal pneumonia. Staphylokinase activates plasminogen to form plasmin, a fibrinolytic protease associated in clot dissolution. Although its significance *in vivo* has not been experimentally proven, it is plausible that staphylokinase functions in bacterial spreading from fibrin clots or fibrin coated abscesses. The role of *S.aureus* proteases *in vivo* has not been defined but they are most likely associated with nutrient uptake, tissue damage and avoidance of host defences.

1.3.4 Capsular Polysaccharide

Approximately 90% of *S.aureus* clinical isolates produce capsular polysaccharide (CP). Eleven serotypes of CP have been identified which can be categorised into two groups: (i) mucoid capsules (serotypes 1 and 2) and (ii) microcapsules (serotypes 3-11). Due to their prevalence among clinical isolates (80%), serotypes 5 and 8 are the best characterised. The *cap5* and *cap8* loci are composed of 16 genes, 12 of which are almost identical whereas the other 4 are type-specific. CP5 and CP8 polysaccharides are very similar with trisaccharide repeating units and only differ in the linkages between the amino sugars and the position of O acetylation.

Capsular polysaccharides are expressed mainly in the post exponential phase of growth. Their production appears to be regulated by environmental factors. Lee *et al.* (1993) demonstrated that enhanced expression of type 8 capsule was obtained by cultivation of *S.aureus* on agar rather than broth, and under conditions of iron limitation and magnesium abundance. Expression of type 5 capsule was increased under conditions of high oxygen tension and reduced in the presence of carbon dioxide (Lee *et al.*, 2000). In addition to environmental factors, the capsular polysaccharides have been found to be positively regulated by the global regulator, *agr* (Lee *et al.*, 2000). The significance of capsular polysaccharide production in staphylococcal disease has been extensively studied. The mucoid capsule was found to contribute to virulence in an intraperitoneal mouse model due to its antiphagocytic

Table 1.1 A summary of toxins secreted by *S.aureus*

Toxin/ gene name	Mechanism of virulence	Location of gene	Association with disease	Regulation
Membrane active toxins				↑=upregulation ↓=down-regulation
α-toxin (*hla*)	Cytolytic pore-forming toxin. Effects erythrocytes, mononuclear immune cells, platelets, epithelial and endothelial cells.	Chromosomal	Cutaneous abscess (Patel *et al.*, 1987) Ocular keratitis (O'Callaghan *et al.*, 1997)	*agr* ↑ *sarA* ↑ *sarH1*↓
β-toxin (*hlb*)	Haemolytic (Hot-cold lysis). Effects sphingomyelin in membranes of erythrocytes.	Chromosomal	Murine mastitis (Foster *et al.*, 1990) Ocular keratitis (O'Callaghan *et al.*, 1997)	*agr* ↑
δ-toxin (*hld*)	Cytolytic pore-forming toxin. Effects a broad sprectrum of cells including erythrocytes and leukocytes.	Located within the *agr* RNAIII transcript		*agr* ↑ *sarA* ↑
Bicomponent toxins				
γ-toxin (*hlg*)	Haemolytic, leukotoxic. Pore-forming toxin.	The *hlg* locus encodes *hlgA*, *hlgB* and *hlgC*. Chromosomal	Endophthalmitis (Supersac *et al.*, 1997)	
Panton Valentine Leukocidin (PVL) (*luk-PV*)	Non-haemolytic, leukotoxic. Lysis of neutrophils and macrophages	The *luk-PV* locus encodes *lukS-PV* and *lukF-PV*	Primary cutaneous infections (Prévost *et al.*, 1995)	
Superantigenic toxins				
Pyrogenic toxins				
TSST-1 (*tsst*-1)	Activation of T cell proliferation and cytokine induction	Carried on pathogenicity islands SAPI-1 and SAPI-2 (Lindsay *et al.*, 1998)	Toxic shock syndrome and Kawasaki disease	*agr* ↑ *sarA* ↑
SEA - J (*entA* - *entJ*)	Stimulation of vomiting centre in brain. Activation of T cell proliferation and cytokine induction	*entA* is carried on a bacteriophage, *entD* on a plasmid and *entB*, *entC* and *entE* are chromosomal (Iandolo, 1989)	Food posioning and Toxic shock syndrome	*entB, entC agr*↑ *ent B sarA* ↑
Exfoliative toxins				
ETA, ETB (*eta*, *etb*)	Esterase activity, splitting of the epidermis	*eta* is chromosmal. *etb* is present on a plasmid	Scalded skin syndrome	*agr* ↑

Table 1.2 A summary of extracellular enzymes of *S.aureus* associated with virulence.

Enzyme / gene name	Activity / substrate	Putative role in virulence	Regulation
Coagulase (*coa*)	Prothrombin activator	Induction of fibrin clots probably protects bacteria from host defense	*agr* ↓
Lipase (*geh*)	Cleaves long chain glycerol esters	Impairment of granulocyte function and generation of FFA* suggested to impair the immune system	*agr* ↑ *sarA* ↓
Nuclease (*nuc*)	Degrades double and single-stranded DNA and RNA		
Staphylokinase (*sak*)	Plasminogen activator	Release of bacteria from fibrin clots or fibrin coated abscesses	*agr* ↑
Proteases			
V8 protease, serine protease (*ssp*)	Glutamic acid specific protease	Immunoglobulin proteolysis, and inactivation of the neutrophil elastase inhibitor. May contribute to evasion of host defenses and tissue degradation. (Arvidson, 2000)	*sarA* ↓ *agr* ↑
Metalloprotease, aureolysin	Cleaves before bulky hydrophobic amino acids. Activates Ssp, activates prothrombin in human plasma and cleaves plasma proteinase inhibitors (Potempa et al., 2000)	unclear	*sarA* ↓
Thiol proteases (3 types identified)	Cleaves most peptide bonds	Type I (12-13 kDa) has strong activity against elastin therefore may be involved in tissue degradation (Arvidson, 2000)	

*FFA – Free fatty acids

properties (Melly *et al.*, 1974). Recent evidence suggests that microcapsule production protects against phagocytosis in a septic arthritis model presumably by masking Fc-receptor and complement sites for PMN recognition (Nilsson *et al.*, 1997).

1.4 Host defense against infections

The first sign of a *S.aureus* invasion is the acute inflammatory response in which polymorphonuclear leukocytes (PMNs), in particular neutrophils, play a major role. The activation of these cells and others such as monocytes, macrophages and lymphocytes involves the production of cytokines and complement factors. Activation of PMNs triggers changes in the surface properties of the cells enabling adherence to activated endothelial cells and migration through the endothelium to the site of infection. PMNs at inflammatory sites have increased phagocytic and cytotoxic capacities (Weiss *et al.*, 2000). Opsonisation of bacteria with antibody and complement factors enables recognition and efficient phagocytosis by PMNs. However, the efficiency of opsonisation can be reduced by the expression of bacterial protein A and capsular polysaccharide (Verhoef, 1997; Weiss *et al.*, 2000). Engulfment by professional phagocytes induces activation of a cellular oxidative response ('respiratory burst') and fusion of cytoplasmic granules with the phagocytic vacuole. The granules contain antibacterial compounds including phospholipase A2 (PLA2), defensins and cathepsin G that can kill staphylococci.

The importance for PMNs to provide host defense against *S.aureus* infections was confirmed in an experimental model of *S.aureus*-induced septic arthritis. Verdrengh *et al.* (1997) demonstrated that depleting mice of PMNs resulted in decreased survival of the mice and increased bacteraemia, indicating the protective role of PMNs against *S.aureus* infections. However, recent evidence suggests that excessive numbers of PMN can also lead to increased bacteraemia as some of the ingested *S.aureus* cells can survive PMN killing and persist within the cell (Gresham *et al.*, 2000). Moreover, this defense mechanism by *S.aureus* appears to be *sar*-dependent, since a *sar*-defective mutant that lacked the expression of several virulence factors was less able to survive and/or avoid clearance in the presence of PMN.

Platelets also have an antimicrobial role particularly in association with endovascular infections. Platelets can internalise bacteria, trigger PMN activation and also release antimicrobial products such as platelet microbicidal proteins (PMPs) and group IIA PLA2 which are cytotoxic toward gram-positive bacteria (Weiss *et al.*, 2000). Although certain strains of *S.aureus* are resistant to PMPs, group IIA PLA2 (an isoform of PLA2) remains an effective antimicrobial agent against most *S.aureus* strains. Encapsulated and nonencapsulated strains are equally sensitive to PLA2. However, bacteria which are macroencapsulated appear to be much more resistant (Weiss *et al.*, 2000). Clumped bacteria, which are largely resistant to phagocytosis, are also sensitive. However, sensitivity is largely dependent on the extent of clumping (Dominiecki and Weiss, 1999). Moreover, extracellular proteases can increase the sensitivity of clumped bacteria to PLA2 by dissolving the proteins holding the bacteria together.

AIMS OF PRESENT STUDY

Park *et al.* (1991) initially demonstrated the specific association of *S.aureus* strain Cowan with soluble elastin. Affinity chromatography, using elastin peptides as the active ligand, identified a 25 kDa protein from *S.aureus* lysostaphin released extracts as the elastin binding protein (EbpS). N-terminal sequencing of the 25 kDa protein enabled the construction of an oligonucleotide probe to identify the *ebpS* gene. The *ebpS* gene was later cloned and sequenced and found to be 609 bp encoding a protein of 202 amino acids (Park *et al.*, 1996). Recombinant EbpS and a polyclonal antibody raised against the recombinant form of the protein inhibited staphylococcal elastin binding suggesting that the 25 kDa molecule represents the cell surface molecule mediating binding of *S.aureus* to elastin. Furthermore, a series of overlapping EbpS fragments and recombinant constructs were used to localise the ligand-binding site to a 21-amino acid region contained within residues 14-34 (Park *et al.*, 1999). This thesis entails a detailed molecular analysis of EbpS. A sequencing error was made in the original publication of the *ebpS* gene. Chapter 2 describes the molecular cloning and sequencing of the full-length *ebpS* gene and the characterisation of the primary structure of the deduced protein sequence. Expression of an N-terminal His-tag fusion protein in *E.coli* is discussed which enabled the generation of polyclonal antibodies for EbpS expression studies. Chapter 3 describes

the construction of mutant strains of *S.aureus* which failed to produce EbpS. Expression studies comparing wild type and isogenic mutant strains allowed the identification of the *ebpS* gene product. Chapter 4 deals with the elastin binding properties of EbpS. The role of EbpS as a membrane bound receptor for soluble elastin is described including its inability to function as an elastin adhesin. Chapter 5 addresses the effect of regulatory loci on EbpS expression at the transcriptional and protein level.

Chapter 2

Cloning and Sequencing of the *ebpS* Locus

2.1 Introduction

When this project was initiated in October 1997 *Staphylococcus aureus* genome sequence data was not publically available. It was only during the second phase of the project that it was possible to analyse the *ebpS* gene and its flanking DNA in a variety of strains. At the outset, the EbpS protein was reported to be a 25 kDa surface protein in SDS-PAGE with an open reading frame of 609 bp. Analysis of the primary structure indicated that the protein lacked a Sec-dependent N-terminal signal sequence. The C-terminus lacked specific sequences such as an LPXTG motif or repeat domains that enable covalently or non-covalently anchored proteins, respectively associate with the cell wall. This led to the hypothesis that EbpS was secreted by a novel mechanism, possibly involving accessory transport proteins such as those of the type III secretion system of Gram-negative bacteria. The lack of an known anchorage domain also suggested that EbpS was associated to the cell surface via a novel mechanism.

The initial objective of the project was to characterise EbpS at the molecular level and subsequently to construct *ebpS*-defective mutants to examine its role as an elastin-binding adhesin and potential virulence factor. The *ebpS* gene was cloned and sequenced from genomic DNA of strain 8325-4. Sequence data predicted an open reading frame that was considerably longer (1461 bp) than what was previously published (609 bp). The 5' end of the 8325-4 *ebpS* sequence (1-600 bp) encoding the ligand-binding domain was as previously reported. Park *et al.* (1996) sequenced the *ebpS* gene from strain Cowan. The *ebpS* locus carried on a genomic fragment from strain Cowan was obtained on a plasmid and sequenced. An *ebpS orf* of 1461 bp was found. It was concluded that a sequencing error had occurred in the published *ebpS* sequence.

This chapter describes the cloning and sequencing of the full-length *ebpS* gene from strain 8325-4 and Cowan and predicts the structure of the EbpS protein.

2.2 Methods

2.2.1 PCR amplification of *ebpS*

The published *ebpS* sequence of *S.aureus* strain Cowan was used to engineer the primers, ebpS1, ebpS2, ebpS3, and ebpS4 (Table 2.3). The positions of the 5' ends of the primers relative to the *ebpS* gene are 59, 605, 1 and 609, respectively where 1 corresponds to the A of the *ebpS* ATG start codon (Figure 2.2 and 2.3). The forward and reverse primers, ebpS1 and ebpS2, which lie within the coding region of the Cowan *ebpS* gene were used to screen several lab strains for the presence of the *ebpS* gene. The exact conditions for amplification were 94°C for 3min, followed by 30 cycles of 94°C for 1min, 48°C for 1min, and 72°C for 1min 30 sec. In order to PCR amplify the 609 bp *ebpS* coding region the forward and reverse primers, ebpS3 and ebpS4 were used. The exact conditions for amplification were 94°C for 3min, followed by 30 cycles of 94°C for 1min, 48°C for 1min, and 72°C for 1min. Primers ebpS3 and ebpS4 were used to generate the 609 bp *ebpS* DIG-labelled probe from strain Cowan. The PCR product was gel purified and DIG-labelled using the PCR DIG-labelling mix from Boehringer Mannheim for use as a probe. PCR reactions were performed using a Perkin-Elmer thermocycler using standard reagents.

2.2.2 Molecular cloning of the *ebpS* locus

Based on the observation that the 609 bp DIG-labelled *ebpS* probe hybridised to a single 5.2 kb PstI genomic fragment from strain 8325-4, a size-selected genomic library in the 5.2 kb region was generated. Genomic DNA from *S.aureus* strain 8325-4 was digested overnight with PstI. A sucrose gradient was used to extract DNA within the 5.2 kb size range (Hartford, 1989). The cloning vector pBluescript KS^+ was digested with PstI and treated with alkaline phosphatase to avoid recircularisation of the vector. Ligation reactions were performed overnight at room temperature. Competent XL1-Blue cells were transformed with the ligation product and plated out on LB agar plates supplemented with ampicillin (100μg/ml) for antibiotic selection, and isopropyl-β-thiogalactopyranoside (IPTG) (0.5mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (40μg/ml) to screen for disruption of the *lacZ* gene in pBluescript KS^+. Colonies were screened by colony blotting using the DIG-labelled 609 bp Cowan specific *ebpS* probe. Plasmid DNA

was purified from positive clones using the Wizard plasmid Mini-prep (Promega), digested with PstI and screened by TAE-agarose gel electrophoresis. The cloned 5.2 kb PstI fragment was designated pKS-5.2.

2.2.3 Sequencing of the *ebpS* locus

Restriction mapping of the plasmid pKS-5.2 enabled the location of the *ebpS* gene within the 5.2 kb genomic DNA insert. The cloned 5.2 kb fragment was digested with EcoRV, yielding a 2.9 kb fragment containing the *ebpS* open reading frame with downstream sequence, which was subcloned into pBluescript KS^+, generating pKS-2.9. The remaining 2.3 kb insert from plasmid, pKS-5.2, containing the upstream sequence of *ebpS*, was religated onto itself generating pKS-2.3. Plasmid DNA (pKS-2.9 and pKS-2.3) used for sequencing was purified using a Qiagen Midi-prep kit. Sequencing reactions were carried out by MWG Biotech (UK). The insert from plasmid pKS-2.9 was sequenced in the forward and reverse directions using the primer walking approach. 1kb of DNA sequence corresponding to the upstream sequence of the *ebpS* gene was generated from the insert of plasmid pKS-2.3 using a reverse primer designed from within the multiple cloning site of pBluescript KS^+.

2.2.4 Analysis of DNA and deduced amino acid sequences

DNA contigs were assembled using the SeqEd® programme, version 1.0.3 (Applied Biosystems Inc.). The Gene Jockey package II (Biosoft) was used to search DNA sequences for endonuclease cleavage sites. The BLAST network service of the National Institute of Health was used to search for homologues in Genbank and protein databases (Altschul *et al.*, 1990). The Predict Protein server (EMBL) was used for secondary structure analysis, Pepstats (UMMS) was used to predict molecular weight and the isoelectric point and ClustalW was used for protein sequence alignments. The Artemis software package (Sanger Centre) was used to examine the *ebpS* locus on large DNA contigs from various *S.aureus* strains being sequenced.

2.2.5 Expression in *E.coli*

2.2.5.1 Cloning of pQE-*ebpS*$_{1-267}$, pQE-*ebpS*$_{1-486}$ and pV4-*ebpS*$_{1-486}$

A 5.2 kb PstI fragment carrying *ebpS* in pBluescript KS$^+$ (30ng) served as a template for PCR reactions. *Pfu* DNA Polymerase was used for maximum accuracy. The entire open reading frame of *ebpS* was PCR-amplified using the forward primer, ebpSNF, and the reverse primer, ebpSNR (Table 2.3). The exact conditions for amplification were 94°C for 3min, followed by 30 cycles of 94°C for 1min, 58°C for 1min, and 72°C for 3min. An 800 bp fragment derived from the 5' end of *ebpS* was PCR-amplified using the forward primer ebpSNF and the reverse primer, ebpSNR$_2$ (Table 2.3). The exact conditions for amplification were 94°C for 3min, followed by 30 cycles of 94°C for 1min, 57°C for 1min, and 72°C for 2min. The ebpSNF primer used in both PCR reactions amplified 6 codons upstream from the Met codon defined by N-terminal sequencing of the 25 kDa form of EbpS purified from *S.aureus*. Thus rEbpS$_{1-486}$ and rEbpS$_{1-267}$ contain 17 extra residues at the N-terminus, the first 11 provided by the pQE30 vector and 6 from the *ebpS* open reading frame that extends upstream from the ATG start codon. The PCR products were digested with BamHI and HindIII and gel-purified. This material was ligated to pQE30 that had been digested with BamHI and HindIII. Competent M15 cells (Qiagen) were transformed with the ligation product and selected by ampicillin (100 μg/ml) and kanamycin (25 μg/ml). *E.coli* strain M15 contains the plasmid pREP4, which constitutively expresses the *lac* repressor at high levels. This allows for stable propagation of expression constructs encoding "toxic" or hydrophobic proteins. pREP4 is maintained in the cell by kanamycin selection. Antibiotic-resistant cells were screened by restriction mapping of the plasmid present and Western blotting was used to detect recombinant protein expression. EbpS was also expressed as a C-terminal His-tag fusion protein using expression vector, pV4 (Van Dyke *et al.*, 1992). The entire *ebpS* open reading frame was PCR amplified using the forward primer, ebpSCF and reverse primer, ebpSCR (Table 2.3). The conditions for amplification were 94°C for 3min, followed by 30 cycles of 94°C for 1min, 49°C for 1min, and 72°C for 3min. The PCR product was digested with BamHI and HindIII, gel-purified and cloned into the BamHI/HindIII site of pV4. The ligation mix was transformed into *E.coli* and positive clones were screened for as described above.

2.2.5.2 Western blotting of *E.coli* cell lysates

Bacteria bearing the expression plasmids pQE-*ebpS*$_{1\text{-}267}$, pQE-*ebpS*$_{1\text{-}486}$ or pV4-*ebpS*$_{1\text{-}486}$ were cultured overnight in 3 ml of LB and diluted by a 1/50 dilution in 10 ml of LB. Strain M15 without the expression plasmid was used as a negative control. Cells were grown until late exponential growth phase (OD_{600} 0.7 – 0.9) before being induced with 1mM IPTG (Biosyth AG) for 4 h at 37°C. A 2 ml aliquot of bacterial cells was pelleted for 5 min at 12,000rpm in a microcentrifuge. Pellets were resuspended in 50µl PBS and an equal volume of 2X final sample buffer. Samples (20µl) were boiled for 5 min and loaded onto a SDS-PAGE gel. Western blotting was performed (as described in Section 3.2.1) using a His probe (INDIA) (1:2000 dilution), anti-EbpS$_{1\text{-}267}$ (1:2000 dilution) or anti-EbpS$_{343\text{-}486}$ (1:1000 dilution) polyclonal antibodies.

2.2.5.3 Purification of rEbpS$_{1\text{-}267}$ and rEbpS$_{1\text{-}486}$

For large scale purification of rEbpS$_{1\text{-}267}$ and rEbpS$_{1\text{-}486}$, a starter culture was grown overnight in 10 ml of LB with ampicillin (100µg/ml) and diluted by a 1/50 dilution into 100 ml of fresh LB with ampicillin. Cells were grown until late exponential growth phase (OD_{600} 0.7 – 0.9) and then induced with 1mM IPTG (Biosyth AG) for 4 h at 37°C. Bacterial cells were pelleted by centrifugation (10 min at 8,000rpm), the pellet was resuspended in 15ml of buffer A (8M Urea, 100mM NaH_2PO_4, 10mM Tris-HCl, pH 8), and gently vortexed for 15min. The lysed cells were then centrifuged at 12,000rpm at 4°C. The supernatant was transferred to a tube containing 4ml of nickel nitriloacetic acid resin (Ni^{2+}-NTA) pre-equilibrated with buffer A and incubated at room temperature for 1h with gentle rotation. The mixture was then transferred to a polypropylene column and washed with 100ml buffer A and buffer B (same as buffer A except pH 6). The tightly bound His-tag fusion protein was then eluted with 10ml buffer C (same as buffer A except pH 4). Fractions containing protein were confirmed by Coomassie blue staining of SDS-PAGE gels. Protein containing fractions were pooled and dialysed twice against 4 litres of PBS at 4°C for 16 h. The yield of rEbpS$_{1\text{-}267}$ and rEbpS$_{1\text{-}486}$ using this protocol was approximately 4mg and 3mg per 100ml of culture, respectively.

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)
Cowan		NCTC 8530	
Cowan Δ*spa*	Δ*spa*::*tet*	DU5889	Sinha *et al*, 1999
Newman		NCTC 8178	Duthie and Lorenz (1952)
FR001	*ebpS*::*erm*	*ebpS*-defective mutant of Newman	This study
FR002	*ebpS*::*erm*	FR001 bearing pCU-*ebpS*$^+$	This study
DU5873 Newman Δ*spa*	Δ*spa*::*tet*	protein A-negative mutant of strain Newman	McDevitt (1995)
FR003	*ebpS*::*erm* , Δ*spa*::*tet*	*ebpS*-defective mutant of DU5873	This study
FR004	*ebpS*::*erm*, Δ*spa*::*tet*	FR003 bearing pCU-*ebpS*$^+$	This study
8325-4 Δ*sar*	*sar*::*kan*	*sar* negative mutant of strain 8325-4	Chan and Foster, 1998b
RN6390 Δ*agr*	*agr*::*tmn*	*agr* negative mutant of strain RN6390	Novick *et al.*, 1993
8325-4 Δ*spa*	*spa*::*kan*	protein A-negative mutant of strain 8325-4	Patel *et al.*, 1989
Newman Δ*spa*	*spa*::*kan*	protein A-negative mutant of strain Newman	This study
Newman Δ*spa* *sar*$^-$	Δ*spa*::*tet sar*::*kan*	*sar*-defective mutant of DU5873	This study
Newman Δ*spa* *agr*$^-$	Δ*spa*::*kan agr*::*tmn*	*agr*-defective mutant of Newman *spa*::*kan*	This study
8325-4		NCTC 8325 cured of prophages	Novick (1967)
8325-4 *fnb*$^-$	*fnbA*::*tet fnbB*::*erm*	*fnbA* and *fnbB*-defective double mutant of 8325-4	Greene *et al.*, 1995
8325-4 *fnb*$^-$ (FnBPA$^+$)	*fnbA*::*tet fnbB*::*erm*	*fnbA* and *fnbB*-defective double mutant of 8325-4 bearing pCU-*fnbA*$^+$	Greene *et al.*, 1995
8325-4 *fnb*$^-$ (FnBPB$^+$)	*fnbA*::*tet fnbB*::*erm*	*fnbA* and *fnbB*-defective double mutant of 8325-4 bearing pCU-*fnbB*$^+$	Greene *et al.*, 1995

Table 2.1 Bacterial strains, continued

Strain	Relevant genotype	Relevant properties	Source/ Reference
DU5875 (8325-4 Δ*spa*)	Δ*spa*::*tet*	Protein A-negative mutant of strain 8325-4	McDevitt (1995)
FR005	*ebpS*::*erm* , Δ*spa*::*tet*	*ebpS*-defective mutant of DU5875	This study
FR006	*ebpS*::*erm*, Δ*spa*::*tet*	FR003 bearing pCU-*ebpS*$^+$	This study
COL		MRSA isolate	
FR017	*ebpS*::*erm*	*ebpS*-defective mutant of COL	This study
FR018	*ebpS*::*erm*,	FR005 bearing pCU-*ebpS*$^+$	This study
P1		Rabbit virulent strain	Sherertz *et al.* (1993)
P1 clfB$^-$	*clfB*::*tet*	*clfB*-defective mutant of P1	F.McAleese (personal communication)
P1 *fnb*$^-$	*fnbA*::*tet fnbB*::*erm*	*fnb*A and *fnb*B-defective double mutant of P1	This laboratory (C.Greene, unpublished)
P1 Δ*spa*	Δ*spa*::*tet*	protein A-negative mutant of strain P1	This study
FR007	*ebpS*::*erm* , Δ*spa*::*tet*	*ebpS*-defective mutant of P1 Δ*spa*	This study
FR008	*ebpS*::*erm*, Δ*spa*::*tet*	FR007 bearing pCU-*ebpS*$^+$	This study
FR009 (Newman Δ*spa*)	Δ*spa*::*tet ebpS-lacZ*	*ebpS-lacZ* transcriptional fusion of strain Newman *spa*::*tet*	This study
FR009b	Δ*spa*::*kan ebpS-lacZ*	*ebpS-lacZ* transcriptional fusion of strain Newman *spa*::*kan*	This study
FR010	Δ*spa*::*tet ebpS-lacZ sar*::*kan*	*sar*-defective mutant of strain FR009	This study
FR011	Δ*spa*::*kan ebpS-lacZ agr*::*tmn*	*agr*-defective mutant of strain FR009b	This study
FR012	Δ*spa*::*kan ebpS-lacZ sigB*::*tet*	*sigB*-defective mutant of strain FR009b	This study

Table 2.1 Bacterial strain, continued

Strain	Relevant genotype	Relevant properties	Source/ Reference
FR013 (8325-4 Δ*spa*)	Δ*spa*::*tet ebpS-lacZ*	*ebpS-lacZ* transcritpional fusion of strain 8325-4 *spa*::*tet*	This study
FR013b	Δ*spa*::*kan ebpS-lacZ*	*ebpS-lacZ* transcritpional fusion of strain 8325-4 *spa*::*kan*	This study
FR014	Δ*spa*::*tet ebpS-lacZ sar*::*kan*	*sar*-defective mutant of strain FR013	This study
FR015	Δ*spa*::*kan ebpS-lacZ agr*::*tmn*	*agr*-defective mutant of strain FR013b	This study
FR016	Δ*spa*::*kan ebpS-lacZ sigB*::*tet*	*sigB*-defective mutant of strain FR013b	This study

Table 2.1 Bacterial strains, continued

Strain	Genotype	Properties	Source
E.coli			
XL1-Blue	[F'*pro*AB, *lacI*^{q} ZΔM15, Tn10(Tc^{r})], *end*A1, *gyr*A96, *hsd*R17, *lac*, *rec*A1, *rel*A1, *sup*E44, *thi*-1	Propagation of plasmids	Stratagene
TOPP-3	[F'*pro*AB, *lacI*^{q} ZΔM15, Tn10(Tc^{r})(Kn^{r})]	Expression of recombinant proteins	Stratagene
M15	(pREP4 *lacI*^{q}) (Kn^{r}) *lacZ*ΔM15, *lac, ara, gal, mtl*	Expression of recombinant proteins	Qiagen, Inc

Table 2.2 List of Plasmids

Plasmid	Features	Marker(s)	Source/ reference
pBluescript KS^+	Used for cloning and sequencing	Ap^{r*}	Stratagene
pUC18/19	Used for cloning and sequencing	Ap^r	New England Biolabs Inc.
pQE30	Expression vector, recombinant proteins fused to polyhistidine tag (N-terminal His-tag)	Ap^r	Qiagen Inc.
pQE-*ebpS*$_{1\text{-}267}$	pQE30 containing the 5' end of the *ebpS* gene corresponding to residues 1-267.	Ap^r	This work
pQE-*ebpS*$_{1\text{-}486}$	pQE30 containing the entire *ebpS* gene	Ap^r	This work
pV4	Expression vector, recombinant proteins fused to polyhistidine tag (C-terminal His-tag)	Ap^r	Van Dyke *et al.*, 1992
pV4-*ebpS*$_{1\text{-}486}$	pV4 containing the entire *ebpS* gene	Ap^r	This work
pCU1	Shuttle vector derived from pC194 and pUC19, 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 the 8325-4 *ebpS* gene plus 300 bp upstream and 200 bp downstream sequence.	Ap^r in *E.coli*, Cm^r in *S.aureus*	This work
pTS2	Derived from pTV1$_{ts}$ (Youngman, 1987). Cm^r (from pC194) linked tp *rep*$_{ts}$ from pE194$_{ts}$, with multiple cloning site from pGem 7Z (f)+, Ts replication, replicates in *S.aureus*	Cm^r	This laboratory (C.O'Connell, unpublished)
pFR1	pUC-18 containing 2.9 kb PCR-amplified *ebpS* locus from pKS-5.2 with insertion of a unique SacII site in the middle of the *ebpS* gene	Ap^r in *E.coli*,	This work
pFR2	Insertion of *ermC* from pE194 into the SacII site of *ebpS* on pFR1	Ap^r in *E.coli*,	This work

Table 2.2 List of plasmids, continued

Plasmid	Features	Marker(s)	Source/Reference
pFR3	pTS2 containing pFR2	Ap^r in E.coli, Em^{r***} and Cm^r in S.aureus	This work
pKS-800	pBluescript KS^+ containing 800 bp of 5' ebpS sequence	Ap^r	This work
pKS-5.2	pBluescript KS^+ containing ebpS on a 5.2 kb 8325-4 genomic fragment	Ap^r	This work
pKS-2.3	pBluescript KS^+ containing 2.3 kb ebpS upstream DNA	Ap^r	This work
pKS-2.9	pBluescript KS^+ containing ebpS and downstream DNA on a 2.9 kb fragment	Ap^r	This work
pUC-4.2	pUC-19 containing ebpS on a 4.2 kb Cowan genomic fragment	Ap^r	Park et al., 1996
pQE-rTE	pQE-30 containing N-terminal truncate of bovine tropoelastin (exons 1-15).	Ap^r	R.P. Mecham, (personal communication)
pAZ106	Promoterless transcriptional lacZ fusion vector	Ap^r in E.coli, Em^r in S.aureus	Kemp et al., 1991
pAZ-ebpS	1kb BamH1/EcoR1 fragment PCR-amplified from pKS-5.2 containing the 5' end of the ebpS gene and upstream sequence in BamH1/EcoR1 pAZ106	Ap^r in E.coli, Em^r in S.aureus	This study

*Ap^r: resistance to ampicillin **Cm^r: resistance to chloramphenicol

*** Em^r: resistance to erythromycin

Table 2.3 List of primers

Primer name	Primer sequence
ebpSNF	5'-CGCGGATCCATAGAAAGGAAG**G**TGGCT**G**TG-3'
ebpSNR	5'-CCCAAGCTTTTATGGAATAACGATTTGTTGACC-3'
ebpSNR$_2$	5'-CCCAAGCTTTTACCACTTATGATGATCTTTAGA-3'
ebpSCF	5'-CCCAAGCTTGATGTCTAATAATTTTAAAGATG-3'
ebpSCR	5'-CGCGGATCCTGGAATAACGATTTGTTGAC-3'
ebpS1	5'-CACATCAAGACCATACGG-3'
ebpS2	5'-CACTTATGATGATCTTTAG-3'
ebpS3	5'-CGGGATCCATGTCTAATTTTAAAGATG –3'
ebpS4	5'-CGGAATTCTTACCACTTATGATGATC –3'
mutF1	5'-CCCAAGCTTTGAATGGTTGCTGCCGTTTC-3'
mutR1	5'-TCCCCGCGGGATGTTTCTGATTGGTTAGCAC-3'
mutF2	5'-TCCCCGCGGCTGGTGCAATGGCTGCTTC-3'
mutR2	5'-CCGGAATTCCTTCTCGGTAATAAACTAGCG-3'
ermCF	5'-TCCCCGCGGAGGTGTCACAAGACACTC-3'
ermCR	5'- TCCCCGCGGAGCTCGTGCTATAATTATAC-3'
compF	5'-CCGGAATTCGGATCAATGTAGACGGAAG-3'
compR	5'-CCCAAGCTTGACAAGACCTTGTTAAATAAC-3'
sarF	5'-AAAGCGTTGATTTGGGTAGTA-3'
sarR	5'-AGTGCCATTAGTGCAAAACCT-3'

- The A of the two ATG codons in the forward primer, ebpSNF, was changed to G (in boldface lettering) to avoid internal initiation of translation as recommended by Qiagen.
- The following restriction sites used are underlined : SacII site CCGCGG, HindIII AAGCTT and EcoRI site GAATTC, BamH1 site GGATCC.

2.3 Results

2.3.1 PCR analysis of the *ebpS* locus

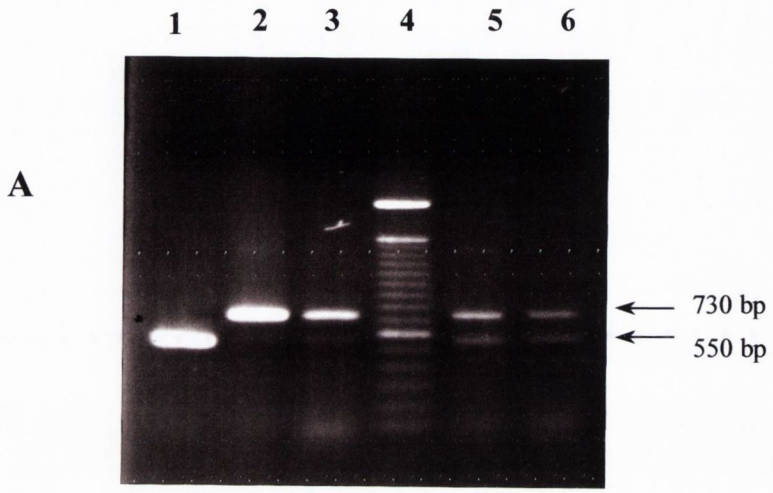
The *ebpS* gene of *S.aureus* strain Cowan was reported to be 609bp in length and to encode a protein of 202 amino acids (Park *et al.*, 1996). Using PCR primers (ebpS1 and ebpS2), which lie within the published Cowan *ebpS* coding region (Figure 2.2), the *ebpS* gene from several laboratory strains was amplified. Each strain showed two PCR products of approximately 550 bp and 730 bp with the exception of Cowan, which gave a single PCR product of 550 bp (Figure 2.1). In order to elucidate if the *ebpS* gene was duplicated in these strains, the larger 730 bp PCR product was extracted from the agarose gel and used as a template in a second PCR reaction under the same conditions. Again two fragments of the same size were observed suggesting a second primer-binding site in a single larger *ebpS* gene (Figure 2.2).

2.3.2 Cloning and sequencing of pKS800

In order to elucidate if the *ebpS* gene in strain 8325-4 was larger than the Cowan *ebpS* gene, the larger PCR product defined by the second primer-binding site was cloned and sequenced. Using PCR primers which flank the published Cowan *ebpS* coding region (ebpS3 and ebpS4; Table 2.3, Figure 2.3) the *ebpS orf* was amplified from strain 8325-4. Two PCR products of 609 bp and approximately 800 bp were observed. The 800 bp PCR product was digested with BamHI and EcoRI , gel-purified and cloned into the BamHI-EcoRI site of pBluescript KS^+ generating pKS800 (Table 2.2). The ligation mix was transformed into *E.coli* XL1-Blue cells and purified plasmids were screened by restriction mapping and Southern blotting using a DIG-labelled probe derived from the 609 bp *ebpS* fragment of strain Cowan (Section 2.2.1). The 800 bp plasmid insert was sequenced in both directions using standard primers flanking the pBluescript KS^+ multiple cloning site. Sequence data predicted an *ebpS* open reading frame of 787 bp in length. The translated product of the 8325-4 *ebpS* gene sequence was 93% identical to the Cowan protein sequence although it appeared to be longer than the Cowan *ebpS* gene of 609 bp. An 18 bp DNA repeat motif at positions 591-609 bp and 769-787 bp of 8325-4 *ebpS* overlapped the reverse primer-annealing sites thus explaining the generation of two PCR products (Figure 2.3). An alignment of the first repeat motif between the two

Figure 2.1 A. PCR based screening for the *ebpS* gene.

The *ebpS* gene was PCR amplified from several laboratory strains using primers ebpS1 and ebpS2 designed from within the Cowan *ebpS* coding region. Lane1, Cowan; lane 2, 8325-4; lane 3, Newman; lane 4, 100 bp ladder; lane 5, M60; lane 6 P10. B. PCR amplification of the larger 730 bp PCR product under the same conditions. Lane 1, 8325-4; lane 2, Newman; lane 3, 100 bp ladder; lane 4, M60; lane 5, P10.


1
2
3
4
5
6
A
730 bp
550 bp

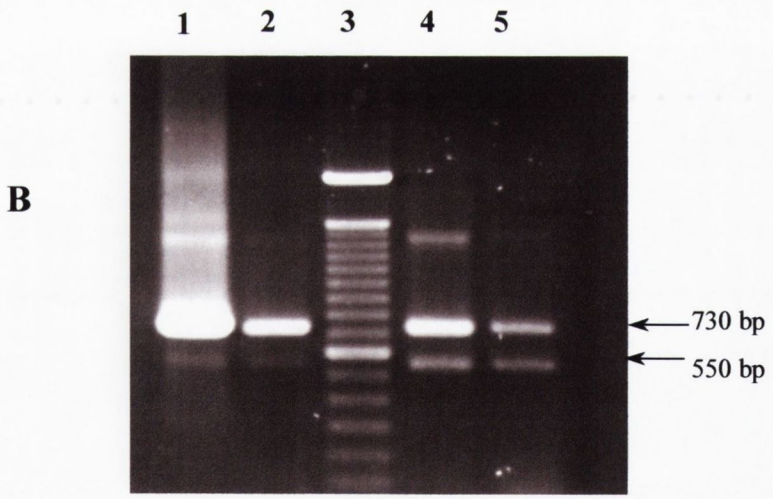
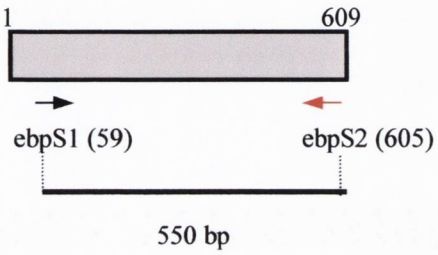

1
2
3
4
5
B
730 bp
550 bp

Figure 2.2 An illustration of the PCR analysis of the *ebpS* locus.

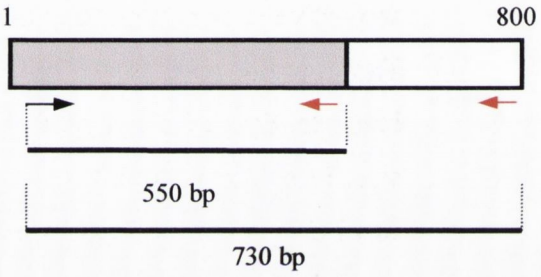
Forward (black arrow) and reverse (red arrow) primers, ebpS1 and ebpS2, respectively, were used to amplifiy the *ebpS* gene from strains Cowan and 8325-4. The coordinates of these primers relative to the *ebpS* sequence are marked in brackets. Strain Cowan yielded a single PCR product of approximately 550 bp in size. In contrast, strain 8325-4 contained a second primer annealing site yielding two PCR products of approximately 550 bp and 730 bp in size. Two scenarios are possible: (i) a second reverse primer-annealing site or (ii) a second forward primer annealing site is located in 8325-4 *ebpS*.

PCR of Cowan *ebpS* sequence



(i)

PCR of 8325-4 *ebpS* sequence



(ii)

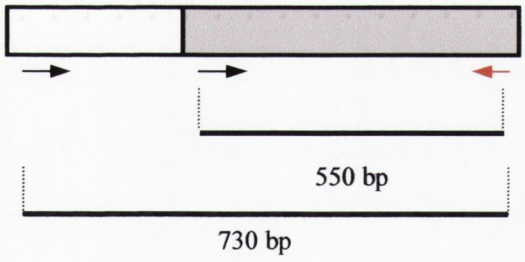
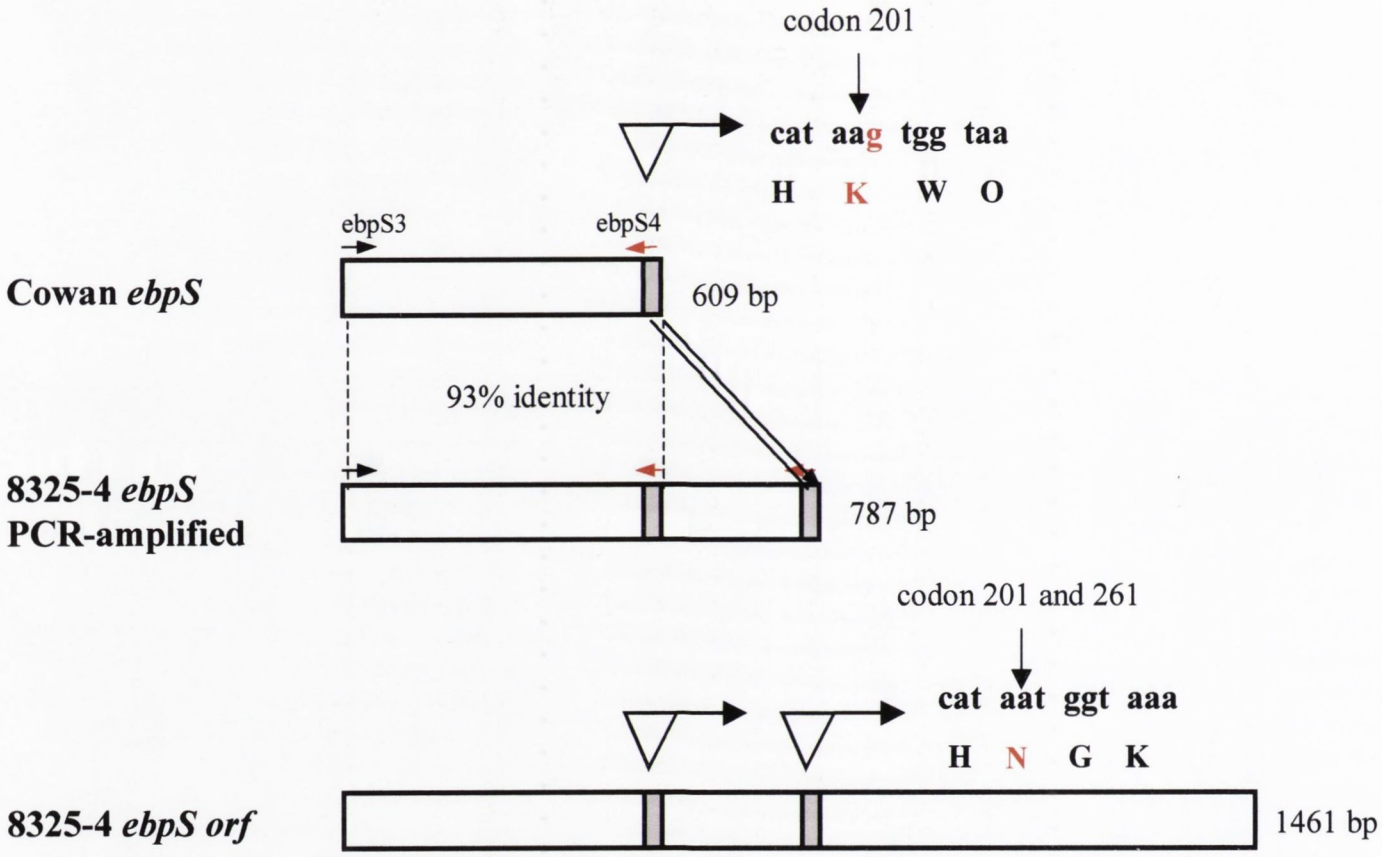


Figure 2.3 Illustration of the sequencing error in the published Cowan *ebpS* sequence. The small arrows represent the forward (black arrow) and reverse (red arrow) primers, ebpS3 and ebpS4, respectively and the sites to which they bind. The grey boxes depict the 18 bp DNA repeat motif resulting in the second reverse primer-annealing site. The published sequence of the Cowan *ebpS* gene contains an insertion of a single guanidine nucleotide at codon 201 resulting in a frameshift and premature termination of the gene two codons downstream. Sequence analysis of the PCR amplified *ebpS* gene of strain 8325-4 predicted an *orf* of 787 bp due to the incorporation of the extra guanidine nucleotide from the reverse primer, ebpS4, resulting in premature translational termination. Sequence analysis of the *ebpS* locus from 8325-4 genomic DNA predicted an *orf* of 1461 bp in length. The absence of the extra guanidine nucleotide in codons 201 and 261 allows translation to continue.


codon 201
cat aag tgg taa
H K W O
ebpS3
ebpS4
Cowan ebpS
609 bp
93% identity
8325-4 ebpS
PCR-amplified
787 bp
codon 201 and 261
cat aat ggt aaa
H N G K
8325-4 ebpS orf
1461 bp

strains revealed the absence of a single guanidine nucleotide at codon 201 in strain 8325-4 which enabled translation to continue, suggesting a difference in the *ebpS* gene of strain 8325-4 and Cowan.

2.3.3 Cloning and sequencing of the *ebpS* gene

It was suggested that the 8325-4 *ebpS* gene is larger than the Cowan *ebpS* gene. However, previous sequencing was carried out from DNA which was PCR-amplified using Cowan *ebpS*-specific primers. To determine the true length of the *ebpS* gene, the gene present on a genomic fragment was cloned and sequenced from strain Cowan and 8325-4. Southern blotting was used to identify a 5.2 kb PstI genomic fragment from strain 8325-4 bearing the *ebpS* gene (Figure 2.4). The 5.2 kb PstI fragment was cloned into pBluescript KS^+ as described in Section 2.2.2. Restriction mapping identified a unique EcoRV site in the 5.2 kb insert and allowed subcloning to form two smaller plasmids, pKS-2.9 and pKS-2.3 (Figure 2.5). pKS-2.9, containing the *ebpS* open reading frame and some downstream DNA, was sequenced. Interestingly, this revealed an *ebpS* open reading frame of 1461 bp encoding a protein of 486 residues, which is significantly larger than the previous prediction of 202 amino acids (Figure 2.6). The Cowan *ebpS* locus was then sequenced from plasmid pUC-4.2 (provided by Dr. Robert P. Mecham). An *orf* of 1461 bp was also found. When translated the predicted protein was 95% identical to that of strain 8325-4. It was concluded that a sequencing error had been made in the original publication of the Cowan *ebpS* sequence. The addition of a single guanidine nucleotide at codon 201 resulted in a frameshift, causing the *orf* to terminate out of frame at codon 203 (Figure 2.3). The ebpS4 primer, designed from the published Cowan sequence, contained the extra guanidine nucleotide explaining the incorrect prediction of a 787 bp *ebpS orf* from the PCR-amplified fragment. The TIGR, Oklahoma and Sanger sequencing projects have recently determined the genomic sequences of strains COL, 8325 and eMRSA-16, respectively. The *ebpS* gene in these strains is consistent with the length of *ebpS* in strains 8325-4 and Cowan.

The inserts in plasmid pKS-2.3 and pKS-2.9 were sequenced to generate sequence data upstream and downstream, respectively of the *ebpS orf* of strain 8325-4. The BLAST network service of the National Institute of Health was used to search for homologues in Genbank and EMBL protein databases (Altschul *et al.*, 1990). The data predicted that the *ebpS* gene is flanked by genes encoding proteins with strong

Figure 2.4 Southern blotting of the *ebpS* locus of strain 8325-4. Genomic DNA was digested with various enzymes and probed with a DIG-labelled 609bp fragment from the 5′ end of the Cowan *ebpS* gene. Lane1, *Kpn*I; lane 2, *Eco*RV; lane 3, *Eco*RI; lane 4, *Hind*III; lane 5, *Bgl*II; lane 6, size standards; lane 7, *Nsi*I; lane 8, *Xba*I; lane 9, *Pst*I; lane 10, *Sma*I.

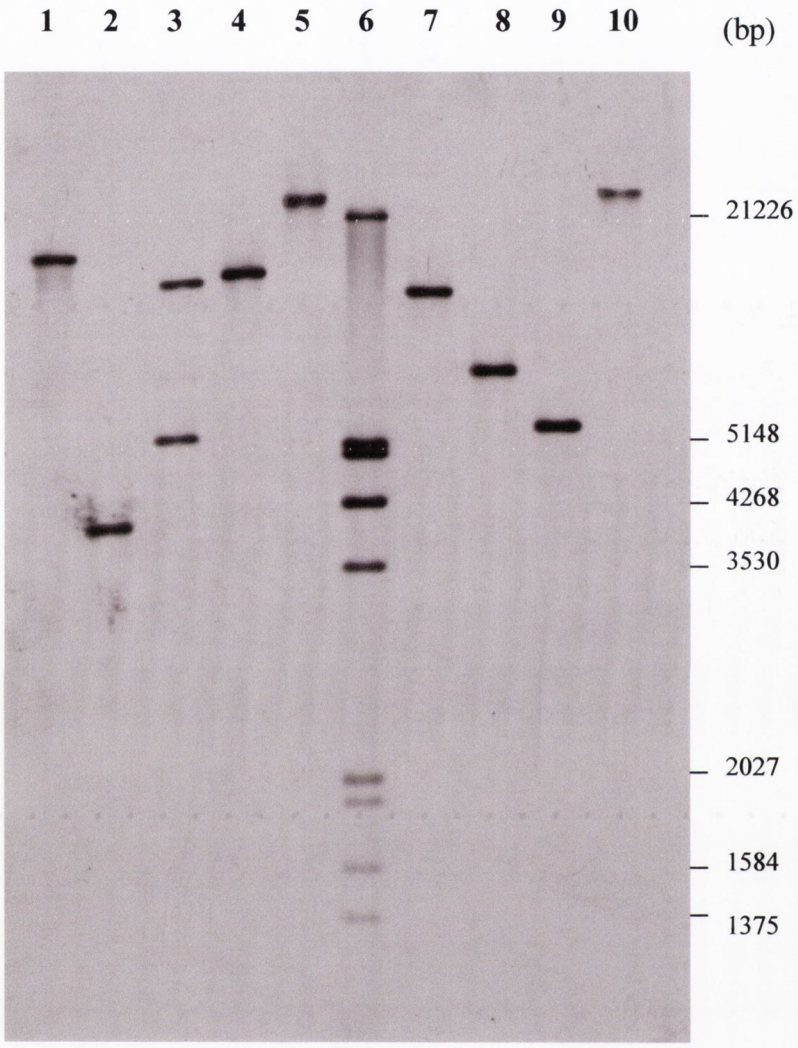

1
2
3
4
5
6
7
8
9
10
(bp)
21226
5148
4268
3530
2027
1584
1375

Figure 2.5 Cloning of the *ebpS* locus from strain 8325-4. The *ebpS* locus, present on a 5.2 kb PstI fragment, was cloned from genomic DNA of strain 8325-4 into pBluescript KS^+. A 2.9 kb EcoRV fragment containing the *ebpS orf* with some downstream sequence was subcloned into pBluescript KS^+ and sequenced. A 2.3 kb EcoRV fragment containing some *ebpS* upstream sequence data was subcloned into pBluescript KS^+ and 1 kb of DNA proximal to *ebpS* was sequenced.

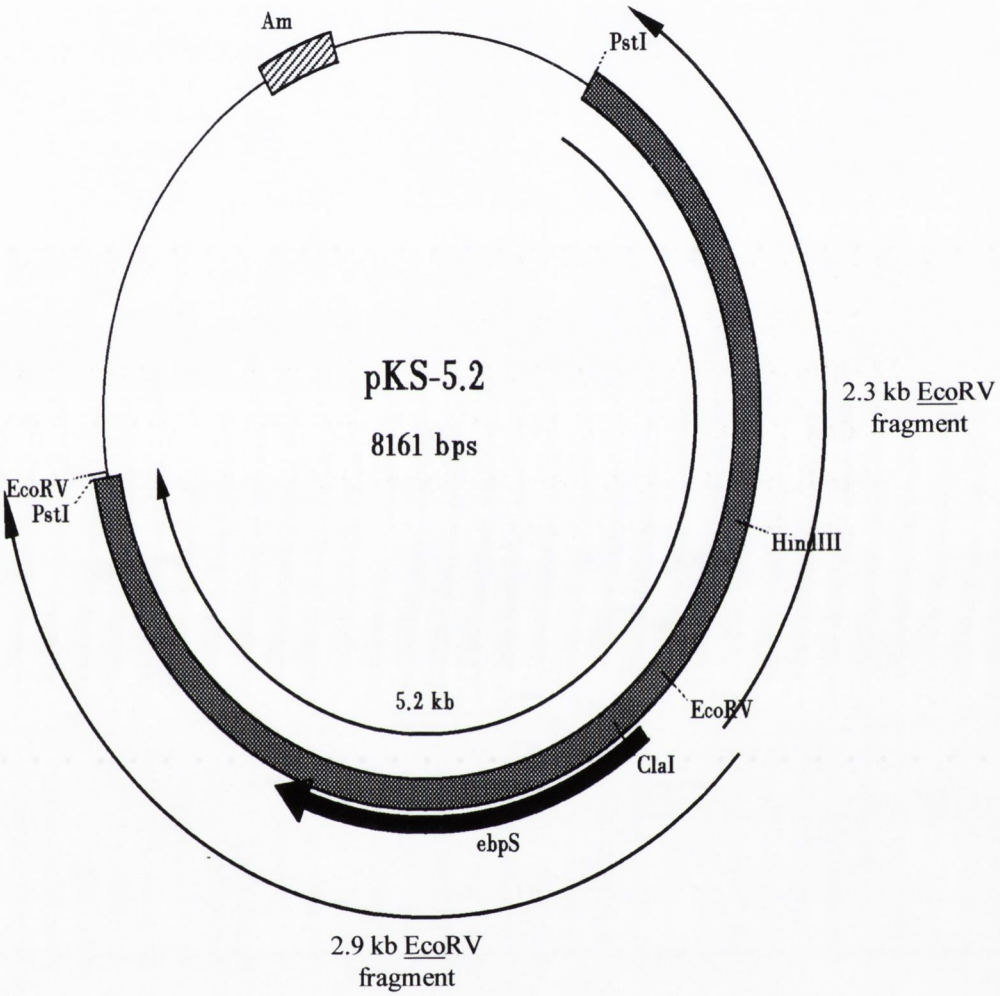

Am
PstI
pKS-5.2
8161 bps
2.3 kb EcoRV
fragment
EcoRV
PstI
HindIII
5.2 kb
EcoRV
ClaI
ebpS
2.9 kb EcoRV
fragment

Figure 2.6 Sequence of *ebpS* and flanking DNA, and translation of the open reading frame. The amino acid sequence is numbered starting at the translation initiation codon. The putative –35 and –10 hexamers and ribosomal binding site are underlined. The start codon is indicated in bold and the ligand-binding domain corresponding to residues 14-34 is underlined. The positions of the LysM module and the three hydrophobic domains (H1, H2 and H3) are highlighted including the six amino acid repeat motif preceding H1 and H2 (underlined). The upstream sequence which bears homology to the putative SarA binding site consensus sequence (Chien *et al.*, 1999) is marked in red.

```
                                                -35                  -10
GATTGAAATAATTTTGAAAAATATACATAAACATATGTCATGTGGGTATATTTTATGTAA

                          RbS
AATCATTGTAATAGAATAGAAAGGAAGATGGCTATGTCTAATAATTTTAAAGATGACTTT
                                                M  S  N  N  N  F  K  D  D  F    9

GAAAAAAATCGTCAATCGATAGACACAAATTCACATCAAGACCATACGGAAGATGTTGAA
 E  K  N  R  Q  S  I  D  T  N  S  H  Q  D  H  T  E  D  V  E                   29

AAAGACCAATCAGAATTAGAACATCAGGATACAATAGAGAATACGGAGCAACAGTTTCCG
 K  D  Q  S  E  L  E  H  Q  D  T  I  E  N  T  E  Q  Q  F  P                   49

CCAAGAAATGCCCAAAGAAGAAAAAGACGCCGTGATTTAGCAACGAATCATAATAAACAA
 P  R  N  A  Q  R  R  K  R  R  R  D  L  A  T  N  H  N  K  Q                   69

GTTCACAATGAATCACAAACATCTGAAGACAATGTTCAAAATGAGGCTGGCACAATAGAT
 V  H  N  E  S  Q  T  S  E  D  N  V  Q  N  E  A  G  T  I  D                   89

GATCGTCAAGTCGAATCATCACACAGTACTGAAAGTCAAGAACCTAGCCATCAAGACAGT
 D  R  Q  V  E  S  S  H  S  T  E  S  Q  E  P  S  H  Q  D  S                  109

ACACCTCAACATGAAGAGGAATATTATAATAAGAATGCTTTTGCAATGGATAAATCACAT
 T  P  Q  H  E  E  E  Y  Y  N  K  N  A  F  A  M  D  K  S  H                  129

CCAGAACCAATCGAAGACAATGATAAACACGATACTATTAAAAATGCAGAAAATAACACT
 P  E  P  I  E  D  N  D  K  H  D  T  I  K  N  A  E  N  N  T                  149

GAGCATTCAACAGTTTCTGATAAGAGTGAAGCTGAACAATCTCAGCAACCTAAACCATAT
 E  H  S  T  V  S  D  K  S  E  A  E  Q  S  Q  Q  P  K  P  Y                  169

TTTACAACAGGTGCTAACCAATCAGAAACATCAAAAAATGAACATGATAATGATTCTGTA
 F  T  T  G  A  N  Q  S  E  T  S  K  N  E  H  D  N  D  S  V                  189

                                                      H1>
AAACAAGATCAAGATGAACCTAAAGAACATCATAATGGTAAAAAAGCAGCAGCTATTGGT
 K  Q  D  Q  D  E  P  K  E  H  H  N  G  K  K  A  A  A  I  G                  209

GCTGGAACAGCAGGTGTTGCAGGTGCAGCTGGTGCAATGGCTGCTTCTAAAGCTAAGAAA
 A  G  T  A  G  V  A  G  A  A  G  A  M  A  A  S  K  A  K  K                  229

CATTCAAATGACGCTCAAAACAAAAGTAATTCTGGCAAGGCGAATAACTCGACTGAGGAT
 H  S  N  D  A  Q  N  K  S  N  S  G  K  A  N  N  S  T  E  D                  249

                                                      H2>
AAAGCGTCTCAAGATAAGTCTAAAGATCATCATAATGGCAAAAAAGGTGCAGCGATCGGT
 K  A  S  Q  D  K  S  K  D  H  H  N  G  K  K  G  A  A  I  G                  269

GCTGGAACAGCAGGTTTGGCTGGAGGCGCAGCAAGTAAAAGTGCTTCTGCCGCTTCAAAA
 A  G  T  A  G  L  A  G  G  A  A  S  K  S  A  S  A  A  S  K                  289

CCACATGCCTCTAATAATGCAAGCCAAAACCATGATGAACATGACAATCATGACAGAGAT
 P  H  A  S  N  N  A  S  Q  N  H  D  E  H  D  N  H  D  R  D                  309

                   H3>
AAAGAACGTAAAAAAGGTGGCATGGCCAAAGTATTGTTACCATTAATTGCAGCTGTACTA
 K  E  R  K  K  G  G  M  A  K  V  L  L  P  L  I  A  A  V  L                  329
```

```
ATTATCGGTGCATTAGCGATATTTGGAGGCATGGCATTAAACAATCATAATAATGGTACA
 I  I  G  A  L  A  I  F  G  G  M  A  L  N  N  H  N  N  G  T  349

AAAGAAAATAAAATCGCGAATACAAATAAAAATAATGCTGATGAAAGTAAAGACAAAGAC
 K  E  N  K  I  A  N  T  N  K  N  N  A  D  E  S  K  D  K  D  369

ACATCTAAAGACGCTTCTAAAGATAAATCAAAATCTACAGACAGTGATAAATCAAAAGAG
 T  S  K  D  A  S  K  D  K  S  K  S  T  D  S  D  K  S  K  E  389

GATCAAGACAAAGCGACTAAAGATGAATCTGATAATGATCAAAACAACGCTAATCAAGCG
 D  Q  D  K  A  T  K  D  E  S  D  N  D  Q  N  N  A  N  Q  A  409

AACAATCAAGCACAAAATAATCAAAATCAACAACAAGCTAATCAAAATCAACAACAGCAA
 N  N  Q  A  Q  N  N  Q  N  Q  Q  Q  A  N  Q  N  Q  Q  Q  Q  429

                              LysM>
CAACAACGTCAAGGTGGTGGCCAAAGACATACAGTGAATGGTCAAGAAAACTTATACCGT
 Q  Q  R  Q  G  G  G  Q  R  H  T  V  N  G  Q  E  N  L  Y  R  449

ATCGCAATTCAATACTACGGTTCAGGTTCACCGGAAAATGTTGAAAAAATTAGACGTGCC
 I  A  I  Q  Y  Y  G  S  G  S  P  E  N  V  E  K  I  R  R  A  469

AATGGTTTAAGTGGTAACAATATTAGAAACGGTCAACAAATCGTTATTCCATAATATAAC
 N  G  L  S  G  N  N  I  R  N  G  Q  Q  I  V  I  P  *        486

TATATAAATTGTAACTGAACTGCTATATACAAACGTGCATATTATAAGTATCTCTAAATA

TTTTGGAGAACTTAAATTGCGCTTATAAGTATGTAGCGGTTTTTTCATTTTTCAAAGTTT

GTTATTTAACAAGGTCTTGTCTCGAATATTGGCATATCAATTTAACTTTTTAAATAGTCA

TCAAAAAGATAAAACACCACAATCAACAAATTTAACGAGGAAGAATAAAAAATAAATCAA
```

similarities to YpdA (64% identity) and RecQ (34% identity) of *Bacillus Subtilis* (Figure 2.11). YpdA is a thioredoxin reductase homologue and RecQ is a DNA unwinding protein. In strain 8325-4 *ebpS* is separated from its neighbouring genes by 255 bp and 400 bp of non-coding sequence downstream and upstream from the *ebpS orf*, respectively. This implies that *ebpS* may be monocistronic however this would need to be verified by transcript mapping. The *ebpS* upstream sequence is very AT-rich. Park *et al.* (1996) identified putative –10 and –35 hexamers at positions –31 and –54, with a spacing of 17 base pairs and a potential ribosome binding site at position –7 (Figure 2.6).

2.3.4 The EbpS protein of *S.aureus*

The translation product of the *ebpS* gene is 486 residues in length with a predicted molecular weight of 53.2 (Pepstats). EbpS has a deduced pI of 6.26 and is devoid of cysteine residues. Analysis of the primary structure of EbpS showed that the protein lacks many of the common features of Gram-positive surface adhesins such as, (i) an N-terminal signal peptide, and (ii) a conserved C-terminal LPXTG motif (required for anchoring the protein to the cell wall) (Figure 2.7). Like many Gram-positive surface adhesins, the ligand-binding domain of EbpS is located in the amino-terminal region exposed on the cell surface. The domain has been localised to a 21-amino acid region contained within residues 14-34 of EbpS (Park *et al.*, 1999). Examination of the amino acid sequence of EbpS identified three large hydrophobic domains in the centre of the molecule, H1 (residues 205-224), H2 (residues 265-280), and H3 (residues 315-342). Hydropathy analysis (Kyte and Doolittle, 1982) yielded values of 1.9, 1.7 and 3.6 implying that each domain has potential to span the cytoplasmic membrane. However, membrane protein prediction algorithms only detected the larger domains H1 and H3 as putative transmembrane regions with an α-helix secondary structure. It is interesting to note that the first two hydrophobic domains are similar (75% residue identity) and are both preceded by six identical amino acid residues (HHNGKK) (Figure 2.6). A corresponding similarity at the DNA level explains the generation of two PCR products using the reverse primer ebpS2.

The BLAST network service of the National Institutes of Health was used to search for sequence homologies. The Brookhaven Protein Data Bank, Genbank™,

EMBL and SWISSPROT protein databases were screened. A conserved domain of 48 residues in length similar to the LysM domain occurs at the extreme C-terminus of the protein. The LysM domain is a widespread module which has been proposed to have a general peptidoglycan binding function (Joris *et al.*, 1992). It was originally identified in bacterial lysins therefore termed the LysM domain for the lysin motif (Ponting *et al.*, 1999). It is predominantly found in cell wall degrading enzymes presumably to anchor the catalytic domains to their substrates. However, it is also found in other bacterial proteins which are cell wall or membrane associated such as *S.aureus* protein A and intimin and MltD of *E.coli*. LysM domains can be found as a single module or repeated at either the N- or C-termini of proteins (Figure 2.8). The alignments of the LysM domains from several proteins showed that EbpS has highest similarity with YpbE of *Bacillus subtilis* with 25 identities for 48 aligned amino acids (52% identical) (Figure 2.9). Recent studies have reported the structure of this domain as having a beta-alpha-alpha-beta secondary structure with the two helices packing onto the same side of an anti-parallel beta sheet (Bateman and Bycroft, 2000). Secondary structure prediction analysis of the entire EbpS molecule predicts a protein with 29% helix, 6 % extended (β-sheet) and 64.4% loop structures.

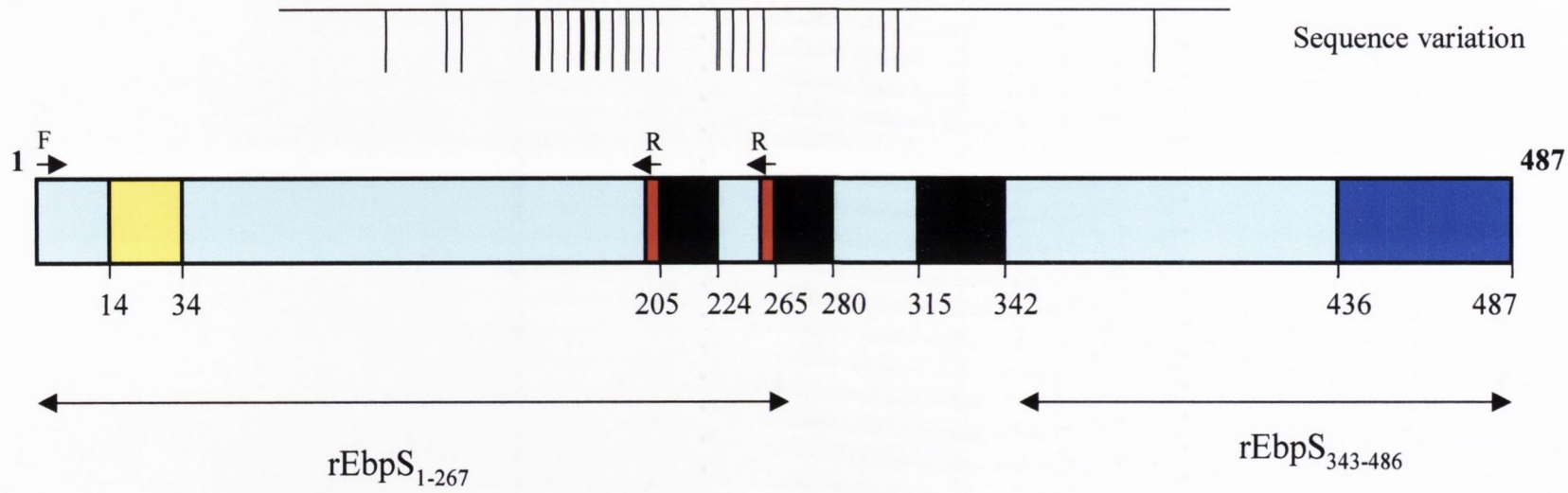
2.3.5 EbpS sequence variation among strains

A comparative alignment of the EbpS protein sequence from strains COL, Cowan, eMRSA-16, and 8325-4 was performed. Figure 2.10 shows an alignment of EbpS from strains 8325-4 and Cowan. The Cowan and 8325-4 EbpS proteins have 25 residue differences (95% identity). Most of the differences (16/25) are clustered between residues 171-205 (also illustrated in the bar diagram in Figure 2.7). The hydrophobic (domains H2 and H3), LysM and ligand binding domains are identical between strains. The hydrophobic domain H1 is 85% identical between strains. The EbpS protein of strain COL is identical to that of 8325-4 (and 8325), whereas the Cowan protein is identical to that of eMRSA-16.

2.3.6 The *ebpS* locus

With the recent availability of several *S.aureus* genome sequences it was possible to examine the *ebpS* locus in different strains and to extend the analysis of flanking sequence. The *ebpS* locus was mapped on a 30 kb contig from the genome

Figure 2.7 Illustration of the primary structure of the EbpS protein. The black boxes located in the centre of the moleculerepresent the hydrophobic domains. The hydropathy plot below ascribes values to each domain. The red bars preceding the first two hydrophobic domains depict the six amino acid repeat and the binding sites in *ebpS* DNA for the reverse primers (R) ebpS2 and ebpS 4 (black arrows). The position of the forward primer (F) ebpS 3 is also marked by a black arrow. The yellow box indicates the ligand binding domain of EbpS located in a 21-amino acid region and the dark blue box at the C-terminus represents the 48 residue LysM domain. The bar above the diagram shows the positions of the sequence variation between strains Cowan and 8325-4.



Hydropathy plot of EbpS (Kyte-Doolittle, 1982)

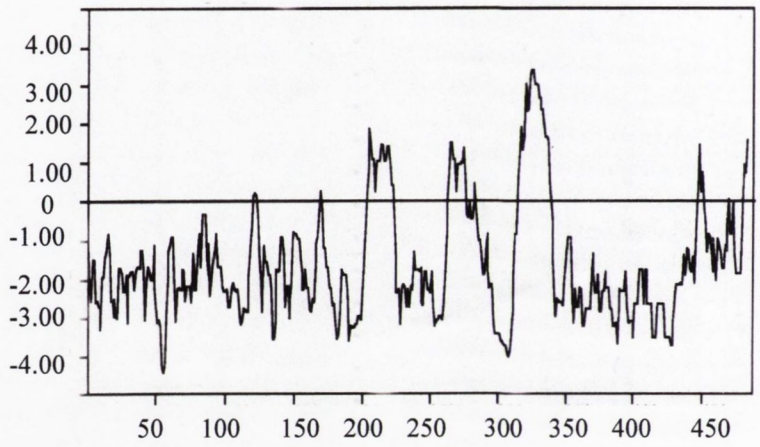


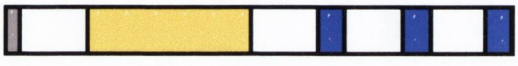
Figure 2.8 Schematic representation of the domain architectures of representative proteins containing the LysM domain. The domain has been found as a single module or repeated at either the N- or C-terminus of proteins and is proposed to function as a peptidoglycan binding domain.

MltD [452 aa]

E.coli

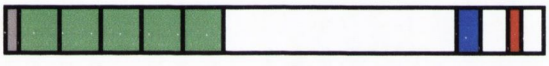
Alys (Autolysin) [670 aa]

Enterococcus faecalis



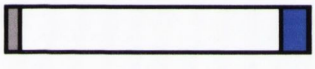
AcmA (N-acetylmuramidase) [437 aa]

Lactococcus lactis



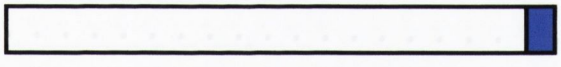
Spa [472 aa]

Staphylococcus aureus



YpbE [270 aa]

Bacillus subtilis



EbpS [486 aa]

Staphylococcus aureus

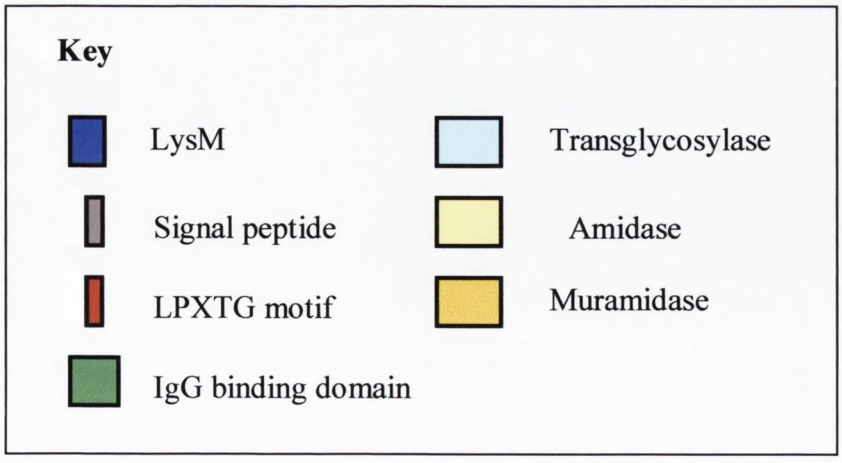


Figure 2.9 Multiple sequence alignment of the conserved residues of the LysM domains between strains. Conserved residues are denoted in red (100% identity), and boxed (67-83% identity).

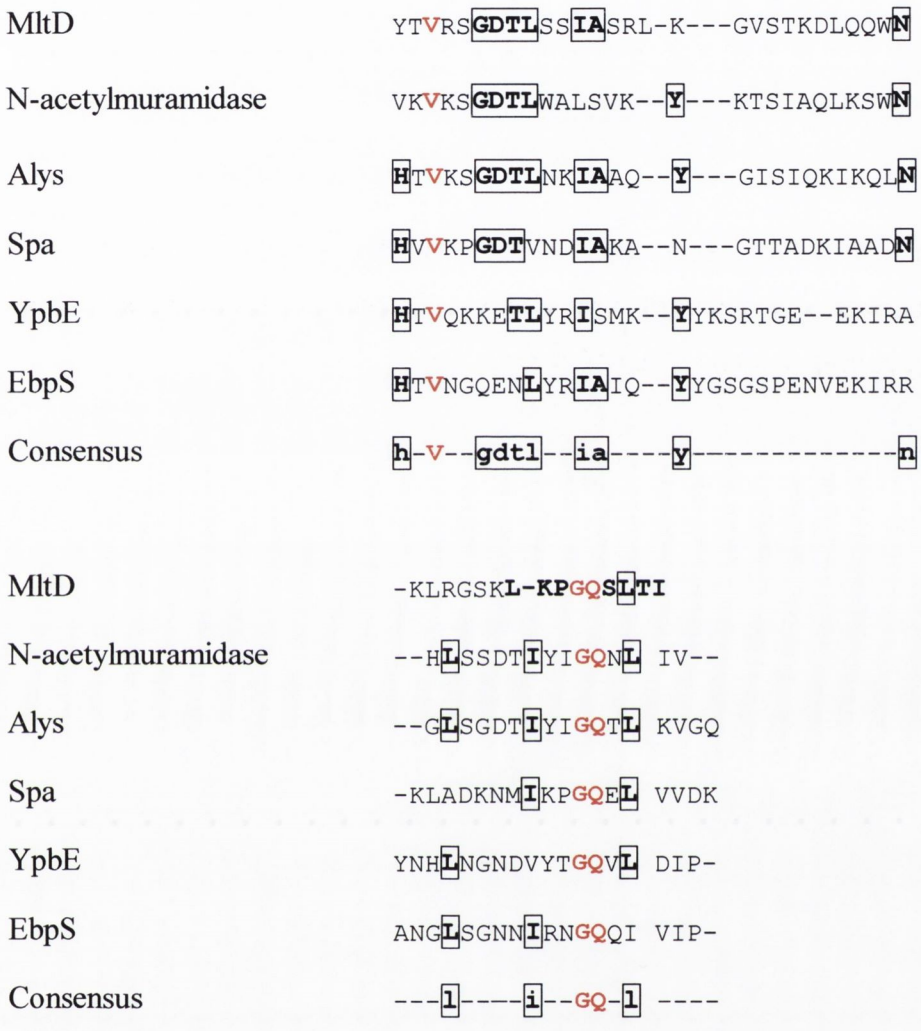

MltD
YTVRSGDTLSSIASRL-K---GVSTKDLQQWN
N-acetylmuramidase
VKVKSGDTLWALSVK--Y---KTSIAQLKSWN
Alys
HTVKSGDTLNKIAAQ--Y---GISIQKIKQLN
Spa
HVVKPGDTVNDIAKA--N---GTTADKIAADN
YpbE
HTVQKKETLYRISMK--YYKSRTGE--EKIRA
EbpS
HTVNGQENLYRIAIQ--YYGSGSPENVEKIRR
Consensus
h-V--gdtl--ia----y----------n
MltD
-KLRGSKL-KPGQSLTI
N-acetylmuramidase
--HLSSDTIYIGQNL IV--
Alys
--GLSGDTIYIGQTL KVGQ
Spa
-KLADKNMIKPGQEL VVDK
YpbE
YNHLNGNDVYTGQVL DIP-
EbpS
ANGLSGNNIRNGQQI VIP-
Consensus
---l----i--GQ-l ----

Figure 2.10 An alignment of the EbpS protein sequence of strains 8325-4 and Cowan. The shaded grey box and underlined region represent the ligand binding domain and LysM domain, respectively. The open boxes denote the three large hydrophobic domains in the centre of the molecule. The locations of the sequence variation between strains are highlighted in red.

```
8325-4    1 MSNNFKDDFE KNRQSIDTNS HQDHTEDVEK DQSELEHQDT IENTEQQFPP
Cowan     1 MSNNFKDDFE KNRQSIDTNS HQDHTEDVEK DQSELEHQDT IENTEQQFPP

8325-4   51 RNAQRRKRRR DLATNHNKQV HNESQTSEDN VQNEAGTIDD RQVESSHSTE
Cowan    51 RNAQRRKRRR DLATNHNKQV HNESQTSEDN VQNEAGTIDD RQVESSHSTE

8325-4  101 SQEPSHQDST PQHEEEYYNK NAFAMDKSHP EPIEDNDKHD TIKNAENNTE
Cowan   101 SQEPSHQDST PQHEEGYYNK NAFAMDKSHP EPIEDNDKHE TIKEAENNTE

8325-4  151 HSTVSDKSEA EQSQQPKPYF TTGANQSETS KNEHDNDSVK QDQDEPKEHH
Cowan   151 HSTVSDKSEA EQSQQPKPYF ATGANQANTS KDKHDDVTVK QDKDESKDHH

8325-4  201 NGKKAAAIGA GTAGVAGAAG AMAASKAKKH SNDAQNKSNS GKANNSTEDK
Cowan   201 SGKKGAAIGA GTAGVAGAAG AMGVSKAKKH SNDAQNKSNS GKVNNSTEDK

8325-4  251 ASQDKSKDHH NGKKGAAIGA GTAGLAGGAA SKSASAASKP HASNNASQNH
Cowan   251 ASEDKSKEHH NGKKGAAIGA GTAGLAGGAA SNSASAASKP HASNNASQNN

8325-4  301 DEHDNHDRDK ERKKGGMAKV LLPLIAAVLI IGALAIFGGM ALNNHNNGTK
Cowan   301 DEHDHHDRDK ERKKGGMAKV LLPLIAAVLI IGALAIFGGM ALNNHNNGTK

8325-4  351 ENKIANTNKN NADESKDKDT SKDASKDKSK STDSDKSKED QDKATKDESD
Cowan   351 ENKIANTNKN NADESKDKDT SKDASKDKSK STDSDKSKDD QDKATKDESD

8325-4  401 NDQNNANQAN NQAQNNQNQQ QANQNQQQQQ QRQGGGQRHT VNGQENLYRI
Cowan   401 NDQNNANQAN NQAQNNQNQQ QANQNQQQQQ QRQGGGQRHT VNGQENLYRI

8325-4  451 AIQYYGSGSP ENVEKIRRAN GLSGNNIRNG QQIVIP
Cowan   451 AIQYYGSGSP ENVEKIRRAN GLSGNNIRNG QQIVIP
```

of strain eMRSA-16 (Sanger Centre) and 8325 (Oklahoma). Putative open reading frames were screened for homologues using the BLAST search tool. The same *ebpS* flanking genes were identified as in strain 8325-4 and variations in the length of the non-coding regions were observed in strain eMRSA-16. In this strain 235 bp and 120 bp of non-coding sequence occur downstream and upstream from *ebpS,* respectively. The 120 bp upstream sequence is identical between strains and the 110 bp downstream sequence is 96% identical between strains.

The gene order of 11 kb surrounding *ebpS* in strains 8325 and eMRSA-16 showed strong similarity to the gene order in the *recQ-cmk* region of *Bacillus subtilis* and a region of *Enterococcus faecalis* (Figure 2.11) suggesting a conservation across the genera. YbpE (*Bacillus subtilis*) and Alys (*Enterococcus faecalis*) showed 52% and 33% identity, respectively, to the C-terminal domain of EbpS. Further sequence homology was not apparent however a single hydrophobic domain of 15 residues was identified in the N-terminal region of YpbE. Hydropathy analysis (Kyte and Doolittle, 1982) yielded a value of 3.7 suggesting that this domain may traverse the membrane (Figure 2.12). Alys is an N-acetylmuramoyl-L-alanine-amidase, whereas the function of YpbE has not yet been determined. A comparative analysis of genes flanking *ebpS*, *ypbE* and *alyS* revealed organisational conservation between strains. Interestingly, a search for homology to EbpS against known genes in GenBank/EMBL and Swissprot databases identified that the Aas surface protein of *Staphylococcus saprophyticus* shows 21% identity (39% positives) to the N-terminus (residues 1-202) of EbpS (Figure 2.12). The region of homology on Aas was localised to the N-terminus of the molecule (residues 140-361) bearing 4/7 of the 40 amino acid repeats. Aas is a cell wall associated surface protein of *S.saprophyticus* which functions as an autolysin and adhesin (Hell *et al.*, 1998). The protein possesses two distinct enzymatic activities including an N-acetylmuramyl-L-alanine amidase and a glucosaminidase. The central domains interconnecting the enzymatically active domains is responsible for fibronectin binding and haemaglutinating activities. The N-terminus of the protein contains seven 40 amino acid contiguous repeats. These repeats are cell wall located but their function is unknown. It has been proposed that they function as peptidoglycan binding domains (Hell *et al.*, 1998).

Figure 2.11 A comparative alignment of the *ebpS* locus from *S.aureus* with DNA from other Gram-positive bacteria. A comparative analysis of the *ebpS* locus showed a conservation of gene order between different Gram-positive species. Within this locus the functions of two proteins are known. Cmk is a cytidine monophosphate kinase and RecQ is a DNA unwinding protein. A Blast analysis was performed of the remaining protein sequences from *Bacillus subtilis* to identify homologs and assign a putative function to these proteins. YpfD is homologous to the 30S ribosomal protein S1, YphC is homologous to a GTP-binding protein and YpdA is homologous to a thioredoxin reductase. *ebpS*, *ypbE* and *alyS* are located in the centre of this alignment and share a LysM domain which is encoded at the 3' end of the gene (marked in black on *alyS* and *ypbE*). The following web sites were used to extract the genomic sequence data: genolist.pasteur.fr/SubtiList/ ; www.sanger.ac.uk/Projects/S_aureus; www.genome.ou.edu/staph.html; www.tigr.org/tdb/mdb/mdbinprogress.html and www.vge.ac.uk.html.

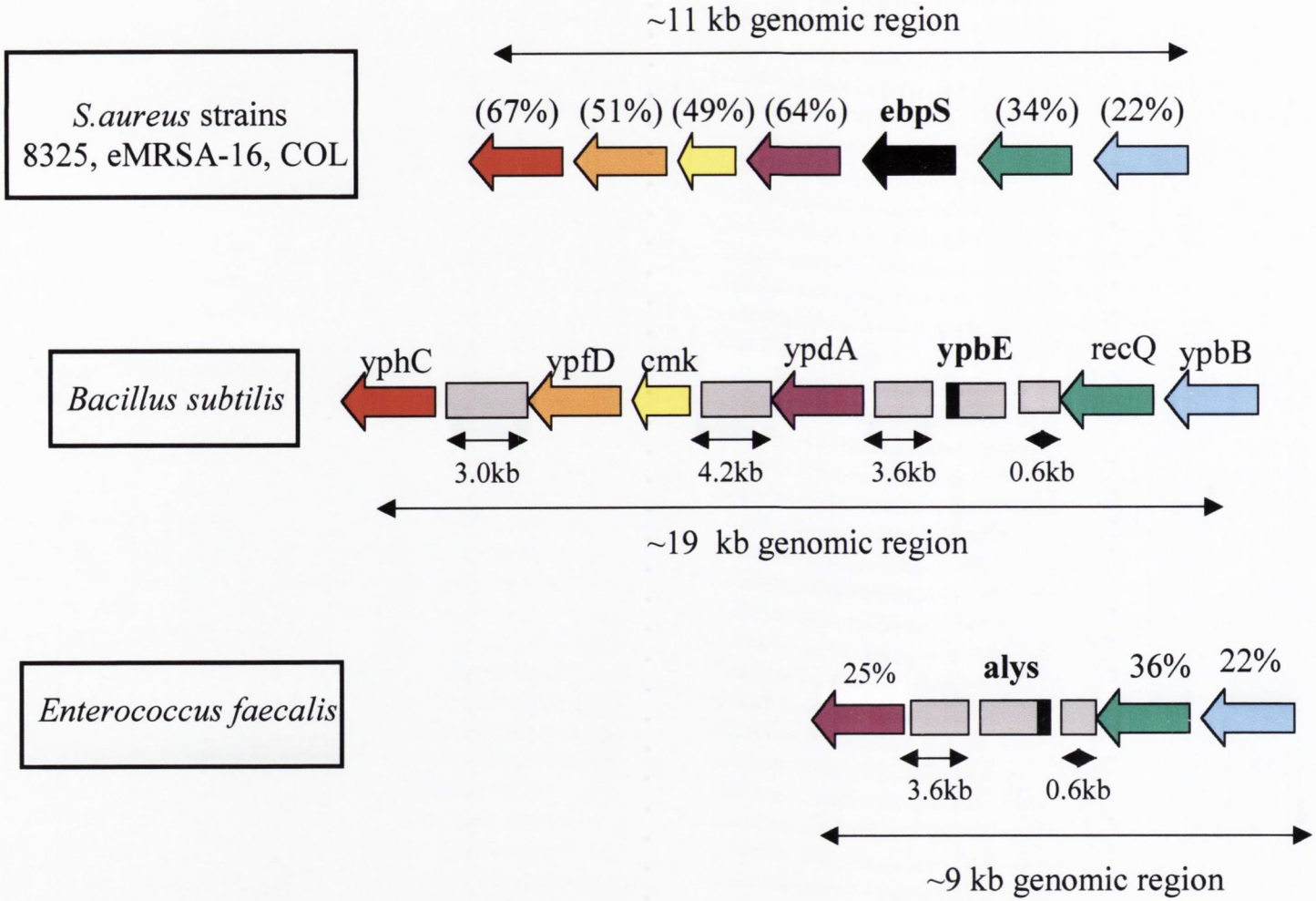
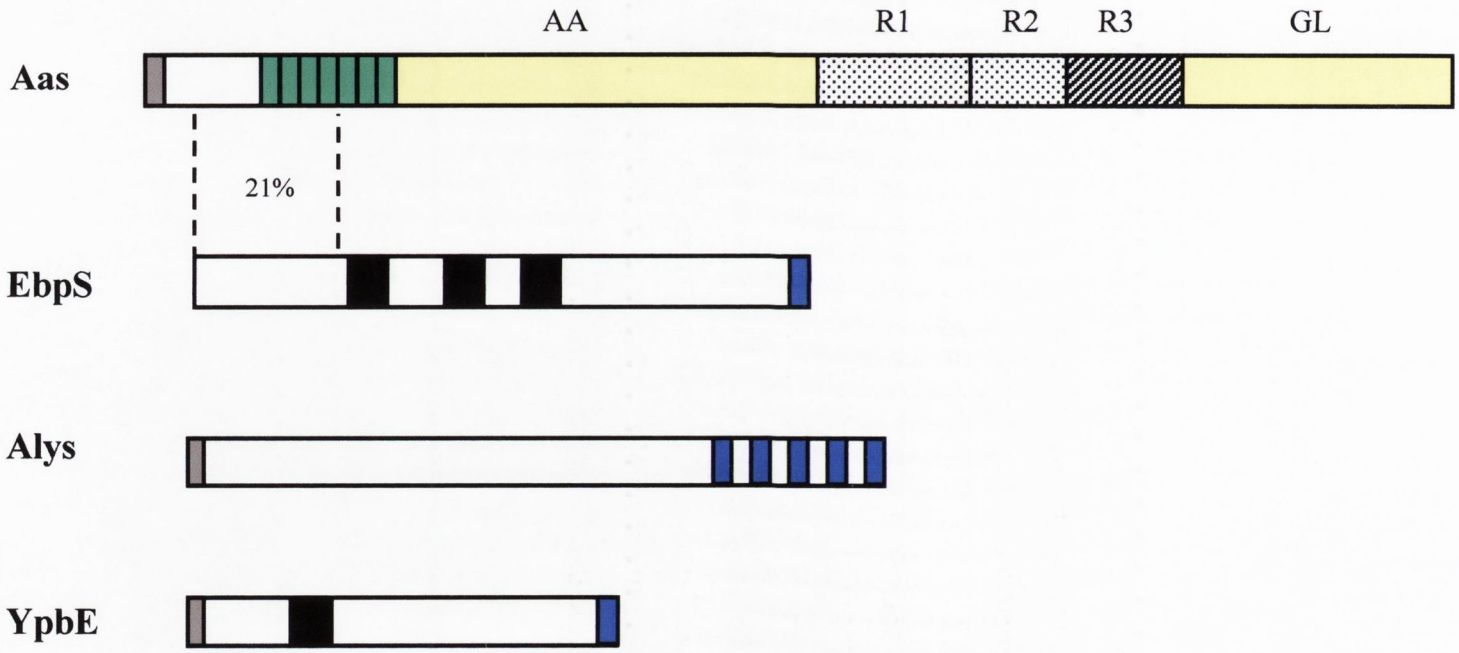

~11 kb genomic region
S.aureus strains
8325, eMRSA-16, COL
(67%)
(51%)
(49%)
(64%)
ebpS
(34%)
(22%)
Bacillus subtilis
yphC
ypfD
cmk
ypdA
ypbE
recQ
ypbB
3.0kb
4.2kb
3.6kb
0.6kb
~19 kb genomic region
Enterococcus faecalis
25%
alys
36%
22%
3.6kb
0.6kb
~9 kb genomic region

Figure 2.12 Alignment of proteins showing homology to EbpS. The black and blue boxes correspond to the hydrophobic and LysM domains, respectively. Aas of *S.saprophyticus* is composed of a signal peptide (grey box), 7 contiguous N-terminal repeats (green boxes), an alanine amidase (AA) and a glucosaminidase (GL). Regions R1-R3 connecting the enzymatic centres contain the ligand binding acitivity. The entire N-terminal domain of EbpS is 21% homologous to the N-terminus of Aas. EbpS (*S.aureus*), Alys (*Enterococcus faecalis*) and YbpE (*Bacillus subtilis*) are located on a chromosomal locus that is conserved between species. These proteins share the same position in this locus and all bear the LysM domain at the C-terminus of the protein. YpbE also contains a single hydrophobic domain in the N-terminus although the function of this protein is unknown.


AA
R1
R2
R3
GL
Aas
21%
EbpS
Alys
YpbE

2.3.7 Expression of *ebpS* in *E.coli*

2.3.7.1 rEbpS$_{1-267}$

A DNA fragment corresponding to N-terminal residues 1-267 of EbpS was PCR-amplified from strain 8325-4 and cloned into the expression vector pQE-30. The N-terminal truncate was expressed in *E.coli* strain M15 as an N-terminal His-tag fusion protein. Due to the presence of a single hydrophobic domain in this protein, rEbpS$_{1-267}$ was insoluble upon purification under standard conditions. Therefore, purification of rEbpS$_{1-267}$ from *E.coli* extracts was carried out under denaturing conditions using Ni^{2+}-NTA chromatography followed by slow dialysis in PBS buffer (Section 2.2.5.3). rEbpS$_{1-267}$ migrates as a 58kDa band on an SDS-PAGE gel (Figure 2.13). Purified rEbpS$_{1-267}$ was then used to generate polyclonal antibodies in rabbits, (R.Downer, personal communication).

2.3.7.2 rEbpS$_{1-486}$

The generation of a full-length rEbpS construct was initiated for mass spectroscopy analysis. The entire 1461 bp *orf* of *ebpS* from strain 8325-4 was PCR-amplified and cloned into the expression vectors pQE-30 and pV4 to generate N and C terminal His-tag fusion proteins, respectively. The recombinant proteins were then expressed in *E.coli* (M15). Western blotting of M15 cell lysates, using His probe and polyclonal antibodies directed against the N or the C-terminus of EbpS, (anti-EbpS$_{1-267}$ and anti-EbpS$_{342-486}$), identified rEbpS migrating on a SDS-PAGE gel as an 83kDa band (Figure 2.14). Native EbpS released from protoplasts of strain Newman Δ*spa* migrated at the same size as the recombinant protein (see chapter 3). The full-length recombinant protein appeared to be susceptible to proteolytic breakdown. This occurred at the C-terminus of the protein as indicated (i) by the loss of the His-tag from the C-terminal fusion protein and (ii) by the inability of anti-EbpS$_{343-486}$ antibodies to recognise the breakdown products detected by anti-EbpS$_{1-267}$ antibodies. Mass spectroscopy was therefore abandoned as (i) the purified N-terminal fusion protein contained high molecular weight breakdown products which were difficult to separate (Figure 2.13) and (ii) the His-tag was cleaved from the C-terminal fusion protein preventing purification by Ni^{2+}-NTA chromatography.

Figure 2.13 Coomassie staining of purified $rEbpS_{1-267}$ (A) and $rEbpS_{1-486}$ (B).

The entire *ebpS* gene and an 800 bp fragment from the 5' end were PCR amplified and expressed in *E.coli* as N-terminal His-tag fusion proteins $rEbpS_{1-486}$ and $rEbpS_{1-267}$, respectively. The recombinant proteins were purified from *E.coli* extracts under denaturing conditions using Ni^{2+}-NTA chromatography and fractionated by 10% (w/v) SDS- PAGE and stained by Coomassie Brilliant Blue. Lanes 2-11 correspond to a 5μl sample of the 0.5 ml fractions 1-10 eluted from the column. Size standards are indicated in lane 1.

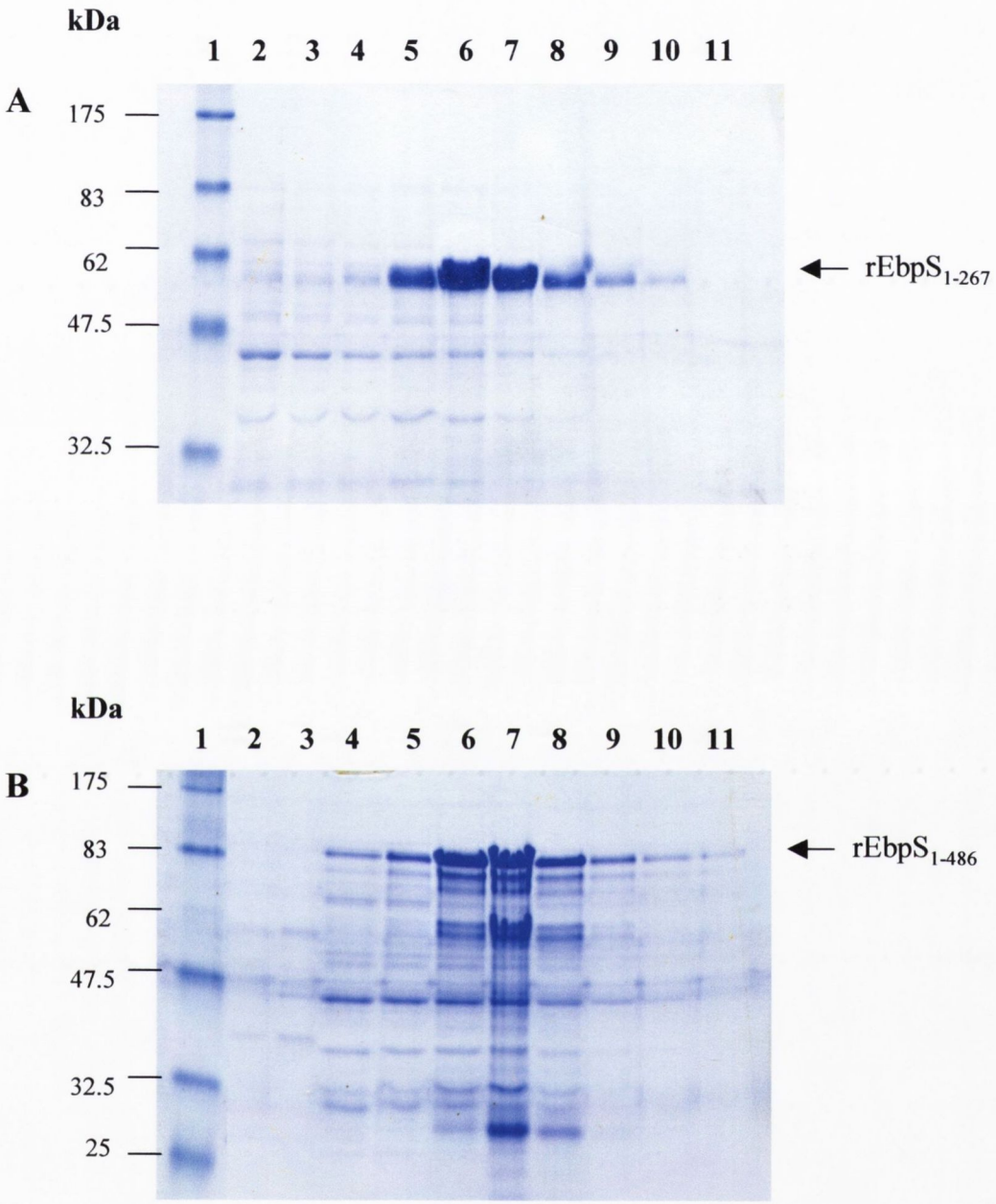
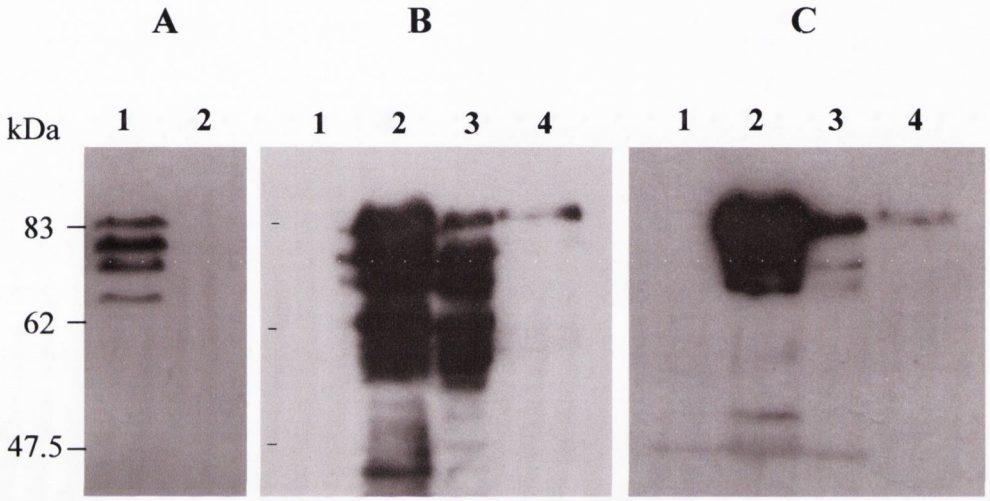

kDa
1 2 3 4 5 6 7 8 9 10 11
A
175
83
62
47.5
32.5
rEbpS1-267
kDa
1 2 3 4 5 6 7 8 9 10 11
B
175
83
62
47.5
32.5
25
rEbpS1-486

Figure 2.14 Western immunoblotting of rEbpS expressed as a His-tag fusion protein in *E.coli* strain M15. rEbpS was expressed as an N-terminal and C-terminal His-tag fusion protein from the expression plasmids pQE-ebpS$_{1-486}$ and pV4-ebpS$_{1-486}$, respectively. Expression of EbpS was induced for 4hr with IPTG. rEbpS was detected by Western blotting of cell lysates using a His probe (**A**) and polyclonal antibodies directed against the N-terminus (anti-EbpS$_{1-267}$) (**B**) or the C-terminus (anti-EbpS$_{342-486}$) (**C**) of EbpS. **A**, lane 1, M15 pQE-ebpS$_{1-486}$; lane 2, M15 pV4-ebpS$_{1-486}$. **B** and **C**, lane 1, M15 ; lane 2, M15 pQE-ebpS$_{1-486}$; lane 3, M15 pV4-ebpS$_{1-486}$; lane 4, lysostaphin released EbpS from *S.aureus* strain Newman. Size standards are indicated on the left hand side.


A
B
C
kDa
1
2
1
2
3
4
1
2
3
4
83
62
47.5

2.4.1 Discussion

The gene encoding the elastin binding protein, EbpS, of *S.aureus* strain Cowan was originally reported to be 609 bp in length. However, cloning and sequencing of the *ebpS* locus on a 5.2 kb genomic fragment from 8325-4 and the 4.2 kb fragment of strain Cowan demonstrated that the *ebpS* gene in fact comprises 1461 bp encoding an EbpS protein of 486 residues. A sequencing error was made in the original publication of the Cowan *ebpS* sequence at codon 201. This resulted in a translational frameshift leading to apparent termination two codons downstream. Park *et al.* (1996) identified the *ebpS* gene product as a 25 kDa surface protein. Since the protein size complemented the proposed 609 bp gene the sequencing data was not questioned. However, the 25kDa protein originally reported as the mature EbpS protein is likely to be an N-terminal truncate of the full-length 83 kDa protein (discussed in chapter 3).

The EbpS protein of strain 8325-4 was found to be very similar in sequence to that of strain Cowan (95% residue identity). The majority of residue differences were located between the ligand binding domain and H1. It was within the DNA that encodes this region that the reverse primers *ebpS*2 and *ebpS*4 used in the PCR analysis of *ebpS*, were designed (residues 196-203). This explains the difference in PCR amplification of *ebpS* between strains Cowan and 8325-4 using primer *ebpS*2. Since the primer was designed from the Cowan published sequence, strain Cowan amplified a strong PCR product of approximately 550 bp in size. The primer annealing site in strain 8325-4 was only 88% identical explaining the weaker PCR product. In addition, the *ebpS*2 primer sequence was located in DNA encoding a six amino-acid repeat motif (HHNGKK) generating two primer annealing sites (residues 196-202 and 267-272). In strain 8325-4, the second primer-binding site was 94% identical to the primer sequence and therefore yielded a strong PCR product of approximately 730 bp. Strain Cowan was unable to amplify a PCR product, presumably because the sequence was only 83% identical.

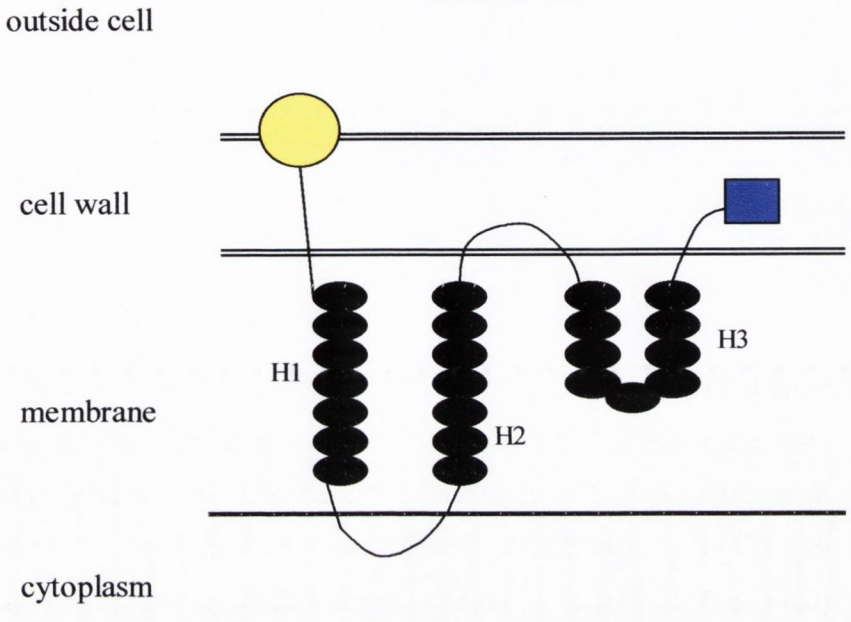
In this chapter the primary structure of full-length EbpS is reported. The absence of an N-terminal signal sequence and an LPXTG motif suggests a protein quite distinct from previously described cell-wall associated proteins of Gram-positive bacteria. Several mechanisms for displaying proteins at the surface of Gram-positive bacteria have been described. Each mechanism is identified by the presence

of particular structural domains in the protein sequence. EbpS showed no similarity to the covalently anchored LPXTG proteins. However, the presence of three large hydrophobic domains, which were sufficiently long to function as membrane spanning anchors, suggested a membrane anchored protein. Hydropathy analysis supported this hypothesis and yielded values of approx 1.9, 1.7 and 3.6 for the three domains which are above the minimum expected value of 1.6 for a membrane spanning domain (Kyte and Doolittle, 1982). Recently, Downer (personal communication) has formally demonstrated that EbpS is a membrane-associated protein. Previous studies have proposed that the N-terminus of the protein is surface exposed (confirmed later in this study) and Downer (personal communication) has shown using PhoA fusions that the C-terminal domain, bearing the LysM module, is cell wall associated. Based on this data, possible models of the topography of EbpS have therefore been proposed (Figure 2.15). The first model predicts that domains H1 and H2 span the membrane while domain H3 is membrane-associated but forms a helical hairpin via a single proline residue which allows the C-terminus to be associated with the cell wall. Membrane and secondary structure prediction algorithms only detected domains H1 and H3 as transmembrane helices. Therefore the second model predicts that domains H1 and H3 traverse the membrane while domain H2 is only loosely associated with the inner cytoplasmic membrane.

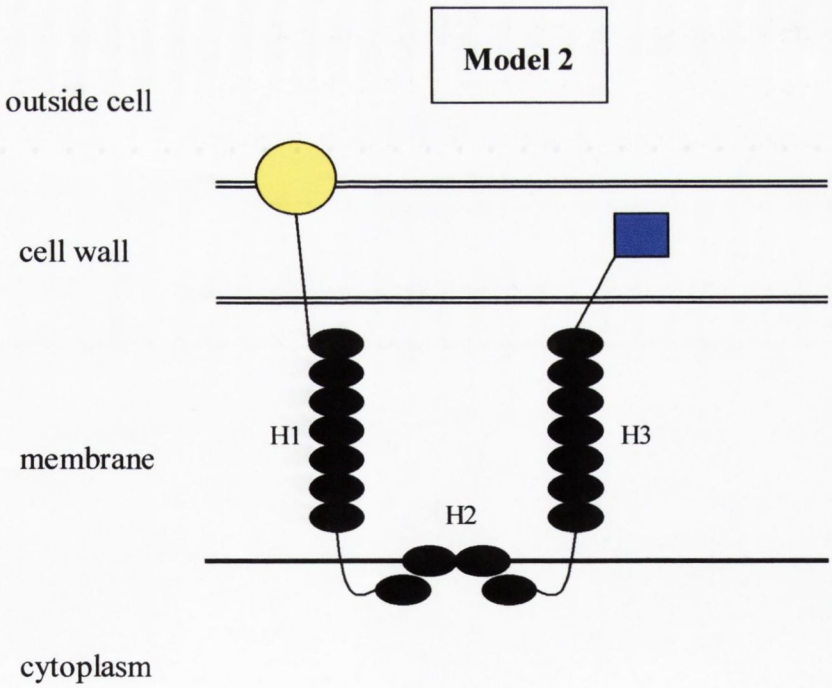
The absence of an N-terminal signal sequence initially suggested an alternative secretory mechanism is used. Other surface proteins have also been found to lack signal peptides. Endolysins are phage-encoded lysins, which are synthesized without a signal sequence. They are fully folded in the cytoplasm before they are secreted to the cell wall peptidoglycan via pore-forming holins. Another example is the LytA autolysin of *S.pneumoniae*. LytA is expressed without a signal peptide and is found to be associated with choline-containing teichoic acids in the cell wall. The export of this protein must therefore occur by a pathway other than the known Sec machinery. However, if EbpS is an integral membrane protein it is not secreted across the membrane and presumably associates with the membrane like other typical membrane proteins. Most bacterial membrane proteins are targeted to the membrane by the signal recognition particle (SRP) system (Fekkes and Driessen, 1999). SRP binds to transmembrane segments of integral membrane proteins and targets them to a peripheral membrane protein which acts as the SRP receptor. SRP-targeted proteins can then be inserted or translocated across the membrane by a Sec-dependent or

Figure 2.15 Two possible models of the topography of EbpS in the cytoplasmic membrane. EbpS has been described as a membrane protein (Downer, personal communication). The N-terminus (yellow circle) is located on the cell surface exposing the ligand binding domain and the C-terminus bearing the LysM domain (blue box) is embedded in the cell wall. This figure illustrates two possible models of the topography of EbpS in the cell membrane. Model 1, The three hydrophobic domains span the membrane but H3 forms a helical hairpin via asingle proline residuewhich projects the C-terminus into the cell wall. Model 2, Hydrophobic domains H1 and H3 traverse the membrane while domain H2 remains only loosely associated with the inner cytoplasmic membrane.

Model 1



Model 2



independent system. Most of the knowledge of this system is based on studies with mammalian and *E.coli* cells therefore its significance in Gram-positive bacteria remains to be elucidated.

Sequence analysis of DNA flanking *ebpS* from strain 8325-4 identified genes showing similarity to genes of *Bacillus subtilis*. Further examination of the *ebpS* locus in strains 8325 and eMRSA-16 identified that the molecular organisation of genes in 11 kb of DNA flanking *ebpS* shared significant similarity to the gene order in *Bacillus subtilis* and *Enterococcus faecalis*. The proteins EbpS, YbpE (*Bacillus subtilis*) and AlyS (*Enterococcus faecalis*), which represent the centre point of this alignment all possess a C-terminal LysM domain. Further sequence similarity between these proteins was not found. YpbE is a 27.5 kDa protein with an as yet unidentified function. The presence of an N-terminal hydrophobic domain large enough to traverse the membrane suggests this protein may be membrane-associated. AlyS represents an autolysin with N-acetylmuramoyl-l-alanine-amidase activity. Interestingly, the *orfs* flanking *ebpS*, *ybpE* and *alyS* showed unmistakable organisational conservation although some gene shuffling was apparent. It has been recently reported that this region (*recQ-cmk* locus in *Bacillus subtilis*) is part of a supraoperon. It mostly encodes enzymes involved in the synthesis of amino acids and is conserved across wide phylogenetic boundaries, albeit with some shuffled gene organisation (Xie *et al.*, 1999). Moreover, a homology search of EbpS against several protein databases identified that EbpS bears some homology to the autolysin/adhesin protein Aas of *S.saprophyticus*. The region of homology was in the N-terminus of both proteins which lies outside any of the functional domains identified in Aas. Hence, the location of *ebpS* in the genome coupled with its homology to cell wall hydrolases suggests an alternative function for EbpS apart from elastin binding activity. However, the identification of this additional function has yet to be elucidated.

In order to study EbpS expression in both *E.coli* and *S.aureus*, it was essential to generate antibodies to detect it. An N-terminal truncate of EbpS ($rEbpS_{1-267}$) was purified as an N-terminal His-tag fusion protein using the pQE30 expression vector. An additional 17 residues were included at the N-terminus of the protein (11 provided by the pQE30 vector and 6 from sequence upstream of the *ebpS orf*). $rEbpS_{1-212}$, which contained the same additional residues as $rEbpS_{1-267}$, was demonstrated to specifically inhibit strain Cowan from binding elastin peptides (Park

et al., 1996). The additional residues did not interfere with the ligand binding activity of $rEbpS_{1-212}$ and presumably also of $rEbpS_{1-267}$. The purified $rEbpS_{1-267}$ protein was used to raise specific antibodies against the N-terminus of EbpS (R.Downer, personal communication). Antibodies were also raised against the C-terminal residues 343-486 of the protein (R.Downer, personal communication).

Full-length recombinant EbpS was expressed in *E.coli* as an N- and C-terminal His-tag fusion protein. Anti-$rEbpS_{1-267}$ antibodies detected EbpS as an 83kDa protein when fractionated on reducing SDS-PAGE. This is a significant deviation from its predicted molecular weight of 53 kDa. Aberrant migration on SDS-PAGE is frequently observed for Gram-positive surface proteins. In some cases this is attributable to multiple repetitive domains (McDevitt *et al.*, 1994, Sela *et al.*, 1993) or a high proline content (Signäs *et al.*, 1989; Sjöbring, 1992). In the case of EbpS, the hydrophilic N-terminus may be partially responsible for the anomalous migration. Park *et al.* (1996) expressed $rEbpS_{1-212}$ as a His-tag fusion protein, which migrated as a 45 kDa protein, nearly double the predicted size of 26kDa. In this study, $rEbpS_{1-267}$ bearing the first hydrophobic domain migrated as a slightly larger 58kDa protein. The small difference in size is attributable to the additional amino acid residues but suggests that the hydrophobic domains are not responsible for the abberrant migration of the 83 kDa full length protein. Purification of full-length rEbpS proved difficult due to proteolysis at the C-terminus of the molecule. Degradation at the C-terminus is also observed with the native protein presumably as a result of the extraction protocol (Section 3.3.4).

Chapter 3

Construction of *Staphylococcus aureus* Mutants Deficient in EbpS by Allelic Replacement and Studies on the Expression of EbpS

3.1 Introduction

3.1.1 Expression of EbpS in *S.aureus*

In the previous chapter the *ebpS* gene was shown to be 1461 bp encoding a protein of 486 amino acids with a predicted molecular weight of 53.2 kDa. It was also shown that recombinant EbpS migrated with an apparent molecular weight of 83 kDa. Park *et al.* (1996) tentatively identified EbpS in lysates of *S.aureus* Cowan as a protein of 25 kDa. In order to identify the EbpS protein in extracts of *S.aureus* it was necessary to isolate an *ebpS* mutant by allele replacement. This mutant would be invaluable in expression studies, in determining the role of EbpS in promoting bacterial interactions with host elastin and in virulence studies.

3.1.2 Construction of *ebpS* isogenic mutants

The ability to selectively disrupt gene function remains a critical element in elucidating information regarding the role of specific proteins in the pathogenesis of staphylococcal infections. This information is particularly useful for identifying target genes for vaccine or antibiotic development. The development of molecular genetic tools have enabled the isolation of isogenic mutants in many *S.aureus* genes. The role of individual genes and their importance in disease can therefore be examined by inactivating or deleting the gene of interest and comparing the isogenic mutant with the parent strain in a suitable *in vivo* disease model. Complementation of the mutation to restore the phenotype would indicate that loss of the phenotype was due to inactivation of the gene and not due to polar effects or to unlinked mutations. An alternative to gene knockouts is antisense technology which allows the selective control and downregulation of specific gene products. This is a particularly useful system for essential genes, which cannot be inactivated by homologuous recombination. This system has been used in *S.aureus* to downregulate alpha-toxin expression (Ji *et al.*, 1999).

In the past, chemical and UV mutagenesis was used to isolate mutants defective in particular phenotypes. However, this procedure was non-specific and likely caused several mutations leading to ambiguous results in testing for virulence in animal models. A variety of techniques have been developed in staphylococci to achieve functional inactivation of gene products. Transposon mutagenesis can be used to isolate insertion mutations in chromosomal genes. Even without knowledge

of the gene or its product it is possible to recognise an insertion mutation by a change in phenotype and then to clone DNA flanking the transposon for sequencing analysis. However, the usefulness of this approach depends upon the randomness with which the transposon inserts into the target genome. In spite of this, the approach has been used to inactivate and clone the *agr* and *sar* regulatory loci of *S.aureus* (Peng *et al.*, 1988; Cheung and Projan, 1994), the fibrinogen binding protein gene (*clfA*) of *S.aureus* (McDevitt *et al.*, 1994) and the serotype 5 capsular polysaccharide loci of *S.aureus* (Lee *et al.*, 1994; Sau *et al.*, 1997).

Another approach includes allele replacement mutagenesis which allows a mutation that has been constructed in a cloned gene to be introduced into the chromosomal copy by homologous recomination. This is a more specific method than transposon mutagenesis but does require the gene of interest to be cloned and sequenced. Plasmid incompatibility was the first method described for isolating mutants by allelic replacement. Patel *et al.* (1987) constructed a mutation in the protein A gene by this approach. A shuttle vector was constructed carrying a mutation in the *spa* gene whereby an internal fragment of the gene was deleted and replaced by an ethidium bromide resistance determinant ($EtBr^r$). The plasmid was transduced into a strain carrying an incompatible chloramphenicol resistance (Cm^r) plasmid. The resulting strain was grown with Cm for 200 generations to eliminate the shuttle plasmid from the population. Colonies that showed a Cm^r and $EtBr^r$ phenotype signified replacement of the wild-type gene with the inactivated copy. Greene *et al.* (1995) used a similar approach except that the shuttle plasmid was temperature sensitive for replication in *S.aureus*. The plasmid could be maintained stably at the permissive temperature of 30°C and eliminated from the population by growth at the restrictive temperature of 43°C. Isogenic mutants have also been isolated using a non-replicating suicide plasmid carrying the mutated gene. Since the plasmid lacks a *S.aureus* replicon, the only way the marker can survive is by recombination with the chromosomal site of homology. McDevitt *et al.* (1993) mutated the coagulase gene in this way. The disadvantage of this approach is that the gene of interest must be present in RN4220 (a restriction-deficent host which must be used as the recipicent).

Transduction is an efficient mechanism for moving mutations (either on plasmids or on the chromosome) between strains of staphylococci. The most

common transducing phages are ϕ11, ϕ85 and ϕ80α. Plasmid DNA carrying the mutated copy of the gene must first be transformed into RN4220, to overcome the restriction barriers, before being transduced into different *S.aureus* strains. Chromosomal markers are transduced at a lower frequency than plasmid DNA and must undergo a double crossover event to be maintained in the recipient strain. Transduction also facilitates the construction of strains with mutations in several different chromosomal genes using different resistance markers. In this way, the role of several virulence genes and regulatory loci can be investigated.

In order to identify the EbpS protein, understand its role as an elastin-binding protein and determine its importance in disease, an *ebpS*-defective mutant was constructed by allele replacement using a temperature sensitive shuttle vector equivalent to that used by Greene *et al.* (1995). The mutation was restored in the complemented mutant bearing the wild-type copy of the *ebpS* gene. Therefore, the loss of phenotype observed in the *ebpS*-defective strain was associated with the *ebpS* mutation. Generalised transduction was used to move the *ebpS* mutation and the shuttle plasmid into different strains.

3.2 Methods

3.2.1 SDS-PAGE and Western blotting

Cultures were grown at 37°C in 20ml of TSB broth in 250ml conical flasks at 200rpm for 18 hours giving stationary phase cultures (OD_{600} of 12). Cells were harvested by centrifugation and washed in an equal volume of PBS. The cell suspensions were adjusted to the same optical density (OD_{600} of 100 units). Cells were pelleted and resuspended in 0.5ml of digestion buffer (30%(w/v) raffinose, 50mM Tris-HCl, 20mM $MgCl_2$, pH7.5). Cell wall proteins were released from stabilised protoplasts by digestion with lysostaphin (0.2 mg/ml) for 30 min at 37°C in the presence of protease inhibitors (Mini-Complete, Boehringer-Mannheim) with occasional shaking. Protoplasts were recovered by centrifugation at 6,000g for 20 min. The supernatant was taken as the cell wall fraction. Protoplasts were resuspended in ice-cold lysis buffer (50mM Tris-HCl, 20mM $MgCl_2$, pH 7.5) with protease inhibitors and DNase (80μg/ml). Protoplasts were lysed on ice by repeated pipetting. Isolated proteins were prepared for electrophoresis by boiling for 5 min in 2x final sample buffer (0.125M Tris-HCl pH6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.002% (w/v) bromophenol blue. Samples (15μl) were analysed by SDS-PAGE (Laemmli, 1970) in 10% (w/v) acrylamide gels in a BRL minigel system. Gels were stained with Coomassie blue or transferred to PVDF western blotting membranes (Boehringer Mannheim) by the semidry system (Bio-Rad) in 48mM Tris-HCl, 39mM glycine and 20% (v/v) methanol. Membranes were blocked for 15 h at 4°C in 10% (w/v) skim milk. Western blots were processed using the BM Chemiluminescence Detection System (POD) of Boehringer Mannheim, according to the manufacturer's instructions. Rabbit polyclonal anti-$EbpS_{1-267}$ and anti-$EbpS_{343-486}$ antibodies were used at a 1/2000 and 1/1000 dilution, respectively, and incubated for 1.5 h at room temperature. Protein A conjugated with horse radish peroxidase (Sigma) diluted 1/2000 was used to detect bound antibody and incubated for 1 h at room temperature.

3.2.2 Mutagenesis of *ebpS* by insertional inactivation

Two sets of forward and reverse primers were used to PCR-amplify two arms of the *ebpS* locus from the template pKS-5.2 (Figure 3.1). Primers mutF1 and mutR1 (Table 2.3) amplified a 1.5 kb fragment containing 0.9 kb upstream DNA and 0.6 kb of the 5′ *ebpS* sequence. Primers mutF2 and mutR2 (Table 2.3) amplified a 1.5 kb fragment containing 0.8kb of the 3′ *ebpS* sequence and 0.7 kb of downstream sequence. Both arms were amplified under the following conditions: 94°C for 3min, followed by 30 cycles of 94°C for 1min, 58°C for 1min, and 72°C for 1min 30sec. The PCR products were digested with their respective restriction endonucleases, gel-purified and directionally ligated to pUC18 digested with HindIII and EcoRI, generating pFR1. The two products were joined by the introduced SacII restriction site simultaneously creating a 16 bp deletion in the *ebpS* gene forming plasmid pFR1. The *ebpS* gene was further disrupted by insertion of an erythromycin resistance marker (*ermC* from pE194 (Horinouchi and Weisblum, 1982)) into the SacII site of the gene on pFR1, (Figure 3.1). The *ermC* gene was PCR-amplified from pE194 using the forward and reverse primers, ermCF and ermCR (Table 2.3). The exact conditions for amplification were 94°C for 3min, followed by 30 cycles of 94°C for 1min, 47°C for 1min, and 72°C for 2min 30sec. *Pfu* DNA polymerase was used. The PCR product was digested with SacII, treated with alkaline phosphatase and gel-purified. This material was ligated with pFR1 digested with SacII, generating pFR2. Plasmid pTS2, with temperature sensitive *S.aureus* replicon and a Cm^r marker was cloned into this construct at the HindIII site to generate plasmid pFR3. This cloning step was carried out in *E.coli*, and transformants were selected on ampicillin (100μg/ml). The shuttle plasmid was then transformed into *S.aureus* RN4220 by electroporation (Schenk and Laddaga, 1992), and Em^r transformants selected at 30°C. A broth culture grown at 30°C with antibiotic (Em 10μg/ml) was diluted 1/100 in fresh TSB medium with antibiotic (Em 10μg/ml) and grown at 42°C overnight. They were then diluted 1/100 and incubated at 42°C with antibiotic for another period. The cultures were then diluted to give approximately 100 CFU per plate on medium containing Em, and incubated overnight at 42°C. Colonies were then replica transferred to fresh Em (10μg/ml) and Cm (10μg/ml) plates. Colonies which had a Em^r and Cm^r phenotype were presumed to have undergone a single crossover event where the plasmid was forced to integrate into the genome at the site of shared

homology (Figure 3.2). Two colonies were independently grown in broth for two cycles of growth at 30°C in the absence of antibiotic. The cultures were then diluted 1/100 and incubated at 42°C without antibiotic for another period. The cultures were diluted to give approximately 100 CFU per plate on medium containing Em, and incubated overnight at 42°C. Colonies were replica plated as before. Colonies which had a Em^r and Cm^s phenotype were presumed to have undergone a double crossover event between the plasmid and the genome, leading to replacement of the wild-type gene with the mutated one, and subsequent loss of the plasmid. Five hundred colonies were screened per culture. Seven representative mutants were selected and genomic DNA isolated. The insertional inactivation of the *ebpS* gene was detected by Southern blotting, by probing HindIII and EcoRI cut genomic DNA from the mutants with a 787 bp DIG-labelled *ebpS* probe designed from the 5' end of the *ebpS* gene using primers ebpS3 and ebpS4 (Table 2.3). The mutation was then transduced into strains Newman, Newman Δ*spa*, 8325-4 Δ*spa*, P1 Δ*spa* and COL using bacteriophage 85 (Asheshov, 1966).

3.2.3 Complementation of the *ebpS* mutation

The $EbpS^+$ phenotype was restored in *ebpS* mutants for use in complementation studies. This was enabled by cloning the *ebpS* locus from strain 8325-4 into the high copy number shuttle plasmid, pCU1. The *ebpS* locus was PCR-amplified from pKS-5.2 template using the forward and reverse primers, compF and compR. The exact conditions for amplification were 94°C for 3min, followed by 30 cycles of 94°C for 1min, 52°C for 1min, and 72°C for 4min. *Pfu* DNA polymerase was used. The PCR product, containing the *ebpS* gene with 300 bp of upstream and 200 bp of downstream DNA, was digested, gel purified and ligated to pCU1 digested with EcoRI and HindIII. The construct was then transformed into strain RN4220 and transduced into the *ebpS* mutants, Newman Δ*spa ebpS*::*erm*, 8325-4 Δ*spa ebpS*::*erm* and COL *ebpS*::*erm*. Transductants were selected on Cm (10μg/ml).

3.3 Results

3.3.1 Construction of *ebpS*::*erm* mutants in strain RN4220

The *ebpS* gene in pFR1 was insertionally inactivated by cloning a 1 kb erythromycin resistance determinant (*ermC*), that had been PCR-amplified from plasmid pE194 into the unique SacII site that had been introduced into the *ebpS* gene by PCR (Section 3.2.2; Figure 3.1). The resulting plasmid, pFR2 (Em^r ,Cm^r) contained 1.5 kb of DNA upstream and 1.5 kb of DNA downstream from the insertion. This was thought to be sufficient to promote efficient homologous recombination with the chromosomal *ebpS* locus. pFR2 was made temperature sensitive when cloned into plasmid pTS2 (Cm^r). The resulting construct (pFR3) was transformed into strain RN4220. Allelic replacement mutants (Em^r ,Cm^s) were isolated through a series of temperature shift experiments to select for a double crossover event (Section 3.2.2; Figure 3.2). Southern blotting was used to screen HindIII and EcoRI digested genomic DNA of putative mutants using a DIG-labelled 787 bp fragment containing 5′ sequence of the *ebpS* gene of strain 8325-4 (Figure 3.3). As expected a single 3.15 kb HindIII/EcoRI fragment hybridised in the wild-type and a single larger 4.0 kb HindIII/EcoRI fragment hybridised in 6/7 of the putative mutants screened, indicating the presence of the mutated copy of the *ebpS* gene in the chromosome. There was no evidence of a single cross-over event in these mutants where the plasmid containing the wild-type copy of the *ebpS* gene remained integrated in the chromosome. If this was the case, an additional 3.15 kb fragment would have hybridised in these lanes.

3.3.2 Transduction of the *ebpS*::*erm* mutation into several strains of *S.aureus* and complementation of *ebpS*

The *ebpS*::*erm* mutation was transduced from strain RN4220 into strain Newman, Newman Δ*spa*, 8325-4 Δ*spa*, P1 Δ*spa* and COL, using bacteriophage 85 and selecting for Em^r (Section 3.2.3). Genomic DNA of putative mutants was digested with HindIII and EcoRI and screened by Southern blotting using the DIG-labelled 787 bp probe, (Figure 3.3). As described previously, a single 3.15 kb HindIII/EcoRI fragment hybridised in the wild-type and a single larger 4.0 kb HindIII/EcoRI fragment hybridised for each putative mutant tested. To restore the $EbpS^+$ phenotype in these mutant strains a construct containing the wild-type copy of

Figure 3.1 Construction of temperature sensitive shuttle vector carrying a mutated copy of the *ebpS* gene. The *ebpS* gene is shown as a solid arrow and the plasmid DNA is underlined. The abbreviations H, S and E, represent the restriction enzymes HindIII, SacII and EcoRI, respectively. The abbreviations H* and E* represent the restriction sites HindIII and EcoRI introduced by PCR, which lie 50 bp in from each end from the natural restriction sites. The forward primers are denoted by mutF1and mutF2 and the reverse primers are denoted by mutR1 and mutR2. **A.** Introduction of a unique SacII restriction site into the middle of the *ebpS* gene by PCR amplification from pKS-5.2, **B.** Ligation of PCR products to pUC18 digested with HindIII and EcoRI (pFR1). Disruption of the *ebpS* gene by insertion of an *erm* marker into the SacII site (pFR2). **C.** Ligation of temperature sensitive plasmid, pTS2, into the HindIII site of pFR2 generating pFR3.

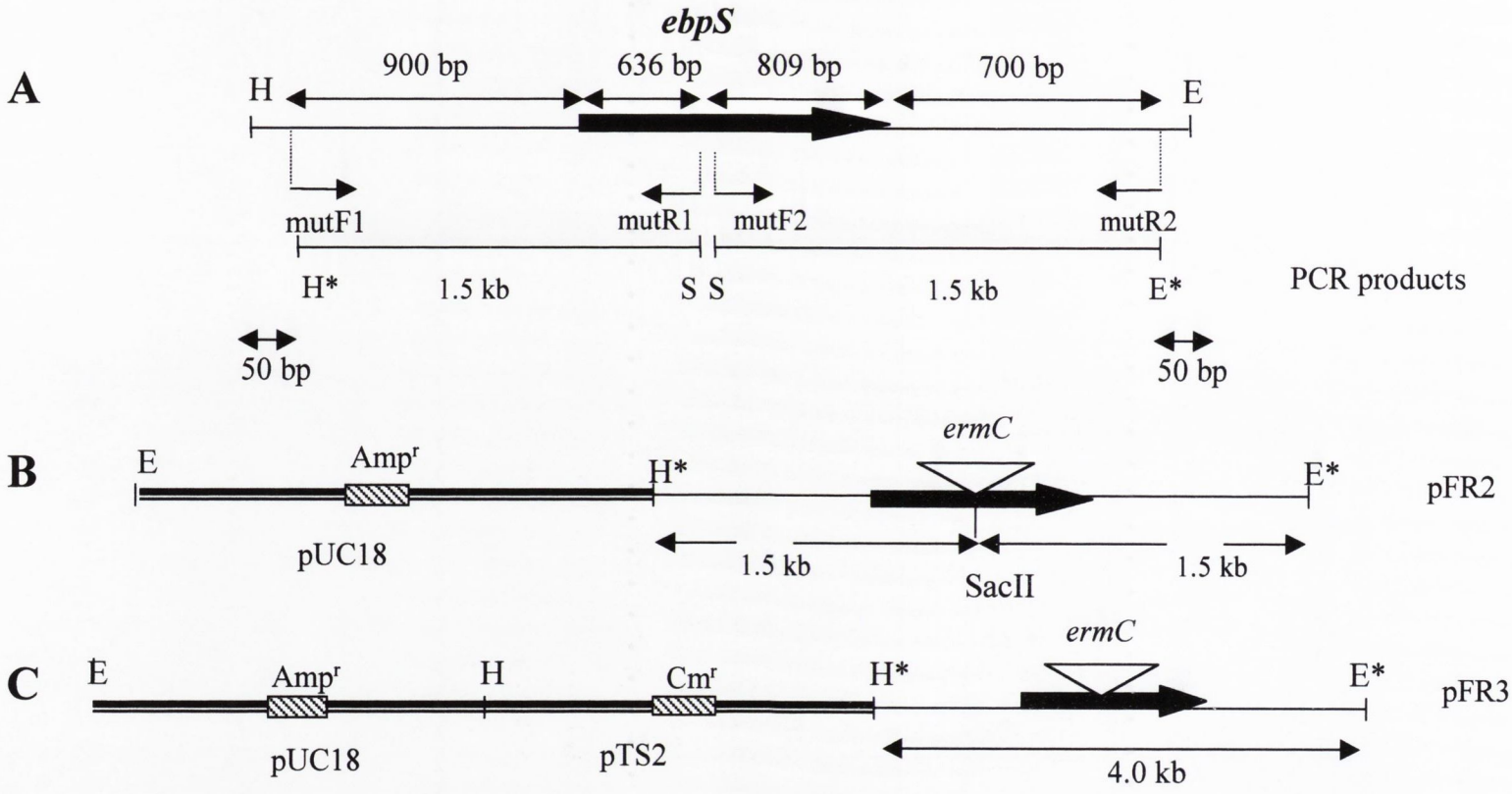

A
ebpS
900 bp
636 bp
809 bp
700 bp
H
E
mutF1
mutR1
mutF2
mutR2
H*
1.5 kb
S S
1.5 kb
E*
PCR products
50 bp
50 bp
B
ermC
E
Amp^r
H*
E*
pFR2
pUC18
1.5 kb
SacII
1.5 kb
C
ermC
E
Amp^r
H
Cm^r
H*
E*
pFR3
pUC18
pTS2
4.0 kb

Figure 3.2 Allele replacement mutagenesis of *ebpS*.

Prediction of the hybridising pattern in the event of a single or double crossover between pFR3 and the chromosome of *S.aureus* RN4220. The *ebpS* gene is shown as a solid arrow and the plasmid DNA as a double line in part A and B. The abbreviations H and E represent the restriction enzymes HindIII and EcoRI, respectively, which are present in the genome. The abbreviations H* and E* represent the restriction sites introduced by PCR (see Figure 3.1) which lie 50 bp in from the natural HindIII and EcoRI restriction sites on the genome. **A**. A single crossover event with homologous recombination occuring at the 5′ end of the chromosomal gene, **B**. A single crossover event with homologous recombination occuring at the 3′ end of the chromosomal gene, **C**. A double crossover event. The integrated plasmid excises by a recombination event on the opposite side of the chromosomal locus so that the wild type gene is removed and the mutant copy remains.

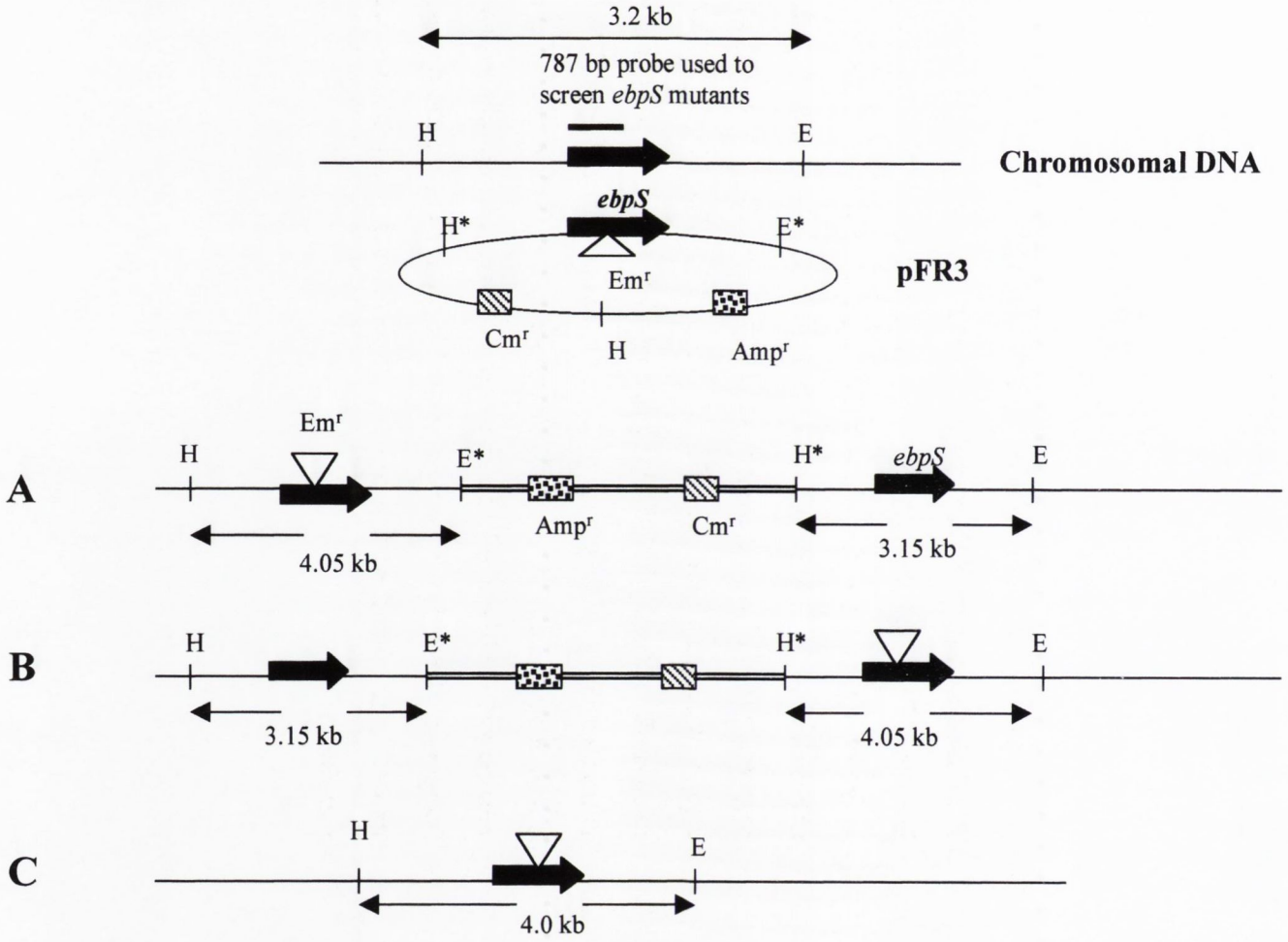
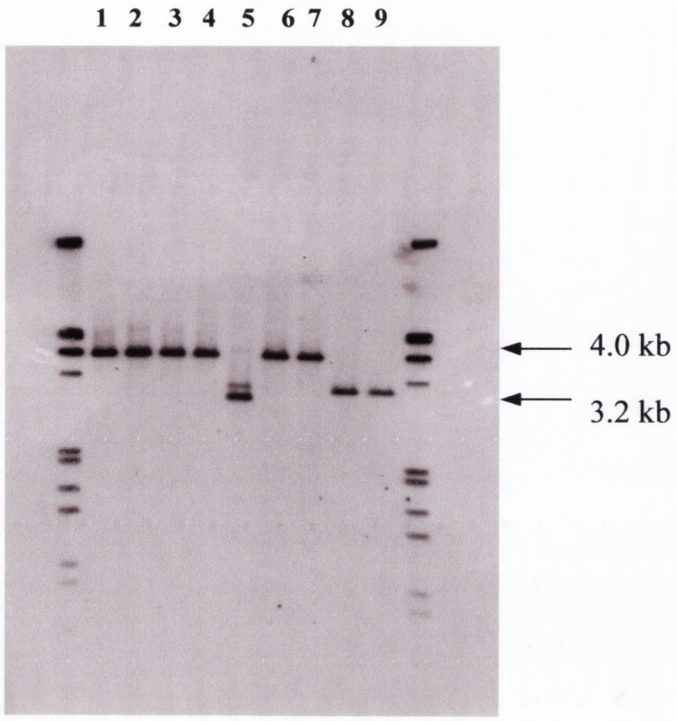

3.2 kb
787 bp probe used to screen ebpS mutants
H
E
Chromosomal DNA
ebpS
H*
E*
Emr
pFR3
Cmr
H
Ampr
A
Emr
H
E*
H*
ebpS
E
Ampr
Cmr
4.05 kb
3.15 kb
B
H
E*
H*
E
3.15 kb
4.05 kb
C
H
E
4.0 kb

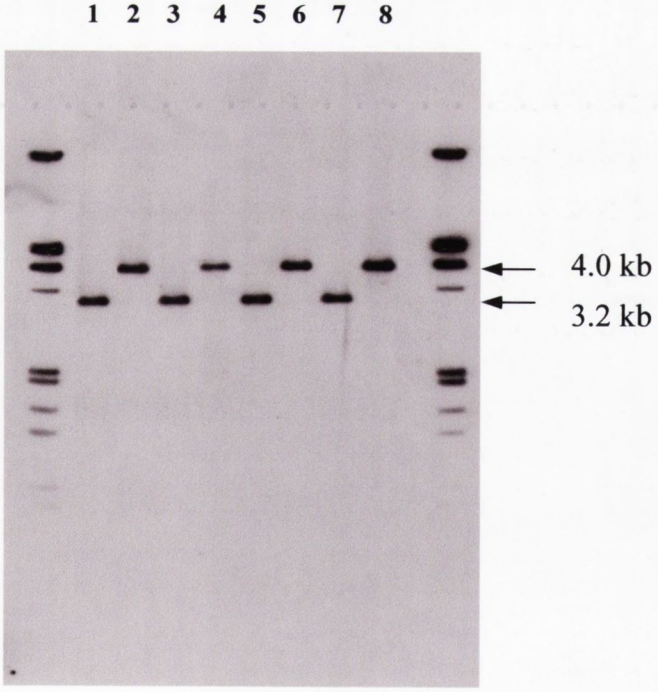
Figure 3.3 A. Southern blotting to confirm insertional inactivation of the *ebpS* gene in RN4220. Lanes 1-9 contain HindIII/EcoRI digested genomic DNA. Lanes 1-7 contain DNA from seven putative mutants of strain RN4220. Lanes 8-9 contain DNA from *S.aureus* RN4220. The probe used to screen the mutants was directed against a 787 bp fragment at the 5′ end of the gene. DIG-labelled size markers are located at the sides.

Figure 3.3 B. Southern blotting to confirm insertional inactivation of the *ebpS* gene in several strains of *S.aureus*. The figure shows the hybridisation of HindIII/EcoRI digested DNA of wild-type and mutant strains to a DIG-labelled 787 bp probe containing 5′ sequence of the *ebpS* gene of strain 8325-4. Lane 1, RN4220; lane 2, RN4220 *ebpS*::*erm*; lane 3, 8325-4 Δ*spa*; lane 4, 8325-4 Δ*spa* *ebpS*::*erm*; lane 5, Newman Δ*spa*; lane 6, Newman Δ*spa* *ebpS*::*erm*; lane 7, COL; lane 8, COL *ebpS*::*erm*.

A



B



the *ebpS* gene with 300 bp of upstream DNA and 200 bp of downstream DNA was cloned into the shuttle vector pCU1. This construct, pCU-*ebpS*$^{+}$, was made in *E.coli* and then transformed into *S.aureus* strain RN4220, from where it was transduced into strains Newman *ebpS*::*erm*, Newman Δ*spa ebpS*::*erm* , 8325-4 Δ*spa ebpS*::*erm*, P1 Δ*spa ebpS*::*erm* and COL *ebpS*::*erm*.

Western blotting was used to compare the wild-type strain with its *ebpS* isogenic mutant, to confirm lack of EbpS expression, and with the complemented mutant, to show the restored EbpS^{+} phenotype, (Figure 3.4). Cells were grown to stationary phase and protoplast fractions were examined for EbpS expression using anti-EbpS$_{343\text{-}486}$ antibodies. Results showed that in strains Newman Δ*spa* and 8325-4 Δ*spa*, the native EbpS protein migrated as an 83 kDa band on an SDS-PAGE gel. The protein was not expressed in the *ebpS*::*erm* mutants and expression of an 83 kDa protein was restored in the complemented mutants bearing the pCU-*ebpS*$^{+}$ plasmid. Collectively, this data indicates that EbpS is expressed as a larger protein than previously predicted migrating at a molecular weight of 83 kDa.

EbpS was found to be overexpressed in the complemented mutant bearing the pCU-*ebpS*$^{+}$ plasmid. pCU-1 is a multi-copy plasmid therefore overexpression of the protein is most likely due to a gene dosage effect. A series of doubling dilutions of the overexpressed protein illustrated that EbpS was expressed approximately 8-fold higher in the complemented strain compared to wild-type levels (Figure 3.5).

3.3.3 EbpS is membrane-bound

EbpS was previously reported to be a 25 kDa protein that was released from the cell by lysostaphin (Park *et al.*, 1996). Lysostaphin is a murolytic enzyme that solubilises cell wall peptidoglycan and EbpS was assumed located in the cell wall although this was not formally proved in that study. However, the primary structure of the EbpS protein sequence was found here to be quite distinct from previously reported cell wall associated proteins (Section 2.3.4). The presence of three large hydrophobic domains in the centre of the molecule is not typical of a cell wall protein but predicted the protein to be membrane anchored. The cellular localisation of EbpS in *S.aureus* was determined to be in the cytoplasmic membrane by extensive fractionation studies (R.Downer, personal communication) although parts of the protein are associated with the cell wall (Figure 2.15). When stable protoplasts of

Figure 3.4 Western immunoblot showing EbpS expression by *S.aureus* strains and their isogenic mutants. Protoplast fractions were made from stationary phase cultures and protein expression was detected with anti-$EbpS_{343-486}$ antibodies. A. lane 1, 8325-4 Δ*spa;* lane 2, 8325-4 Δ*spa ebpS*::*erm*; lane 3, 8325-4 Δ*spa ebpS*::*erm* pCU-*ebpS*$^{+}$. B. lane 1, Newman Δ*spa;* lane 2, Newman Δ*spa ebpS*::*erm*; lane 3, Newman Δ*spa ebpS*::*erm* pCU-*ebpS*$^{+}$.

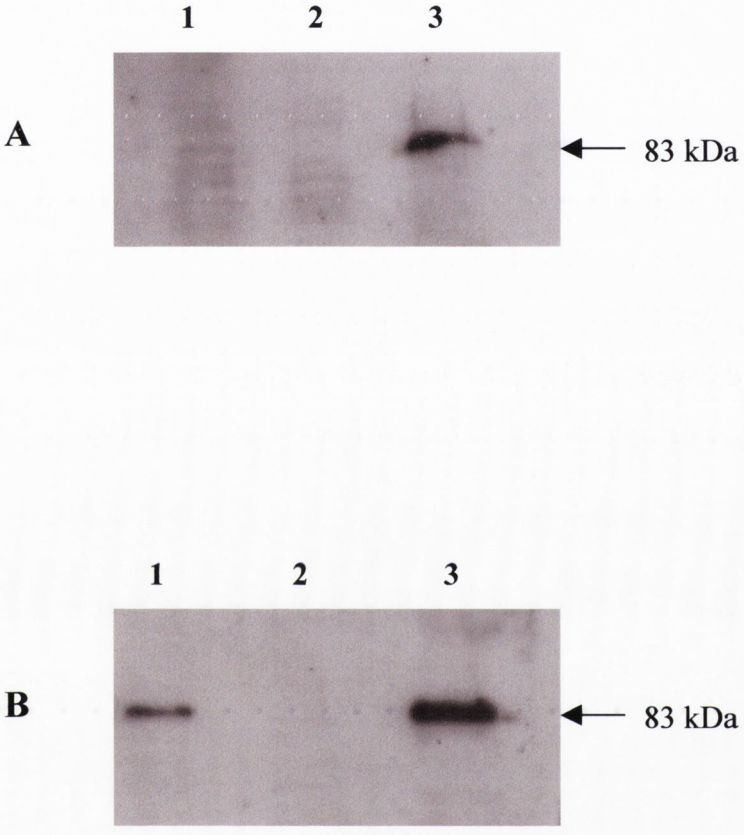
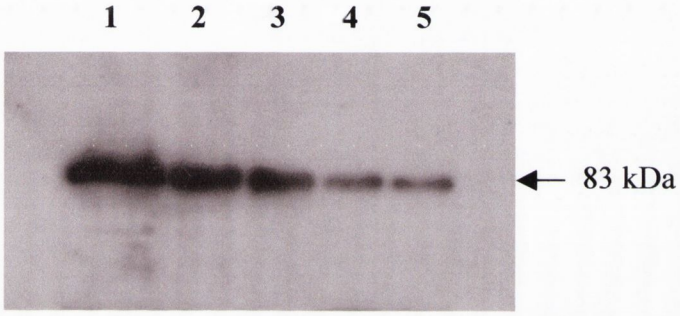

1
2
3
A
83 kDa
1
2
3
B
83 kDa

Figure 3.5 EbpS is overexpressed in the complemented mutant.

Cells were grown to stationary phase and protoplast fractions were examined for EbpS expression using anti-$EbpS_{343-486}$ antibodies. Lanes 1-4, doubling dilutions of Newman Δ*spa ebpS*::*erm* pCU-*ebpS*$^{+}$: lane 1, 20 μl, lane 2, 10 μl, lane 3, 5 μl, lane 4, 2.5 μl; lane 5, Newman Δ*spa* (20μl).


1
2
3
4
5
83 kDa

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 (R.Downer, personal communication). Figure 3.6 illustrates the association of EbpS with the membrane fraction. Western immunoblotting studies characterising EbpS expression examined total protoplast lysates for EbpS protein.

3.3.4 Expression of EbpS in *S.aureus*

The expression of EbpS was studied in several laboratory and clinical strains of *S.aureus* (Figure 3.7). Cells were grown to stationary phase and the stabilised protoplasts were analysed for EbpS expression using anti-$EbpS_{343-486}$ antibodies. EbpS migrated in SDS-PAGE with an apparent molecular weight of 83 kDa in all strains tested except strain Cowan Δ*spa* and a nasal isolate (Figure 3.7B, lane 2). In these strains the protein appeared slightly smaller of ~ 80 kDa. The difference in protein size is probably attributable to the difference in protein sequence between strains (Section 2.3.5).

Previous studies reported that EbpS was released from strain Cowan as a 25kDa protein following lysostaphin digestion, (Park *et al.*, 1996). In contrast, in our laboratory EbpS was demonstrated to be a membrane-associated protein (R.Downer, personal communication) migrating at 83kDa or 80 kDa in the case of strain Cowan Δ*spa*. A closer examination of the lysostaphin digestion conditions used in the previous study revealed that it was carried out in the absence of raffinose and $MgCl_2$ and involved a prolonged incubation time of three hours. Under these conditions protoplasts are no longer intact and cell lysis occurs. As a result protoplasts lyse releasing membrane and cytoplasmic proteins which can interact with proteins solubilised from the cell wall. It is therefore most likely that the 25 kDa EbpS protein previously described is an N-terminal truncate cleaved from the body of the EbpS protein by bacterial proteases during prolonged incubation in the absence of protease inhibitors. The N-terminus of the 25 kDa protein is known to be intact because (i) the 25kDa EbpS protein retained ligand binding activity and (ii) N-terminal protein sequencing of the 25kDa protein predicted the correct amino acid sequence (Park *et al.*, 1996).

Figure 3.6 Western blotting showing EbpS associated with the protoplast fraction.

Cell wall and protoplast fractions from stationary phase cells of strain Newman Δ*spa* were analysed for EbpS activity using anti-$EbpS_{343-486}$ antibodies. Molecular weight standards are indicated on the right.

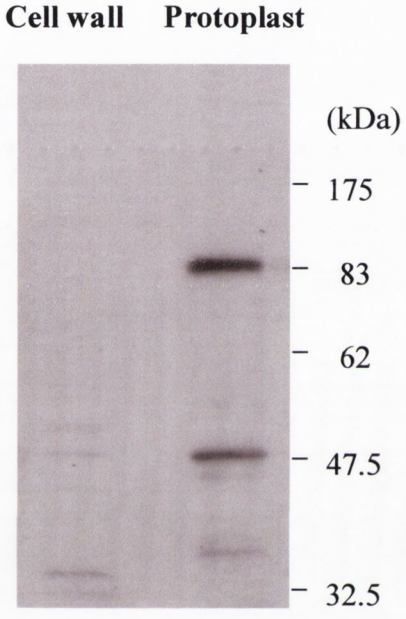
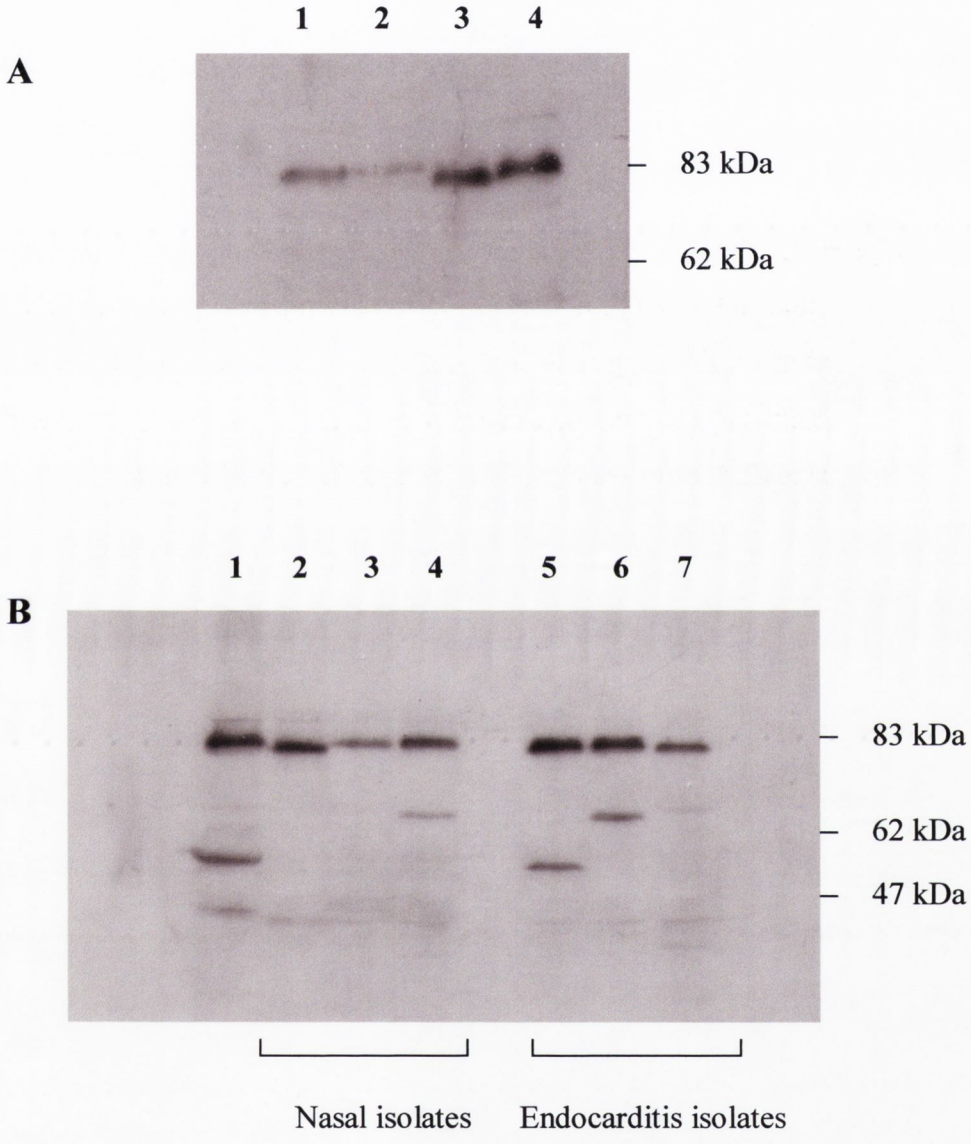

Cell wall
Protoplast
(kDa)
175
83
62
47.5
32.5

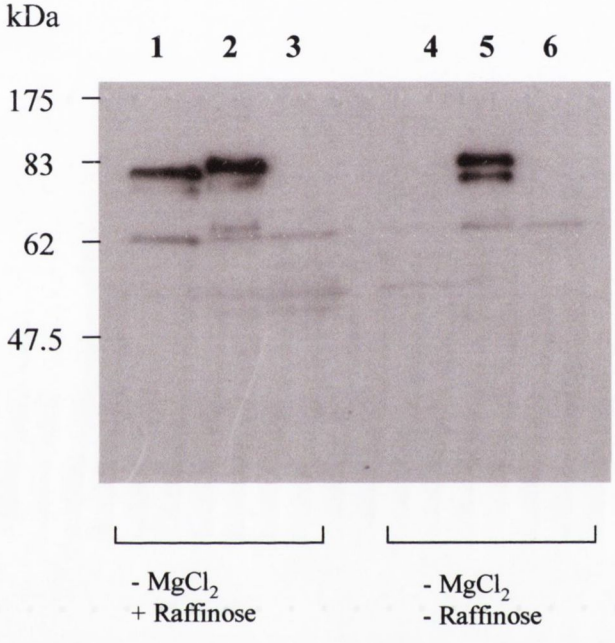
Figure 3.7 EbpS expression in various *S.aureus* laboratory strains (A) and clincial isolates (B). Protein expression was examined in protoplast fractions of stationary phase cells using anti-$EbpS_{343-486}$ antibodies. A. Lane 1, Newman Δ*spa*; lane 2, 8325-4 Δ*spa*; lane 3, Cowan Δ*spa*; lane 4, P1 Δ*spa*. B. Lane 1, Newman Δ*spa*; lanes 2-4, nasal isolates; lanes 5-7, endocarditis isolates.


A
1 2 3 4
83 kDa
62 kDa
B
1 2 3 4 5 6 7
83 kDa
62 kDa
47 kDa
Nasal isolates
Endocarditis isolates

EbpS expression was examined in the presence and absence of raffinose and $MgCl_2$ in the digestion buffer. After removal of solubilised cell wall material by incubation with lysostaphin for 30 min in the presence of raffinose and $MgCl_2$, EbpS was detected only in the protoplast fraction of strain Cowan Δ*spa* and Newman Δ*spa* as an 80-83 kDa protein (Figure 3.7). In the absence of $MgCl_2$, that is required for stabilising protoplasts, EbpS was found in the cell wall fraction of both strain Cowan Δ*spa* and Newman Δ*spa* but this is likely to be contaminated with cytoplasmic proteins and membrane fragments (Figure 3.8). However, in the absence of both raffinose and $MgCl_2$ in the digestion buffer, EbpS was not detected in the cell wall fraction of strain Cowan Δ*spa* with either anti-$EbpS_{1-267}$ or anti-$EbpS_{342-486}$ antibodies. Under the same conditions, EbpS of strain Newman Δ*spa* was detected as an 83kDa protein. This suggests that strain Cowan Δ*spa* has higher levels of protease activity compared to strain Newman Δ*spa*. The 25kDa protein observed by Park *et al.* (1996) was not detected by either procedure. However it is implied that proteolysis is responsible for generating this N-terminal 25kDa truncate of EbpS.

Anti-$EbpS_{1-267}$ antibodies detected an additional band of approximately 75 kDa. This represented a N-terminal breakdown product as (i) it was not detected in the *ebpS*-defective mutant (ii) and it was not detected by C-terminal antibodies. r$EbpS_{1-486}$ was also found to be susceptible to proteolysis at the C-terminus of the protein (Section 2.3.7.2).

Figure 3.8 Western immunoblotting of EbpS following lysostaphin digestion in the absence of $MgCl_2$. Lanes 1-6 represent cell wall proteins isolated from *S.aureus* by lysostaphin treatment for 30 min in the absence of $MgCl_2$ and in the presence (Lanes 1-3) and absence (Lanes 4-6) of raffinose, an osmotic stabiliser. EbpS was detected using anti-$EbpS_{1-267}$ antibodies. Lanes 1 and 4, strain Cowan Δ*spa*; Lanes 2 and 5, strain Newman Δ*spa*; Lanes 3 and 6, strain Newman Δ*spa ebpS*::*erm*.


kDa
1 2 3 4 5 6
175
83
62
47.5
- MgCl2
+ Raffinose
- MgCl2
- Raffinose

3.4 Discussion

The *ebpS* gene was inactivated by insertion of an erythromycin marker and the *in vitro* constructed *ebpS*::*erm* mutation was introduced into the *S.aureus* chromosome by recombinational allele replacement. Southern blot hybridisation confirmed the insertion showing a single larger 4.0 kb EcoRI and HindIII fragment corresponding to the predicted size increase compared to a 3.15 kb fragment in the wild type strain. Plasmid excision and loss was verified by chloramphenicol sensitivity and the absence of a second hybridising band of wild-type size in the mutant strain.

The *ebpS*::*erm* mutation was moved into several different strains by transduction and verified by Southern blotting. Western immunoblotting demonstrated that strains carrying the mutation in *ebpS* had an EbpS$^-$ phenotype. In addition, inactivation of the *ebpS* gene enabled identification of the *ebpS* gene product as a protein that migrates in SDS-PAGE with an apparent molecular weight of 83 kDa. Expression was restored by providing the wild-type gene in *trans* on a multicopy plasmid. This result suggests that *ebpS* is transcribed from a promoter and is monocistronic.

EbpS expression was studied in several *S.aureus* strains (laboratory and clincial) by Western immunoblotting of protoplasts made from stationary phase cells. In each strain a protein of 80-83 kDa was detected. This is a significant deviation from the molecular weight of 53 kDa predicted from the EbpS amino acid sequence data. Other surface proteins of *S.aureus* have been found to migrate slower than predicted. For example, the fibronectin-binding proteins (McGavin *et al.*, 1997) and ClfA (Hartford *et al.*, 1997) show an apparent molecular weight in SDS-PAGE of approximately twice the predicted value. The reason for this is not known. Expression studies with the recombinant EbpS protein suggested that the N-terminus may play a role in the aberrant migration (Section 2.4.1). A slightly smaller protein of 80 kDa was detected in strain Cowan by antibodies generated against the N- and C-terminus of the protein. The EbpS protein sequence of strain Cowan is 95% similar to that of strain 8325-4 (Section 2.3.5). Slight variations in protein size (*i.e.* 80 or 83 kDa) are most likely attributable to the sequence variation between strains.

Park *et al.* (1996) previously reported that EbpS was released from the surface of strain Cowan as a 25 kDa protein following lysostaphin digestion implying a cell

wall associated protein. However, the lysostaphin digestion conditions used to isolate the 25 kDa protein did not contain raffinose and $MgCl_2$. Schneewind *et al.* (1992) documented optimum lysostaphin digestion conditions for isolating cell wall proteins from stabilised protoplasts. Most importantly, $MgCl_2$ and raffinose are required for generating stable and viable protoplasts. In their absence, protoplasts are leaky and lysostaphin-digested cell wall fractions are contaminated with membrane and cytoplasmic material. Recent analysis of the cell envelope of *S.aureus* indicated that EbpS is associated with the cytoplasmic membrane and not the cell wall (R.Downer, personal communication). The discrepancy between these results can be explained by the difference in digestion conditions used. In the absence of raffinose and $MgCl_2$ the cell wall fraction is contaminated with membrane and cytoplasmic proteins. Hence, EbpS found in the cell wall fraction was due to membrane contamination. This is supported by the study of EbpS expression carried out in strains Newman Δ*spa* and Cowan Δ*spa* in the presence and absence of $MgCl_2$ and raffinose. In the presence of both $MgCl_2$ and raffinose EbpS is detected only in the protoplast fraction as an 80-83 kDa protein. However, in the absence of $MgCl_2$ EbpS is detected in the cell wall fraction of both strains due to membrane contamination.

Moreover, Park *et al.* (1996) identified the *ebpS* gene product in strain Cowan as a 25 kDa protein. The discrepancy in EbpS protein size can also be explained by the difference in digestion conditions used. In the presence of $MgCl_2$ and raffinose EbpS is detected as an 80-83 kDa protein however, omitting both components from the digestion buffer renders EbpS undetectable in strain Cowan Δ*spa*. It is possible that contamination of cytoplasmic proteases may have caused complete proteolysis of the protein. Although the 25 kDa protein was not detected under the latter digestion conditions it is most likely that this form of the protein, previously described as full-length EbpS, represents a breakdown product of the mature 83 kDa protein. Since Park *et al.* (1996) demonstrated that the 25 kDa protein retained ligand binding activity and contained the correct N-terminal amino acid sequence suggests that it represents an N-terminal truncate.

Both native and recombinant forms of the full-length protein exhibited proteolysis at the C-terminal domain. The recombinant protein appeared to be subject to more breakdown than the native form. In contrast, a C-terminal truncate of EbpS ($rEbpS_{343-486}$) showed little proteolysis (R.Downer, personal communication)

suggesting that the breakdown products observed in the full-length protein were promoted by the different purification procedures and that treatment with denaturing agents rendered the rEbpS more susceptible to proteolysis. Alternatively, one could speculate that the full length rEbpS protein has a different secondary structure at the C-terminus than $rEbpS_{343-486}$ which is more susceptible to proteolysis than the C-terminal truncate.

EbpS was detected as an 83 kDa protein by antibodies directed against both the N- and C-terminus of the protein. It is interesting to note that native 8325-4 EbpS released from the *S.aureus* cell membrane migrated at the same size as the full-length recombinant protein. This observation suggests that the native protein is targeted to the membrane compartment without any processing occurring.

Chapter 4

Studies on the Elastin Binding Ability of *Staphylococcus aureus*

4.1 Introduction

4.1.1 Elastin

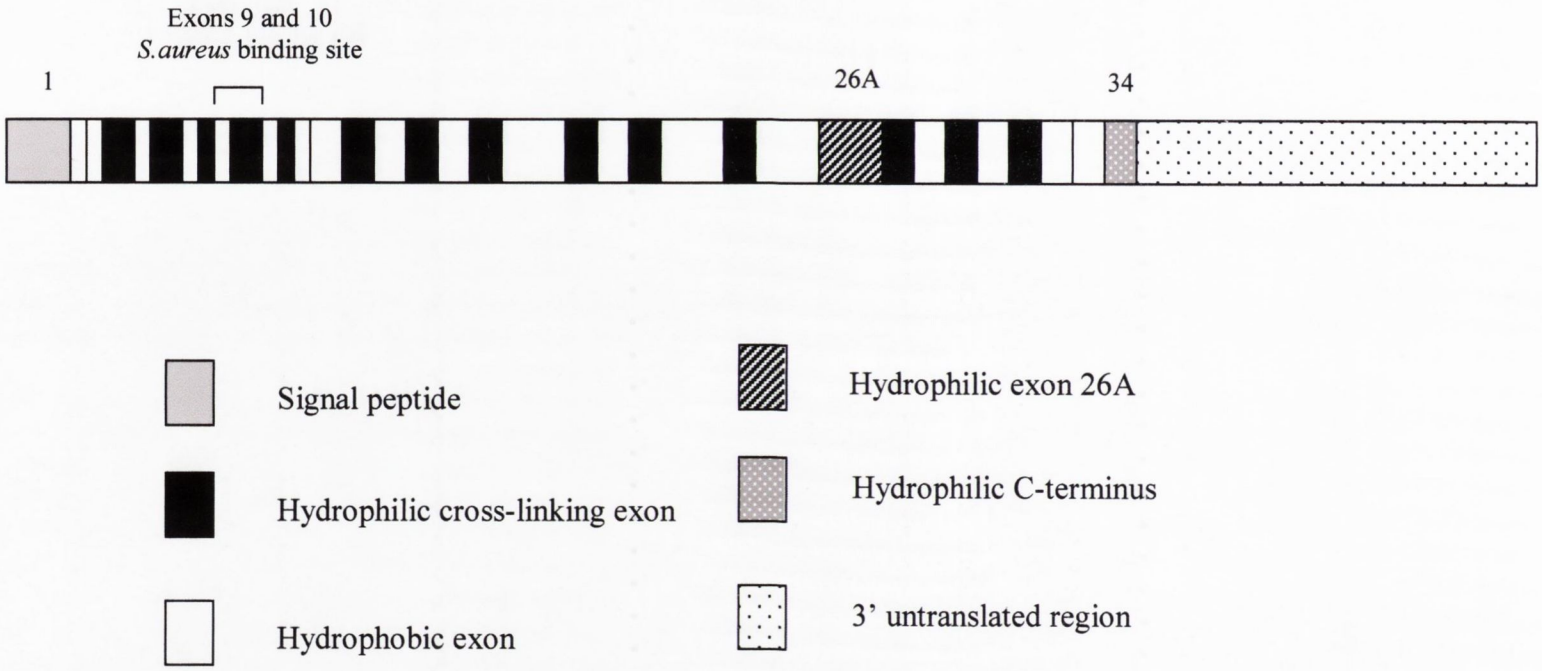
Elastic fibers are among the most complex structures of the extracellular matrix. They are composed mainly of elastin, microfibrillar proteins and lysyl oxidase. The structure of elastic fibers has been found to vary in different tissues. In elastic ligaments, lung and skin, the fibers are small, forming interwoven rope-like filaments. In the aorta, elastic fibers are organised as concentric sheets or lamellae. In elastic cartilage, the fibers form a three-dimensional honeycomb arrangement. Elastin is the major protein of elastic fibers. It is an extremely hydrophobic and insoluble protein with 70% of its residues being non-polar. Its principle function is to provide elasticity and resilience to tissues. However, elastin also promotes cell adhesion and elastin peptides have been shown to be chemotactic (Varga *et al.*, 1989).

Tropoelastin is the soluble precursor of elastin. It is firstly secreted from the cell into the extracellular space where it is rapidly rendered insoluble by crosslink formation between protein monomers at lysine residues by the enzyme lysyl oxidase. Fibroblasts, endothelial cells and smooth muscle cells have all been found to synthesize tropoelastin. The amino acid sequence of tropoelastins from various sources including human (Indik *et al.*, 1987) and bovine (Yeh *et al.*, 1989) have been deduced from DNA sequence and are very similar. Tropoelastin is made with an N-terminal 26-amino-acid signal peptide and in human is secreted as an approximately 72 kDa protein. Hydrophobic and cross-linking domains are encoded by separate and alternating exons (Bashir *et al.*, 1989; Indik *et al.*, 1987; Figure 4.1).

As a result of the cross-linking and consequent insolubility, structural analysis of mature elastin has been greatly hampered but now the secondary structure of insoluble human elastin has been described (Debelle *et al.*, 1998). The results were similar to those estimated for its solubilised form, κ-elastin, and for insoluble bovine elastin (Debelle *et al.*, 1995).

Human elastin consists of a three-dimensional repetition of globular β-class tropoelastin molecules which are closely packed and crosslinked by their helical domains (≈10% α-helices) while their hydrophobic 'elastic' regions consist of buried short and/or distorted antiparallel β-strands (≈35%) alternating with external

Figure 4.1 Structure of human tropoelastin cDNA. The human gene has 34 exons encoding alternating hydrophobic and cross-linking domains designated by the white and black boxes, respectively. Exon 26A is an unusual highly hydrophilic domain which is only found in human tropoelastin and whose function is unknown. Exon 34 is highly conserved amongst species (>70%) and codes for a basic C-terminus in addition to a large 3'-untranslated region which has been proposed to contain regulatory elements (Vrhovski and Weiss, 1998).


Exons 9 and 10
S.aureus binding site
1
26A
34
Signal peptide
Hydrophilic cross-linking exon
Hydrophobic exon
Hydrophilic exon 26A
Hydrophilic C-terminus
3' untranslated region

undefined substructures (≈55%). Elastin can be solubilised by non-specific hydrolysis with weak acids or alkali resulting in a mixture of peptides of varying lengths. Oxalic acid is used to form α-elastin and KOH is used to form κ-elastin (Mecham and Lange, 1982).

4.1.2 Bacterial interactions with elastin

Some microbial pathogens can colonise the host to initiate infection by adhering to components of the extracellular matrix. In the case of *Staphylococcus aureus*, adherence is mediated by surface protein adhesins called MSCRAMMs. *S.aureus* can adhere to many ECM proteins including fibrinogen, fibronectin, laminin, vitronectin and collagen. However, adherence of *S.aureus* or indeed any microbial pathogen, to solid-phase elastin has not previously been shown. Park *et al.* (1991) previously demonstrated that *S.aureus* is capable of binding to soluble elastin (tropoelastin) mediated by a 25 kDa elastin-binding protein, EbpS. A series of overlapping EbpS fragments and recombinant constructs were used to localise the ligand binding site to a 21-amino acid region contained within residues 14-34 (Park *et al.*, 1999). The ability of a recombinant EbpS construct containing residues 1-34 to neutralise the inhibitory activity of anti-$EbpS_{1-212}$ antibodies on *S.aureus* binding soluble tropoelastin further demonstrated the importance of this region in binding elastin. Critical residues were further defined by testing the ability of overlapping synthetic peptides corresponding to residues 14-36 to inhibit binding of *S.aureus* to tropoelastin. A hexameric sequence Thr^{18}-Asn-Ser-His-Gln-Asp^{23} was shared by all active constructs and loss of activity by substition of Asp^{23} with Asn in the active synthetic peptides provided further evidence for the importance of this sequence in elastin binding. However the inability of this synthetic hexamer to inhibit *S.aureus* elastin binding suggests that the EbpS binding domain is conformational and requires flanking residues for elastin recognition. The bacterial binding domain on tropoelastin is distinct from the binding site of the mammalian elastin-binding protein and is contained within a 30 kDa fragment at the amino terminus of the molecule. The region encoded by exons 9/10 of tropoelastin appears to contain part of the *S.aureus* binding domain (R. Mecham, personal communication; Figure 4.1) because polyclonal antibodies directed against exons 9/10 of tropoelastin inhibited binding of *S.aureus* to tropoelastin. However, synthetic peptides corresponding to

exons 9/10 did not inhibit binding. This suggests that the interaction between *S.aureus* and tropoelastin requires the exon 9/10 region as well as other N-terminal domains.

In this study, a comparison of *S.aureus* strain Newman and the isogenic *ebpS* mutant was performed to confirm EbpS as an elastin-binding protein. Residual binding of the *ebpS* mutant suggested a second elastin-binding surface component. Ligand affinity blotting was used in an attempt to identify cell wall associated surface proteins capable of interacting with biotinylated elastin peptides. The ability of *S.aureus* to promote adhesion to immobilised elastin was tested.

4.2 Methods

4.2.1 Colony immunoblotting

S.aureus strain Newman Δ*spa*, Newman Δ*spa ebpS*::*erm* (FR003) and Newman Δ*spa ebpS*::*erm* pCU-*ebpS*$^{+}$ (FR004) were grown to early exponential (OD_{600} of 1.0) and stationary phase (OD_{600} of 12) in 20ml of TSB in a 250 ml flask at 37°C with shaking at 200 rpm. Cells were harvested by centrifugation at 7,000rpm for 15 min and washed in 10ml of PBS. Cells were standardised to an OD_{600} of 100 units and 10 µl from this suspension and dilutions was dotted onto a nylon membrane (Protran) and allowed to dry for 30min. The membrane was blocked at room temperature with 5% (w/v) skim milk in PBS for 1 h. Western blots were processed as described in Section 3.2.1.

4.2.2 Iodination of Tropoelastin

Full-length human recombinant tropoelastin (rTE) was kindly provided by Prof. Mecham. rTE was iodinated by adding 80µl of PBS to 20µl of a 1mg/ml solution of rTE (20µg) and incubating with 1mCi of $Na^{125}I$ for 10 min at room temperature in an microfuge tube coated with 30µg of insoluble Iodogen. Unincorporated radioactivity was separated from the radiolabelled protein by PD-10 gel chromatography using PBS as the eluting buffer. The gel column was initially saturated with 5ml of BSA (Sigma) (1mg/ml) before addition of the radiolabelled protein to prevent non-specific binding of rTE. The ^{125}I-rTE was eluted in a 500µl fraction giving an approximate protein concentration of 40µg/ml.

4.2.3 Binding of ^{125}I-rTE to *S.aureus* strain Newman Δ*spa*

The following protocol was adapted for use from Park *et al.* (1991).
Bacteria were grown in 20 ml of TSB for 18 h and then cells were pelleted and washed in PBS. For all soluble binding assays, 1x 10^8 CFU of *S.aureus* strain Newman Δ*spa*, Newman Δ*spa ebpS*::*erm* and Newman Δ*spa ebpS*::*erm* pCU-*ebpS*$^{+}$ was used. The total volume of the reaction was 300µl and TSB was used as the assay buffer. Cells were added to microfuge tubes containing 40ng of ^{125}I-rTE and incubated for 1 hr at room temperature with vortexing. For competition assays excess unlabelled α-elastin peptides (30µg) were added to the reaction mix. The reaction

was terminated by centrifugation for 8 min at 12,000rpm in a microfuge and the supernatant discarded. The pellet was resuspended in 800µl of TSB, transferred to a new microfuge tube and recentrifuged for 8 min to remove unbound tropoelastin and to minimise background due to radiolabelled tropoelastin sticking to the sides of the plastic microfuge tubes. The pellets were washed once more with 1ml of TSB and recentrifuged. The bottom of the microfuge tube was cut off and radioactivity associated with the pellet was measured with an LKB 1280 Ultro gamma counter.

4.2.4 Adhesion assay to immobilised kappa-elastin

To coat the microtitre plate wells, (Porvair), κ-elastin (Elastin Product Company) was dissolved in 0.1M sodium bicarbonate buffer before it was added at a 50µl volume to the wells at a range of concentrations (0.001 - 1mg/ml). The κ-elastin was then air-dried under UV light (366 nm) for 18hr at room temperature (Hinek *et al.*, 1999). Control wells contained sodium bicarbonate buffer only. The plates were then blocked with 5% (w/v) BSA (filtered) for 2h at room temperature. *S.aureus* cells were grown to the appropriate growth phase, washed in 10mM Tris-HCl pH 7 and resuspended to an OD_{600} of 1 unit ($1x10^8$ 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 5mM solution in dimethyl sulfoxide, which was diluted 1:10 in 10mM Tris-HCl to give a working solution of 0.5mM. Bacterial cultures ($1x10^8$ CFU/ml) were incubated with 2.5µg/ml of SYTO-13 (2.5µM) at room temperature for 15 min in the dark. Following the staining procedure and washing of the microtitre plate with PBS, 100µl of a prestained cell suspension (1×10^7 CFU) were added to the protein-coated ELISA wells and incubated in the dark for 2h at room temperature. After further washing with PBS, the amount of cells bound was determined using an LS-50B Luminescence spectrometer with excitation and emission at 488 and 509nm, respectively. To validate the assay, ELISA wells were coated with 5µg/ml fibrinogen (Calbiochem) and the ability of strains Newman and Newman *clfA::erm* to promote adherence was tested.

4.2.5 rEbpS$_{1-267}$ binding immobilised elastin

Microtitre plates (Sarstedt) were coated with a 50 µl volume of κ-elastin (2.5, 1.25 and 0.625mg/ml) and blocked as described previously. Following two steps of

washing with PBS (200 µl/well), 100µl of different concentrations of purified $rEbpS_{1-267}$ (10, 5, 1 and 0.5µg/ml) were added and the plates incubated for 2h at room temperature. Control wells contained no rEbpS. After three washing steps with PBS, the plates were incubated with anti-$EbpS_{1-267}$ antibodies (1:2500 diln) for 1 h followed by further washing and incubation with goat-anti-rabbit-HRP (DAKO, 1:2000 dilution) for 1h. Bound protein was detected by addition of the enzyme substrate TMB (tetramethylbenzidine), and measured using a microtitre plate reader (Labsystems, multiskan EX) at an optical reading of 450nm.

4.2.6 Biotinylation of α-elastin peptides

Alpha-elastin peptides were biotinylated using a commercially available kit (Pierce, EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit). Elastin peptides (5mg) were incubated with a 20-fold molar excess of sulfo-NHS-biotin reagent (0.8mg) in 1ml of PBS for 2h at 4°C. Biotinylated proteins were then separated from free biotinylating reagent using a desalting column (molwt cut off 5,000). The 10ml desalting column was firstly equilibrated with 30ml of PBS. The sample was then added to the column and eluted in 0.5ml fractions with PBS. A BioRad protein assay was used to determine the protein concentration.

4.2.7 Ligand Blotting

S.aureus strain P1 Δ*spa*, Newman Δ*spa*, and Newman Δ*spa ebpS*::*erm* were grown to stationary phase in 20ml of TSB. Cells were pelleted by centrifugation, washed in 10ml of PBS and the cell density was standardised to an OD_{600} of 100 units.The cell wall and protoplast fractions were isolated as described previously (Section 3.2.1). Cell wall and protoplast samples (15µl) were fractionated by 10% (w/v) SDS-PAGE and transferred to PVDF western blotting membranes (Boehringer Mannheim) by the semidry system (Bio-Rad) as described previously. Membranes were blocked for 15 h at 4°C with 50ml of blocking buffer that contained 5% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20 in Tris-buffered saline (5mM Tris, 150mM NaCl, pH7.5). The blots were washed twice with blocking buffer and incubated for 2h at room temperature with biotinylated α-elastin (10pg/ml) in blocking buffer. After washing three times with blocking buffer, the blots were incubated with a 1:2000 dilution of Streptavidin conjugated to horseradish

peroxidase. Membranes were developed using the BM Chemiluminescence Detection System (POD) of Boehringer Mannheim, according to the manufacturer's instructions.

4.3 Results

4.3.1 Colony immunoblotting

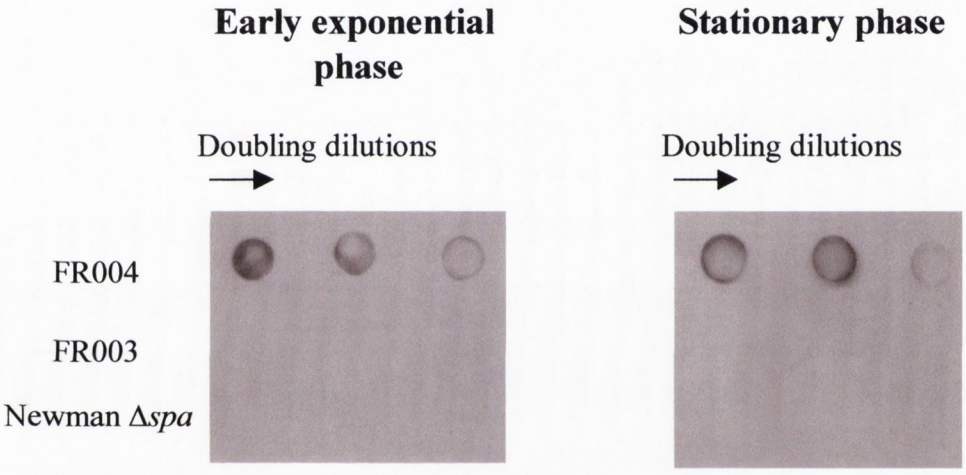
Previous experimental evidence strongly suggested that EbpS is exposed on the cell surface of *S.aureus*. The ligand-binding domain occurs at the N-terminus of the molecule (residues 14-34) and $rEbpS_{1-212}$ and Fab fragments of anti-$rEbpS_{1-212}$ IgG were shown to inhibit *S.aureus* binding to soluble elastin. In this study, whole cell immunoblotting was used to demonstrate that the N-terminal domain of EbpS is surface exposed (Figure 4.2). *S.aureus* strain Newman Δ*spa,* Newman Δ*spa ebpS*::*erm* (FR003) and Newman Δ*spa ebpS*::*erm* (pCU-*ebpS*$^+$) (FR004) were grown to the mid-exponential and stationary phase of growth and probed with anti-$EbpS_{1-267}$ antibodies. EbpS was not detected on the surface of the wild-type or *ebpS* mutant strain. However, a weak reaction occurred with the complemented mutant from both phases of growth. Since EbpS is overexpressed in the complemented mutant (Section 3.3.2), this would suggest that elevated expression of the protein is required to detect EbpS on the cell surface by antibody labelling. EbpS was also demonstrated to be expressed as a surface protein in the heterologous host *Lactococcus lactis* as determined by colony immunoblotting using anti-$EbpS_{1-267}$ antibodies which provides further evidence that the N-terminus of EbpS is surface exposed (R.Downer, personal communication).

4.3.2 Binding of I^{125} recombinant tropoelastin to *S.aureus* strain Newman Δ*spa*

Previous studies with *S.aureus* strain Cowan have shown that EbpS mediates bacterial binding to the soluble elastin precursor (tropoelastin) and to soluble degradation products of elastin (α-elastin peptides). To investigate whether EbpS represented the sole elastin binding activity on the surface of *S.aureus*, the ability of stationary phase cells of *S.aureus* Newman Δ*spa*, FR003 (*ebpS*::*erm*) and FR004 (*ebpS*::*erm* pCU-*ebpS*$^+$) were compared in a soluble I^{125}-rTE binding assay (Figure 4.3). The *ebpS* mutant showed a 70% reduction in I^{125}-rTE binding compared to the parent strain and binding was further reduced by incubating with excess unlabelled elastin peptides. The elastin-binding activity of the parent strain was reduced to the same level as the *ebpS* mutant upon incubation with excess unlabelled elastin peptides. The complementing plasmid pCU-*ebpS*$^+$ restored elastin binding to the

Figure 4.2 Dot blotting of *S.aureus* strain Newman Δ*spa*.

S.aureus strain Newman Δ*spa* and its isogenic mutants were grown to early exponential or late stationary phase of growth, washed in PBS, and standardised to an OD_{600} of 100 units. Doubling dilutions of the cell suspension were dotted onto Protran membrane and probed with anti-$EbpS_{1-267}$ antibodies. FR003 represents Newman Δ*spa ebpS::erm* and FR004 represents Newman Δ*spa ebpS::erm* (pCU-*ebpS*$^{+}$).


Early exponential phase
Stationary phase
Doubling dilutions
Doubling dilutions
FR004
FR003
Newman Δspa

same level as the wild-type strain. This data is representative of three separate experiments and suggests that EbpS is the major elastin binding factor of *S.aureus* strain Newman in stationary phase, and that a second elastin binding site with lower affinity is present.

The ability of strain Cowan to bind I^{125}-rTE throughout the growth cycle was carried out by Park (unpublished data). Results showed that elastin binding was growth phase-dependent. Binding was observed in early exponential phase and showed a gradual increase throughout the growth cycle with maximum binding occurring during the postexponential phase followed by a 45% decrease in stationary phase. Due to the lack of I^{125}-rTE, a time course for strain Newman binding could not be performed. Only late exponential (t = 6 h) and late stationary (t = 18 h) phase cells were tested. Figure 4.3 illustrates the results of the binding assay. Cells from both growth phases exhibited a similar affinity for soluble elastin which is consistent with Western blotting data showing similar EbpS protein levels during these phases of growth (Section 5.3.1).

4.3.3 *S.aureus* adherence to immobilised elastin

4.3.3.1 Development of an adherence assay

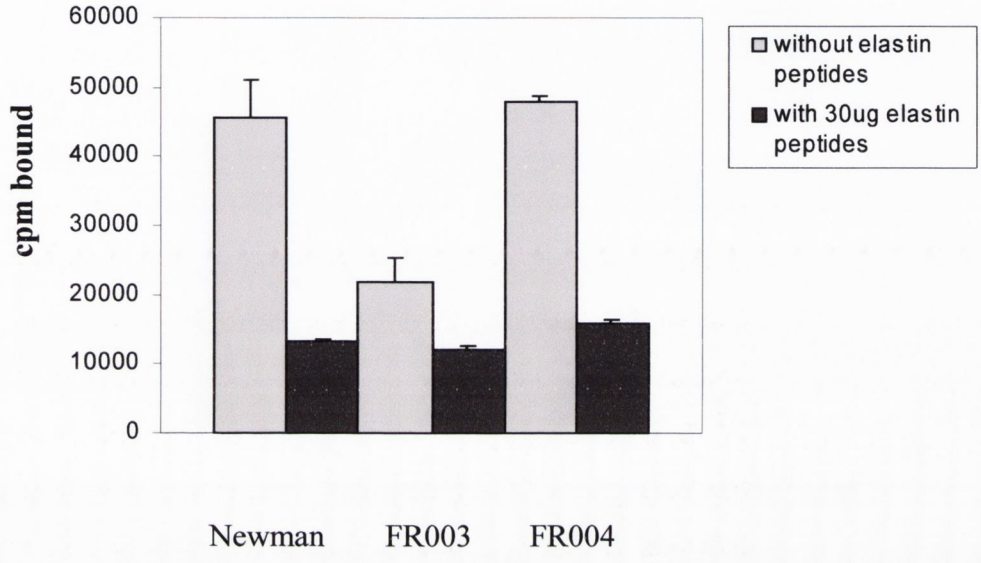
Since EbpS promotes the interaction of *S.aureus* cells with soluble elastin it is plausible that it may also stimulate bacterial adherence to immobilised elastin. To investigate the possible role of EbpS as an adhesin, the ability of *S.aureus* cells to adhere to κ-elastin immobilised on a plastic surface was investigated.

ELISA-based assays are routinely used to demonstrate adherence of *S.aureus* to immobilised collagen, fibrinogen and fibronectin. Bacterial cell adherence is measured by staining cells with crystal violet and reading the optical density at 450nm (Wolz *et al.*, 1996). However, the development of an ELISA-based elastin adherence assay proved both time-consuming and laborious. Difficulties arose with the ligand, coating conditions and methods of bacterial detection.

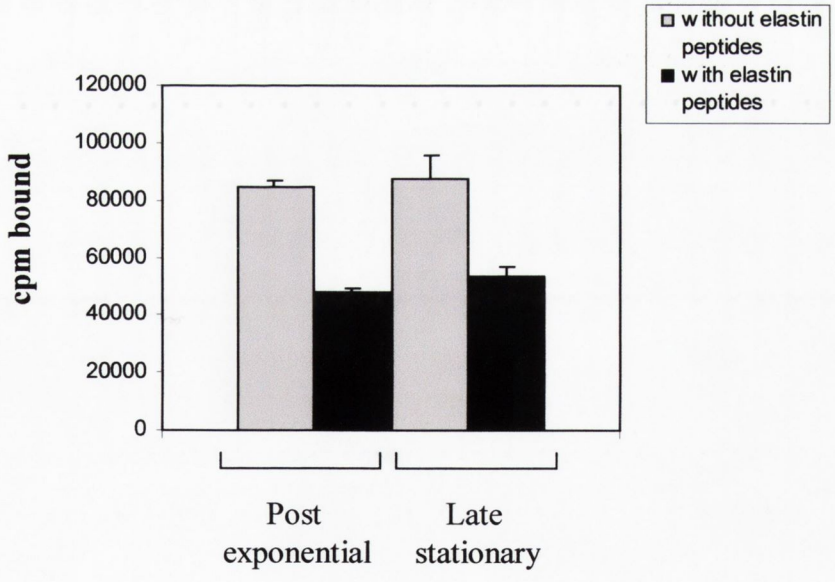
Firstly, it was decided to use recombinant bovine tropoelastin (rTE) as the ligand. Prof. Robert Mecham kindly provided a pQE30 construct expressing bovine tropoelastin (exons 1-15) with an N-terminal His-tag. However, due to its hydrophobic nature, purification of rTE from *E.coli* proved difficult. The expected molecular weight of the N-terminal truncate was 32 kDa. However in this study the

Figure 4.3 A.Binding of radioiodinated human tropoelastin to stationary phase strain Newman Δ*spa* and isogenic mutants. B. The ability of late exponential (t = 6 h) and stationary phase (t = 18 h) strain Newman Δ*spa* to bind radioiodinated human tropoelastin. *S.aureus* cells (1 x 10^8 CFU) were incubated with 40ng of I^{125}-labelled elastin peptides in the absence or presence of excess unlabelled elastin peptides (30μg) for 1hr at room temperature. FR003 represents Newman Δ*spa ebpS::erm* and FR004 represents Newman Δ*spa ebpS::erm* (pCU-*ebpS*+). Radioactivity associated with cells is presented as the mean cpm bound ± S.D. of triplicate determinations. Each experiment was performed three times with similar results.

A


60000
50000
40000
30000
20000
10000
0
cpm bound
without elastin peptides
with 30ug elastin peptides
Newman
FR003
FR004

B


120000
100000
80000
60000
40000
20000
0
cpm bound
without elastin peptides
with elastin peptides
Post exponential
Late stationary

expressed protein occured as high molecular weight multimers (~85 kDa) in SDS-PAGE when analysed with His-probe and anti-tropoelastin antibodies. This is indicative of aggregate formation. Several different growth conditions (addition of glucose, growth at 25 and 30°C, varying IPTG concentrations and induction times) and different strains of *E.coli* (XL1, TOPP-3, and M15) were examined in an attempt to reduce aggregate formation with little success. Full-length recombinant bovine tropoelastin was then provided by Prof. Robert Mecham. Coating of ELISA wells was successful as determined by anti-tropoelastin antibodies. Optimum coating buffer was 0.02% sodium carbonate buffer with an overnight incubation temperature of 4°C. However, storage of this protein caused problems since the tropoelastin supplied was susceptible to proteolysis. An alternative ligand to tropoelastin was κappa elastin peptides (κ-elastin), a solubilised form of elastin prepared from bovine neck ligament by boiling in NaOH followed by hydrolysis in KOH (Mecham and Lange, 1982). The use of κ-elastin as the ligand offered the advantages that it is available in large quantities, it is not readily susceptible to proteolysis and degradation and it is more similar to mature elastin because it contains the interpeptide crosslinks. Despite this, difficulties arose in the coating of 96 well ELISA plates with κ-elastin.

Several strategies were employed in attempts to coat the ELISA wells with this protein such as (i) different coating buffers and (ii) different incubation temperatures (4°C, 37°C, room temperature). However all approaches were unsuccessful in promoting binding of κ-elastin to the plastic surface. Finally, a technique used for elastin adherence assays with mammalian cells was adopted (Hinek *et al.*, 1999). Binding of κ-elastin to the plastic surface was determined by detection using anti-tropoelastin antibodies. The technique involves coating ELISA wells with elastin peptides in a 0.1 M sodium bicarbonate buffer and incubating under UV light at room temperature for 18 hours.

The next step in the development of the assay was to establish a method for detecting bacterial adherence. Since κ-elastin coated wells stained with crystal violet, the method used in the detection of bacterial attachment to immobilised fibrinogen (Wolz *et al.*, 1996), an alternative detection system was needed. The SYTO-13 probe is a high affinity nucleic acid stain that easily penetrates both gram-positive and

gram-negative bacteria. Labelled bacteria emit a bright green fluorescent signal after excitation at 488 nm. This can be detected with a fluorescence spectrometer.

4.3.3.2 Optimum staining conditions of *S.aureus* with SYTO-13

The SYTO-13 probe has previously been used for detecting *S.aureus* cells in Flow Cytometry (Mason *et al.*, 1998). Since the fluorescence is measured by a different detection system in this study it was necessary to determine the optimum probe concentration and time of incubation. *S.aureus* Newman Δ*spa* cells were grown to the stationary phase of growth, washed in 10mM Tris-HCl pH 7 and resuspended to an OD_{600} of 1 unit ($1x10^8$ CFU/ml). Bacterial cultures were incubated at room temperature in the dark with a range of SYTO-13 probe concentrations (20μM, 10μM, 5μM and 2.5μM) (Section 4.2.4). Following the labelling procedure, 1 x 10^7 CFU of bacterial cells were added to the microtiter wells and fluorescence was measured at intervals over a 2 h time period using a luminescence spectrometer with emission and excitation at 488 and 509 nm, respectively (Figure 4.4). An incubation time of 15 min and a probe concentration of 2.5μM were found to be optimum corresponding to a 1:200 (vol/vol) dilution of the SYTO-13 working solution. These conditions were used in subsequent adherence experiments.

4.3.3.3 Validation of fluorescence detection system

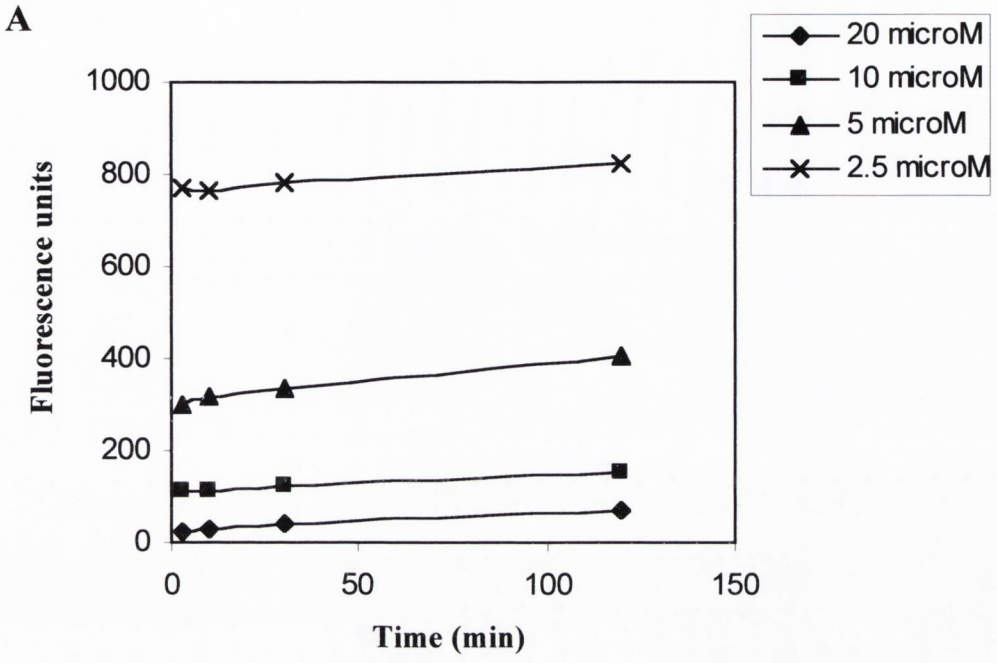
Since the SYTO-13 detection probe has not previously been used in an ELISA-based adherence assay, it was essential to perform a control assay for example by testing the ability of stationary phase strain Newman and Newman *clfA*::*erm* to adhere to immobilised fibrinogen. ClfA has been reported as the primary fibrinogen adhesin of *S.aureus* in stationary phase of growth (McDevitt *et al.*, 1994). Various concentrations of the SYTO-13 probe were used. The assay was performed as described in Section 4.2.4. As expected the wild-type strain promoted binding to fibrinogen compared to the mutant strain which gave only background levels of fluorescence (Figure 4.4). These results indicate that SYTO-13 is a suitable labelling detection system and again confirmed that higher fluorescence units correlated with a probe concentration of 2.5μM.

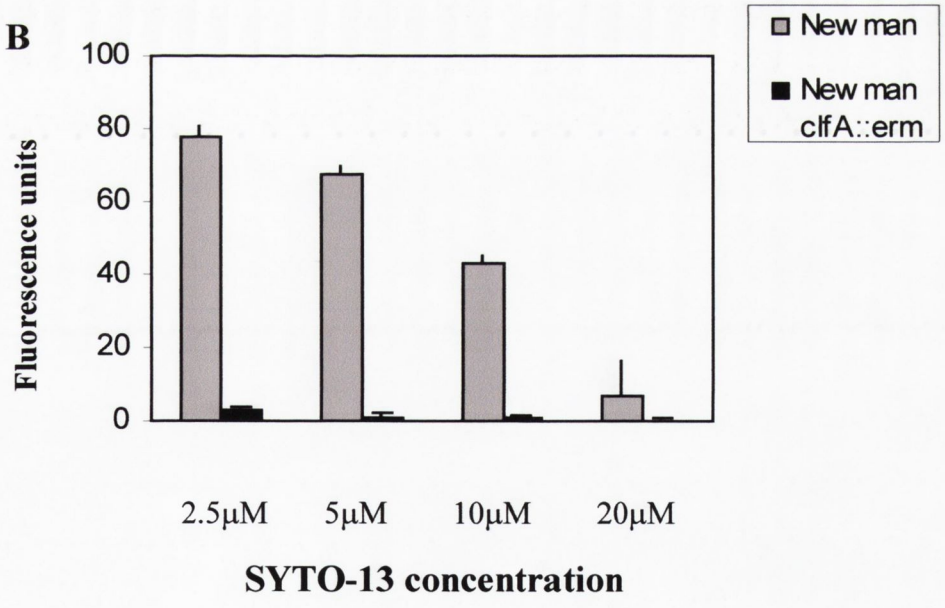
Figure 4.4 A. Optimisation of SYTO-13 staining conditions for *S.aureus.*

Stationary phase *S.aureus* strain Newman Δ*spa* was incubated with a range of concentrations of SYTO-13 and fluorescence was measured at different incubation times as described in Section 4.3.3.2.

B. Validation of SYTO-13 probe for use in *S.aureus* adhesion assays.

Stationary phase *S.aureus* strain Newman and Newman *clfA*::*erm* were prestained with a range of concentrations of SYTO-13 and tested in their ability to adhere to immobilised fibrinogen. The coating concentration of fibrinogen was 5μg/ml.


A
20 microM
10 microM
5 microM
2.5 microM
Fluorescence units
1000
800
600
400
200
0
0
50
100
150
Time (min)


B
New man
New man clfA::erm
Fluorescence units
100
80
60
40
20
0
2.5μM
5μM
10μM
20μM
SYTO-13 concentration

4.3.3.4 Adherence of *S.aureus* to immobilised elastin

The ability of several *S.aureus* strains to bind immobilised κ-elastin was investigated. In the first assays, microtitre plate wells were coated with κ-elastin (50μg/well) and bacterial cell adherence was monitored over the growth cycle including early exponential (t=1.5h), mid-exponential (t=3h) and late stationary phase of growth (t=18h). Strains Newman, 8325-4 and COL cells from early exponential phase showed very weak elastin binding activity. In contrast, early exponential phase cells of strains P1 and Cowan promoted strong adherence (Figure 4.5). To further understand the elastin-binding ability of *S.aureus*, strain P1 was investigated more closely. Binding of strain P1 to elastin occurred during early exponential phase (OD_{600} ≈0.2 – 1.0) but was rapidly diminished as cells progressed into late exponential phase of growth (OD_{600} ≈3.0) (Figure 4.6). Microtitre wells were coated with a range of concentrations of κ-elastin and early exponential phase P1 cells showed an elastin concentration-dependent increase in adherence (Figure 4.7). Maximum adherence was observed at a κ-elastin concentration of approximately 500μg/ml (or 25μg/well). Adherence was still observed at a concentration of 1-2μg/ml (or 0.05-0.1μg/well). Strain P1 Δ*spa* was compared with the wild type strain P1 in its ability to bind elastin (Figure 4.7). A similar binding profile was observed.

4.3.3.5 *S.aureus* adherence to elastin is not EbpS-dependent

In order to determine whether EbpS is responsible for the attachment of *S.aureus* to immobilised elastin, the *ebpS*::*erm* mutation and complementing plasmid were transduced into strain P1 Δ*spa* generating P1 Δ*spa ebpS*::*erm* (FR007) and P1 Δ*spa ebpS*::*erm* (pCU-*ebpS*$^{+}$) (FR008), respectively. Western blotting and adherence to fibrinogen was used to confirm the isogenic mutants (Figure 4.8). The wild-type strain was compared with the *ebpS*-defective mutant and the complemented mutant in its ability to bind to immobilised κ-elastin (Figure 4.8). There was no significant difference in elastin-binding capacity between strains indicating that EbpS is not the major adhesin molecule of early exponential phase *S.aureus* cells which promotes attachment to immobilised elastin. The EbpS protein was also overexpressed in *Lactococcus lactis* (R.Downer, personal communication) and compared with the parental strain in its ability to promote attachment to immobilised κ-elastin (Figure

Figure 4.5 Adherence of several laboratory strains to immobilised κ-elastin.

ELISA plastes were coated with κ-elastin (50μg/well), and 1 x 10^7 CFU of various *S.aureus* strains grown to early exponential phase and prestained with SYTO-13 were added to the wells. Bound cells were detected using a luminescence spectrometer (Section 4.2.4).

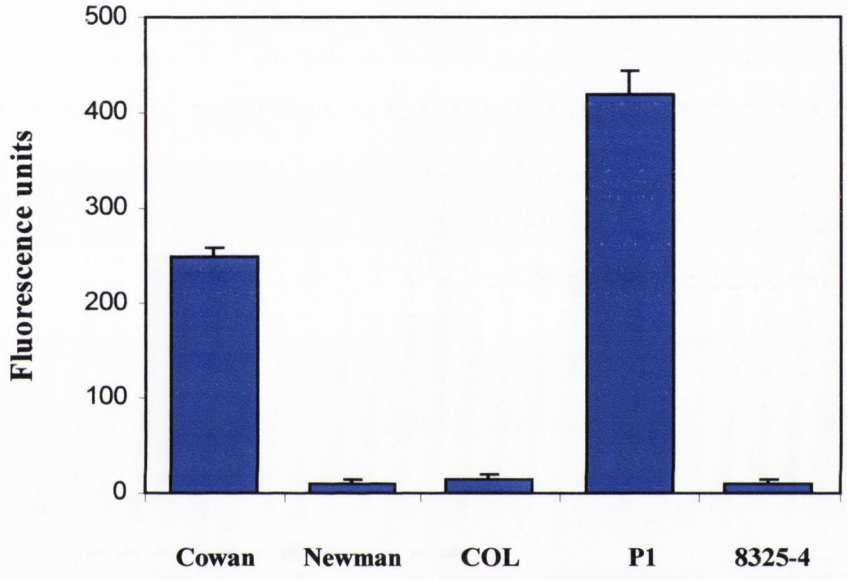

Fluorescence units
500
400
300
200
100
0
Cowan
Newman
COL
P1
8325-4

Figure 4.6 Adherence of *S.aureus* strain P1 to immobilised elastin peptides is growth phase dependent. Strain P1 was grown to different stages of growth and tested for its ability to bind immobilised κ-elastin (50μg/ml). Bound bacteria were measured as described in Section 4.2.4.

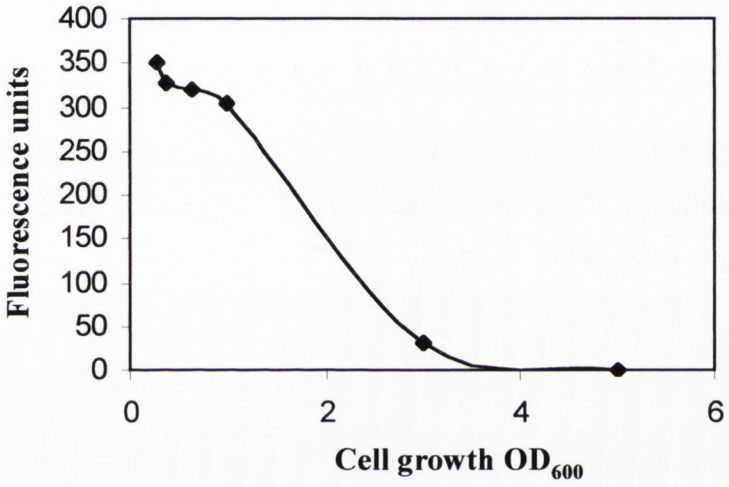
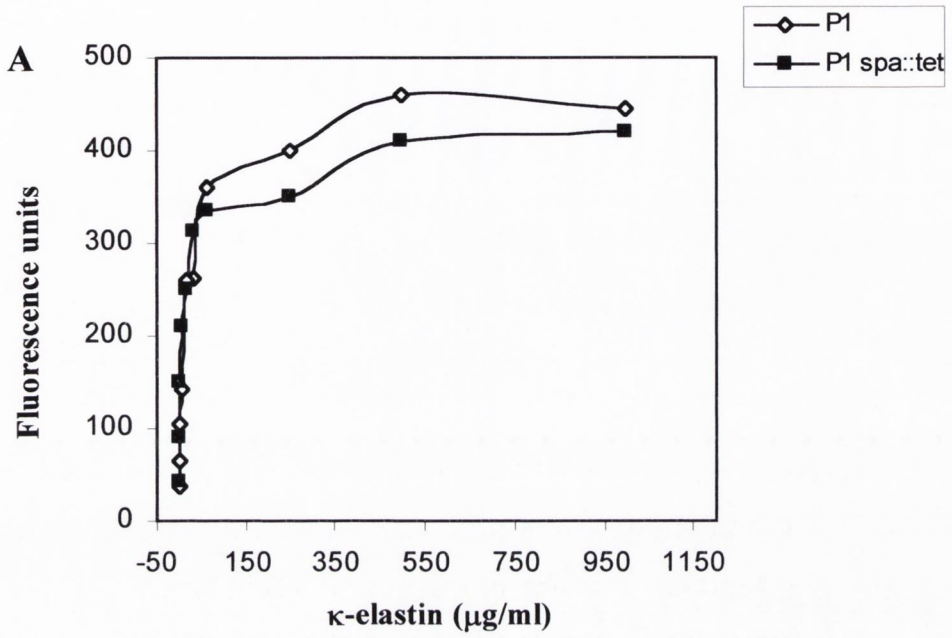
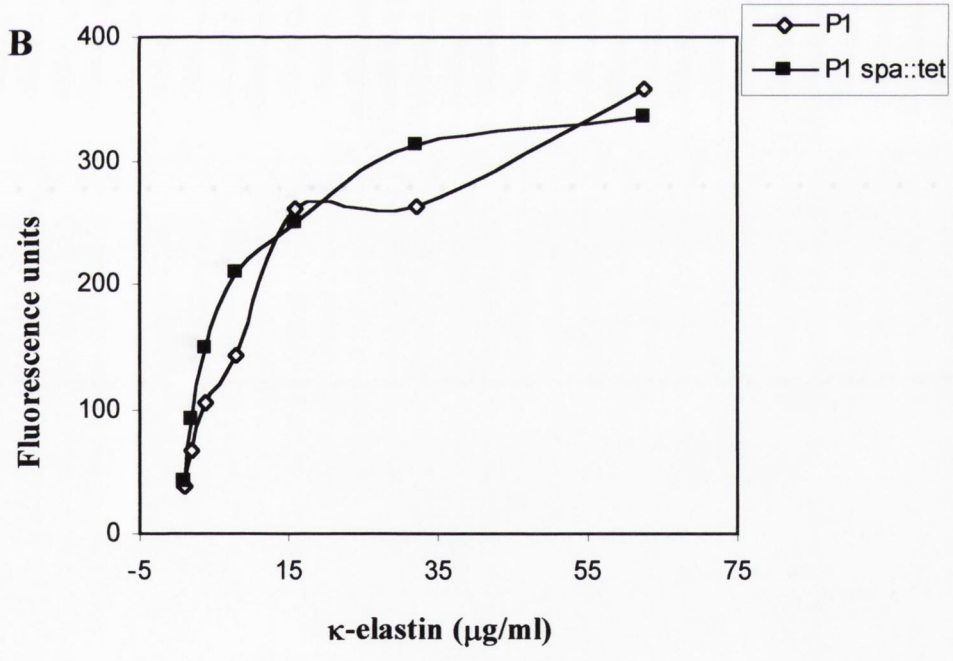

400
350
300
250
200
150
100
50
0
Fluorescence units
0
2
4
6
Cell growth OD600

Figure 4.7 Adherence of *S.aureus* strain P1 and P1 Δ*spa* to immobilised κ-elastin. ELISA plates were coated with increasing amounts of κ-elastin and incubated with a fixed concentration of early exponential phase cells (1 x 10^7 CFU). Panel B represents the first part of the binding curve from panel A.


A
P1
P1 spa::tet
Fluorescence units
500
400
300
200
100
0
-50
150
350
550
750
950
1150
κ-elastin (μg/ml)


B
P1
P1 spa::tet
Fluorescence units
400
300
200
100
0
-5
15
35
55
75
κ-elastin (μg/ml)

4.9). In this host, Downer (personal communication) demonstrated that the N-terminal ligand binding domain is surface exposed although the ability of this strain to bind soluble I^{125}-rTE was not examined. In this strain EbpS expression levels were greater than that observed in the complemented mutant of strain Newman Δ*spa*, (FR004). However, as with strain FR004, no adherence was observed. Thus it appears that EbpS is not an elastin-adhesin and that the adherence detected here is promoted by another surface component.

4.3.4 rEbpS binds to immobilised Elastin

Previous studies reported that an N-terminal His-tagged protein truncate of recombinant EbpS ($rEbpS_{1-212}$) interacted specifically with elastin peptides immobilised onto an affinity column. In this study the ability of $rEbpS_{1-267}$ to interact specifically with κ-elastin-coated ELISA wells was investigated as a control for the bacterial cell adhesion assays. A range of concentrations of rEbpS were tested with various concentrations of immobilised κ-elastin. Bound rEbpS was detected using anti-$EbpS_{1-267}$ antibodies. The negative control value (BSA instead of an elastin-coated well) was subtracted from the sample values. The data in Figure 4.10 indicate that rEbpS can bind to immobilised elastin peptides in a dose-dependent manner, even though it apparently cannot promote bacterial adhesion.

4.3.5 Identification of a putative elastin adhesin

The initial strategy used in an attempt to identify the κ-elastin adhesin expressed by strain P1 was to screen several known surface proteins such as ClfA, SdrC, SdrD and SdrE expressed on the surface of *Lactococcus lactis* (strains supplied by L.O'Brien). However no adherence was observed. Another approach was to test mutants of strain P1 lacking expression of different surface proteins. Surprisingly, inactivation of *fnbA* and *fnbB* in strain P1 reduced binding to background levels (Figure 4.11). To further investigate the role of the FnBPs in promoting adherence to elastin, strain 8325-4 was compared to the complemented mutants 8325-4 *fnb*$^-$ pCU-FnBPA$^+$ and 8325-4 *fnb*$^-$ pCU-FnBPB$^+$ in their ability to bind immobilised κ-elastin. As described previously wild type strain 8325-4 showed weak binding to immobilised κ-elastin. However, overexpression of FnBPA or FnBPB on a multicopy plasmid significantly increased the elastin binding ability of strain 8325-4,

Figure 4.8 The adherence of strain P1 Δ*spa* to immobilised κ-elastin is EbpS-independent. A. Western immunoblotting of P1 Δ*spa* (lane 1), FR007 (*ebpS*::*erm*; lane 2) and FR008 (*ebpS*::*erm* pCU-*ebpS*$^{+}$; lane 3) using anti-$EbpS_{343-486}$ antibodies. B. An adherence assay comparing the ability of stationary phase cells of strain P1 Δ*spa* and its isogenic mutants to bind fibrinogen (5μg/ml). C. An adherence assay comparing the ability of early exponential phase cells of strain P1 Δ*spa* and its isogenic mutants to bind κ-elastin. ELISA plates were coated with increasing amounts of κ-elastin and a fixed concentration of cells (1×10^7 CFU).

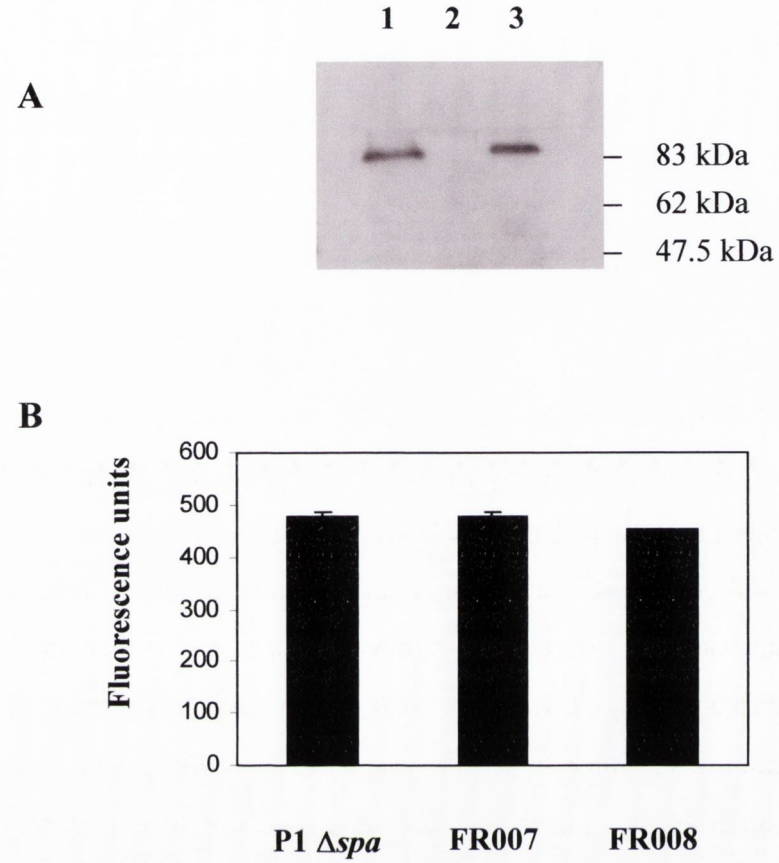
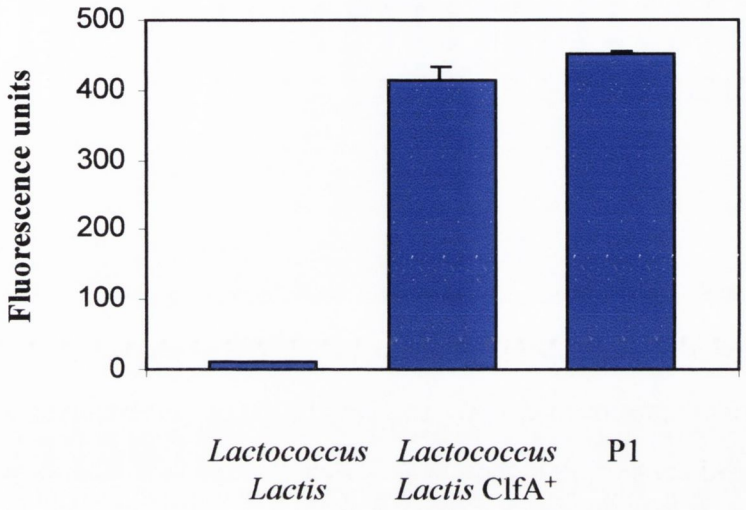

A
1 2 3
83 kDa
62 kDa
47.5 kDa
B
Fluorescence units
600
500
400
300
200
100
0
P1 Δspa
FR007
FR008
C
Fluorescence units
400
350
300
250
200
150
100
50
0
-50
150
350
550
750
950
1150
κ-elastin (μg/ml)
P1
FR007
FR008

Figure 4.9 Adherence of *Lactococcus lactis* and its derivatives to immobilised fibrinogen (A) and κ-elastin (B). ELISA plates were coated with a fixed concentration of fibrinogen (5μg/ml @100 μl/well) and κ-elastin (62.5 μg/ml @50 μl/well) and the ability of stationary phase *Lactococcus lactis* cells and early exponential phase P1 cells to bind was detected using the SYTO-13 probe.

A


Fluorescence units
500
400
300
200
100
0
Lactococcus Lactis
Lactococcus Lactis ClfA+
P1

B

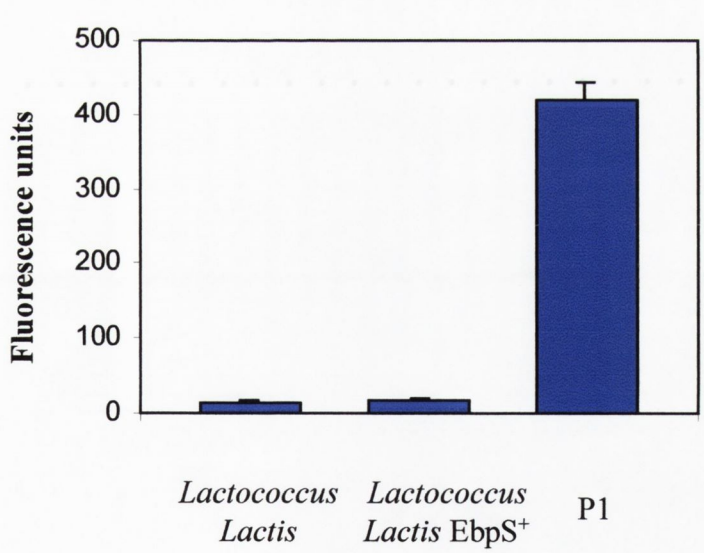

Fluorescence units
500
400
300
200
100
0
Lactococcus Lactis
Lactococcus Lactis EbpS+
P1

Fig 4.10 Binding of recombinant EbpS (residues 1-267) to immobilised κ-elastin using an ELISA-based assay. A range of concentrations of rEbpS were tested against various concentrations of immobilised κ-elastin. The negative control is no elastin in the wells. Binding was detected using anti-$EbpS_{1\text{-}267}$ antibodies as described in Section 4.2.5. The data is presented as the mean $\pm$ SD of triplicate measurements.

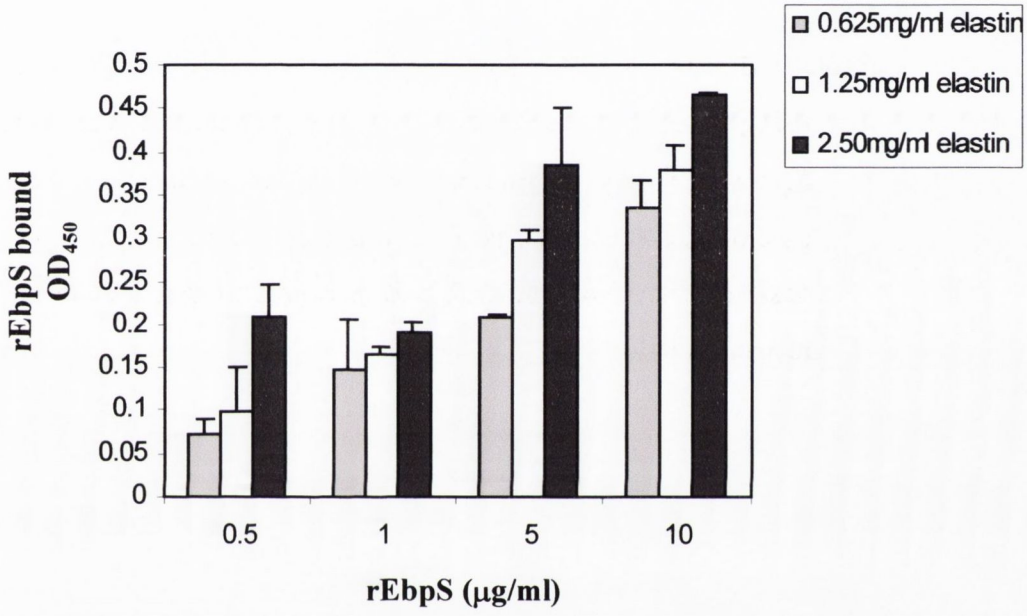
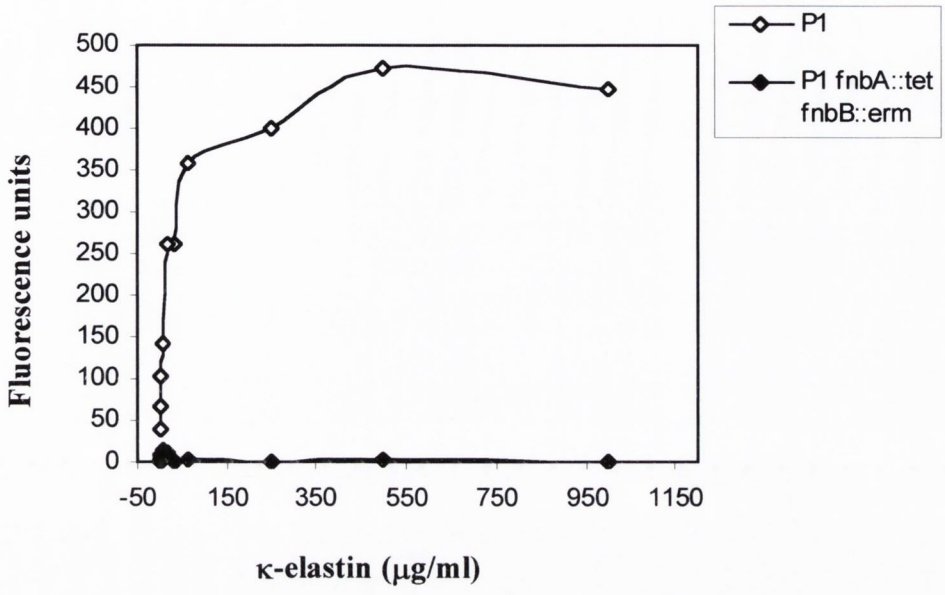

0.625mg/ml elastin
1.25mg/ml elastin
2.50mg/ml elastin
rEbpS bound
OD450
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.5
1
5
10
rEbpS (μg/ml)

Figure 4.11 Adherence of *S.aureus* strain P1 and its *fnb*-defective mutant to immobilised κ-elastin. ELISA plates were coated with increasing concentrations of κ-elastin and a fixed concentration of early exponential phase cells (1 x 10^7 CFU). A. The ability of strain P1 and P1 *fnbA::tet fnbB::erm* to bind κ-elastin. B. An enlargement of the exponential section of the binding curve from panel A.


P1
P1 fnbA::tet fnbB::erm
Fluorescence units
500
450
400
350
300
250
200
150
100
50
0
-50
150
350
550
750
950
1150
κ-elastin (μg/ml)

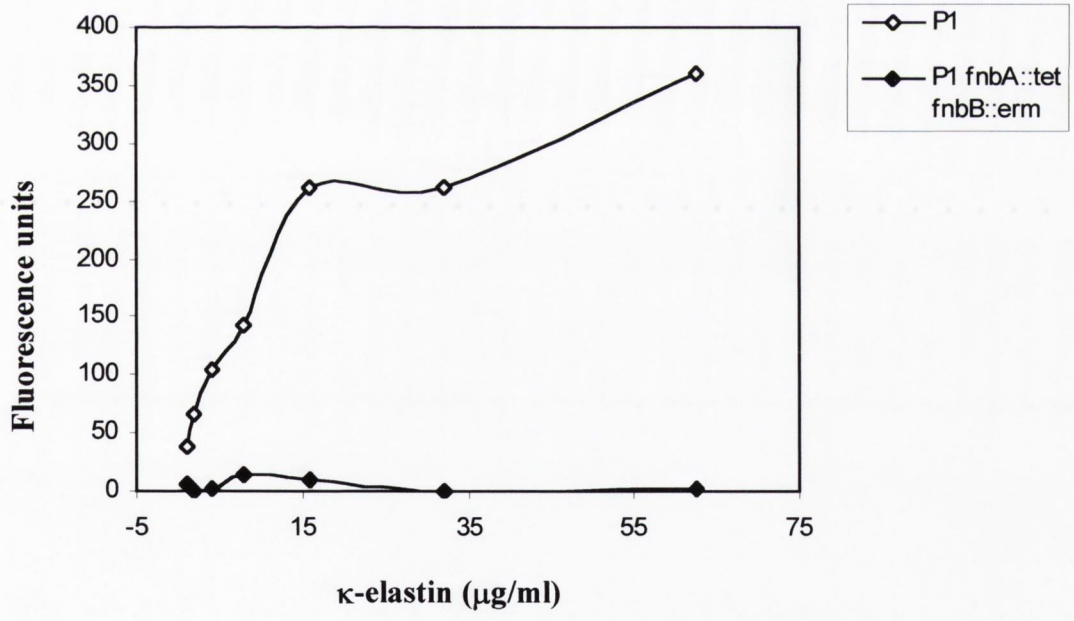
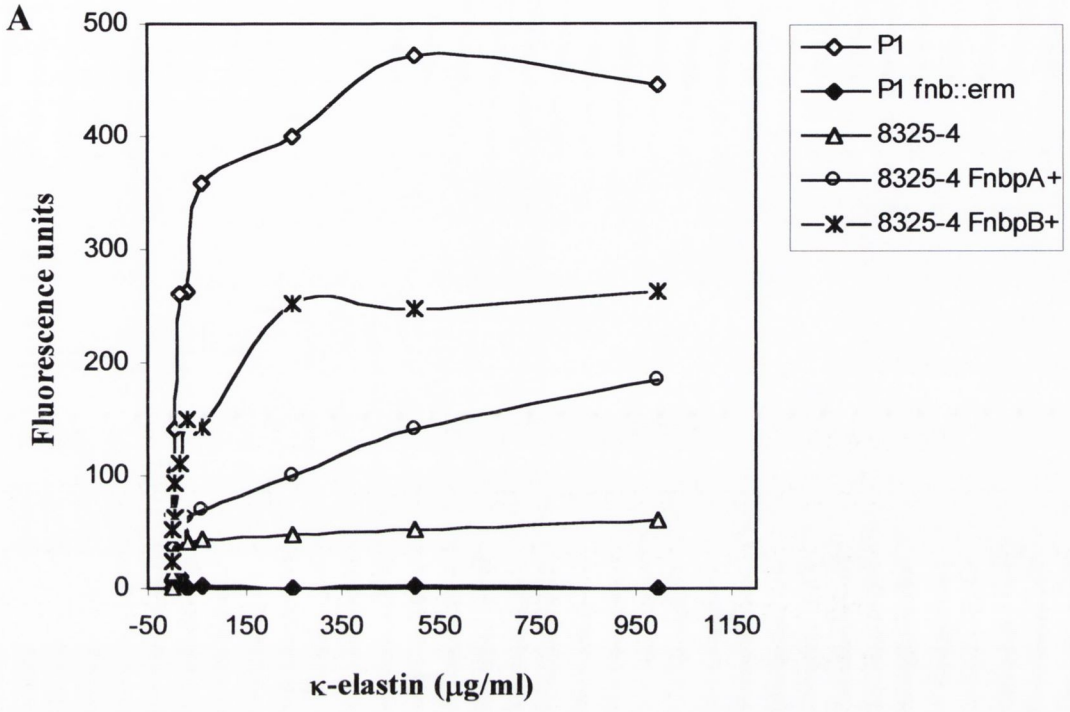
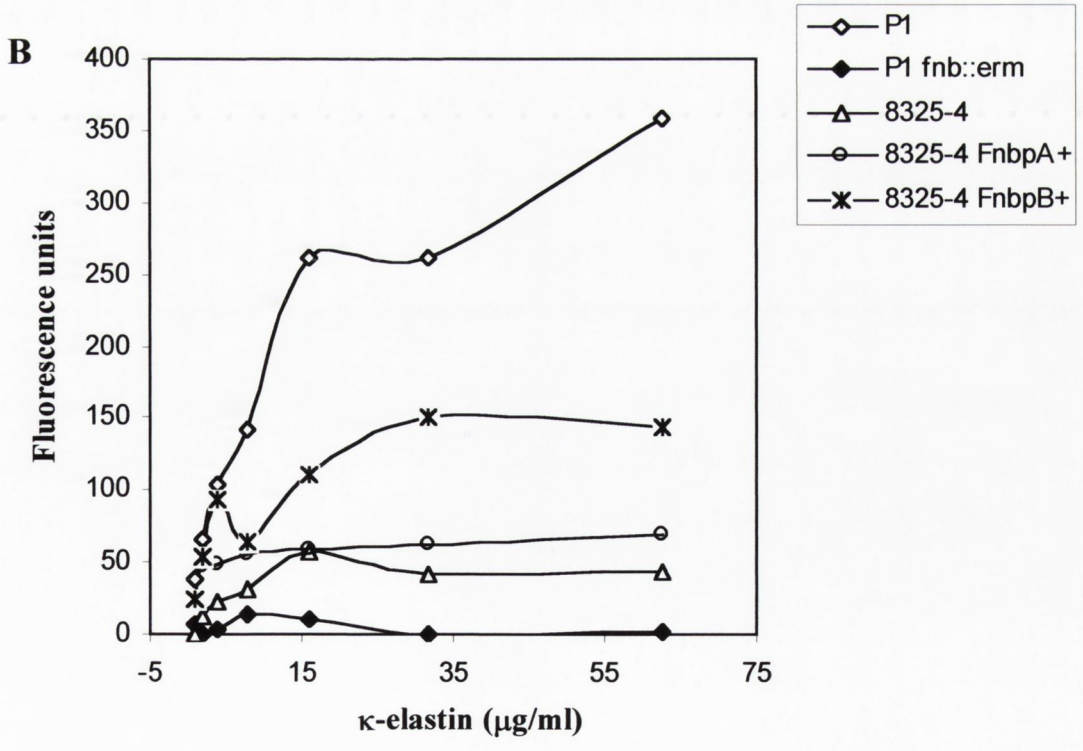

P1
P1 fnbA::tet fnbB::erm
Fluorescence units
400
350
300
250
200
150
100
50
0
-5
15
35
55
75
κ-elastin (μg/ml)

Figure 4.12 The role of fibronectin binding proteins (FnBPA and FnBPB) in promoting the adherence of *S.aureus* to immobilised elastin peptides. ELISA plates were coated with increasing concentrations of κ-elastin and a fixed concentration of early exponential phase cells (1 x 10^7 CFU). Panel B represents an enlargement of the exponential section of the binding curve from panel A. Strains 8325-4 FnbpA$^+$ and 8325-4 FnbpB$^+$ correspond to a double *fnbA* and *fnbB* knockout mutant of strain 8325-4 overexpressing the FnbpA or FnbpB protein, respectively on a multicopy shuttle plasmid.


A
500
400
300
200
100
0
Fluorescence units
-50
150
350
550
750
950
1150
κ-elastin (μg/ml)
P1
P1 fnb::erm
8325-4
8325-4 FnbpA+
8325-4 FnbpB+


B
400
350
300
250
200
150
100
50
0
Fluorescence units
-5
15
35
55
75
κ-elastin (μg/ml)
P1
P1 fnb::erm
8325-4
8325-4 FnbpA+
8325-4 FnbpB+

strongly suggesting that the FnBPs represent elastin adhesins (Figure 4.12). FnBPB appeared to promote higher levels of adherence compared to FnBPA.

4.3.6 Clinical data

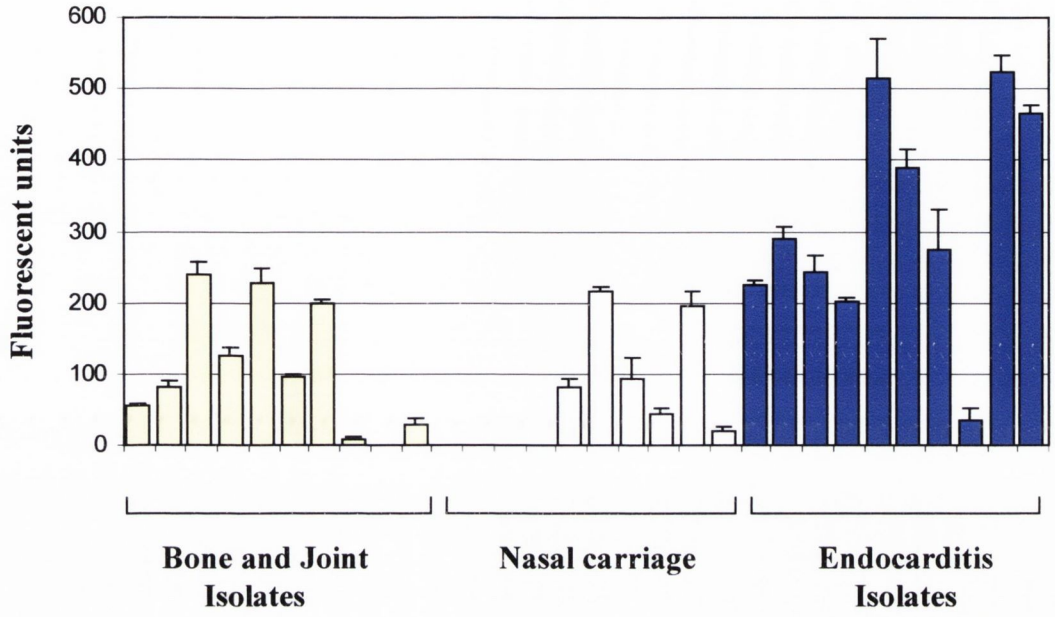
To determine whether there was a correlation between elastin-binding activity and disease type a panel of 30 clinical isolates were tested in their ability to adhere to immobilised κ-elastin using the ELISA-based assay established in this study. Isolates were categorised into three groups according to the clinical history of the patient from whom they were isolated: nasal carriage in the absence of *S.aureus* disease (n=10); native-valve endocarditis (n=10); and primary septic arthritis and/or osteomylitis (n=10). Isolates were from individuals with community-acquired carriage or infection and each group represented a mixed selection from Oxford and Auckland. Adherence of the bacterial group associated with endocarditis infection (100% bound) was greater than that for the nasal (40% bound) and bone and joint (90% bound) infections (Figure 4.14). The adherence of the endocarditis strains (removing E8) ranged from 48% to 124% of the mean adherence for *S.aureus* strain P1. A dotplot illustrates that the mean adherence of the endocarditis group is greater than the other groups tested (Figure 4.13).

4.3.7 Ligand Blotting

Ligand blotting was employed to compare the profile of elastin binding proteins of strains P1 Δ*spa*, Newman Δ*spa* and Newman Δ*spa ebpS*::*erm* (Figure 4.14). Cell wall and protoplast fractions from stationary phase cells were fractionated on an SDS-PAGE gel, transferred to nylon membranes and probed with biotinylated elastin peptides (10pg/ml) (Figure 4.14). In *S.aureus* extracts a protein of approximately 120kDa located predominantly in the cell wall fraction and to a lesser extent in the protoplast fraction of each strain was found to interact with biotinylated α-elastin. Detection of an identical protein band in strain Newman Δ*spa ebpS*::*erm* verified that the 120kDa protein was not the EbpS protein. Mutants of strain Newman lacking either *sdr*C, *sdr*D, *sdr*E or *map* were examined (strains supplied by L.O'Brien and M.Höök). In addition, strain P1 was compared against its *fnb*-defective mutant. Inactivation of these genes did not eliminate the 120kDa protein. Interestingly, the native 83kDa EbpS protein in the protoplast fraction did not

Figure 4.13 Adherence of clinical isolates to immobilised elastin.

ELISA plates were coated with 50µl of a 1mg/ml solution of elastin peptides (50 µg/well) and *S.aureus* clinical isolates, grown to early exponential phase were added (1 x 10^7 CFU/well). Each point represents the mean $\pm$ SD of triplicate measurements. Binding assays were only performed once. A dotplot illustrates that the mean adherence of the endocarditis group is greater than the other clinical groups tested.


Fluorescent units
600
500
400
300
200
100
0
Bone and Joint Isolates
Nasal carriage
Endocarditis Isolates

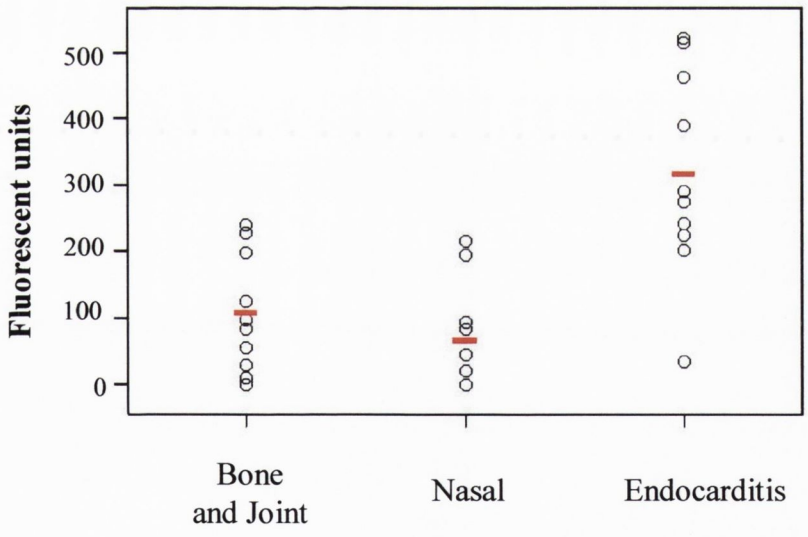
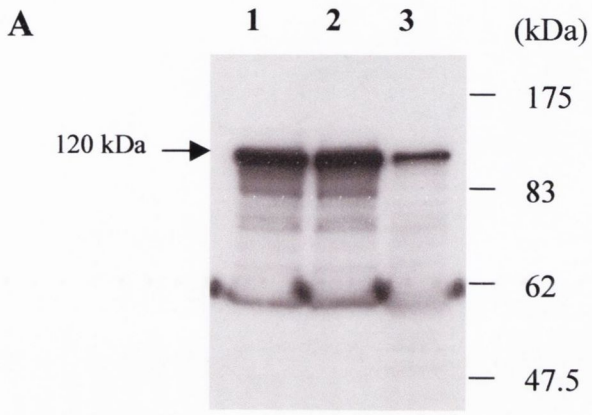
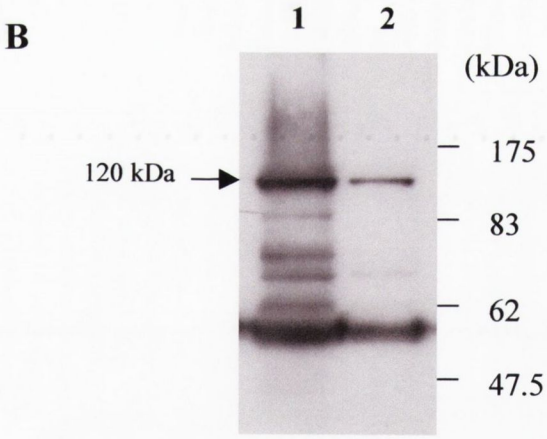

Fluorescent units
500
400
300
200
100
0
Bone and Joint
Nasal
Endocarditis

Figure 4.14 Ligand blot of stationary phase extracts from *S.aureus* strains using biotinylated α-elastin. A. Lane 1, FR003 (*ebpS::erm*) (cell wall fraction); lane 2, Newman Δ*spa* (cell wall fraction); lane 3, Newman Δ*spa* (protoplast fraction). B. Lane 1, P1 Δ*spa* (cell wall fraction); lane 2, P1 Δ*spa* (protoplast fraction). Molecular mass standards are indicated on the right. The weaker bands below the ~120 kDa reactive band may represent degradation products of the 120 kDa protein, non-specific binding or proteins with a weak affinity for α-elastin.


A
1
2
3
(kDa)
120 kDa
175
83
62
47.5


B
1
2
(kDa)
120 kDa
175
83
62
47.5

interact with the biotinylated α-elastin. A possible explanation for this is that protein conformation is important for binding. Denaturation (following SDS-PAGE) and immobilisation on a membrane may result in failure of EbpS to refold to its native conformation.

4.4 Discussion

The first step in the infection process is colonisation, which involves bacterial cell adherence to matrix components mediated by specific surface receptors. Several surface proteins of *S.aureus* contribute to the pathogenesis of this organism by binding to components of the extracellular matrix. For example, binding to collagen mediated by the collagen binding protein has been implicated in osteomylelitis and septic arthritis (Switalski *et al.*, 1993; Patti *et al.*, 1994). Moreillon *et al.* (1995) demonstrated that inactivation of the fibrinogen-binding protein gene, *clfA*, yielded mutants with defective ability to induce experimental endocarditis. *S.aureus* is known to infect elastin-containing tissue such as the lung, heart valves and skin. The ability of *S.aureus* to interact with elastin may also play a role in the pathogenesis of this bacterium. This chapter describes experiments intended to determine if EbpS is an adhesin for immobilised elastin and to confirm its role as a binding determinant for soluble elastin.

Park *et al.* (1991) first described the interaction between soluble elastin and *S.aureus*. Binding of radiolabelled tropoelastin to *S.aureus* strain Cowan was shown to be specific, reversible and mediated by a 25 kDa surface protein, EbpS. In this study EbpS was shown to be an 80-83 kDa protein (Section 3.3.2). The 25 kDa protein must be an N-terminal fragment of the larger molecule. Colony immunoblotting confirmed that the N-terminus of EbpS is exposed on the cell surface. Since the ligand binding domain is located at the extreme N-terminus of the protein (residues 14-34) it is probable that only a small portion of the protein is surface displayed. Therefore, a possible explanation for weak antibody binding is that there are too few epitopes on the surface of cells.

A soluble I^{125}-rTE bacterial binding assay comparing wild-type strain Newman Δ*spa* with its *ebpS*-defective isogenic mutant conclusively showed that EbpS is the primary factor determining binding of soluble elastin to bacterial cells. Complementation of strain FR003 (*ebpS::erm*) with pCU-$ebpS^{+}$ restored EbpS production and the elastin binding capacity to at least the same level as shown by Newman Δ*spa* (Figure 4.2). Western blotting and colony immunoblotting studies have demonstrated that EbpS is overexpressed in the complemented mutant compared to the wild-type strain in the stationary growth phase (Section 3.3.4), yet the binding levels were essentially the same. This suggests that elevated amounts of

protein did not enhance elastin-binding activity. It is possible that additional EbpS molecules may be inactive or are masked by other surface proteins obstructing access of elastin peptides.

The ability of strain Cowan to bind I^{125}-rTE at different stages of the growth cycle was carried out by P.W.Park (personal communication). Binding to soluble elastin was found to be growth phase dependent with maximum binding during the post exponential phase and a 45% decrease in binding capacity during stationary phase. Here, the elastin binding ability of *S.aureus* strain Newman Δ*spa* was monitored during late exponential and stationary phases of growth. A similar level of binding activity was observed for both stages of growth (Figure 4.2). Expression of EpbS was examined in strain Newman Δ*spa*, both at the transcriptional level and at the level of protein expression (described in chapter 5). Transcription of *ebpS* was switched on early in exponential phase showing a gradual increase in the level of the *lacZ* reporter until it appeared to terminate during transition into stationary phase of growth. Western blotting of strain Newman detected similar EbpS protein levels during the post exponential and stationary phases of growth explaining the similar elastin binding profile observed at these stages. The reduction in elastin binding activity in the stationary phase of growth in strain Cowan may be due to (i) removal of protein from the cell surface by proteolysis or (ii) capsule production hindering EbpS accessibility.

Many surface proteins of *S.aureus* are capable of binding both soluble and immobilised forms of the host ligand, *e.g.* the fibrinogen binding proteins (ClfA and ClfB), the fibronectin binding proteins (FnBPA and FnBPB), and the collagen binding protein (Cna). Therefore, the ability of EbpS to bind soluble elastin suggested a role for EbpS as an adhesin. To test this hypothesis, an ELISA-based assay was developed using κ-elastin as the immobilsed ligand and a fluorescent nucleic acid stain, SYTO-13, was employed to measure bound bacteria. *S.aureus* cells were capable of attaching to immobilised elastin but binding was strain specific. Strains 8325-4, Newman and COL failed to bind, yet paradoxically Newman can promote binding of elastin peptides in a soluble binding assay. Adherence was only observed with strains Cowan, P1 and the clinical isolates. Further adhesion studies were performed with strain P1. Binding was observed during early exponential phase but diminished rapidly as cells progressed into mid-exponential phase of growth.

Several factors initially suggested that EbpS could be an elastin MSCRAMM: (i) the EbpS elastin-binding site is surface exposed, (ii) EbpS is capable of binding soluble elastin, (iii) EbpS is expressed and detected on the cell surface and (iv) $rEbpS_{1-267}$ is capable of interacting with immobilised elastin peptides in a dose-dependent manner. However, inactivation of the *ebpS* gene did not affect the ability of strain P1 Δ*spa* to bind immobilised elastin, indicating that EbpS is not an elastin-binding adhesin. This is supported by the observation that overexpression of EbpS in *S.aureus* and *Lactococcus lactis* did not promote adherence to immobilised elastin. A possible explanation for this is that the binding domain of EbpS is very closely associated with the bacterial surface, perhaps even in clefts and may only be accessible to soluble peptides and tropoelastin. It is therefore possible that surface-associated EbpS does not have the flexibility to interact with the immobilised ligand but only allows the interaction with soluble elastin. The possibility that the binding site for EbpS on the elastin peptides (*i.e.* exons 9 and 10 of the tropoelastin molecule) is not exposed when the peptides are immobilised on a plastic surface is unlikely because $rEbpS_{1-267}$ could bind. However, this needs to be verified by showing that the binding is specific, for example by inhibition with a synthetic peptide bearing the ligand-binding domain.

Since EbpS is unlikely to be the major elastin MSCRAMM, it seems likely that *S.aureus* expresses another adhesin, which promotes adherence to elastin in cells from early exponential phase. Here, the fibronectin binding proteins (FnBPs) have been identified as playing a putative role in adhesion. The double $FnBPA^-$ and $FnBPB^-$ mutant of strain P1 did not adhere to κ-elastin. Furthermore, complementation of the poor binding strain 8325-4 with plasmids overexpressing FnBPA and FnBPB conferred elastin adherence. FnBPB appeared to promote higher levels of adherence compared to FnBPA. This is most likely due to higher levels of FnBPB expression as transcriptional studies have shown that the *fnbB* promoter expresses a threefold higher level of transcriptional activity than the *fnbA* promoter (Greene *et al.*, 1995). Alternatively, the FnBPB protein might have a higher affinity for κ-elastin. Collectively, this data suggests that the FnBPs play a role in promoting bacterial attachment to immobilised elastin peptides. However, further characterisation of this interaction at the molecular level would be required to support these initial studies. For example, to ensure the interaction is specific soluble

recombinant FnBPs (rFnBPs), anti-FnBP antibodies and soluble κ-elastin (or tropoelastin) should inhibit bacterial binding. The elastin-binding site could be localised to a domain of FnBP by generating a series of overlapping fragments of rFnBPs and testing their ability to either bind directly to or block bacteria binding to immobilised κ-elastin. EbpS interacts with the N-terminus of the tropoelastin molecule. If a synthetic peptide of EbpS bearing the elastin-binding site inhibited the FnBP-elastin interaction this would suggest that EbpS and FnBP compete for the same ligand-binding site on elastin.

The differences in binding patterns between strains may be attributable to different levels of FnBP expression. Strain Cowan and P1 express high levels of FnBPs (Sinha *et al.*, 1999) and correspondingly promote strong binding to immobilised elastin peptides. Strain 8325-4 only binds weakly to elastin despite possessing both FnBP genes. This poor level of binding can be explained by low levels of expression of FnBPs, as detected by Western ligand affinity blotting (Bisognano *et al.*, 1997). Sinha *et al.* (1999) also found strain 8325-4 to be poorly invasive of human epithelial cells due to low levels of expression of FnBPs. Similarly, strains Newman and COL also showed poor binding to elastin-coated ELISA wells. Strain Newman expresses similar levels of FnBPs as strain Cowan and can bind soluble fibronectin, but it does not adhere efficiently to fibronectin-coated surfaces (Lindberg *et al.*, 1990; Vaudaux *et al.*, 1998) suggesting weak FnBP expression levels or perhaps masking by other surface components such as capsular polysaccharide. The expression of FnBPs has not been studied in strain COL but one could speculate that weak expression levels or masking might also account for the poor adherence levels of this strain to elastin-coated plates. Thus, it appears that a high level of FnBP expression is required for adherence to immobilised elastin.

The ability of the FnBPs to interact with another host ECM protein apart from the cognate ligand is not unique. Recent studies have shown that the N-terminal A region of FnBPA binds specifically to the C-terminal residues of the γ-chain of fibrinogen, a site that is also recognised by ClfA. The multivalent interactions between FnBPs and ECM proteins is likely to be advantageous for the organism in colonising different sites of the host tissue.

In order to further understand the importance of the *S.aureus*-elastin interaction, the ability of a panel of clinical isolates to adhere to elastin was tested.

The clinical isolates were collected from two geographically distant countries and from community-acquired disease to reduce the chance of studying organisms derived from a restricted number of clones. The strains were categorised according to the clinical history including carriage isolates (nasal) and strains associated with invasive disease (bone and joint or endocarditis). The ELISA-based assay was used to evaluate bacterial adherence to immobilised elastin. A comparison of the three groups (n=10 within each group) demonstrated that adherence of those strains associated with endocarditis was significantly higher than the other groups tested (Figure 4.13). This suggests that the ability of *S.aureus* to bind elastin is related to disease type. The same isolates were also tested in their ability to bind to immobilised fibronectin (Peacock *et al.*, 2000). However, no difference in adherence levels was observed between the three groups (i.e. nasal, endocarditis and septic arthritis isolates). *S.aureus* is known to bind to several components of the endovascular tissue such as platelets, fibrinogen, fibronectin, collagen and endothelial cells. Adherence to some of these components has been suggested to correlate with its ability to induce infective endocarditis (Yeaman *et al.*, 1992 (platelets); Moreillon *et al.*, 1995 (ClfA)). Once attached to the vegetation surface *S.aureus* cells can persist at this location to propagate the infection. The collagen adhesin, Cna, on the other hand was found to have minor importance for adherence to the vegetation surface but was proposed to be a virulence factor by enabling bacteria to persist within the vegetative lesion (Hienz *et al.*, 1996). Elastin is a major component of the extracellular matrix of endovascular tissue. The ability of *S.aureus* to adhere to elastin may participate in the pathogenesis of endocarditis infection either by initial adherence to induce IE or once IE is established (with exposure of elastin) binding to elastin may be a virulence factor by enabling bacterial persistence, similar to the collagen binding adhesin.

The identification of the FnBPs as elastin binding proteins raised the question of the purity of the elastin peptide samples used (α-elastin and κ-elastin). Since *S.aureus* is known to bind low concentrations of fibronectin, the possibility of fibronectin contamination of the elastin peptide samples was suggested. However, experts in the elastin field which routinely use the α-elastin and κ-elastin preparations from the same source have never found fibronectin contamination as determined by Western immunoblotting using anti-fibronectin antibodies (Robert

Mecham and Alek Hinek, personal communication). The procedures used to solubilise both forms of elastin peptides suggest inactivation of other matrix proteins (Mecham and Lange, 1982). First, elastin is purified by boiling in NaOH, which is proposed to dissociate and inactivate all non-covalently associated elastic fiber matrix components. Second, the insoluble elastin is hydrolyzed in weak acid for alpha and weak base for kappa elastin peptides. The FnBP recognition site in fibronectin is structural, comprised of several type I repeat modules. Loss of a single repeat would inactivate the *S.aureus* binding site. Moreover, in the case of κ-elastin, the presence of fibronectin N-terminal fragments (bearing the *S.aureus* binding domain) would be separated as the final purification step involves chromatography to separate the high molecular weight material from any lower molecular weight degradation products. Due to time limitations the question of fibronectin contamination of the elastin peptide samples was not properly addressed by Western immunoblotting using anti-fibronectin antibodies. However, a recombinant form of tropoelastin bearing the *S.aureus* binding domain (pQE-rTE; Table 2.2) was expressed and purified from *E.coli* (Appendix, Figure 1) and when immobilised on ELISA wells was shown to promote P1 bacterial adherence in a dose dependent and FnBP-dependent manner (Appendix, Figure 2). The interaction of surface expressed FnBP proteins with recombinant tropoelastin strongly suggests that the adherence of strain P1 to elastin peptides is mediated by an interaction with elastin and not with fibronectin. However, Western immunoblotting of the elastin peptide samples with anti-fibronectin antibodies would be required to conclusively demonstrate that this interaction is not due to fibronectin contamination.

In summary, binding to soluble or immobilised elastin appears to be an important function of *S.aureus*. Bacterial adherence to immobilised elastin is restricted to the early exponential phase of growth and is possibly mediated by FnBPs. It is not a function shared by all strains but the ability of clincial isolates (in particular endocarditis strains) to promote attachment suggests the significance of this interaction in disease. Binding to soluble elastin appears to be a property of most *S.aureus* strains. Park *et al.* (1991) reported that all *S.aureus* strains tested (4/4) but not all staphylococcal species were capable of binding to soluble elastin. This is consistent with the observation that *ebpS* occurs in all *S.aureus* strains (Smeltzer *et al.*, 1997). Binding to soluble elastin is mediated by EbpS and occurs throughout the

growth cycle but predominantly during post exponential and stationary phases of growth. The presence of another receptor for soluble elastin in stationary phase cells was predicted from the observation of the residual binding in the *ebpS::erm* mutant of strain Newman Δ*spa* in the soluble I^{125}–rTE binding assay which could be eliminated by excess cold elastin peptides. It is possible that this residual binding is mediated by the FnBPs. This could be easily resolved by testing the ability of a Newman *fnbA::tet fnbB::erm ebpS::erm* mutant to bind soluble rTE.

Ligand blotting of *S.aureus* cell wall and protoplast fractions identified a 120 kDa protein that was capable of binding to biotinylated elastin peptides. Binding was observed using a low concentration of elastin (10pg/ml) and under stringent conditions (0.05% Tween 20 in 5% BSA blocking buffer) to reduce non-specific interactions. However, the identity of this protein and its role as an elastin receptor is unknown. Affinity chromatography of lysostaphin extracts prepared from surface labelled *S.aureus* cells could be employed to identify the putative elastin binding protein using elastin peptides as the active ligand. Surface-labelled molecules which bind to the affinity resin could be eluted with 1% (w7v) SDS buffer which has been shown to have an inhibitory effect on rTE binding whole cells (Park *et al.*, 1991).

The ligand affinity blot failed to detect native EbpS released from the *S.aureus* cells. A possible explanation is that conformation is important for EbpS activity. Park *et al.* (1999) performed secondary structure prediction analysis on the amino-terminal region of EbpS. The region was predicted to fold into amphipathic alpha-helices except for the regions including residues 14-23, which is where the elastin-binding site resides. These predictions suggest that the elastin-binding domain lies in a pocket and is stabilised by the flanking residues in EbpS for interaction with the ligand elastin. EbpS might take on a different secondary structure when immobilised on a nylon membrane rendering it incapable of binding to the ligand.

The significance of EbpS as a putative virulence factor in staphylococcal infections is unclear. The inability of EbpS to function as an elastin adhesin suggests it is not involved in colonisation of elastin tissue but may have another role in contributing to infection. A murine septic arthritis model (Bremell *et al.*, 1991) and skin infection model (Mölne and Tarkowski, 2000) were tested to investigate the importance of EbpS *in vivo* (E.Josefsson, personal communication). No difference in virulence was observed between wild type strain Newman and its isogenic mutants in

the septic arthritis model. However, in the skin infection model preliminary studies suggested that the *ebpS*-defective mutant was less likely to cause dermatitis than the wild type strain. In this study, 10^8 or 10^7 CFU of Newman wild type or *ebpS* mutant were injected intradermally. There were 6-7 mice in each group. The dermatitis, measured as the size of the abscess/infiltrate of inflammatory cells (histiocytes and neutrophils), was more severe in the wild type injected mice compared to the mutant strain suggesting that EbpS may play a role in the virulence of skin infections. A reduction in virulence may be due to the inability of bacteria to bind soluble elastin or may be attributable to another phenotype of EbpS, which has not yet been identified. Although a trend was apparent, further studies will be required to test a larger sample number to determine if the difference is statistically significant.

5.1 Introduction

The potency of *Staphylococcus aureus* as a human pathogen can be attributed to the co-ordinated and temporally regulated expression of a large number of virulence factors that facilitate the colonisation, multiplication and spread of the bacterium (Iandolo, 1990). In general, it has been observed that the cell wall-associated proteins, including the MSCRAMMs, are expressed during logarithmic growth, corresponding to early infection, and not post exponentially. It appears that covalent anchoring of the LPXTG proteins to the cell wall can only occur during cell wall assembly, hence protein production is optimal during exponential phase of growth. Several of these MSCRAMMs have been found to promote attachment to the extracellular matrix (Patti *et al.*, 1994) and play a role in evading the immune response, (Verhoef *et al.*, 1979). As the cells enter stationary phase of growth the expression of many surface proteins including FnBP (Proctor *et al.*, 1982), ClfB (Ní Eidhin *et al.*, 1998), and protein A (Bjkorklind and Arvidson, 1980) is reduced. Reduced expression of surface-located proteins is a combination of cessation of transcription, shedding from the surface by autolysis and/or proteolysis. Simultaneously, exoprotein expression increases (Arvidson, 1983) with some exceptions including enterotoxin A (Tremaine *et al.*, 1993) and coagulase (Duthie, 1954). This modulation of virulence determinant biosynthesis has been proposed to enable bacteria under nutritional stress to detach from the site of infection and migrate to seed at a new site.

The regulation of virulence factor expression in *S.aureus* involves several global regulatory loci of which *agr* and *sar* are best characterised, (Recsei *et al.*, 1986; Peng *et al.*, 1988; Cheung *et al.*, 1992; Morfeldt *et al.*, 1996). Other putative regulators such as *xpr, sae*, *sigB* and *sarH1* have been described but not well characterised (Hart *et al.*, 1993; Giraudo *et al.*, 1994; Wu *et al.*, 1996; Tegmark *et al.*, 2000). Inactivation of the *sar* or *agr* locus results in a less virulent phenotype than the parental strain in several animal models reflecting their importance *in vivo* (Abdelinour *et al.*, 1993; Cheung *et al.*, 1992; Cheung *et al.*, 1994; Recsei *et al.*, 1986).

The *agr* locus consists of two divergent transcripts RNAII and RNAIII, initiated from two distinct promoters, P2 and P3, respectively (Peng *et al.*, 1988) (Figure 5.1). The P2 promoter drives the RNAII molecule, which encodes a four

gene operon, *agrBDCA*, all of which are required for transcriptional activation of the *agr* system (Novick *et al.*, 1995). AgrC and AgrA correspond to the sensing and activating components of a two component regulatory system (Novick *et al.*, 1995; Stock *et al.*, 1989). AgrD encodes an autoinducer propeptide, which is processed to the active autoinducing peptide (AIP) and possibly exported from the cell by the 26kDa membrane bound AgrB, (Ji *et al.*, 1997). The AIP is unlike other peptides responsible for autoinduction in Gram-positive bacteria (Novick, 1999). It contains a thiolactone bond between the conserved cysteine residue and the carboxy-terminal carboxy forming a thioester ring structure, which is essential for *agr* activation (Ji *et al.*, 1997). Linear synthetic peptides with identical sequences were found to be devoid of activity (Ji *et al.*, 1995).

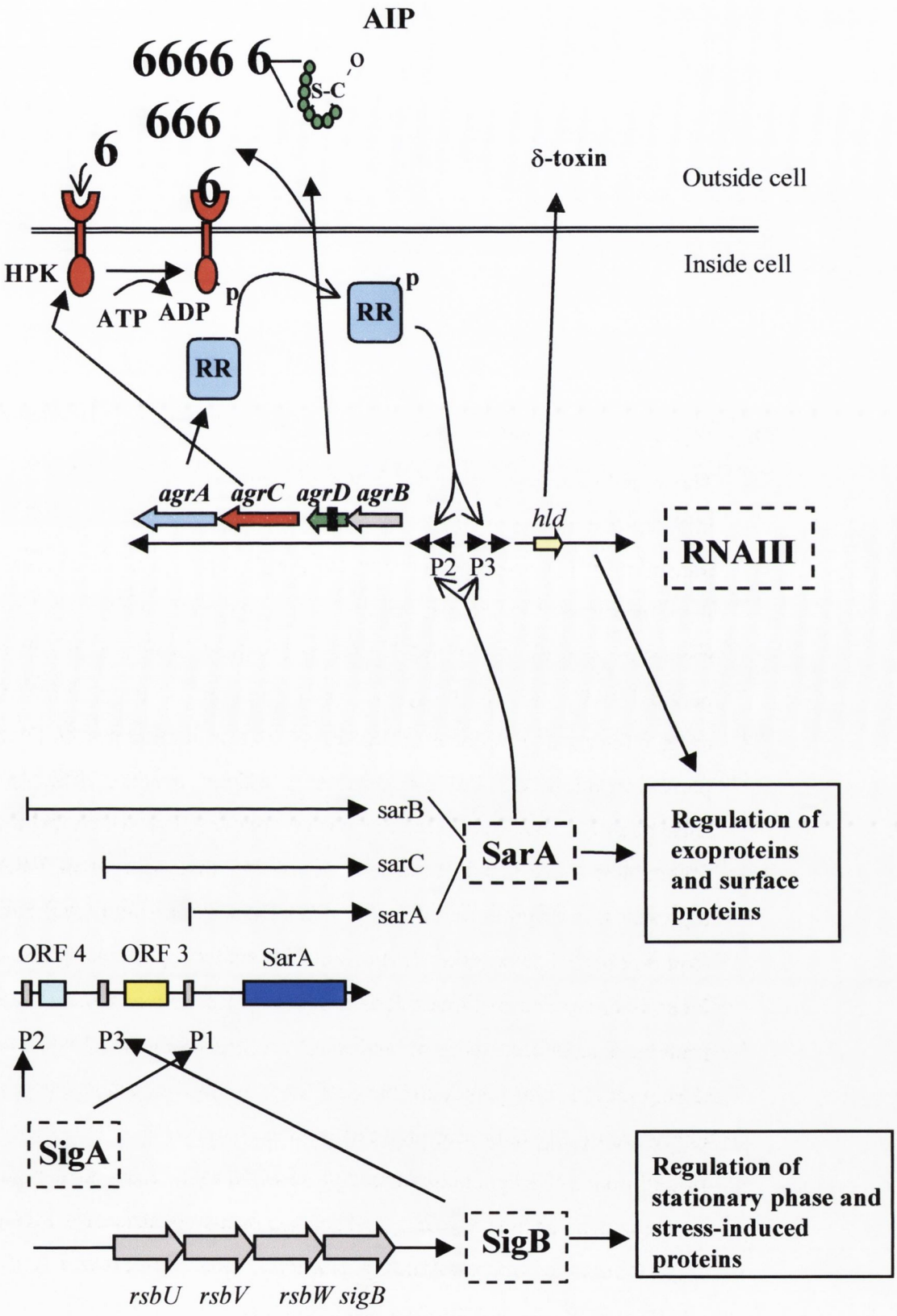
AgrC is a 46kDa transmembrane protein, which most likely binds to the autoinducing peptide (Ji *et al.*, 1995). It is composed of six or seven transmembrane helices and a C-terminal cytoplasmic domain containing the conserved histidine common to the histidine protein kinase moiety of bacterial signal receptors (Lina *et al.*, 1998). The ligand-binding site of AgrC is proposed to be located in the third extracellular loop of the protein (Lina *et al.*, 1998). When the octapeptide reaches a critical concentration, AgrC becomes phosphorylated, probably at this histidine residue. This phosphorylation event has been shown to occur both *in vivo* and *in vitro* (Lina *et al.*, 1998). AgrA is a 34kDa cytoplasmic protein (Peng *et al.*, 1988) which is proposed to be activated by transphosphorylation of its conserved aspartate residue by the activated sensor, AgrC. The primary role of AgrA is to upregulate both of the major *agr* promoters, P2 and P3. However, there is also evidence that AgrA is directly responsible for the transcriptional activation of *coa* (Lebeau *et al.*, 1994). A direct interaction between AgrA and the *agr* promoters has not been shown (Morfeldt *et al.*, 1996). However, it is possible that only activated AgrA will bind or perhaps another factor such as SarA is jointly required (Cheung and Projan, 1994).

The P3 promoter drives transcription of a 514nt transcript, RNAIII, which is the effector molecule of *agr*-specific target gene regulation (Novick *et al.*, 1993) (Figure 5.1). Within this transcript is encoded the 26 amino acid δ-haemolysin peptide (*hld*) which is not involved in regulation (Arvidson *et al.*, 1990). During exponential phase, the autoinducing peptide accumulates extracellularly and increases in concentration as a function of population density. When the peptide

Figure 5.1 The global regulatory loci *agr, sar* and *sigB* of *S.aureus*.

The *agr* locus is composed of two divergent transcripts RNAII and RNAIII, initiated from two distinct promoters, P2 and P3, respectively. The RNAII transcript encodes four gene products, AgrB, AgrD, AgrC, and AgrA. AgrA and AgrC correspond to the activating and sensing components of a two component regulatory system. AgrB and AgrD are involved in generating an autoinducing peptide (AIP) which functions as a quorum sensing mechanism. When extracellular AIP reaches a critical concentration it activates its ligand, AgrC by phosphorylation of its cytoplasmic histidine residue. This phosphate is subsequently transferred to the response regulator AgrA, which together with SarA upregulates the P2 and P3 promoters driving transcription of RNAIII. RNAIII represses transcription of surface proteins (Spa, FnbpA and Coa) and upregulates expression of haemolysins (α-haemolysin) and exoproteins (TSST-1). The *sar* locus is composed of three overlapping transcripts all of which encode the SarA protein. The transcripts *sarA* and *sarB* are SigA-dependent and are transcribed during exponential phase. In contrast, *sarC* is SigB-dependent and preferentially expressed during stationary phase of growth.

In addition to upregulating transcription of RNAIII, SarA also regulates protein expression independently of *agr*. The *sigB* locus consists of a four gene operon. SigB levels are maximal during stationary phase of growth to function as a transcription factor for stress-induced proteins.


AIP
S-C
δ-toxin
Outside cell
Inside cell
HPK
ATP
ADP
p
RR
agrA
agrC
agrD
agrB
hld
P2
P3
RNAIII
sarB
sarC
sarA
SarA
Regulation of exoproteins and surface proteins
ORF 4
ORF 3
SarA
P2
P3
P1
SigA
SigB
Regulation of stationary phase and stress-induced proteins
rsbU
rsbV
rsbW
sigB

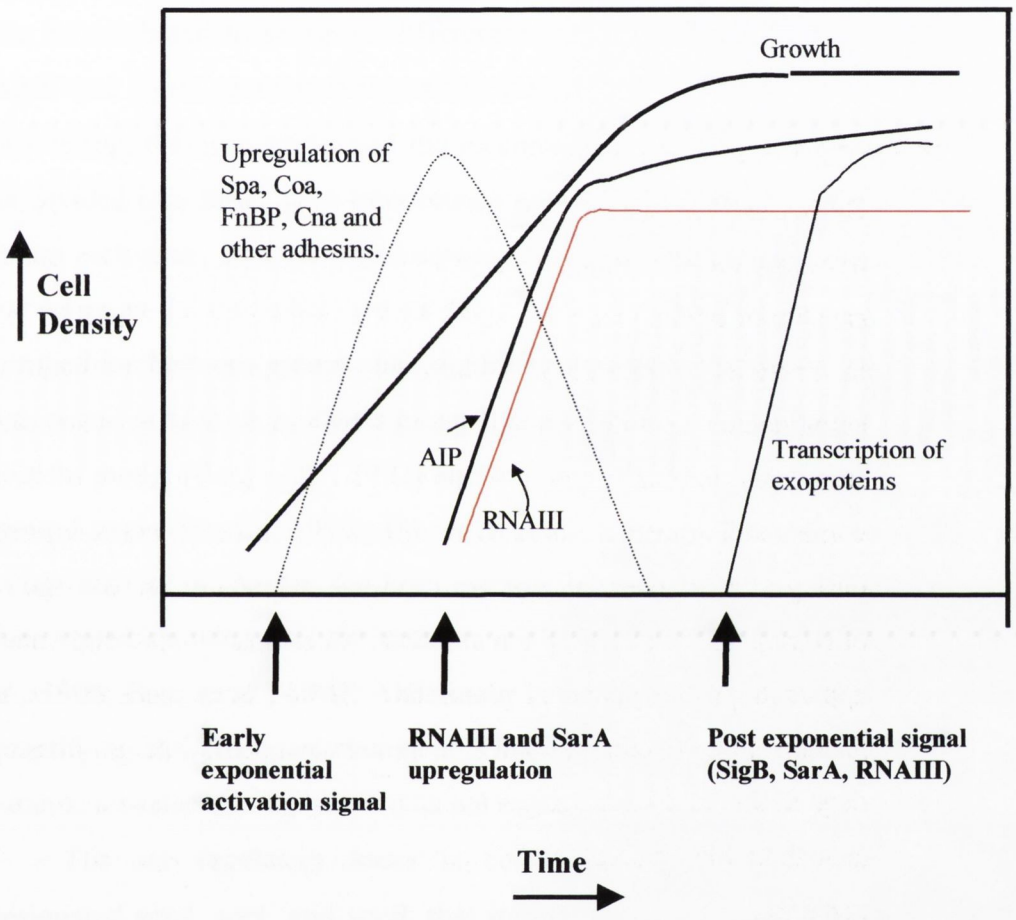
reaches a critical concentration the synthesis of RNAIII is activated. The induction of RNAIII immediately downregulates transcription of surface protein genes (protein A, fibronectin binding protein, coagulase) and causes upregulation of exoprotein genes (hemolysins, exotoxins) in the post exponential phase of growth. This density-dependent mechanism thus ensures a rapid regulation of virulence gene expression via RNAIII, in response to bacterial population density. Maximum amounts of RNAIII are not seen until the post-exponential phase of growth when synthesis of exoproteins is upregulated (Figure 5.2). The delay in transcriptional activation of certain exoproteins, irrespective of the presence of RNAIII earlier in the growth cycle, suggests the requirement of a second activation signal (Vandenesch *et al.*, 1991) and/or requires that RNAIII be modified after transcription.

RNAIII regulates most target genes at the level of transcription, by a mechanism that has yet to be determined. It is postulated that RNAIII indirectly regulates target genes by effecting the function of regulatory proteins from outside the *agr* locus possibly by forming a nucleoprotein complex (Novick *et al.*, 1999). This hypothesis is consistent with the identification of a regulatory protein, SarH1, which has been described to antagonise RNAIII–dependent activation of the *hla* promoter resulting in repression of *hla* expression (Tegmark *et al.*, 2000). RNAIII has also been shown to regulate translation of at least one gene product, α-haemolysin. Hybridisation of the 5' end of RNAIII with the *hla* mRNA 5' leader region induces a conformational change in the folding of the mRNA allowing the ribosome binding site to become accessible for translation initiation (Novick *et al.*, 1993; Morfeldt *et al.*, 1995).

A model of RNAIII secondary structure has been proposed. The RNA molecule is characterised by 14 hairpin structures and three long-range interactions that bring the 3' and 5' ends of RNAIII into close proximity (Benito *et al.*, 2000). The 5' side of the central domain contains the regulatory region involved in the activation of alpha-toxin translation. The 3' end of RNAIII is an independent structural domain, which is important for repressing protein A gene expression (Benito *et al.*, 2000). This stem-loop domain is highly conserved among RNAIII molecules from other staphylococcal species (Benito *et al.*, 2000). Prior to this study it was postulated that complementary base pairing within the RNAIII molecule was responsible for the delay in δ-haemolysin translation by 1 h following transcription

Figure 5.2 Temporal regulation of the virulence response in *S.aureus.*

During early exponential phase of growth an activation signal(s) upregulates the transcription of matrix binding surface proteins (including adhesins and proteins capable of binding soluble matrix molecules (e.g. EbpS, Coa) which are involved in bacterial colonisation of host tissue. As the cell density increases the autoinducing peptide accumulates and when a critical concentration is reached stimulates the transcription of RNAIII, the effector molecule of the *agr* locus. A corresponding increase in SarA (transcribed from *sarA* and *sarB*) is also observed during the exponential phase of growth. Upregulation of RNAIII and SarA downregulates surface proteins such as Coa, Spa, FnbpA and Cna. As the cells approach post exponential phase RNAIII and SarA activate the expression of haemolysins, exotoxins and other extracellular proteins involved in tissue degradation and bacterial dissemination. SigB is expressed in stationary phase of growth and is involved in the upregulation of *sarC* and proteins involved in stress response.


Growth
Upregulation of Spa, Coa, FnBP, Cna and other adhesins.
Cell Density
AIP
RNAIII
Transcription of exoproteins
Early exponential activation signal
RNAIII and SarA upregulation
Post exponential signal (SigB, SarA, RNAIII)
Time

(Balaban and Novick, 1995). Toe-printing experiments contradict this theory indicating that the *in vitro* structure of RNAIII allows translation of its *hld* gene product (Benito *et al.*, 2000). Thus, the reason for the delay in *hld* translation is unknown.

A study of various strains of *S.aureus* and other staphylococci revealed that the soluble autoinducer from some strains could inhibit the *agr* response in other strains, representing a novel type of bacterial interference which inhibits virulence expression rather than growth (Ji *et al.*, 1997; Otto *et al.*, 1999). This phenomenon can be attributed to sequence differences in highly variable domains in the AgrD, AgrB and AgrC proteins representing the regions of specificity of the autoinducer processing, the propeptide, and the receptor-ligand interaction. *S.aureus* strains can be divided into three main interference groups. The autoinducer produced within a group activates RNAIII transcription within that group but inhibits RNAIII transcription in the other groups (Ji *et al.*, 1997). This represents a means for competition between groups. The significance of peptide-based bacterial interference was tested *in vivo*. A synthetic group II peptide was tested in a mouse subcutaneous abscess model (Barg *et al.*, 1992) and found to dramatically attenuate infection by a group I strain (Novick, 1999). However, it should be pointed out that the importance of *agr* activity *in vivo* has not been extensively studied. Several animal models have been tested showing that *agr* mutants are greatly attenuated in virulence (Foster *et al.*, 1990; Barg *et al.*, 1992; Abdelinour *et al.*, 1993). In contrast, an *in vivo* study quantifying RNAIII transcription in *S.aureus* infecting the lungs of cystic fibrosis patients revealed that *agr* activity is not essential (Goerke *et al.*, 2000).

The *sar* regulatory locus is composed of three overlapping transcripts designated *sarA*, *sarC* and *sarB*, that initiate from three promoters P1, P2 and P3, respectively (Cheung *et al.*, 1992) (Figure 5.1). Production of the three transcripts is regulated temporally (Figure 5.2). The *sarB* and *sarA* transcripts can be detected mainly during the exponential phase (Bayer *et al.*, 1996), while *sarC* is initiated from the σ^B-dependent P3 promoter (Deora *et al.*, 1997; Manna *et al.*, 1998), and thus is detected in the post-exponential phase of growth (Bayer *et al.*, 1996; Manna *et al.*, 1998). These transcripts all encompass the major *sarA* open reading frame, which codes for SarA, a regulatory DNA binding protein. Transcriptional and gel shift studies have shown that SarA is capable of binding DNA fragments containing *cis*-

regulatory elements for the P2 and P3 promoters of the *agr* locus (Cheung *et al.*, 1997a; Heinrichs *et al.*, 1996; Morfeldt *et al.*, 1996). Mutations of *sar* result in reduced expression of both RNAII and RNAIII in stationary phase of growth, demonstrating that the binding of SarA to the *agr* promoters acts as a transcriptional activator (Cheung *et al.*, 1997a; Heinrichs *et al.*, 1996). There is also evidence that SarA can directly regulate virulence genes by interaction with upstream promoter regions (Blevins *et al.*, 1999). Hence, SarA can modulate virulence determinant expression by an *agr*-dependent mechanism as in the case of α- haemolysin (Cheung *et al.*, 1997a) but also by *agr*-independent mechanisms as in the case of protein A, Cna and V8 serine protease (Cheung *et al.*, 1997b, Blevins *et al.*, 1999, Foster and Höök, 1998). In addition, *sar* plays a role as a signal transduction regulatory element by modulating virulence determinant production in response to aeration (Chan *et al.*, 1998b). Further support for the importance of *sar* as a regulator of virulence determinants comes from the observation that a *sar agr* double mutant has more greatly reduced virulence than an *agr* mutant (Cheung *et al.*, 1994).

The identification of the primary binding site for SarA in the interpromoter region of *agr* is controversial. A recent report published by Chien *et al.* (1999) proposed a conserved binding motif essential for SarA-dependent gene regulation.
In contrast, two other reports of SarA interactions with the *agr* region have different conclusions regarding the binding sites for SarA (Rechtin *et al.*, 1999; Morfeldt *et al.*, 1996). Morfeldt *et al.* (1996) proposed that SarA probably interacts with the regulatory regions containing the 7 bp heptad repeats immediately upstream of the P3 and P2 promoters. Rechtin *et al.* (1999) claimed that SarA interacts with three high affinity binding sites composed of two half sites each. Some of these sites overlap with the heptad repeated sequences observed by Morfeldt *et al.* (1996) and one half site contains the binding site described by Chien *et al.* (1999). The discrepancies between these reports can probably be explained by the nature of the SarA protein used for the DNase I footprinting analysis. The SarA protein used for DNase I footprinting analysis by Chien *et al.* (1999) was expressed as an N-terminal GST fusion. The N-terminus of SarA has been found to be very sensitive to modifications, which may interfere with results. Rechtin *et al.* (1999) expressed SarA in its full-length unmodified form and Morfeldt *et al.* (1996) used native SarA protein purified from *S.aureus* extracts by DNA-affinity chromatography. A

consensus sequence for the SarA binding site is still not obvious. Further characterisation of other target genes directly regulated by SarA (*e.g. cna*) will further advance the understanding of SarA-DNA-binding.

Analysis of DNA-binding proteins with an affinity for promoter fragments of differentially regulated genes enabled the identification of a new regulator, SarH1 which belongs to a family of Sar homologs (Tegmark *et al.*, 2000). SarH1 is a 29.9 kDa protein with a high degree of identity to SarA in its N-terminal (32% identical) and C-terminal (34% identical) domain. Northern blot analysis using a *sarH1*-specific probe suggested that the *sarH1* locus expresses three transcripts of approximately 1000, 1500 and 3000 nt in size. However, primer extension analysis allowed only the identification of the promoter regions for the two smaller transcripts, P1 and P2, respectively. Expression of *sarH1* is regulated by *sar* and *agr*. In wild type strain 8325-4 the level of *sarH1* expression from the P1 promoter is low throughout the growth cycle. However, in a *sar* or *agr* mutant *sarH1* levels are significantly increased. The temporal expression of *sarH1* from the P1 promoter in a *sar* or *agr* mutant is similar to that of the SigA-dependent *sarA* and *sarB* transcripts from the *sar* locus. Expression is maximal during the early exponential phase of growth and declines during late exponential phase. The P2 promoter was demonstrated to be SigB-dependent. Therefore expression of the ~1500 nt transcript was low during exponential phase and increased during post exponential phase. Hence, similar to *sar*, *sarH1* is transcribed from both SigA and SigB-dependent promoters (Deora *et al.*, 1997; Manna *et al.*, 1998). Inactivation of *sarH1* has demonstrated that certain regulatory effects on virulence genes, which were previously attributed to *sar* and *agr*, are in fact due to *sarH1*. For example, analysis of *hla* and *spa* transcription in *sarH1*, *sar* and *agr* mutants and *sarA sarH1* and *sarH1 agr* double mutants revealed that *sarH1* represses *hla* transcription and activates *spa* transcription. The repression of *hla* by *sarH1* was only observed when *agr* was intact. As mentioned previously, RNAIII is thought to activate *hla* transcription by interacting with other regulatory proteins (Novick *et al.*, 1999). Tegmark *et al.* (2000) proposes that SarH1 represses *hla* expression by interacting with RNAIII and hence reducing activation of *hla* transcription. SarH1 was found to be an activator of *spa* transcription contrary to previous findings that RNAIII and SarA repress *spa* transcription. In a *sar* or *agr* mutant *spa* levels are increased. Inactivation of *sarH1* in an *agr* and *sar* mutant results in decreased *spa* transcription.

This proves that upregulation of *spa* expression in a *sar* and *agr* mutant is not due to the lack of the proposed repressor, SarA or RNAIII, but to the upregulation of the activator, SarH1. However, both RNAIII and SarA also appeared to have a suppressive effect on *spa* transcription independently of *sarH1*.

The SarH1 protein is proposed to be a DNA-binding protein. The mechanism in which it regulates *agr*-independent gene transcription has not been described but due to sequence similarity with SarA, it is possible that SarH1 also binds to promoter regions of target genes to regulate transcription levels.

A recently discovered alternative sigma factor of *Staphylococcus aureus*, SigB (σ^B), plays an important role in stress responses and the regulation of virulence factors. The *sigB* gene is encoded within a four gene operon (*rsbU, rsbV*, *rsbW*, *sigB*) (Figure 5.1) with significant predicted amino acid similarity and gene arrangement to *rsbU, rsbV, rsbW*, and *sigB* in *Bacillus Subtilis* (Kullik and Giachino, 1997; Wu *et al.*, 1996). Similar to most sigma factors, posttranslational control via protein complexes is important in controlling SigB activity. RsbW has been shown to function as an anti-sigma factor of SigB by binding to SigB thus inhibiting SigB-dependent transcription (Miyazaki *et al.*, 1999). In *Bacillus subtilis*, the RsbU phosphatase controls the σ^B activity in response to physical stress by altering RsbW binding (Völker *et al.*, 1995a, 1995b). A strong amino acid sequence similarity (64%) between RsbU in *B.subtilis* and *S.aureus* suggests a common function for this protein. In *S.aureus* 8325-4, σ^B activity is low compared to other strains tested. An 11bp deletion in the *rsbU* gene in strain 8325-4 could be responsible for this low σ^B activity as in the absence of RsbU, RsbW is permanently bound to SigB preventing SigB-dependent transcription (Kullik and Giachino, 1997). Most studies characterising *sigB* have utilised strain 8325-4 as the host strain hence careful interpretation of these results is important.

The SigB protein of *S.aureus* has been evaluated as a stress response and stationary phase sigma factor. It is expressed during stationary phase and upon heat shock (Kullik and Giachino, 1997). In addition, interruption of the *S.aureus sigB* gene caused reduced pigmentation, accelerated sedimentation, and increased sensitivity to hydrogen peroxide during the stationary growth phase. SigB has further been shown to effect transcription of the *sar* locus. The *sarC* transcript, which is preferentially expressed during stationary phase, is dependent on SigB for

transcription (Deora *et al.*, 1997; Manna *et al.*, 1998). In a *sigB* mutant, *sarC* was no longer transcribed, yet elevated SarA expression levels were observed (Cheung *et al.*, 1999). Interestingly, this coincided with hyper-production of alpha-haemolysin and upregulation of fibrinogen binding capacity mediated by ClfA and coagulase (Cheung *et al.*, 1999). As *agr* is not altered in a *sigB* mutant, and SarA has been shown to bind to the *hla* promoter region, Cheung *et al.* (1999) have postulated that an increase in *hla* expression occurs by a SarA-dependent mechanism. The regulation of ClfA and coagulase has not been extensively studied. However, one could speculate that σ^B-dependent genes act in concert with SarA to modulate these targets. A separate study carried out by Chan *et al.* (1998b) reported that SarA upregulated *sigB* expression in a growth-phase-dependent manner. Inactivation of the *sar* locus led to reduced *sigB* expression in stationary phase.

The *sae* locus encodes a two component regulatory system, comprising *sae*R and *sae*S, which encode a response regulator and a histidine protein kinase, respectively (Giraudo *et al.*, 1999). Inactivation of the *sae* locus led to diminished levels of alpha- and beta- haemolysins, DNase, coagulase and slightly reduced levels of protein A. Northern blot analysis showed that *sae* regulates target genes at the transcriptional level (Giraudo *et al.*, 1997). Inactivation of *sae* did not affect the expression of the *agr* or *sar* genes implying that *sae* regulates target gene expression in an *agr* and *sar* independent manner. The virulence of a *sae* mutant was lower than that of the parental strain in an intraperitoneal infection model in mice. An *sae agr* double mutant showed further reduced virulence in the same infection model (Giraudo *et al.*, 1996, Rampone *et al.*, 1996).

The roles of several loci in the co-ordinated regulation of virulence determinant gene expression have been characterised in *S.aureus*. Evidence shows that most regulators described (*agr*, *sar*, *sarH1* and *sigB*) modulate the effect of each other to determine the level of expression of individual virulence genes. Environmental stimuli have also been demonstrated to control virulence gene expression (Chan *et al.*, 1998a). Therefore the mechanisms of regulation of staphylococcal virulence genes are complex and involve an interactive, hierarchial regulatory cascade, which is only partially understood. Entry into the genomic era will hopefully help to identify other regulatory proteins and ultimately accelerate our understanding of staphylococcal regulation.

5.2 Methods

5.2.1 Culture growth conditions used to examine regulation of EbpS expression

To investigate the role of *sar* on EbpS expression cultures were grown under different conditions of aeration but other factors such as inocculum (1:100 dilution of starter culture), temperature (37°C), agitation (200 rpm), broth (TSB) and incubation time (18 hours) were kept constant. Under high and medium aeration, cultures were grown in 20ml and 100ml of TSB broth in a 250 ml flask, respectively. Under low aeration, cultures were grown in 30 ml of TSB broth in a 50 ml screw-cap tube (Greiner). Stationary phase cells were washed in 10 ml of PBS and standardised to an OD_{600} of 50 units. Western blotting was employed to analyse protoplast fractions for EbpS expression as described in Section 3.2.1

5.2.2 Construction of *ebpS-lacZ* reporter-gene fusion

The pAZ-*ebpS* fusion plasmid was constructed by cloning a 1kb PCR fragment (containing 900bp *ebpS* upstream sequence and 120bp 5' *ebpS* sequence) into the BamHI/EcoRI site of the suicide vector pAZ106. The *ebpS-lacZ* fusion plasmid (10-20μg DNA) was introduced into *S.aureus* strain RN4220 by electroporation (Schenk and Laddaga, 1992) and recombinants selected on erythromycin (10μg/ml). Since the *lacZ* vector does not contain a *S.aureus* replicon, the plasmid is forced to undergo a single-crossover integrational event with the host chromosome. The integrant was transferred into *S.aureus* 8325-4 Δ*spa* and Newman Δ*spa* by phage transduction, using ϕ85 as the carrier, selecting on erythromycin (10μg/ml). In order to study the role of regulatory loci on *ebpS* transcription, the *ebpS-lacZ* fusion was also transferred by phage transduction into the regulatory mutants of *S.aureus*, *agr*::*tmn* (Novick *et al.*, 1993), *sar*::*kan* (Chan *et al.*, 1998b) and *sigB*::*tet* (Chan *et al.*, 1998c). All fusions were blue when streaked on TSA plates containing X-Gal (40μg/ml), indicating expression of the *lacZ* gene, whilst the parental strain was white on X-Gal. The fusion strains were further validated by testing (i) antibiotic resistance markers and (ii) the haemolytic pattern on sheep blood plates. The *agr* mutants were non-haemolytic when grown on sheep blood plates and an increased expression of α-haemolysin was indicative of the *sigB* mutants. PCR was used to ensure inactivation of the *sar* locus in strains FR010 and FR014. The forward and reverse primers, sarF

and sarR, were designed based on the published sequence (Table 2.3) and used to amplify the complete *sar* operon (1349 bp). A larger PCR product of 2838 bp was indicative of a disrupted *sar* locus due to the insertion of a 1489 bp kanamycin resistance cassette (Chan *et al.*, 1998b). The conditions for amplification were as follows: 94°C for 3min, followed by 30 cycles of 94°C for 1min, 56°C for 1min, and 72°C for 3min. The integrational events were also confirmed by Southern blot analysis using a DIG-labelled probe bearing 787 bp of the 5' *ebpS* sequence of strain 8325-4. The *lacZ* fusion was constructed to contain an intact copy of the *ebpS* gene plus a truncated copy fused to the *lacZ* gene, stably integrated into the chromosome of *S.aureus*.

5.2.3 β-galactosidase assays

β-galactosidase assays were performed using 4-MUG (4-methyllumbelliferyl β-D-galactopyranoside) as the substrate (Chan *et al.*, 1998a). Fusion strains were grown overnight with the appropriate antibiotics. For aerobic growth cells were diluted 1:100 into 100 ml of fresh TSB (without antibiotics) in a 250 ml flask, allowed to grow to mid-exponential phase (OD_{600} ~1) and used to inoculate 100 ml of prewarmed TSB (without antibiotics). Samples (0.5ml) were taken in duplicate at regular time intervals throughout the growth cycle. Cells were harvested by centrifugation at 14,000rpm for 5min and the pellet was snap frozen and stored at –70°C. Pellets were thawed, resuspended in 0.5ml ABT (100mM NaCl, 60mM K_2HPO_4, 40mM KH_2PO_4, 0.1% (v/v) Triton 100) and incubated at 37°C for 10min. 50µl of 4-MUG (4mg/ml) was then added and allowed to incubate at 25°C for 60min followed by immediate addition of 0.5 ml of 0.4M Na_2CO_3. Dilutions were made in a 96 well plate using ABTN (1:1 of 0.4M Na_2CO_3 and ABT). 225µl of ABTN was added to each well. For each sample 25µl was added to the first well, mixed and 25µl was subsequently taken from this well and added to the next well giving a 1/10 dilution. This was repeated to give a 1/100 dilution taking 25µl from the last well to ensure consistent volumes. β-galactosidase activity was measured using an luminesence spectrophotometer (LS-50B) at slit size 2.5cm with absorption and emission at 365nm and 455nm respectively. FLWinlab (Perkin Elmer) was the software package used to analyse the data. A standard curve was generated using a range of concentrations of 4-MU (Sigma). A 1mM stock of 4-MU was made up in

DMSO and dilutions were made in ABTN buffer to give the following concentrations: 10μM, 2.5μM, 1μM, 500nM, 250nM, 100nM, 50nM and 10nM. 250μl of each concentration was added to each well. The measured values were used to plot a standard curve on Cricket graph and the equation of a straight line, $y = mx + c$ was calculated. This equation was used to calculate the amount of pmoles of MU (x) produced from sample counts (y). A rearrangement of the above equation yields $x = \frac{y - c}{m}$.

Then by substituting in the values from the equation of the line obtained from the standard curve it is possible to calculate x, the amount of pmoles of MU produced.

The following equation was used to determine β-galactosidase activity:

β-galactosidase activity = U/(D x 60 x OD)

where

U = pmoles of MU produced (derived from the standard curve)

OD = OD_{600} at given time point

60 = time of incubation at 25°C

D = dilution factor.

For example, 1050 (the volume of sample (0.5ml) + ABT (0.5ml) + MUG (50μl)) / 25 (volume sampled x dilution factor (1 for neat, 10 for 1/10 diln, 100 for 1/100 diln etc.) x 2 (to calculate per ml sampled since 0.5ml was only taken).

Therefore,	1050 / 25 x 2	= 1/84	for neat
	1050 / 250 x 2	= 1/840	for 1/10
	1050 / 2500 x 2	= 1/8400	for 1/100

5.2.4 Preparation of protease-rich culture supernatant

S.aureus strain FR010 (Newman Δ*spa*::*tet ebpS-lacZ sar*::*kan*) and FR014 (8325-4 Δ*spa*::*tet ebpS-lacZ sar*::*kan*) were grown at 37°C in 100ml of TSB broth in 250ml conical flasks at 200 rpm for 18 hours representing stationary phase cultures (OD_{600} of 12). 30 ml of cells were pelleted by centrifugation at 7,000rpm for 15 min. The supernatant was concentrated 10-fold in a stirred-cell ultrafiltration chamber equipped with a 10,000-molecular-mass cutoff membrane (Amicon). Chloramphenicol (50μg/ml) was added to overnight cultures (100ml) of strain FR009 (Newman Δ*spa*::*tet ebpS-lacZ*) and FR013 (8325-4 Δ*spa*::*tet ebpS-lacZ*) and incubated at room temperature for 1 hour. 30 ml of cells were then supplemented with a 1/10 volume of 10 X concentrated supernatant of FR010 and FR014, respectively and incubated at 37°C for 1.5 h. Cells were harvested by centrifugation at 7,000rpm for 15min and protoplasts were isolated as described previously (Section 3.2.1). Western blotting was used to examine the stability of the EbpS protein in the protoplast fractions using anti-$EbpS_{1\text{-}267}$ and anti-$EbpS_{343\text{-}486}$ antibodies.

5.3 Results

5.3.1 Expression of EbpS throughout the growth cycle

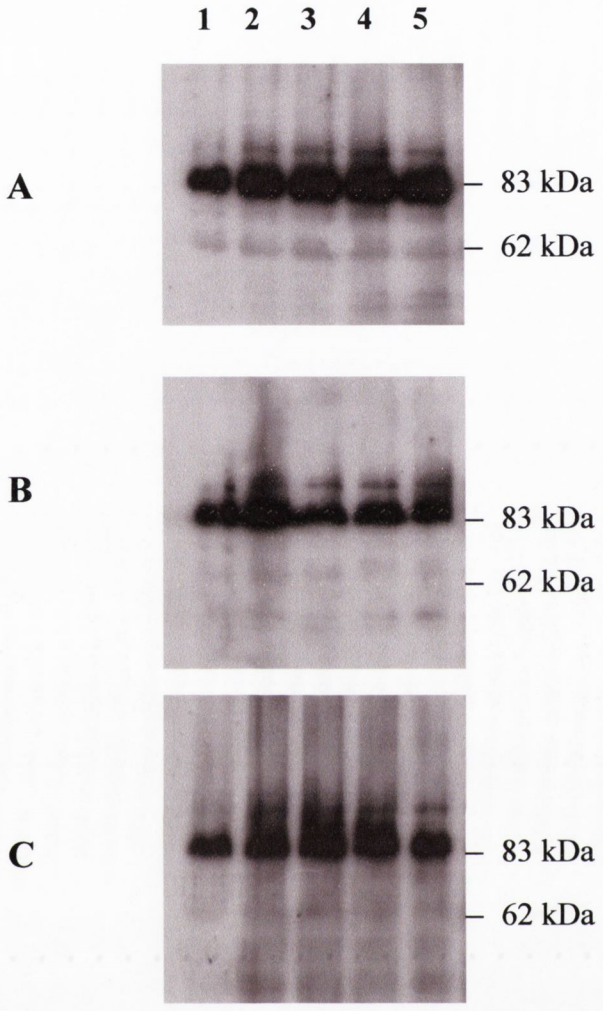
Western immunoblotting using polyclonal antisera directed against the C-terminus of EbpS (anti-$EbpS_{343-486}$) was used to monitor the expression of EbpS throughout the growth cycle in strain Newman Δ*spa*. Cultures were grown at 37°C in 20ml of TSB broth in a 250ml flask for 18 hours with aeration giving stationary phase cultures (OD_{600} of 12). Cells were diluted 1:100 into prewarmed 20ml fresh TSB broth and grown for 2, 4, 6 and 8 hours representing early (t = 2, OD_{600} ~0.4), mid (t = 4, OD_{600} ~3) and late (t = 6 and 8, OD_{600} ~6 and 8) exponential phase cultures, respectively. The cells were washed and adjusted to the same optical density (OD_{600} of 100 units). Western blotting was carried out as described in Section 3.2.1. The data in Figure 5.3 (panel A) clearly shows that the 83kDa EbpS protein was present in the protoplast fraction throughout the growth cycle.

5.3.2 Regulation of EbpS expression in *S.aureus*

The regulation of *Staphylococcus aureus* virulence factors involves several regulatory loci of which *sar* and *agr* are best characterised. To examine whether these loci are involved in the regulation of *ebpS* expression, the production of EbpS from wild-type strain Newman Δ*spa* was compared by Western blotting with strains carrying mutations inactivating *sar* or *agr*. EbpS expression was initially monitored throughout the growth cycle to determine the effect of *sar* and *agr* on EbpS expression and membrane localisation, (Figure 5.3, panel B, C). The cell wall peptidoglycan was solubilised by lysostaphin and the protoplast fraction was analysed for EbpS expression using anti-$EbpS_{343-486}$ antibodies. A similar expression profile was observed in the regulatory mutants as compared to the wild-type strain, *i.e.* EbpS was expressed in early exponential phase and was still detected in stationary phase of growth. Although levels of protein expression could not be directly compared between strains, results demonstrated that under aerobic growth, *sar* or *agr* have no effect on the localisation of EbpS to the cytoplasmic membrane.

A direct comparison of the regulatory mutants of strain Newman Δ*spa* (*sar* and *agr*) grown to stationary phase and under the same growth conditions (i.e. high aeration) showed that inactivation of the *agr* locus had no significant effect on EbpS expression (Figure 5.4). A slight reduction in EbpS expression was observed in the

Figure 5.3 Time course of EbpS expression in strain Newman Δ*spa* and regulatory mutants. Cells were grown for 2, 4, 6, 8 and 18 hr representing early (t = 2), mid (t = 4) and late exponential (t = 6 and 8) and late stationary (t = 18) phase cultures. Protoplast fractions were analysed for EbpS production using anti-$EbpS_{343-486}$ antibodies. Lanes 1-5 correspond to points on the graph, inset. Panel A, Newman Δ*spa*; Panel B, Newman Δ*spa sar*::*kan*, Panel C, Newman Δ*spa agr*::*tmn*.


1 2 3 4 5
A
83 kDa
62 kDa
B
83 kDa
62 kDa
C
83 kDa
62 kDa

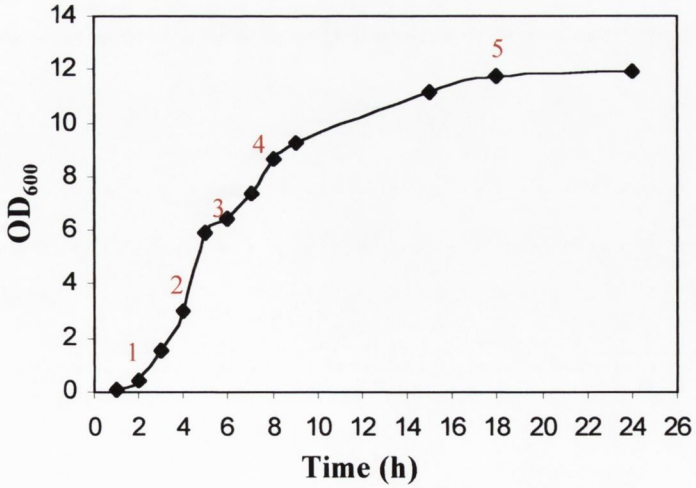
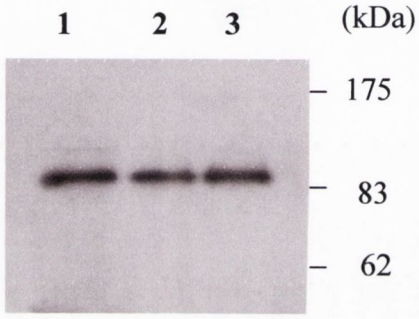
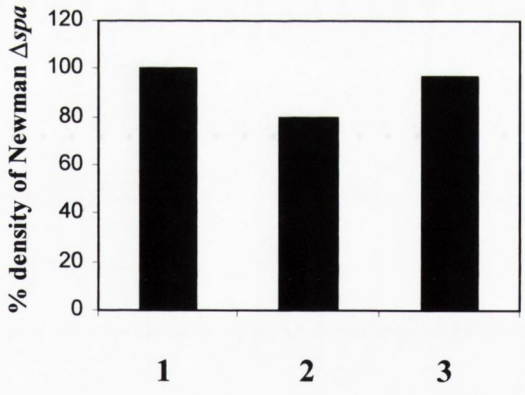

14
12
10
8
6
4
2
0
OD600
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
1
2
3
4
5

Figure 5.4 Western immunoblotting of Newman Δ*spa* and regulatory mutants under aerobic growth conditions. Lane 1, Newman Δ*spa*; lane 2, Newman Δ*spa sar*::*kan*, lane 3, Newman Δ*spa agr*::*tmn*. Cells were grown to stationary phase of growth, washed and standardised to an OD_{600} of 50 units. Protoplast fractions were examined for EbpS expression using anti-$EbpS_{343-486}$ antibodies. Molecular weight markers are indicated on the right hand side. Densitometry was carried out on protein bands. Strain Newman Δ*spa* was taken as 100% and the intensity of bands in regulatory mutants was calculated as a percentage of this strain.


1
2
3
(kDa)
175
83
62


% density of Newman Δspa
120
100
80
60
40
20
0
1
2
3

sar mutant strain. Densitometry (Gelscan) of the protein bands calculated a 20% decrease in EbpS expression in the *sar* mutant compared to the parental strain.

5.3.3 Transcriptional activity of *ebpS*

In order to measure the activity of the *ebpS* promoter and to study transcriptional regulation, a chromosomally located *ebpS-lacZ* transcriptional fusion was constructed to contain an intact copy of the *ebpS* gene plus a truncated copy of *ebpS* fused to the *lacZ* gene. The 5' part of *ebpS* (120 bp) including 900 bp of upstream sequence and likely to contain all *cis*-acting regulatory sites, was cloned upstream of the promoterless *lacZ* gene in pAZ106 to generate plasmid pAZ-*ebpS* (Figure 5.5). Transformation of *S.aureus* RN4220 with the pAZ-*ebpS* plasmid resulted in integration of the suicide plasmid into the chromosomal *ebpS* gene as shown in Figure 5.5. Southern blotting confirmed the integration event using a DIG-labelled probe comprising a 787bp fragment from the 5′ end of the *ebpS* sequence (Figure 5.6). A 10kb band hybridised in the wild-type strain. Following a single cross-over integrational event between the plasmid (pAZ-*ebpS*) and the host *ebpS* locus two hybridising bands of 12.5kb and 7kb were observed due to the presence of an EcoRI site in the multiple cloning site of the *lacZ* gene of pAZ-*ebpS* (Figure 5.5). The relative intensities of the hybridising bands indicated that the 12.5kb band corresponds to the full-length intact *ebpS* gene and the weaker 7kb band corresponds to the partial *ebpS* sequence fused to the *lacZ* gene. The *ebpS-lacZ* fusion was transduced into strains Newman Δ*spa* and 8325-4 Δ*spa* generating FR009 and FR013, respectively. Transductants were selected on erythromycin (10μg/ml) and X-Gal (40μg/ml).

The transcriptional activity of *ebpS-lacZ* was monitored in strains FR009 and FR013 throughout the growth cycle by measuring the amount of β-galactosidase produced. In strain FR009 (*ebpS-lacZ*), *ebpS* expression was found to be switched on in early exponential phase (t =2 h) achieving a maximum of approximately 450 MUG units in late exponential phase (t =4.5 h). A reduction in expression was observed upon entry into stationary phase with residual activity of 150 MUG units (Figure 5.7). In strain FR013 (*ebpS-lacZ*) a similar transcriptional profile was found (550 MUG units) however, the residual activity was found to increase to 450 MUG

Figure 5.5 Strategy for the construction of the chromosomally located *ebpS-lacZ* fusion in *S.aureus*. The *ebpS* promoter region used to generate the transcriptional fusion was amplified by PCR. BamH1 and EcoR1 restriction sites were introduced to facilitate subsequent cloning into pAZ106. Plasmid pAZ-*ebpS* was constructed in *E.coli* and subsequently electroporated into *S.aureus* strain RN4220 where a single-crossover integrational event occured between the plasmid (pAZ-*ebpS*) and the host genome (RN4220). This resulted in an intact copy of the *ebpS* gene plus a truncated copy fused to the *lacZ* gene, stably inserted into the chromosome of *S.aureus* (RN4220 *ebpS-lacZ*).

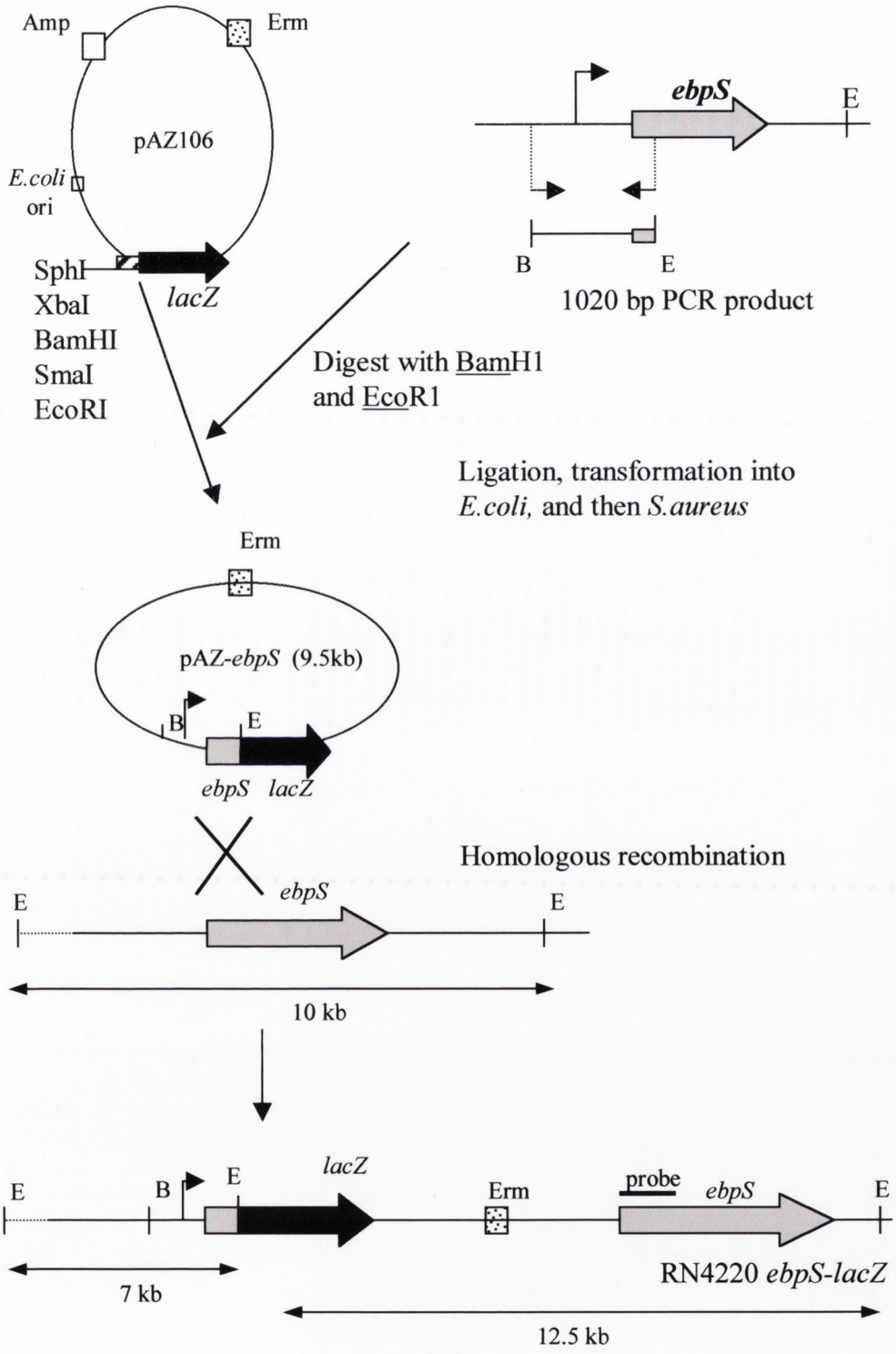
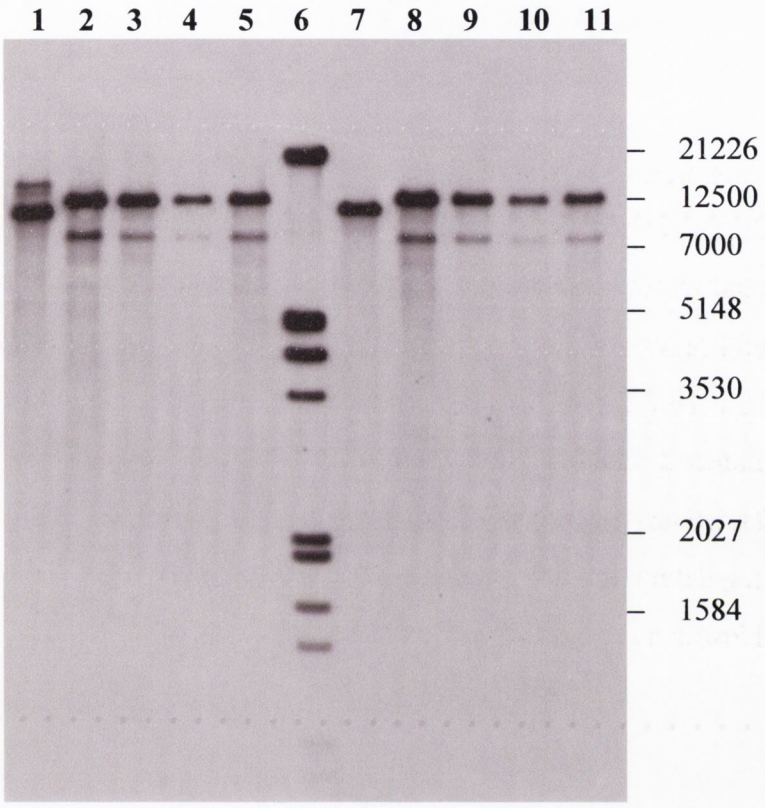

Amp
Erm
pAZ106
E.coli
ori
SphI
XbaI
BamHI
SmaI
EcoRI
lacZ
ebpS
E
B
E
1020 bp PCR product
Digest with BamH1
and EcoR1
Ligation, transformation into
E.coli, and then S.aureus
Erm
pAZ-ebpS (9.5kb)
B
E
ebpS lacZ
Homologous recombination
ebpS
E
E
10 kb
E
B
E
lacZ
Erm
probe
ebpS
E
RN4220 ebpS-lacZ
7 kb
12.5 kb

Figure 5.6 Southern blot analysis of *ebpS-lacZ* fusion strains. Genomic DNA was digested with EcoR1 and probed with a DIG labelled 787bp *ebpS* fragment to confirm the integrational event. Lanes 1 and 7 represent strains 8325-4 Δ*spa* and Newman Δ*spa* respectively. The presence of additional weaker bands in lane 1 of approx 14 kb is indicative of partial digestion of the genomic DNA. Lanes 2 and 8 represent the *lacZ* fusion strains 8325-4 Δ*spa ebpS-lacZ* and Newman Δ*spa ebpS-lacZ,* respectively. Lanes 3-5 and 9-11 represent the regulatory mutants *sar*, *agr* and *sigB* in strains 8325-4 Δ*spa ebpS-lacZ* and Newman Δ*spa ebpS-lacZ,* respectively.


1 2 3 4 5 6 7 8 9 10 11
21226
12500
7000
5148
3530
2027
1584

units upon entry into stationary phase (Figure 5.8). These results are based on three independent experiments.

5.3.4 Transcriptional regulation of *ebpS*

To determine if known regulatory loci play a role in the transcriptional regulation of *ebpS,* the *ebpS-lacZ* fusion was transduced into the single *sar, agr* and *sigB* mutants of strain Newman Δ*spa* (FR010, FR011, FR012) and 8325-4 Δ*spa* (FR014, FR015, FR016), respectively. All fusions were erythromycin resistant (10μg/ml) and showed the correct haemolytic profile on sheep blood plates. Southern blotting confirmed that the *ebpS-lacZ* fusions were intact (Figure 5.6) and PCR analysis was used to ensure inactivation of the *sar* locus in strains FR010 and FR014 (Figure 5.10).

The effect of the regulatory mutations on *ebpS* transcriptional activity was measured by changes in β-galactosidase levels (Figure 5.7 and 5.8). The *agr* and *sigB* mutations had no effect on the transcriptional activity of *ebpS* in strains FR011 and FR012. *ebpS* expression rapidly increased at approximately 2 hours of growth reaching a peak in post exponential phase followed by a decline as cells entered stationary phase (Figure 5.7). Expression of *ebpS* in FR010 (*ebpS-lacZ sar::kan*) was reduced in post exponential phase approximately three to four-fold the level in FR009 (*ebpS-lacZ*). A similar reduction in *ebpS* transcription levels was observed in FR014 (*ebpS-lacZ sar::kan*) (Figure 5.8). There was no apparent difference in the lag period before *ebpS* expression between the wild type and *sar* mutant. These results are representative of three independent experiments.

Since the *ebpS-lacZ* fusion strains were constructed to contain an intact copy of the wild type *ebpS* gene, EbpS protein levels could be examined in each of the fusion strains by Western immunoblotting (Figure 5.9). Cells were grown to stationary phase under the same growth conditions used in the *lacZ* assays (*i.e.* medium aeration) and under limited oxygen conditions (Section 5.2.1). Protoplast fractions were examined for EbpS expression using anti-$EbpS_{343\text{-}486}$ antibodies. There was no significant difference in EbpS expression in strain FR009 or FR013 compared to its *agr* and *sigB* regulatory mutants following growth under both conditions of aeration (Figure 5.9). However, a reduction in EbpS expression was observed in the *sar* mutants. EbpS expression was reduced approximately two-fold in the *sar* mutant of

strain FR009 following aerobic growth as determined by densitometry (Gelscan) of the protein bands (Figure 5.9, panel A). A further reduction in protein expression in the *sar* mutant compared to the parent strain was observed following growth of cells under limited oxygen conditions (Figure 5.9, panel B). Densitometry quantified a four-fold reduction in EbpS expression in the *sar* mutant compared to the parent strain. A similar reduction in EbpS levels was observed in the *sar* mutant of strain FR013 (Figure 5.9, panel C) indicating that *sar* is a positive regulator of EbpS. To ensure that the reduction in EbpS expression in the *sar* mutant was not an artifact of the fusion strains Western immunoblotting was carried out in regulatory mutants lacking the *lacZ* insertion. As expected, under limited oxygen levels EbpS expression was reduced in the *sar* mutant of strain Newman Δ*spa* and 8325-4 Δ*spa* compared to the parent strain (Figure 5.10).

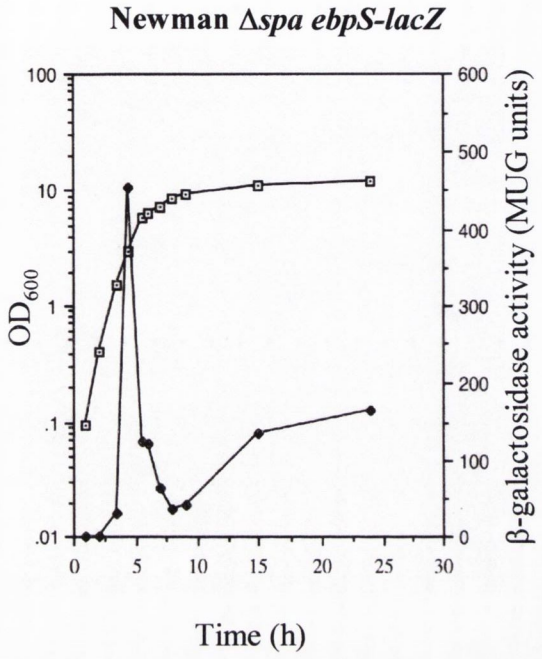
5.3.5 Cell surface-located EbpS is unaffected by protease activity in a *sar* mutant

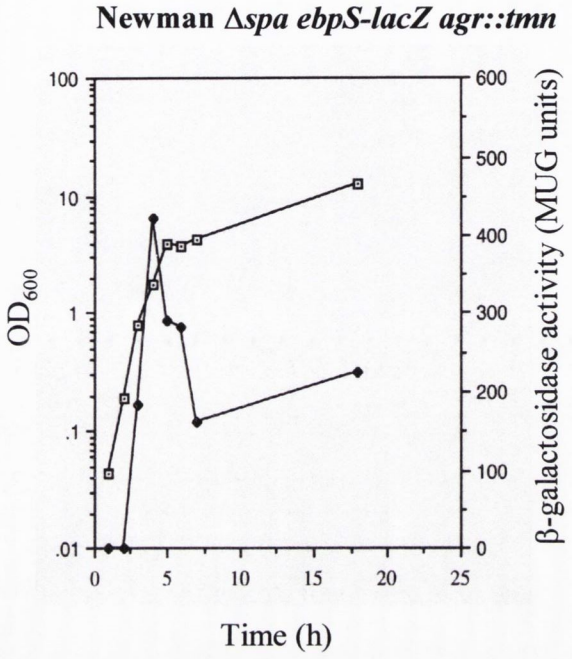
A recent report showed that a mutation of the *sar* locus resulted in increased production of several proteases, including V8 serine protease and a metalloprotease, aureolysin (Chan *et al.*, 1998b). It is therefore plausible that the reduction in EbpS levels in a *sar* mutant might be due to an increase in protease activity. This question was addressed by incubating concentrated culture supernatants from stationary phase strains FR010 (*ebpS-lacZ sar::kan*) and FR014 (*ebpS-lacZ sar::kan*) for 1.5 hours with non-growing stationary phase cells from strains FR009 (*ebpS-lacZ*) and FR013 (*ebpS-lacZ*), respectively. Western blotting of protoplast fractions revealed that the increased levels of proteases in the *sar* mutant strains had no effect on the 83 kDa EbpS protein (Figure 5.11). Probing of the same samples of strain FR009 with anti-$EbpS_{1\text{-}267}$ antibodies also showed that there was no enhancement of the 70 kDa truncated forms of the protein (Figure 5.11, panel B). This confirmed that the reduction in EbpS protein levels in a *sar* mutant was not due to proteolysis but to transcriptional regulation.

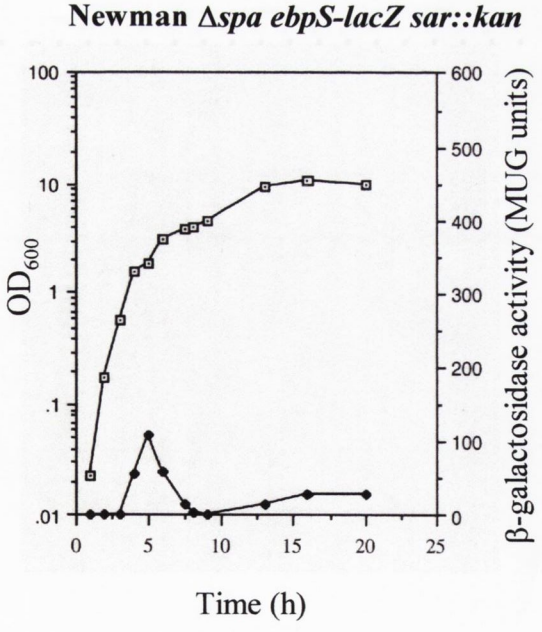
5.3.6 Northern blot analysis of *ebpS* expression

To confirm that inactivation of the *sar* locus effects *ebpS* mRNA levels Northern blotting was carried out by our collaborators to examine the difference in

Figure 5.7 Expression of the *ebpS-lacZ* gene fusion during aerobic growth of *S.aureus* strain Newman Δ*spa* and its regulatory mutants. Growth conditions are as described in Section 5.2.3. Samples were taken at various times throughout the growth cycle and *ebpS* expression levels were monitored by measuring β-galactosidase activity.


Newman Δspa ebpS-lacZ
OD600
β-galactosidase activity (MUG units)
Time (h)


Newman Δspa ebpS-lacZ agr::tmn
OD600
β-galactosidase activity (MUG units)
Time (h)


Newman Δspa ebpS-lacZ sar::kan
OD600
β-galactosidase activity (MUG units)
Time (h)

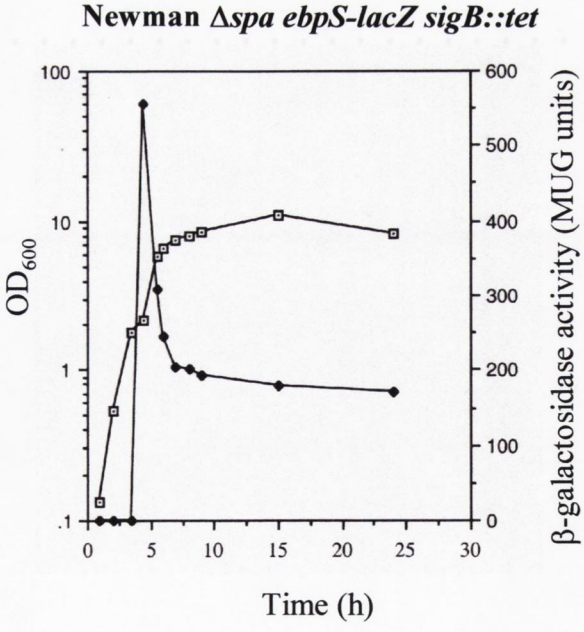
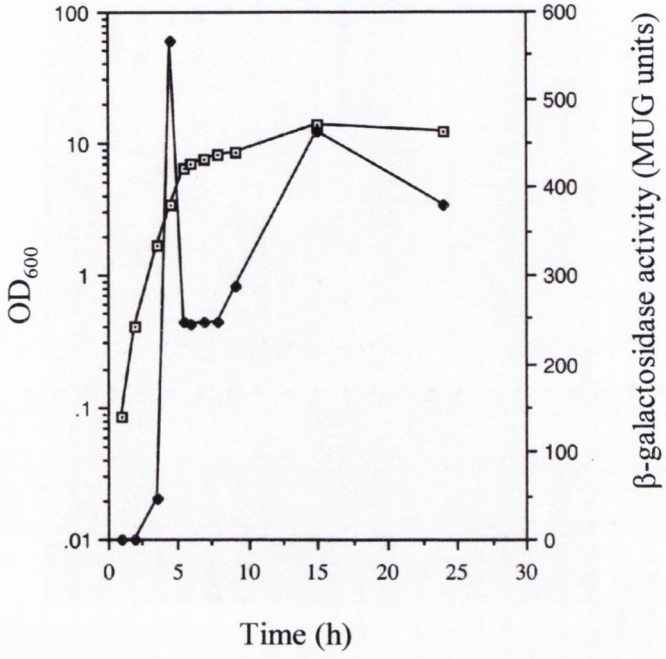

Newman Δspa ebpS-lacZ sigB::tet
OD600
β-galactosidase activity (MUG units)
Time (h)

Figure 5.8 Expression of the *ebpS-lacZ* gene fusion during aerobic growth of *S.aureus* strain 8325-4 Δ*spa* and its regulatory mutants. Growth conditions are as described in Section 5.2.3. Samples were taken at various times throughout the growth cycle and *ebpS* expression levels were monitored by measuring β-galactosidase activity.

8325-4 Δ*spa ebpS-lacZ*



8325-4 Δ*spa ebpS-lacZ sar::kan*

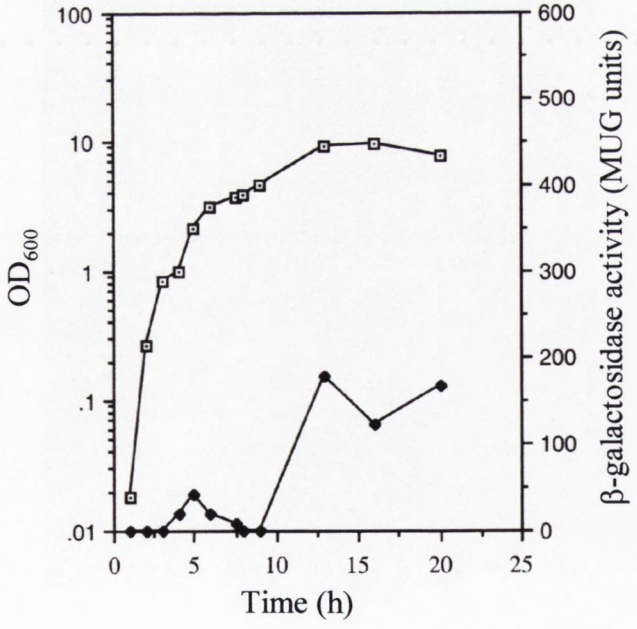
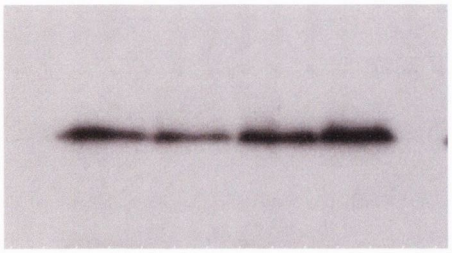
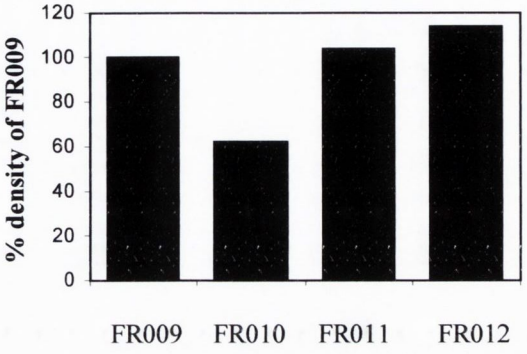


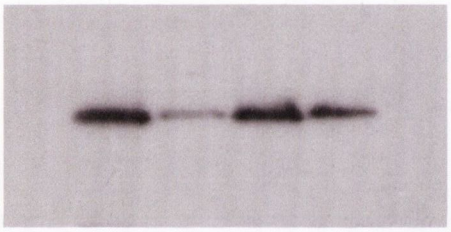
Figure 5.9 Western immunoblotting of *ebpS-lacZ* fusion strains and their regulatory mutants following growth under different conditions of aeration. EbpS expression was studied in strains FR009 (A and B) and FR013 (C) grown to stationary phase under aerobic (A) and limited oxygen (B and C) growth conditions. Densitometry was carried out on protein bands. Wild type strains FR009 and FR013 were taken as 100% and intensity of bands in regulatory mutants was calculated as a percentage of the wild type strain. A and B. Lane 1, FR009 (*ebpS-lacZ*); lane 2, FR010 (*ebpS-lacZ sar*::*kan*); lane 3, FR011 (*ebpS-lacZ agr*::*tmn*) ; lane 4, FR012 (*ebpS-lacZ sigB*::*tet*). C. Lane 1, FR013 (*ebpS-lacZ*); lane 2, FR014 (*ebpS-lacZ sar*::*kan*); lane 3, FR015 (*ebpS-lacZ agr*::*tmn*); lane 4, FR016 (*ebpS-lacZ sigB*::*tet*). The slight decrease in EbpS expression in the *sigB* mutant of FR009 (panel B, lane 4) is an artifact of the experiment.

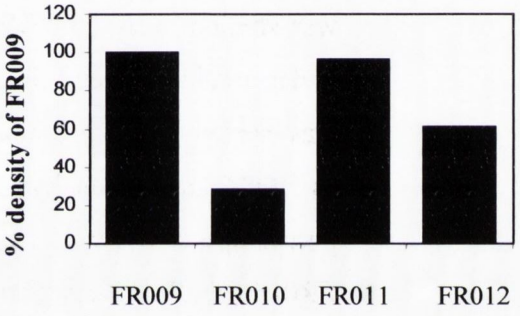
A 1 2 3 4




% density of FR009
120
100
80
60
40
20
0
FR009
FR010
FR011
FR012

B




% density of FR009
120
100
80
60
40
20
0
FR009
FR010
FR011
FR012

C

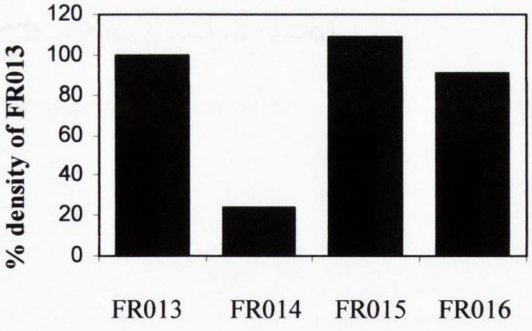

% density of FR013
120
100
80
60
40
20
0
FR013
FR014
FR015
FR016

Figure 5.10 (A) Western immunoblotting of *S.aureus* strains Newman Δ*spa* and 8325-4 Δ*spa* and their corresponding *sar* mutants grown to stationary phase of growth under limited oxygen conditions. EbpS expression was examined in the protoplast fractions using anti-$EbpS_{343-486}$ antibodies. (B) PCR amplification of the *sar* locus. Primer sequences and conditions for amplification are as described in Section 5.2.2. Lane 1, Newman Δ*spa* ; lane 2, Newman Δ*spa sar*; lane 3, 8325-4 Δ*spa* ; lane 4, 8325-4 Δ*spa sar*. DNA molecular weight markers are marked on the left hand side of panel B.

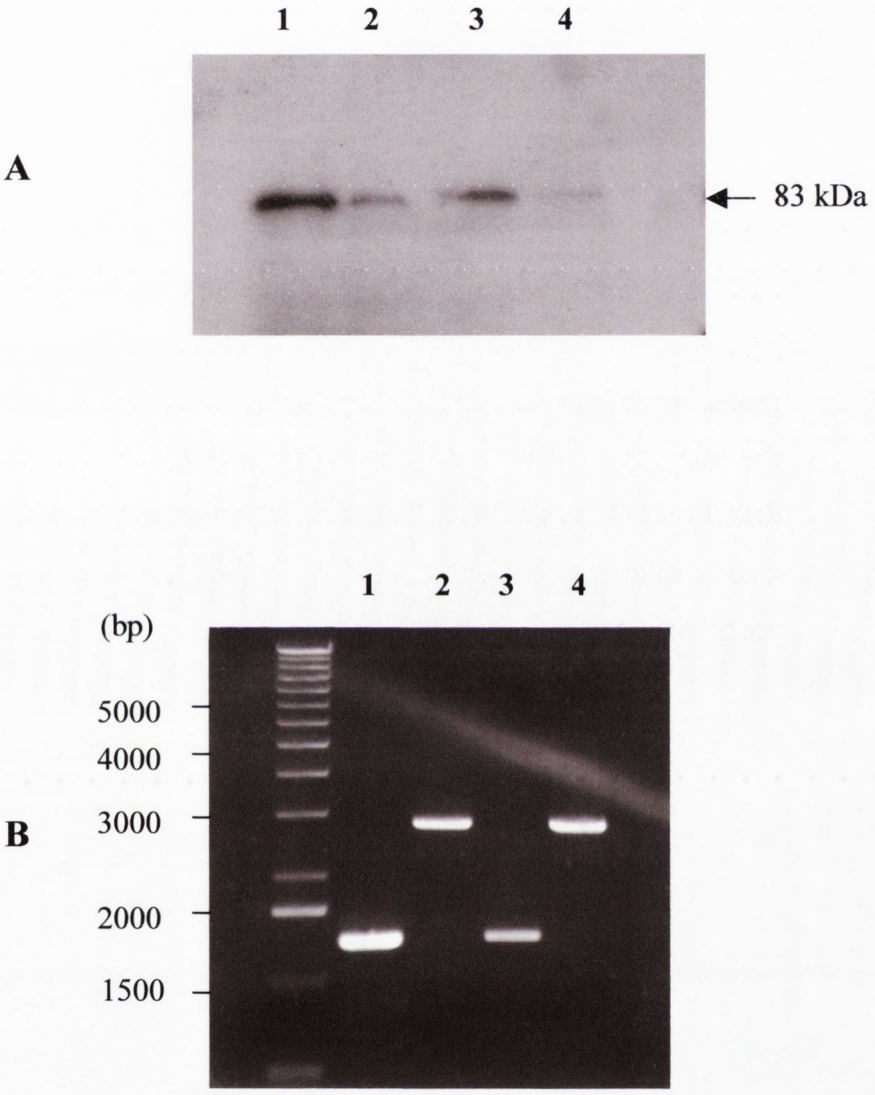
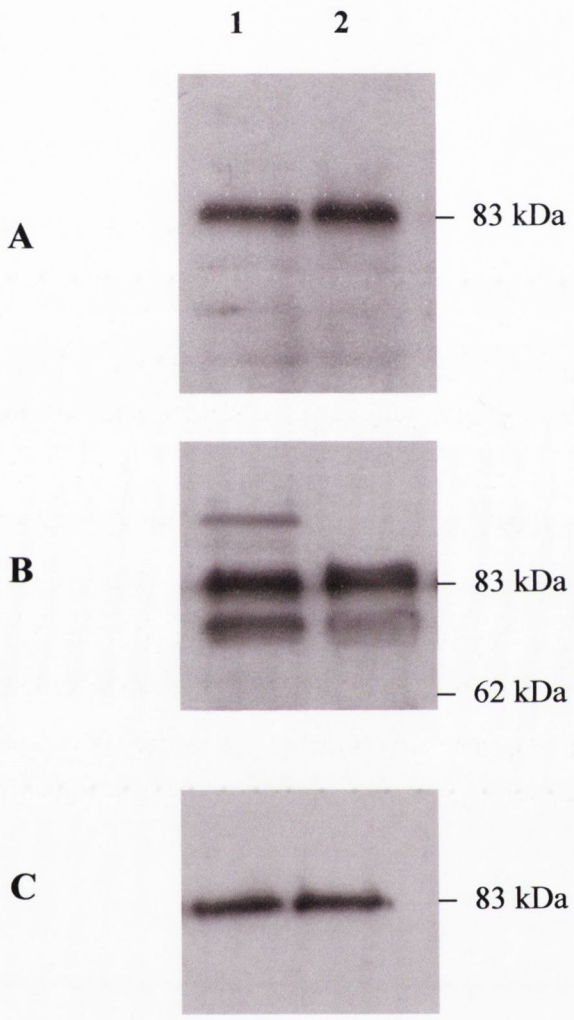

A
1
2
3
4
83 kDa
B
1
2
3
4
(bp)
5000
4000
3000
2000
1500

Figure 5.11 Effect of concentrated stationary phase supernatants from *sar* mutants on EbpS expression. Concentrated stationary phase culture supernatant from strain FR010 (*ebpS-lacZ sar*) was incubated for 1.5 h with chloramphenicol treated FR009 cells (*ebpS-lacZ*) grown to stationary phase (panel A and B), and supernatant from strain FR014 (*ebpS-lacZ sar*) was incubated with chloramphenicol treated cells of strain FR013 (panel C). Protoplasts were examined for EbpS presence using anti-$EbpS_{343-486}$ (panel A and C) and anti-$EbpS_{1-267}$ antibodies (panel B). Lane 1, without supernatant; lane 2, with supernatant. The additional high molecular weight band in lane 1, panel B represents a crossreactive contaminant which is proteolytically degraded upon addition of concentrated supernatant from the *sar* mutant strain (lane 2).


1
2
A
83 kDa
B
83 kDa
62 kDa
C
83 kDa

mRNA levels between strain 8325-4 and 8325-4 *sar::kan* (M.Smeltzer, personal communication). RNA was extracted from cells grown to different phases of growth (early, mid and late exponential) and different concentrations (3, 1 and 0.3μg) were probed for *ebpS* activity using an *ebpS* specific probe (Figure 5.12). No difference in *ebpS* mRNA levels was observed during early exponential growth (OD_{600} 0.3) which is consistent with the *lacZ* transcriptional assays. However, during mid (OD_{600} 1.0) and post (OD_{600} 3.0) exponential phase of growth a four-fold and two-fold reduction, respectively in mRNA levels was observed in the *sar* mutant strain compared to strain 8325-4. This provides further evidence that *sar* regulates *ebpS* expression at the transcriptional level.

5.3.7 Identification of SarA binding motif

Recently, Chien *et al.* (1999) mapped a *sarA* binding site to a 29 bp AT-rich sequence within the *agr* P2-P3 promoter region by DNase footprinting analysis. Analysis of other SarA-regulated genes conveyed similar sequence data. An alignment of sequences from the promoter regions of *hla, spa, fnbA, fnbB*, and *sec* revealed an apparent 26 bp consensus sequence (Chien *et al.*, 1999). Analysis of the *ebpS* upstream sequence reveals a region of strong identity (18/26) to the proposed SarA binding site (Figure 5.12). Since the *ebpS* promoter region has not yet been mapped the distance of this AT-rich region from the transcriptional start site cannot be predicted. However, the 3‘ end of the *ebpS* sequence is located 20bp upstream of its translational start site (Figure 2.6), which lies within the distance of other known SarA regulated genes (Chan *et al.*, 1998b). For example, the 3’ ends of the *tst*, *hlb* and V8 protease sequences are located 145, 61 and 9 bp upstream of their translational start sites.

Figure 5.13 Alignment of a putative common SarA recognition sequence from promoters of *sar* target genes. Chien *et al.* (1999) identified a conserved sequence in the promoter regions of genes regulated by SarA. This sequence is proposed to include one of the SarA binding sites in the *agr* P2-P3 interpromoter region. Analysis of the DNA sequence upstream from *ebpS* identified a sequence showing 18/26 similarities. The position of this sequence upstream from the *ebpS* translational start codon is highlighted in Figure 2.6. The consensus sequence is marked in blue and the nucleotides in *ebpS*, which bear homology to the consensus sequence, are marked in red.

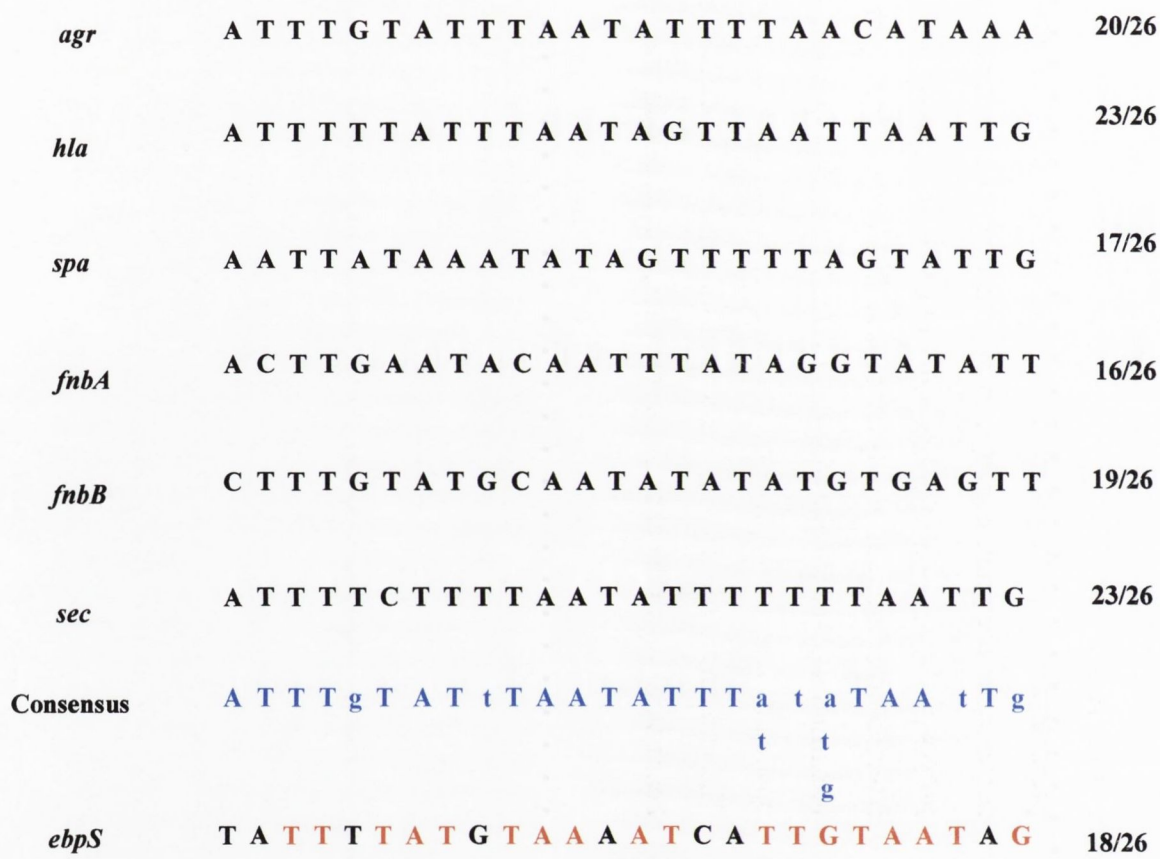
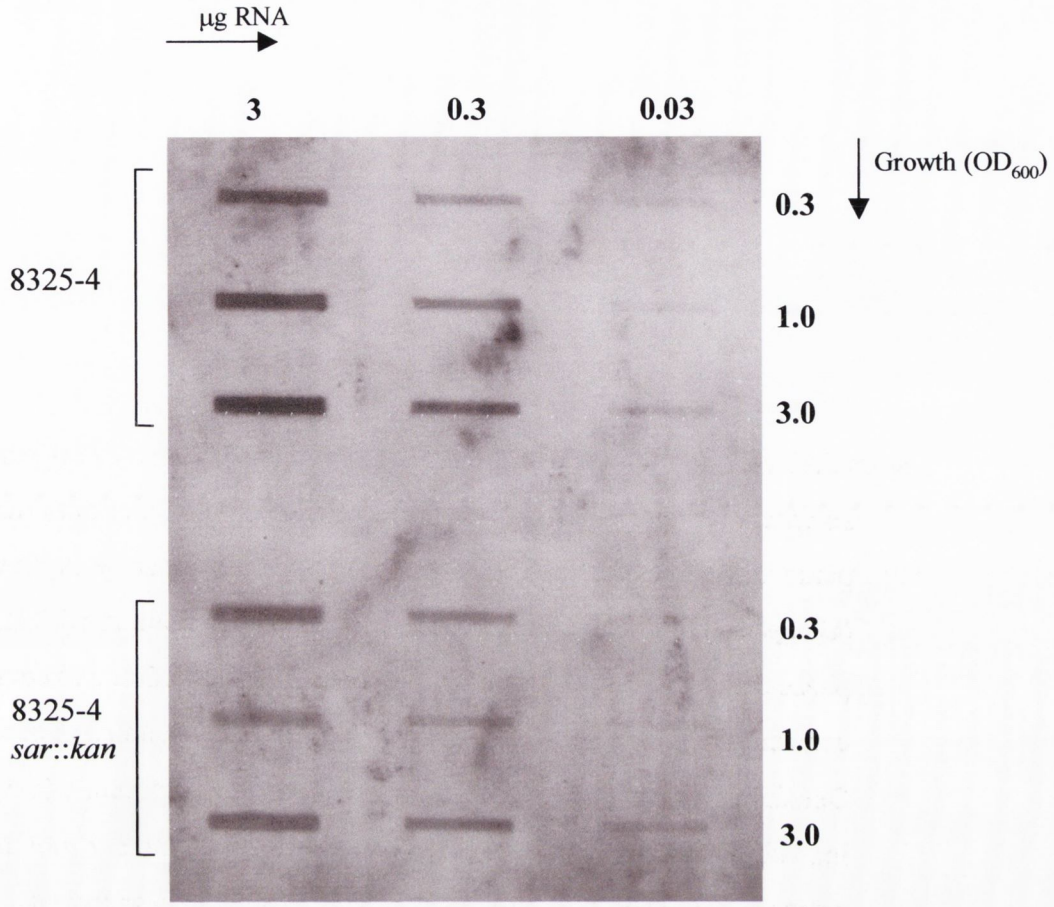
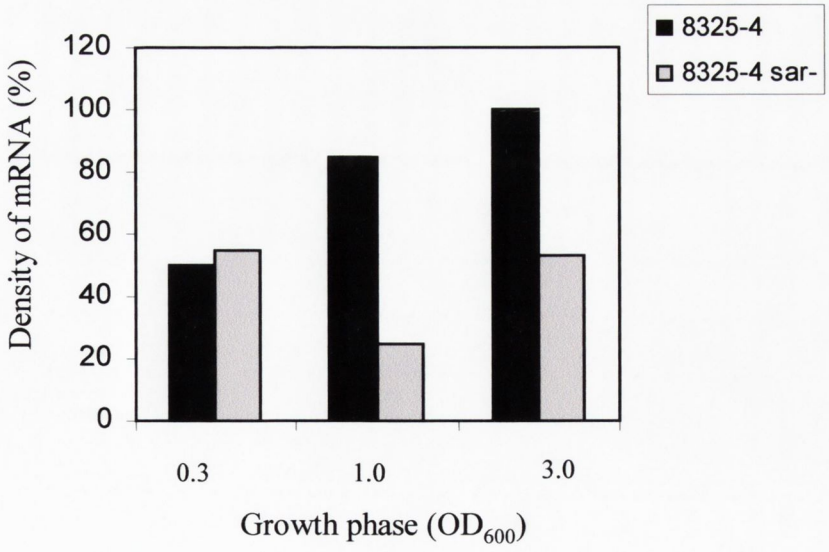

agr
A T T T G T A T T T A A T A T T T T A A C A T A A A
20/26
hla
A T T T T T A T T T A A T A G T T A A T T A A T T G
23/26
spa
A A T T A T A A A T A T A G T T T T T A G T A T T G
17/26
fnbA
A C T T G A A T A C A A T T T A T A G G T A T A T T
16/26
fnbB
C T T T G T A T G C A A T A T A T A T G T G A G T T
19/26
sec
A T T T T C T T T T A A T A T T T T T T T T A A T T G
23/26
Consensus
A T T T g T A T t T A A T A T T T a t a T A A t T g
t t
g
ebpS
T A T T T T A T G T A A A A T C A T T G T A A T A G
18/26

Figure 5.12 Northern blot analysis of *ebpS* expression in strain 8325-4 and 8325-4 *sar::kan*. Northern blotting was performed by our collaborators (M.Smeltzer, personal communication). mRNA was extracted from early exponential phase (OD_{600} 0.3), mid exponential (OD_{600} 1.0) and late exponential (OD_{600} 3.0) phase cells of *S.aureus* strain 8325-4 and 8325-4 *sar::kan* and decreasing concentrations were probed for *ebpS* expression using an *ebpS* specific probe. Densitometry was carried out on mRNA bands corresponding to 3μg of RNA loaded to compare mRNA levels between wild type and *sar* mutant. RNA extracted from strain 8325-4 grown to late exponential phase was taken as 100% and the intensity of the other bands was calculated as a percentage of the wild type strain.


µg RNA
3
0.3
0.03
Growth (OD_{600})
8325-4
0.3
1.0
3.0
8325-4
sar::*kan*
0.3
1.0
3.0


8325-4
8325-4 sar-
Density of mRNA (%)
120
100
80
60
40
20
0
0.3
1.0
3.0
Growth phase (OD_{600})

5.4 Discussion

The ability of *Staphylococcus aureus* to cause infection is probably related to its ability to respond to changing environments in the host. The adaptive response is highly co-ordinated and controlled by regulatory loci, of which *agr*, *sar* and to a lesser extent *sigB* are best characterised. In this chapter EbpS expression was studied and the role of global regulatory loci on *ebpS* expression was examined both at the level of transcription and protein expression.

Firstly, *ebpS* expression was monitored throughout the growth cycle by Western immunoblotting and transcriptional *lacZ* assays. Western immunoblotting of strain Newman Δ*spa* revealed that EbpS was expressed during early exponential phase (t = 2h) and remained on the cell surface into late stationary phase (t = 18h). Transcriptional analysis using an *ebpS-lacZ* gene reporter complemented this expression profile in both strain FR009 (Newman Δ*spa ebpS-lacZ*) and FR013 (8325-4 Δ*spa ebpS-lacZ*). Transcription of *ebpS* was switched on in early exponential phase and a sharp decline in β-galactosidase activity during post-exponential phase implies reduced transcription. It is possible that *ebpS* transcription is switched on earlier in the growth cycle but activity was not detected as too few cells were examined (0.5 ml of cells). A larger volume of cells could be sampled to accurately predict expression levels at the beginning of the growth cycle.

A similar peak transcriptional level of approximately 450-550 MUG units was observed for both strains FR009 and FR013. However in the case of strain FR013, a steady increase in β-galactosidase occurred as the cells progressed into stationary phase. It is possible that this may reflect an artifact of the experiment or even accumulation of the β-galactosidase product, yet this phenomenon was solely restricted to strain 8325-4 Δ*spa* and its derivatives. Assuming *ebpS* transcription terminates in post-exponential phase, β-galactosidase activity would be expected to decline or exhibit residual levels of activity as observed in strain FR009. An increase in β-galactosidase activity in strain FR013 following the decline in transcription in post-exponential phase therefore suggests a re-initiation in *ebpS* transcription. If time allowed this hypothesis could be tested by monitoring *ebpS* transcription in strain FR013 throughout the growth cycle by Northern blotting to determine if *ebpS* mRNA levels continue to increase during stationary phase.

The temporal expression of *ebpS* is similar to that of other surface proteins of *S.aureus*. In general, the expression of surface proteins is rapidly induced during early-exponential phase (*e.g.* Spa, Coa, FnbpA, FnbpB, ClfB, Cna) followed by down-regulation at the transcriptional level as the cells enter the transition to stationary phase of growth. Despite the termination of *ebpS* transcription in post exponential phase, EbpS was still present on the cell surface of late stationary phase cells as indicated by Western blotting and colony blotting (Section 4.3.1). In addition, there was no dramatic difference in the ability of post exponential and stationary phase cells to bind soluble elastin which is consistent with there being a similar number of EbpS molecules on the cell surface during both growth phases (Section 4.3.2). Since EbpS is associated with the cytoplasmic membrane of *S.aureus* it is unlikely to be lost or reduced from the cell surface by cell wall turnover. It appears to be stable and quite resistant to proteolysis with the majority of the protein in the full-length form. Although a small amount of breakdown was evident at the C-terminus of the molecule, this may have occurred during the formation of protoplasts.

The role of regulatory loci on *ebpS* expression was examined. Using the *lacZ* transcriptional reporter fusion it was shown that inactivation of the *sar* locus in strains FR010 (Newman Δ*spa ebpS-lacZ sar::kan*) and FR014 (8325-4 Δ*spa ebpS-lacZ sar::kan*) resulted in reduction of *ebpS* transcription in post exponential phase by three to four-fold compared to the parental strains FR009 and FR013, respectively following aerobic growth (medium aeration). The temporal expression of *ebpS* was unaltered in the *sar* mutant in agreement with the protein expression data shown in Figure 5.3. Northern blotting detected a similar three to four-fold difference in *ebpS* mRNA levels between wild type and *sar* mutant grown to mid exponential phase under the same growth conditions (M.Smeltzer, personal communication). This difference was reduced to two-fold when post exponential phase cells were used. This is consistent with results obtained from Western immunoblot analysis of the fusion strains grown to stationary phase. Under the same growth conditions (*i.e.* aerobic) a two-fold difference in EbpS protein levels between wild type and *sar* mutant was detected. Since the reduction in *ebpS* expression in a *sar* mutant was demonstrated at both the transcriptional and protein level this suggests that SarA plays a role as a transcriptional activator of *ebpS* expression.

Interestingly, the effect of *sar* on *ebpS* regulation was found to vary with changes in aeration. Following growth in highly aerated cultures (20ml of culture in a 250ml flask) there was a 20% reduction in EbpS expression between wild type and *sar* mutant. After growth in medium aerated cultures (100ml of culture in a 250ml flask) a two-fold decrease in *ebpS* transcription in a *sar* mutant was demonstrated by both Western and Northern blotting. By further reducing the level of aeration in the growth culture a further reduction in EbpS synthesis was observed in the *sar* mutants of strains FR010 and FR014 compared to parental strains as determined by Western immunoblotting. Densitometry quantified the reduction as four-fold. A similar reduction in EbpS expression was observed in *sar* mutants of strains lacking the *lacZ* fusion following growth under limited oxygen demonstrating that the effect of the *sar* mutation on *ebpS* transcription was independent of the presence of the *lacZ* gene. Collectively, these results suggest that *sar*-mediated regulation of *ebpS* is affected by aeration levels. SarA has previously been shown to act as a signal transduction regulatory component in response to aeration stimuli (Chan *et al.*, 1998b). For example, *sar* is involved in regulation of *hla* by both direct and indirect mechanisms. Inactivation of the *sar* locus leads to a 60% reduction in *hla* expression in stationary phase under aerobic growth conditions. However, following microaerobic growth a further reduction in expression was observed compared to the wild type strain (Chan *et al.*, 1998b). This suggests that SarA expression is responsive to reduced oxygen levels. A change in SarA levels under limited oxygen conditions may either directly or indirectly upregulate *hla* expression. A similar mechanism may be responsible for the increased upregulation of *ebpS* expression following growth under limited oxygen conditions.

Inactivation of the *sar* locus only partially attenuated the expression of EbpS. A similar phenomenon was observed in a *sar* mutant with TSST-1 and SEB during stationary phase showing 50% and 30% transcriptional activity of the parent strain level, respectively (Chan *et al.*, 1998b). Expression of these proteins is co-regulated by *sar* and *agr*, (Compagnone-Post *et al.*, 1991; Recsei *et al.*, 1986), hence inactivation of the *agr* locus causes a further reduction in transcription activity. Since *ebpS* expression is not fully down-regulated in a *sar* mutant this suggests that another factor might be involved in promoting activation of *ebpS* gene transcription. Alternatively, the residual activity in a *sar* mutant may represent the level of

transcription from the RNA polymerase in the absence of any transcriptional activators.

A recent report has described a new global regulator SarH1, which is a homolog of SarA. Expression of SarH1 was found to be regulated by both *sar* and *agr* showing increased levels in *sar* and *agr* mutants. Inactivation of *sarH1* has demonstrated that this locus is responsible for certain regulatory effects on virulence genes, which were previously attributed to *sar* and *agr*. For example, SarH1 has been demonstrated to act as a repressor of *hla* transcription. SarA and RNAIII were previously reported as positive regulators of *hla* expression as in a *sar* and *agr* mutant, *hla* levels were reduced. Inactivation of *sarH1* in an *agr* mutant had no effect on *hla* levels however, inactivation of *sarH1* in a *sar* mutant restored *hla* expression to the same levels as in the wild type. This proves that repression of *hla* expression in a *sar* mutant was not due to the lack of the proposed activator, SarA, but to the upregulation of the repressor, *Sar*H1. The effect of *sarH1* on *ebpS* expression has not been investigated. *ebpS* expression is reduced in a *sar* mutant, however it is possible that reduced expression might be due to increased levels of the repressor SarH1. This could easily be tested in a *sarH1 sar* double mutant. If *ebpS* expression levels were restored in the double mutant, it would be indicative of SarH1 being a negative regulator of *ebpS*.

Recent evidence has shown that *sar* is a pleiotropic repressor of proteases in *S.aureus*, including V8 serine protease (*ssp*) and a metalloprotease (Chan *et al.*, 1998). It has been proposed that the overproduction of proteases in a *sar* mutant may represent a general mechanism by which bacteria rapidly downregulate adhesive molecules. This is exemplified by the ability of Ssp and aureolysin to reduce FnbP and ClfB respectively, on the cell surface (McGavin *et al.*, 1997; McAleese, personal comunication). The affect of proteases on cell surface EbpS was investigated by incubating concentrated supernatant from a stationary phase *sar* mutant with killed wild type cells. The level of EbpS was not affected by proteolysis and additional truncated forms were not observed. It is likely that only a small portion of the N-terminus is projected from the cell surface making it less susceptible to cleavage by extracellular proteases compared to LPXTG anchored proteins with more exposed domains. This confirms that a reduction in EbpS expression in a *sar* mutant is not a result of an increase in protease levels but due to either (i) the absence of the SarA regulatory molecule required to activate *ebpS* transcription or (ii) the upregulation of

SarH1 to repress *ebpS* transcription. A corresponding reduction in *ebpS* expression in a *sar* mutant at the level of transcription as determined by transcriptional *lacZ* assays and Northern blot analysis further supports this hypothesis.

The mechanism by which SarA (or SarH1) regulates *ebpS* transcription is not known. The fact that *ebpS* expression was unaffected in an *agr* and *sigB* mutant suggests that activation is by an *agr*-independent and *sigB*-independent mechanism. However, this would need to be confirmed in a *sar agr* and a *sar sigB* double mutant. A similar reduction in *ebpS* expression in the double mutants as was shown in the single *sar* mutant would confirm that *sar* regulation of *ebpS* is by an *agr*-independent and *sigB*-independent mechanism. The effector molecule of the *sar* locus is a DNA-binding protein designated SarA. Purified native and recombinant SarA protein has been shown to bind to sites within the *agr* promoter region affecting RNAII and RNAIII transcription (Morfeldt *et al.*, 1996; Rechtin *et al.*, 1999). Morfeldt *et al.* (1996) proposed that SarA might function as a regulatory molecule by affecting the DNA bending of target promoters. A 3bp deletion in the spacer region between the –10 and –35 boxes in the P3 promoter region of the *agr* locus allowed transcription of RNAIII independent of the upstream regulatory sequences (Morfeldt *et al.*, 1996). Chien *et al.* (1999) mapped a SarA binding site to a 29bp sequence within the *agr* regulatory region by DNase footprinting studies. Analysis of other *sar*-regulated genes revealed similar sequences, allowing the construction of a consensus sequence. A region of strong similarity (18/26 identities) to the SarA consensus sequence occurs upstream of *ebpS* suggesting that perhaps SarA interacts directly with the *ebpS* promoter region. However, the 3' end of the SarA motif is located only 20 bp upstream of the *ebpS* translational start codon which would imply that binding of SarA to this region may obstruct RNA polymerase accessibility rather than induce a stronger interaction with the DNA. Alternatively, the identified sequence upstream of *ebpS* may not represent a SarA binding site. More recent studies suggest that SarA is capable of binding multiple DNA targets, implying that there is no clear consensus as to what constitutes a SarA binding site (Rechtin *et al.*, 1999; Blevins *et al.*, 1999). A possible explanation for the discrepancies between the studies in identifying binding sites for SarA could be due to the different types of protein used. Chien *et al.* (1999) used an N-terminal GST-SarA fusion protein, Rechtin *et al.* (1999) expressed SarA in its full-length unmodified form and Morfeldt *et al.* (1996) used native SarA protein. Therefore, the significance of this SarA consensus sequence determined by

Chien *et al.* (1999) in the SarA-mediated regulation of *ebpS* remains to be established experimentally.

The *sar* locus has previously been shown to activate and repress target genes during post exponential phase of growth. In this study we postulate a role for *sar* in early exponential phase in the activation of *ebpS* transcription. Preliminary studies at the transcriptional and protein level support this hypothesis. However, complementation of the *sar* phenotype to restore *ebpS* transcription would be required to conclusively identify *sar* as a positive regulator of *ebpS*. In order to further understand the role of *sar* in the regulation of *ebpS* expression a collaboration has been established with Mark Smeltzer. This study will attempt to investigate if SarA directly interacts with the promoter region of *ebpS* by using DNase footprinting and gel shift analysis. Alternatively, SarA may regulate *ebpS* expression via an additional regulatory protein or indirectly via SarH1.

Chapter 6

Discussion

At the outset of this project the elastin binding protein of *S.aureus* (EbpS) was reported as a 25 kDa protein encoded by a 609 bp gene (Park *et al.*, 1996). In this study the corrected sequence of the *ebpS* gene is presented. It comprises of 1461 bp and encodes an EbpS protein of 486 residues. Western immunoblotting detects EbpS as a protein which migrates in SDS-PAGE with an apparent molecular weight of 83 kDa. It is most likely that the 25 kDa protein described previously is an N-terminal truncate cleaved from the body of the EbpS protein by bacterial proteases. This may have occured during prolonged incubation in lysostaphin digestion buffer lacking raffinose and $MgCl_2$ to stabilise the protoplasts.

Analysis of the cell envelope of *S.aureus* indicated that EbpS is strongly associated with the cytoplasmic membrane (R.Downer, personal communication). Examination of the amino acid sequence and analysis by secondary structure prediction algorithms suggests that there are three hydrophobic domains in EbpS with potential to span the cytoplasmic membrane. The N-terminal ligand binding domain of EbpS is surface exposed which is consistent with the ability of EbpS to mediate bacterial binding to soluble elastin. The C-terminus of the molecule bearing a putative peptidoglycan binding domain (LysM) is also cell wall associated as determined by PhoA fusions (R.Downer, personal communication). It is possible that EbpS is an integral membrane protein bearing two or three transmembrane domains. The construction of PhoA fusions could address this hypothesis and additionally determine the orientation of the hydrophobic domains in the membrane. EbpS does not have an N-terminal signal sequence, indicating that it is incorporated into the membrane by a Sec-independent mechanism. The protein is targeted to the membrane without any processing occurring as the same size protein released form stabilised protoplasts is detected by both anti-$EbpS_{1\text{-}267}$ and anti-$EbpS_{343\text{-}486}$ antibodies. It is possible that EbpS is incorporated into the membrane by a SRP-dependent mechanism typical of most integral membrane proteins.

Data from Western immunoblotting and transcriptional *lacZ* fusions demonstrate that EbpS is expressed during exponential growth and is exposed on the cell surface throughout the growth cycle. This was paralleled by the ability of cells to bind soluble elastin (P.W Park, personal communication). Inactivation of the *sar* locus led to reduced *ebpS* transcription and translation suggesting a role for SarA as

an *ebpS* transcriptional activator in early exponential phase. This decrease was independent of proteases that are upregulated by the *sar* mutation and is aeration dependent. A decrease in oxygen available for growth correlated with a further reduction in *ebpS* expression in the *sar* mutant compared to the parental strain. This suggests that reduced oxygen affects *ebpS* expression in the absence of SarA. In wild type cells the presence of SarA appeared to inhibit *ebpS* repression following growth under limiting oxygen conditions possibly by binding to the promoter region to upregulate *ebpS* expression. SarA has previously been shown to act as a signal transduction regulatory component in response to aeration stimuli (Chan *et al.*, 1998b). Under microaerobic conditions *hla* expression was also further reduced in a *sar* mutant. Many virulence factor genes are repressed under microaerobic growth such as *hla* and *tsst-1*, possibly due to the decrease in growth yield or the reduced quorum sensing potential. Dorman (1991) reported that anaerobic growth could regulate gene transcription by altering the DNA supercoiling properties of target promoters. One could speculate that the *sar* locus selectively upregulates target genes, including *ebpS* and *hla*, which are required for growth or virulence under limiting oxygen conditions.

Inactivation of *ebpS* by allele replacement confirmed that EbpS represents the major receptor of *S.aureus* for soluble elastin. The ability of membrane bound proteins to interact with matrix molecules has not been described before in *S.aureus*. Most matrix binding surface proteins or adhesins expressed by Gram-positive bacteria are associated with the cell wall. However, in *Streptococcus sanguis* a lipoprotein SsaB, which is anchored to the cell membrane and only weakly surface exposed, has adhesive functions (Ganeshkumar *et al.*, 1993). One can only speculate about the *in vivo* significance of a bacterial surface protein binding to soluble elastin. It is conceivable that membrane-bound EbpS is an environment sensing protein, recognising elastin peptides to detect that the bacterium is in an environment where tissue destruction is occuring. Binding to soluble elastin may upregulate tissue destructive enzymes (e.g. elastases) to allow dissemination of the infection in which case a cytoplasmic part of the EbpS protein presumably interacts with a regulatory protein in the cytoplasm. Elastin peptides are also chemotactic (Varga, 1989). Therefore it is possible that bacterial binding to elastin peptides may reduce the migration of PMNs to the site of infections in an attempt to evade the immune

response. Alternatively, binding to soluble tropoelastin may interfere with elastin matrix assembly. However, as yet all this remains a matter of speculation. Further studies need to be performed to ascribe a phenotype to EbpS, either with the *ebpS* mutant or the wild-type bacteria growing in the presence of elastin peptides.

Based on current information, the surface of the *S.aureus* cell wall appears to be complex. It is composed of proteins with different binding functions and enzymatic activities. The ability of cells to attach to different components of the extracellular matrix (ECM) has already been attributed to a variety of surface adhesins (Höök and Foster, 2000). However, the ability of Gram-positive bacteria to adhere to elastin or to immobilised degradation products (elastin peptides) has not previously been described. The EbpS protein has previously been categorised as an MSCRAMM. These proteins are involved in promoting bacterial attachment to components of the ECM thereby contributing to the ability of bacteria to colonise host tissue. However in order to be classified as a true MSCRAMM each protein must promote adhesion to the pure ligand immobilised on an inert surface and also bind to the soluble ligand. In this study, an ELISA based adherence assay was developed to investigate the elastin binding activity of *S.aureus*. Bacterial cells of some strains of *S.aureus* were shown to promote adherence to immobilised elastin peptides but this interaction was EbpS-independent. Therefore, although EbpS recognises soluble elastin, it cannot be regarded as a true MSCRAMM. A possible explanation for this discrepancy may be that the flexibility of EbpS on the cell surface is restricted and it only allows interaction with the soluble ligand. Perhaps projection of the ligand-binding site away from the cell surface might allow cells to adhere to immobilised elastin peptides in an EbpS-dependent manner. This could be tested by constructing an in-frame insertion of the R domain of ClfA downstream from the EbpS ligand binding site or by inserting the EbpS ligand binding domain at the N-terminus of an LPXTG-anchored protein such as Cna.

Bacterial adherence to immobilised elastin peptides was found to be strain specific and growth phase dependent. A screen of several known surface MSCRAMMs identified the fibronectin binding proteins (FnBPs) as the putative elastin receptor. To ensure that this interaction was elastin specific and not due to fibronectin contamination purified recombinant tropoelastin (rTE) was used as the

ligand. Strain P1 was capable of adhering to immobilised rTE in a dose dependent and FnBP dependent manner strongly suggesting that the *S.aureus*-elastin interaction is specific. Further studies will be carried out using recombinant proteins and heterologous expression of FnBPs in Lactococcus lactis to define the binding domain on both the FnBPs and the tropoelastin molecule. The fibronectin binding proteins of *Staphylococcus aureus* have already been shown to promote bacterial adherence to fibronectin and fibrinogen (Section 1.3.1.3.2). The ability of these proteins to adhere to elastin matrix molecules may be additive advantage to the organism during infection in colonising elastin rich tissue.

The inability of EbpS to function as an MSCRAMM suggests another role for this protein. A genomic approach was taken to search for sequence or structural similarities in other known proteins. Analysis of the DNA flanking *ebpS* showed that *ebpS* resides in a locus which is conserved among Gram-positive bacteria such as *Bacillus subtilis* and *Enterococcus faecalis*. Identification of proteins which occupy the same position as EbpS include Alys, an autolysin with N-acetyl-muramoyl amidase activity and YpbE, a protein of unknown function. Moreover, a homology search of several protein databases showed that EbpS bears similarity to the autolysin Aas of *S.saprophyticus*. The regions of similarity between EbpS and these proteins did not involve the known functional domains indicative of an autolysin but rather domains involved in peptidoglycan recognition. It is unlikely that EbpS is an autolysin because inactivation of *ebpS* does not induce cell clumping which is typical of loss of autolytic function. AlyS and Aas both bear several putative peptidoglycan-binding repeats typical of wall associated autolysins. YpbE on the other hand contains a single hydrophobic domain in the N-terminal region with the potential to span the cytoplasmic membrane. Like EbpS, this protein may be membrane associated and could share a similar topology to EbpS. Due to its location in a conserved region of the *S.aureus* genome and its similarity to proteins involved in cell wall hydrolysis one could speculate that EbpS may be involved in cell wall biosynthesis. With the availability of isogenic *ebpS*-defective mutants it would be possible to examine the effect of loss of EbpS on cell wall biosynthesis to test this hypothesis.

Appendix

Figure 1. Coomassie stained SDS-PAGE gel of purified recombinant tropoelastin (rTE). An N-terminal truncate of rTE (exons 1-15) was expressed from plasmid pQE-rTE (Table 2.2) in *E.coli* as an N-terminal his-tagged fusion protein and purified by Ni^{2+} affinity chromatography (Section 2.2.5.3). The higher molecular weight band of approximately 42 kDa corresponds to a dimer form of rTE and the 28 kDa band corresponds to the monomeric form. A single breakdown product of the monomeric form is evident. Protein molecular weight markers are indicated on the right.

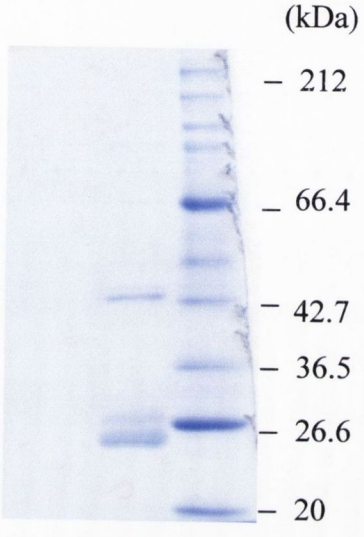
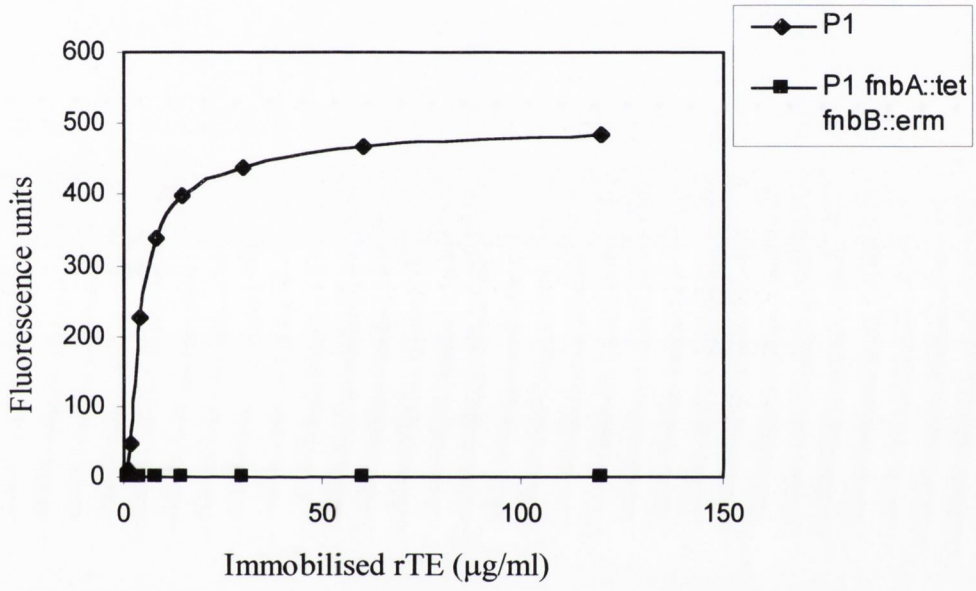

(kDa)
212
66.4
42.7
36.5
26.6
20

Figure 2. The role of fibronectin binding proteins (FnBPA and FnBPB) in promoting the adherence of *S.aureus* to immobilised recombinant bovine tropoelastin (rTE). rTE, which represents an N-terminal truncate of tropoelastin (exons 1-15), was expressed as an N-terminal his-tag fusion protein and purified by Ni^{2+} affinity chromatography. ELISA plates were coated with increasing concentrations of rTE by incubating at 4°C for 18 hr. Following blocking and washing steps the adherence of early exponential phase bacterial cells was examined as described in Section 4.2.4.


P1
P1 fnbA::tet fnbB::erm
600
500
400
300
200
100
0
Fluorescence units
0
50
100
150
Immobilised rTE (µg/ml)

References

Abdelinour, A., S. Arvidson, T. Bremell, C. Ryden, and A. Tarkowski. (1993) The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. Infect. Immun. **61:** 3879-3885.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. **215:** 403-410.

Arvidson, S. (2000) Extracellular enzymes. *In* Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A. and Rood, J.I. (ed.), Gram-Positive Pathogens. ASM Press, Washington D.C. p.379-385.

Arvidson, S., Janzon, L., Lofdahl, S. (1990) The role of the delta-lysin gene (*hld*) in the *agr*-dependent regulation of exoprotein synthesis in *Staphylococcus aureus*. *In* Novick, R.P. (ed.), Molecular Biology of the Staphylococci. New York, VCH Publishers; pp.419-431.

Arvidson, S.O. (1983) Extracellular enzymes from *Staphylococcus aureus*. *In* Easmon C.F., Adlam, C. (ed.), Staphylococci and Staphylococcal Infections. Vol 2. Academic Press, London. pp.745.

Asheshov, E.H. (1966) Loss of antibiotic resistance in *Staphylococcus aureus* resulting from growth at high temperature. J. Gen. Microbiol. **42:** 403-410.

Augustin, J., Rosenstein, R., Wieland, B., Schneider, U., Schnell, N., Engelke, G., Entian, K.-D., and Götz, F. (1992) Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis*. Eur. J. Biochem. **204:** 1149-1154.

Balaban, N., and Novick, R.P. (1995) Autocrine regulation of toxin synthesis by *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **92:** 1619-1623.

Barg, N., Bunce, C., Wheller, L., Reed, G., Musser, J. (1992) Murine model of cutaneous infection with gram-positive cocci. Infect. Immun. **60:** 2636-2640.

Barg, N.L., and Harris, T. (1997) Toxin-mediated syndromes. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp.527-543.

Bateman, A., and Bycroft, M. (2000) The structure of a LysM domain from *E.coli* membrane-bound lytic murein transglycosylase D (MltD). J. Mol. Biol. **299:** 1113-1119.

Bayer, M.G., Heinrichs, J.H., and Cheung, A.L. (1996) The molecular architecture of the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. **178:** 4563-4570.

Benito, Y., Kolb, F.A., Romby, P., Lina, G., Etienne, J., and Vandenesch, F. (2000) Probing the structure of RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. RNA **6:** 668-679.

Betley, M.J., and Mekalanos, J.J. (1985) Staphylococcal enterotoxin A is encoded by phage. Science **229:** 185-187.

Bhakdi, S., Grimminger, F., Suttorp, N., Walmrath, D. and Seeger, W. (1994) Proteinaceous bacterial toxins and pathogenesis of sepsis syndrome and septic shock: the unknown connection. Med. Microbiol. Immunol. **183:** 119-144.

Bibel, D.J., Greenberg, J.H., Cook, J.L. (1977) *Staphylococcus aureus* and the microbial ecology of atopic dermatitis. Can. J. Microbiol. **23:** 1062-1068.

Bisognano, C., Vaudaux, P.E., Lew, D.P., Ng, E.Y.W., and Hooper, D.C. (1997) Increased expression of fibronectin binding proteins by fluoroquinolone-resistant *Staphylococcus aureus* exposed to subinhibitory levels of ciprofloxacin. Antimicrob. Agents. Chemother. **41:** 906-913.

Bjkorklind, A., and Arvidson, S. (1980) Mutants of *Staphylococcus aureus* affected in the regulation of exoprotein synthesis. FEMS. Microbiol. Lett. **7:** 203-206.

Blevins, J.S., A.F. Gillaspy, T.M. Rechtin, B.K. Hurlburt and M.S. Smeltzer. (1999) The staphylococcal accessory regulator (*sar*) represses transcription of the *Staphylococcus aureus* collagen adhesin gene (*cna*) in an *agr*-independent manner. Mol. Microbiol. **33:** 317-326.

Bóden-Wäsfelt, M.K., and Flock, J.-I. (1995) Incidence of the highly conserved *fib* gene and expression of the fibrinogen-binding (Fib) protein among clinical isolates of *Staphylococcus aureus*. J. Clin. Microbiol. **33:** 2347-2352.

Bóden, M.K., and Flock, J.-I. (1989) Fibrinogen-binding protein/clumping factor from *Staphylococcus aureus*. Infect. Immun. **57:** 2358-2363.

Bóden, M.K., and Flock, J.-I. (1994) Cloning and characterisation of a gene for a 19 kDa fibrinogen-binding protein from *Staphylococcus aureus*. Mol. Microbiol. **12:** 599-606.

Bohach, G.A., and Foster, T.J. (2000) *Staphylococcus aureus* exotoxins. *In* Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A. and Rood, J.I. (ed.), Gram-Positive Pathogens. ASM Press, Washington D.C. pp. 367-378.

Bozzini, S., Visai, L., Pignatti, P., Petersen, T.E., and Speziale, P. (1992) Multiple binding sites in fibronectin and the staphylococcal fibronectin receptor. Eur. J. Biochem. **207:** 327-333.

Bremell, T., and Tarkowski, A. (1995) Preferential induction of septic arthritis and mortality by superantigen-producing staphylococci. Infect. Immun. **63:** 4185-4187.

Bremell, T., Lange, S., Holmdahl, R., Rydén, C., Hansson, G.K., and Tarkowski, A. (1994) Immunopathological features of rat *Staphylococcus aureus* arthritis. Infect. Immun. **60:** 2976-2985.

Bremell, T., Lange, S., Yacoub, A., Rydén, C., and Tarkowski, A. (1991) Experimental *Staphylococcus aureus* arthritis in mice. Infect. Immun. **59:** 2615-2623.

Casolini, F., Visai, L., Joh, D., Conaldi, P.G., Toniolo, A., Höök, M., and Speziale, P. (1998) Antibody response to fibronectin-bindig adhesin FnbpA in patients with *Staphylococcus aureus* infections. Infect. Immun. **66:** 5433-5442.

Chan, P.F., and Foster, S.J. (1998a) The role of environmental factors in the regulation of virulence determinant expression in *Staphylococcus aureus* 8325-4. Microbiology. **144:** 2469-2479.

Chan, P.F., Foster, S.J. (1998b). Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus.* J. Bacteriol. **180:** 6232-6241.

Chan, P.F., Foster, S.J., Ingham, E., and Clements, M.O. (1998c) The *Staphylococcus aureus* alternative sigma factor σ^B controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. J. Bacteriol. **180:** 6082-6089.

Chesney, P.J. (1997) Toxic shock syndrome. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 509-525.

Cheung, A.L., Bayer, M.G., and Heinrichs, J.H. (1997a) *sar* genetic determinants necessary for transcription of RNAII and RNAIII in the *agr* locus of *Staphylococcus aureus.* J. Bacteriol. **179:** 3963-3971.

Cheung, A.L., and Projan, S.J. (1994) Cloning and sequencing of *sarA* of *Staphylococcus aureus*, a gene required for the expression of *agr*. J. Bacteriol. **176:** 4168-4172.

Cheung, A.L., Chien, Y.T., and Bayer, A.S. (1999) Hyperproduction of alpha-hemolysin in a *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus.* Infect. Immun. **67:** 1331-1337.

Cheung, A.L., Eberhardt, K., and Heinrichs, J.H. (1997b) Regulation of Protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus*. Infect. Immun. **65:** 2243-2249.

Cheung, A.L., Eberhardt, K.J., Chung, E., Yeaman, M.R., Sullam, P.M., Ramos, M., and Bayer, A.S. (1994) Diminshed virulence of *sar* / *agr* mutant of *Staphylococcus aureus* in rabbit model of endocarditis. J. Clin. Invest. **94:** 1815-1822.

Cheung, A.L., Koomey, J. M., Butler, C. C., Projan, S.J. and Fischetti, V.A. (1992) Regulation of exoprotein expression in *Staphylococcus aureus* by a locus *sar* distinct from *agr*. Proc. Natl. Acad. Sci. USA **89:** 6462-6466.

Cheung, A.L., M.R.Yeaman, P.M. Sullam, M.D. Witt, and A.S. Bayer. (1994) Role of the *sar* locus of *Staphylococcus aureus* in induction of endocarditis in rabbits. Infect. Immun. **62:** 1710-1725.

Chien, Yt., Manna, A.C., Projan, S.J., and Cheung, A. (1999) SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. J. Biol. Chem. **274:** 37169-37176.

Christensen, G.D., Baddour, L.M., Madison, B.M. et al. (1990) Colonial morphology of staphylococci on Memphis agar: phase variation of slime production, resistance to β-lactam antibiotics, and virulence. J. Infect. Dis. **161:** 1153-1169.

Compagnone-Post, P., Malyankar, U., and Khan, S.A. (1991) Role of host factors in the regulation of the enterotoxin B gene. J. Bacteriol. **173:** 1827-1830.

Coulter, S.N., Schwan, W.R., Ng, E.Y.W., Langhorne, M.H., Ritchie, H.D., Westbrock-Wadman, S., Hufnagle, W.O., Folger, K.R., Bayer, A.S., and Stover, C.K. (1998) *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. Mol. Microbiol. **30:** 393-404.

Crass, B.A., and Bergdoll, M.S. (1986) Involvement of staphylococcal enterotoxins in non-menstrual toxic shock syndrome. J. Clin. Microbiol. **23:** 1138-1139.

Debelle, L., Alix, A.J.P., Jacob, M.P., Huvenne, J.P., Berjot, M., Sombret, B., and Legrand, P. (1995) Bovine elastin and κ-elastin secondary structure determination by optical spectroscopies. J. Biol. Chem. **270:** 26099-26103.

Debelle, L., Alix, A.J.P., Wei, S.M., Jacob, M.P., Huvenne, J.P., Berjot, M., and Legrand, P. (1998) The secondary structure and architecture of human elastin. Eur. J. Biochem. **258:** 533-539.

Deora, R., Tseng, T., and Misra, T.K. (1997) Alternative transcription factor sigma σ^{SB} of *Staphylococcus aureus*: characterisation and role in transcription of the global regulatory locus *sar*. J. Bacteriol. **179:** 6355-6359.

Diaz, E., Lopez, R., and Garcia, J.L. (1992) Role of the major pneumococcal autolysin in the atypical response of a clinical isolate of *Streptococcus pneumoniae*. J. Bacteriol. **174:** 5508-5515.

Dominiecki, M.E., and Weiss, J. (1999) Antibacterial action of extracellular mammalian group IIA phospholipase A2 against grossly clumped *Staphylococcus aureus*. Infect. Immun. **67:** 2299-2305.

Dorman, C.J. (1991) DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infect. Immun. **59:** 745-749.

Duthie, E.S. (1954) The production of free staphylococcal coagulase. J. Gen. Microbiol. **10:** 427-.

Duthie, E.S., and Lorenz, L.L. (1952) Staphylococcal coagulase: mode of action and antigenicity. J. Gen. Microbiol. **6:** 95-107.

Dziewanoska, K., Patti, J.M., Deobald, C.F., Bayles, K.W., Trumble, W.R., and Bohach, G.A. (1999) Fibronectin binding protein and host cell tyrosine kinase are required for internalisation of *Staphylococcus aureus* by epithelial cells. Infect. Immun. **67:** 4673-4678.

Fekkes, P., and Driessen, A.J.M. (1999) Protein targeting to the bacterial cytoplasmic membrane. Microbiol. Mol. Biol. Rev. 161-173.

Ficker, L.A., and Seal, D. (1997) Diseases of the Eye. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 441-453.

Flock, J.I., Hienz, S.A., Heimdahl, A., Schennings, T. (1996) Reconsideration of the role of fibronectin binding in endocarditis caused by *Staphylococcus aureus*. Infect. Immun. **64:** 1876-1878.

Forsgren, A. (1970) Significance of protein A production by staphylococci. Infect. Immun. **2:** 672-673.

Foster, T.J., Höök, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. Trends. Microbiol. **6:** 484-488.

Foster, T.J., O'Reilly, M., Phonimdaeng, P., Cooney, J., Patel, A.H., and Bramley, A.J. (1990) Genetic studies of virulence factors of *Staphylococcus aureus*. Properties of coagulase and gamma-toxin and the role of alpha-toxin, beta-toxin and protein A in the pathogenesis of *S.aureus* infections. *In* Novick, R.P. (ed.), Molecular Biology of the Staphylococci, VCH Publishers, Cambridge, U.K. pp. 403-417.

Ganeshkumar, N., Arora, N., and Kolenbrander, P.E. (1993) Saliva-binding protein (SsaB) from *Streptococcus sanguis* 12i. J. Bacteriol. **175:** 572-574.

Gemmell, C.G., Tree, R., Patel, A. O'Reilly, M. and T.J. Foster. (1991). Susceptibility to opsonophagocytosis of protein A, alpha-haemolysin and beta-toxin deficient mutants of *Staphylococcus aureus* isolated by allele-replacement. Zentralblatt fur Bakteriologie (Supplement 21): 273-277, Gustav Fischer Verlag., Stuttgart.

Gentry, L.O. (1997) Osteomyelitis and other infections of bones and joints. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 455-473.

Gillapsy, A.F., Patti, J.M., Pratt, F.L., Jr, Iandolo, J.J., and Smeltzer, M.S. (1997) The *Staphylococcus aureus* collagen adhesin-encoding gene (Cna) is within a discrete genetic element. Gene **196:** 239-248.

Gillaspy, A.F., Lee, C.Y., Sau, S., Cheung, A.L., and Smeltzer, M.S. (1998) Factors affecting the collagen binding capacity of *Staphylococcus aureus*. Infect. Immun. **66:** 3170-3178.

Giraudo, A.T., Calzolari, A., Cataldi, A.A., Bogni, C., Nagel, R.(1999) The *sae* locus of *Staphylococcus aureus* encodes a two-component regulatory system. FEMS Microbiol. Lett. **177:** 15-22.

Giraudo, A.T., Cheung, A.L., Nagel, R. (1997) The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. Arch. Microbiol. **168:** 53-58.

Giraudo, A.T., Rampone, H., Calzolari, A., Nagel, R. (1996) Phenotypic characterisation and virulence of a *sae- agr-* mutant of *Staphylococcus aureus.* Can. J. Microbiol. **42:** 120-123.

Giraudo, A.T., Raspanti, C.G., Calzolari, A., and Nagel, R. (1994) Characterisation of a Tn551-mutant of *Staphylococcus aureus* defective in the production of several exoproteins. Can. J. Microbiol. **40:** 677-681.

Goerke, C., Campana, S., Bayer, M.G., Doring, G., Botzenhart, K., Wolz, C. (2000) Direct quantitative transcript analysis of the *agr* regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile *in vitro.* Infect. Immun. **68:** 1304-1311.

Gouda, H., Shiraiski, M., Takahashi, H., Kalo, K., Torigoe, H., Arala, Y., and Shimada, I. (1998) NMR study of the interaction between the B domain of staphylococcal Protein A and the Fc protein of immunoglobulin G. Biochemistry **37:** 129-136.

Greene, C., McDevitt, D., Francois, P., Vaudaux, P.E., Lew, D.P., and Foster, T.J. (1995) Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes. Mol. Microbiol. **17:** 1143-1152.

Gresham, H.D., Lowrance, J.H., Caver, T.E., Wilson, B.S., Cheung, A.L., Lindberg, F.P. (2000) Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. J. Immunol. **164:** 3713-3722.

Hart, M.E., Smeltzer, M.S., and Iandolo, J.J. (1993) The extracellular protein regulator (*xpr*) affects exoprotein and *agr* mRNA levels in *Staphylococcus aureus.* J. Bacteriol. **175:** 7875-7879

Hartford, O. (1989) The response of *Bacillus subtilis* to oxidative stress. PhD thesis.

Hartford, O., Francois, P., Vaudaux, P., and Foster, T.J. (1997) The dipeptide repeat region of the fibrinogen-binding protein (clumping factor) is required for functional expression of the fibrinogen-binding domain on the *Staphylococcus aureus* cell surface. Mol. Microbiol. **25:** 1065-1076.

Hartford, O., McDevitt, D., and Foster, T.J. (1999) Matrix-binding proteins of *Staphylococcus aureus*: functional analysis of mutant and hybrid molecules. Microbiology 145: 2497-505.

Hartlieb, J., Kohler, N., Dickinson, R.B., Chhatwal, G.S., Sixma, J.J., Hartford, O.M., Foster, T.J., Peters, G., Kehrel, B.E., and Herrmann, M. (2000) Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. Blood **15:** 2149-2156.

Hawiger, J., Timmons, S., Kloczewiak, M., Strong, D.D., and Doolittle, R.F. (1982) γ and α chains of human fibrinogen possess sites reactive with human platelet receptors. Proc. Natl. Acad. Sci. USA **79:** 2068-2071.

Heilmann, C., Hussain, M., Peters, G., Götz, F. (1997) Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. Mol. Microbiol. **24:** 1013-1024.

Heinrichs, J.H., M.G. Bayer, and A.L. Cheung. (1996) Characterisation of the *sar* locus and its interactions with *agr* in *Staphylococcus aureus*. J. Bacteriol. **178:** 418-423.

Hell, W., Meyer, H-G.W., and Gatermann, S.G. (1998) Cloning of *aas*, a gene encoding a *Staphylococcus saprophyticus* surface protein with adhesive and autolytic properties. Mol. Microbiol. **29:** 871-881.

Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E., and Holden, D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. Science **269:** 400-403.

Herrmann, M., Hartlieb, J., Kehrel, B., Montgomery, R.R., Sixma, J.J. and Peters, G. (1997) Interaction of von Willebrand factor with *Staphylococcus aureus*. J. Infect. Dis. **176:** 984-991.

Herrmann, M., Lai, Q.J., Albrecht, R.M., Mosher, D.F., and Proctor, R.A. (1993) Adhesion of *Staphylococcus aureus* to surface-bound platelets: role of fibrinogen/fibrin and platelet integrins. J. Infec. Dis. **167:** 312-322.

Hienz, S.A., Schennings, T., Helmdahl, A., Flock, J-I. (1996) Collagen binding of *Staphylococcus aureus* is a virulence factor in experimental endocarditis. J. Infect. Dis. **174:** 83-88

Hinek, A., Jung, S., and Rutka, J.T. (1999) Cell surface aggregation of elastin receptor molecules caused by suramin amplified signals leading to proliferation of human glioma cells. Acta. Neuropathol. **97:** 399-407.

Homonylo-McGavin, M., Krajewska-Pietrasik, D., Rydén, C., and Höök, M. (1993) Identification of a *Staphylococcus aureus* extracellular matrix-binding protein with broad specificity. Infect. Immun. **61:** 2479-2485.

Höök, M. and Foster, T.J. (2000) Staphylococcal surface proteins. *In* Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A. and Rood, J.I. (ed.), Gram-Positive Pathogens. ASM Press, Washington D.C. pp. 386-391.

Horinouchi, S., Weisblum, B. (1982) Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibodies. J. Bacteriol.**150:** 804-14.

House-Pompeo, K., Xu, Y., Job, D., Speziale, P., and Höök, M. (1996) Conformational changes in the fibronectin binding MSCRAMMs are induced by ligand binding. J. Biol. Chem. **271:** 1379-1384.

Hudson, M.C., Ramp, W., and Frankenburg, K.P. (1999) *Staphylococcus aureus* adhesion to bone matrix and bone-associated biomaterials. FEMS Microb. Lett. **173:** 279-284.

Hynes, R. (1993) Fibronectins. *In* Kreis, T., and Vale, R. (ed.), Guidebook to the Extracellular Matrix and Adhesion Proteins. Oxford University Press. pp. 56-58.

Iandolo, J.J. (1989) Genetic analysis of extracellular toxins of *Staphylococcus aureus.* Annu. Rev. Microbiol. **43:** 375-402.

Iandolo, J.J. (1990) *In* Iglewski, B.H., and Clark, V.L. (ed.), Molecular Basis of Bacterial Pathogenesis. Academic press, Inc., New York. pp.399-426.

Indik, Z., Yeh, H., Ornstein-Goldstein, N., Sheppard, P., Anderson, N., Rosenbloom, J.C., Peltonen, L., and Rosenbloom, J. (1987) Alternative splicing of human elastin mRNA indicated by sequence analysis of cloned genomic and complementary DNA. Proc. Natl. Acad. Sci. USA. **84:** 5680-5684.

Ing, M.B., Baddour, L.M., and Bayer, A.S. (1997) Bacteremia and infective endocarditis: pathogenesis, diagnosis and complications. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 331-354.

Jacobson, M.A., Gellerman, H., Chambers, H. (1988) *Staphylococcus aureus* bacteremia and recurrent staphylococcal infection in patients with acquired

immunodeficiency syndrome and AIDS-realted complex. Am J Med. **85:** 172-176.

Ji, G., Beavis, R., Novick, R.P (1997) Bacterial interference caused by autoinducing peptide variants. Science **276:** 2027-2030.

Ji, G., Beavis, R.C., and Novick, R.P. (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc. Natl. Acad. Sci. USA **92:** 12055-12059.

Ji, Y., Marra, A., Rosenberg, M., and Woodnutt, G. (1999) Regulated antisense RNA eliminates alpha-toxin virulence in *Staphylococcus aureus* infection. J. Bacteriol. **181:** 6585-6590.

Joh, D., Speziale, P., Gurusiddappa, S.aureus, Manor, J., and Höök, M. (1998) Multiple specificities of the staphylococcal and streptococcal fibronectin-binding microbial surface components recognising adhesive matrix molecules. Eur. J. Biochem. **258:** 897-905.

Jönsson, K., McDevitt, D., Homonylo-McGavin, M., Patti, J.M., and Höök, M. (1995) *Staphylococcus aureus* expresses a major histocompatibility complex class II analog. J. Biol. Chem. **270:** 1-4.

Jönsson, K., Signäs, C., Müller, H.-P., and Lindberg, M. (1991) Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterisation of the second gene. Eur. J. Biochem. **202:** 1041-1048.

Joris, B., Englebert, S., Chu, C.P., Kariyama, R., Daneo-Moore, L., Shock, G.D. and Ghuysen, J.M. (1992) Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin. FEMS Microbiol. Lett. **70:** 257-264.

Josefsson, E., McCrea, K.W., Ní Eidhin, D., O'Connell, D., Cox, J., Höök, M., and Foster, T.J. (1998) Three new members of the serine-aspartate repeat region multigene family of *Staphylococcus aureus*. Microbiology **144:** 3387-3395.

Josefsson, E., O'Connel, D., Foster, T.J., Durussel, I., and Cox, J.A. (1998) The binding of calcium to the B-repeat seqment of SdrD, a cell surface protein of *Staphylococcus aureus*. J. Biol. Chem. **273:** 31145-31152.

Kauffman, C.A., and Bradley, S.F. (1997) Epidemiology of community-acquired infection. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 287-308.

Kemp, E.H., Sammons, R.L., Moir, A., Sun, D., and Setlow, P. (1991) Analysis of transcriptional control of the *gerD* spore germination gene of *Bacillus subtilis* 168. J. Bacteriol. **173:** 4646-4652.

Kloos, W. (1997) Taxonomy and systematics of staphylococci indigenous to humans. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 113-137.

Kluytmans, J., van Belkum, A., and Verburgh, H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin. Microbiol. Rev. **10:** 505-520.

Kornblum, J., Kreiswirth, B.N., Projan, S.J., Ross, H., and Novick, R.P. (1990) *Agr*: a polycistronic locus regulation exoprotein synthesis in *Staphylococcus aureus*. *In* R.P Novick (ed). Molecular biology of the staphylococci. VCH Publishers, New York, N.Y. pp.373-402.

Kullik, I., and Giachino, P. (1997) The alternative sigma factor sigma B in *Staphylococcus aureus*: regulation of the *sigB* operon in response to growth phase and heat shock. Arch. Microbiol. **167:** 151-159.

Kuusela, P. (1978) Fibronectin binds to *Staphylococcus aureus*. Nature **276:** 718-720.

Kuypers, J.M., and Proctor, R.A. (1989) Reduced adherence to traumatized rat heart valves by a low-fibronectin-binding mutant of *Staphylococcus aureus*. Infect. Immun. **64:** 1876-1878.

Kyte, J., and Doolittle, R.F. (1982) Simple method for displaying the hydropathy character of a protein. J. Mol. Biol. **157:** 105-132.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.

Lautenschlager, S., Herzog, C., and Zimmerli, W. (1993) Course and outcome of bacteremia due to *Staphylococcus aureus*: evaluation of different clinical case definitions. Clin. Infect. Dis. **16:** 567-573.

Lebeau, C., Vandenesch, F., Greeland, T., Novick, R.P., and Etienne, J. (1994) Coagulase expression in *Staphylococcus aureus* is positively and negatively modulated by an *agr*-independent mechanism. J. Bacteriol. **176:** 5534-5536.

Lee, C.Y., and Lee, J.C. (2000) Staphylococcal capsule. *In* Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A. and Rood, J.I. (ed.), Gram Positive Pathogens. ASM Press, Washington D.C. pp. 361-366.

Lee, J.C., Takeda, S., Livolsi, P., and Paoletti, L.C. (1993) Effects of *in vitro* and *in vivo* growth conditions on expression of type 8 capsular polysaccharide by *Staphylococcus aureus*. Infect. Immun. **61:** 1853-1858.

Lee, J.C., Xu, S., Albus, A. and Livolsi, P.J. (1994) Genetic analysis of type 5 capsular polysaccharide expression by *Staphylococcus aureus*. J. Bacteriol. **176:** 4883-4889.

Lina, G., Jarraud, S., Ji, G., Greenland, T., Pedraza, A., Etienne, J., Novick, R.P., Vanenesch, F. (1998) Transmembrane topology and histidine protein kinase activity of AgrC, the *agr* signal receptor in *Staphylococcus aureus*. Mol. Microbiol. **28:** 655-662.

Lindberg, M., Jönsson, K., Müller, H.P., Jonsson, H., Signäs, C., Höök, M., Raja, R., Giuseppe, R., and Anantharamaiah, G.M. (1990) Fibronectin-binding proteins in *Staphylococcus aureus*. *In* Molecular Biology of the Staphylococci. Novick, R. (ed.). New York: VCH, pp. 343-356.

Lindsay, J.A., Ruzin, A., Ross, H.F., Kurepina, N., Novick, R.P. (1998) The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. Mol. Microbiol. **29:** 527-543

Lowe, A.M., Beattie, D.T., and Deresiewicz, R.L. (1998) Identification of novel staphylococcal virulence genes by *in vivo* expression technology. Mol. Microbiol. **27:** 967-796.

Mahan, M.J., Slauch, J.M., and Mekalanos, J.J. (1993) Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:** 686-688.

Mamo, W., Bóden, M., and Flock, J.-I. (1994) Vaccination with *Staphylococcus aureus* fibrinogen binding proteins (FgBPs) reduces colonisation of *S.aureus* in a mouse mastitis model. FEMS Immunol. Med. Microbiol. **10:** 47-54.

Manna, A.C., Bayer, M.G., and Cheung, A.L. (1998) Transcriptional analysis of different promoters in the *sar* locus in *Staphylococcus aureus*. J Bacteriol **180:** 3828-3836.

Mason, D.J., Shanmuganathan, S., Mortimer, F.C., and Gant, V.A. (1998) A fluorescent gram stain for flow cytometry and epifluorescence microscopy. App. Env. Microbiol. **64:** 2681-2685.

Mazmanian, S.K., Liu, G., Ton-That, H. and Schneewind, O. (1999) *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science. **285:** 760-763.

Mazmanian, S.K., Liu, G., Jensen, E.R., Lenoy, E. and Schneewind, O. (2000) *Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. Proc. Natl. Acad. Sci. USA **97:** 5510-5515.

McCrea, K.W., Hartford, O., Davis, S., Eidhin, D.N., Lina, G., Speziale, P., Foster, T.J., Hook, M. (2000) The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology **146:** 1535-1546.

McDevitt, D., and Foster, T.J. (1995) Variation in the size of the repeat region of the fibrinogen receptor (clumping factor) of *Staphylococcus aureus* strains. Microbiology **141:** 937-943.

McDevitt, D., Francois, P., Vaudaux, P., and Foster, T.J. (1994) Molecular characterisation of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. Mol. Microbiol. **11:** 237-248.

McDevitt, D., Francois, P., Vaudaux, P., and Foster, T.J. (1995) Identification of the ligand binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. Mol. Microbiol. **16:** 895-907.

McDevitt, D., Nanavaty, T., House-Pompeo, K., Bell, E., Turner, N., McIntire, L., Foster, T., and Höök, M. (1997) Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. Eur. J. Biochem. **247:** 416-424.

McDevitt, D., Vaudaux, P., and Foster, T.J. (1992) Genetic evidence that bound coagulase of *Staphylococcus aureus* is not clumping factor. Infect. Immun. **60:** 1514-1523.

McDevitt, D., Wann, E.R., and Foster, T.J. (1993) Recombination at the coagulase locus of *Staphylococcus aureus*. Plasmid integration and amplification. J. Gen. Microbiol. **139:** 695-706.

McGavin, M.J., Gurusiddappa, S., Lingren, P.-E., Lindberg, M., Raucci, G., and Höök, M. (1993) Fibronectin receptors from *Streptococcus dysgalactiae* and *Staphylococcus aureus*: Involvement of conserved residues in ligand binding. J. Biol. Chem. **268:** 23946-23953.

McGavin, M.J., Zahradka, C., Rice, K., and Scott, J.E. (1997) Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease. Infect. Immun. **65:** 2621-2628.

Mecham, R.P., and Lange, G. (1982) Antibodies to insoluble and solubilised elastin. Meth. Enzymol. **82:** 744-747.

Mei, J-M., Nourbakhsh, F., Ford, C.W., and Holden, D.W. (1997) Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. Mol. Microbiol. **26:** 399-407.

Melly, M.A., Duke, L.J., Liau, D.-F., and Hash, J.H. (1974) Biological properties of the encapsulated *Staphylococcus aureus*. Infect. Immun. **10:** 389-397.

Mermel, L.A., Stolz, S.M., and Maki, D.G. (1993) Surface antimicrobial activity of heparin-bonded and anti-septic-impregnated vascular catheters. J. Infect. Dis. **167:** 920-924.

Miyazaki, E., Chen, J-M., Ko, C., and Bishai, W.R. (1999) The *Staphylococcus aureus rsbW* (*orf159*) gene encodes an anti-sigma factor of SigB. J. Bacteriol. **181:** 2846-2851.

Mölne, L., and Tarkowski, A. (2000) An experimental model of cutaneous infection induced by superantigen-producing *Staphylococcus aureus*. J. Invest. Dermatol. 114: 1120-1125.

Moreillon, P., Entenza, J.M., Francioli, P., McDevitt, D., Foster, T.J., Francois, P., and Vaudaux, P. (1995) Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. Infect. Immun. **63:** 4738-4743.

Morfeldt, E., Taylor, D., von Gabain, A., and Arvidson, S. (1995) Activation of alpha-toxin in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. EMBO J. **14:** 4569-4577.

Morfeldt, E., Tegmark, K., and Arvidson, S. (1996) Transcriptional control of the *agr*-dependent virulence gene regulator, RNAIII, in *Staphylococcus aureus*. Mol. Microbiol. **21:** 1227-1237.

Mosher, D.E., and Proctor, R.A. (1980) Binding and factor XIIIa-mediated cross-linking of a 27-kilodalton fragment of fibronectin to *Staphylococcus aureus*. Science **209:** 927-929.

Moxon, R., and Tang, C. (2000) Challenge of investigating biologically relevant functions of virulence factors in bacterial pathogens. Phil. Trans. R. Soc. Lond. B. **355:** 643-656.

Navarre, W.W., and Schneewind, O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cel wall envelope. Microbiol. Mol. Biol. Rev. **63:** 174-229.

Náwas, T., Hawwari, A., Hendrix, E., Hebden, J., Edelman, R., Martin, M., Campbell, W., Naso, R., Schwalbe, R., and Fattom, A.I. (1998) Phenotypic and genotypic characterisation of nosocomial *Staphylococcus aureus* isolates from trauma patients. J. Clin. Microbiol. **36:** 414-420.

Nguyen, T., Ghebrehiwet, B., Peerschke, E.I. (2000) *Staphylococcus aureus* protein A recognises platelet gC1qR/p33: a novel mechanism for staphylococcal interactions with platelets. Infect. Immun. **68:** 2061-2068.

Ní Eidhin, D., Perkins, S., Francois, P., Vaudaux, P., Höök, M., and Foster, T.J. (1998) Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. Mol. Microbiol. **30:** 245-257.

Nilsson, I.M., Hartford, O., Foster, T., and Tarkowski, A. (1999) Alpha-toxin and gamma-toxin jointly promote *Staphylococcus aureus* virulence in murine septic arthritis. Infect. Immun. **67:** 1045-1049.

Nilsson, I.-M., Lee, J.C., Bremell, T., Rydén, C. and Tarkowski, A. (1997) The role of staphylococcal polysaccharide microcapsule expression in septicemia and septic arthritis. Infect. Immun. **65:** 4216-4221.

Nilsson, I-M., Patti, J.M., Bremell, T., Höök, M., and Tarkowski, A. (1998) Vaccination with a recombinant fragment of collagen adhesin provides protection against *Staphylococcus aureus*-mediated septic death. J. Clin. Invest. **101:** 2640-2649.

Noble, W.C. (1997) Staphylococcal carriage and skin and soft tissue infection. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 401-412.

Novick, R.P. (1967) Properties of a cryptic high frequency transducing phage in *Staphylococcus aureus*. Virology **33:** 155-166.

Novick, R.P. (1999) Regulation of pathogenicity in *Staphylococcus aureus* by a peptide-based density-sensing system. *In* Cell-Cell Signalling in Bacteria. Dunny, G.M. and Winans, S.C. (ed.), ASM, Washington D.C pp.129-143.

Novick, R.P., Projan, S., Kornblum, J., Ross, H., Kreiswirth, B., Moghazeh, S. (1995) The *agr* P-2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. **248:** 446-458.

Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B., and Moghazeh, S. (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12:** 3967-3975.

O'Callaghan, R.J., Callegan, M.C., Moreau, J.M., Green, L.C., Foster, T.J., Hartford, O.M., Engel, L.S., and Hill, J.M. (1997) Specific roles of alpha-toxin and beta-toxin during *Staphylococcus aureus* corneal infection. Infect. Immun. **65:** 1571-1578.

O'Connell, D.P., Nanavaty, T., McDevitt, D., Gurusiddappa, S.aureus, Höök, M., and Foster, T.J. (1998) The fibrinogen-binding MSCRAMM (Clumping factor) of *Staphylococcus aureus* has a Ca^{2+}-dependent inhibitory site. J. Biol. Chem. **273:** 6821-6829.

Olsen, B.R., and Ninomiya, Y. (1994) Collagens. *In* Guidebook to the Extracellular Matrix and Adhesion Proteins. Kreis, T., and Vale, R. (ed.). Oxford University Press. pp. 32-47.

Otto, M., Sussmuth, R., Vuong, C., Jung, G., Gotz, F. (1999) Inhibition of virulence factor expression in *Staphylococcus aureus* by the *Staphylococcus epidermidis agr* pheromone. FEBS Lett. **450:** 257-262.

Palma, M., Haggar, A., and Flock, J-I. (1999) Adherence of *Staphylococcus aureus* is enhanced by an endogenous secreted protein with broad binding activity. J. Bacteriol. **181:** 2840-2845.

Palma, M., Nozohoor, S., Schennings, T., Heimdahl, A., and Flock, J.-I. (1996) Lack of the extracellular 19-kilodalton fibrinogen-binding protein from *Staphylococcus aureus* decreases virulence in experimental wound infection. Infect. Immun. **64:** 5284-5289.

Park, P.W., Roberts, D.D., Grosso, L.E., Parks, W.C., Rosenbloom, J., Abrams, W.R., and Mecham, R.P. (1991) Binding of elastin to *Staphylococcus aureus*. J. Biol. Chem. **266:** 23399-23406.

Park, P.W., Rosenbloom, J., Abrams, W.R., Rosenbloom, J., and Mecham, R.P. 1996. Molecular cloning and expression of the gene for elastin-binding protein (*ebpS*) in *Staphylococcus aureus*. J. Biol. Chem. **271:** 15803-15809.

Park, P.W., Broekelmann, T.J., Mecham, B.R., and Mecham, R.P. (1999) Characterisation of the elastin binding domain in the cell-surface 25 kDa elastin-binding protein of *Staphylococcus aureus*. J. Biol. Chem. **274:** 2845-2850.

Patel, A., Nowlan, P., Weavers, E.A. and Foster, T.J. (1987) Virulence of protein A and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. Infect. Immun. **55:** 3101-3110.

Patel, A.H., Foster, T.J., and Pattee, P.A. (1989) Physical and genetic mapping of the protein A gene in the chromosome of *Staphylococcus aureus* 8325-4. J. Gen. Microbiol. 135: 1799-1807.

Patti, J.M., Allen, B.L., McGavin, M.J., Hook, M. (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. Annu. Rev. Microbiol. **48:** 585-.

Patti, J.M., Boles, J.O., Höök, M. (1993) Identification and biochemical characterisation of the ligand binding domain of the collagen adhesin from *Staphylococcus aureus*. Biochemistry **32:** 11428-11435.

Patti, J.M., Bremell, T., Krajewska-Pietrasik, D., Abdelnour, A., Tarkowski, A., Rydén, C., and Höök, M. (1994) The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. Infect. Immun. **62:** 152-161.

Patti, J.M., House-Pompeo, K., Boles, J.O., Garza, N., Gurusiddappa, S., and Höök, M. (1995) Critical residues in the ligand-binding site of the *Staphylococcus aureus* collagen-binding adhesin (MSCRAMM). J. Biol. Chem. **270:** 12005-12011.

Patti, J.M., Jonsson, H., Guss, B., Switalski, L.M., Wiberg, K., Linberg, M., and Höök, M. (1992) Molecular characterisation and expression of a gene encoding a *Staphylococcus aureus* collagen adhesin. J. Biol. Chem. **267:** 4766-4772.

Peacock, S.J., Day, N.P., Thomas, M.G., Berendt, A.R., Foster, T.J. (2000) Clinical isolates of *Staphylococcus aureus* exhibit diversity in *fnb* genes and adhesion to human fibronectin. J. Infect. Dis. **41:** 23-31.

Peacock, S.J., Foster, T.J., Cameron, B.J., and Berendt, A.R. (1999) Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. Microbiology **145:** 3477-3486.

Peng, H.-L., Novick, R.P., Kreiswirth, B., Kornblum, J., and Schlievert, P. (1988) Cloning, characterisation, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J. Bacteriol. **170:** 4365-4372.

Peterson, P.K., Verhoef, J., Sabath, L.D., and Quie, P.G. (1977) Effect of protein A on staphylococcal opsonisation. Infect. Immun. **15:** 760-764.

Phonimdaeng, P., O'Reilly, M., Nowlan, P., Bramley, A.J., and Foster, T.J. (1990) The coagulase of *Staphylococcus aureus* 8325-4: Sequence analysis and virulence of site-specific coagulase-deficient mutants. Mol. Microbiol. **4:** 393-404.

Pöhlmann-Dietze, P., Ulrich, M., Kiser, K.B., Döring, G., Lee, J.C., Fournier, J.M., Botzenhart, K., and Wolz, C. (2000) Adherence of *Staphylococcus aureus* to endothelial cells: Influence of capsular polysaccharide, global regulator *agr*, and bacterial growth phase. Infect. Immun. **68:** 4865-4871.

Ponting, C.P., Aravind, L., Schultz, J., Bork, P., and Koonin, E.V. (1999) Eukaryotic signalling domain homologues in archaea and bacteria. Ancient ancestry and horizontal gene transfer. J. Mol. Biol. **289:** 729-745.

Prévost, G., Couppie, P., Prévost, P., Gayet, S., Petiau, P., Cribier, B., Monteil, H., and Piémont, Y. (1995) Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. J. Med. Microbiol. **42:** 237-245.

Proctor, R.A., Mosher, D.F., and Olbrantz, P.J. (1982) Fibronectin binding to *Staphylococcus aureus*. J. Biol. Chem. **257:** 14788-14793.

Rampone, H., Martinez, G.L., Giraudo, A.T., Calzolari, A., Nagel, R. (1996) *In vivo* expression of exoprotein synthesis with a *sae* mutant of *Staphylococcus aureus*. Can. J. Vet. Res. **60:** 237-240.

Rechtin, T.M., Gillaspy, A.F., Schumacher, M.A., Breenan, R.G., Smeltzer, M.S., and Hurlburt, B.K. (1999). Characterisation of the SarA virulence gene regulator of *Staphylococcus aureus*. Mol. Microbiol. **33:** 307-316.

Recsei, P., Kreiswirth,B., O'Reilly, M., Schlievert, P., Gruss, A. and Novick, R.P. (1986) Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. Mol. Gen. Genet. **202:** 58-61

Rhem, M.N., Lech, E.M., Patti, J.M., McDevitt, D., Höök, M., Jones, D.B., and Wilhelmus, K.R. (2000) The collagen-binding adhesin is a virulence factor in *Staphylococcus aureus* keratitis. Infect. Immun. **68:** 3776-3779.

Rich, R.L., Demeler, B., Ashby, K., Deivanayagam, C.C.S., Petrich, J.W., Patti, J.M., Narayana, S.V.L., and Höök, M. (1998) Domain structure of the *Staphylococcus aureus* collagen adhesin. Biochemistry **37:** 15423-15433.

Ringden, O. (1985) Induction of immunoglobulin secretion by protein A from *Staphylococcus aureus* in human blood and bone marrow B cells. Scan. J. Immunol. **22:** 17-26.

Roos, K., and Scheld, W.M. (1997) Central nervous system infections. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 413-439.

Ruggeri, Z.M. (1994) Fibrinogen. *In* Kreis, T., and Vale, R. (ed.), Guidebook to the Extracellular Matrix and Adhesion Proteins. Oxford University Press. p. 52-53.

Rupp, M.E. (1997) Infections of intravascular catheters and vascular devices. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 379-399.

Sabat, A., Kosowska, K., Poulsen, K., Kasprowicz, A., Sekowska, A., van Den Burg, B., Travis, J., Potempa, J. (2000) Two allelic forms of the aureolysin gene (*aur*) within *Staphylococcus aureus*. Infect. Immun. **68:** 973-976

Sakata, N., Jakab, E., and Wadström, T. (1994) Human plasma fibronectin possesses second binding site(s) to *Staphylococcus aureus* on its C-terminal region. J. Biochem. **115:** 843-848.

Sau, S., Bhasin, N., Wann, E.R., Lee, J.C., Foster, T.J. and Lee, C.Y. (1997) The *Staphylococcus aureus* allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. Microbiology **143:** 2395-2405.

Sawai, T., Tomono, K., Yanagihara, K., Yamamoto, Y., Kaku, M., Hirakata, Y., Koga, H., Tashiro, T., Kohno, S. (1997) Role of coagulase in a murine model of hematogenous pulmonary infection induced by intravenous injection of *Staphylococcus aureus* enmeshed in agar beads. Infect. Immun. **65:** 466-471.

Schenk, S. and Laddaga, R.A. (1992) Improved method for electroporation of *Staphylococcus aureus*. FEMS Microbiol. Lett. **94:** 133-138.

Schneewind , O., Model, P., and Fischetti, V.A. (1992) Sorting of Protein A to the staphylococcal cell wall. Cell **70:** 267-281.

Schneewind, O., and Ton-That, H. (1999) Anchor structure of staphylococcal surface proteins. IV. Inhibitors of the cell wall sorting reaction. J. Biol. Chem. **274:** 24316-24320.

Sela, S., Aviv, A., Tovi, A., Burstein, I., Caparon, M.G., and Hanski, E. (1993) Protein F: an adhesin of *Streptococcus pyogenes* binds fibronectin via two distinct domains. Mol. Microbiol. **10:** 1049-1055

Sherertz, R.J., Carruth, W.A., Hampton, A.A., Byron, M.P., and Solomon, D.D. (1993) Efficacy of antibiotic-coated catheters in preventing subcutaneous *Staphylococcus aureus* infection in rabbits. J. Infect. Dis. **167:** 98-106.

Signäs, C., Raucci, G., Jönsson, K., Lindgren, P-E., Anantharamaiah, G.M., Höök, M., and Lindberg, M.(1989) Nucleotide sequenceof the gene for a fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. Proc. Natl.Acad. Sci. USA **86:** 699-703.

Sinha, B., Francois, P.P., Nüβe, O., Foti, M., Hartford, O.M., Vaudaux, P., Foster, T.J., Lew, D.P., Herrmann, M., Krause, K-H. (1999) Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin α5β1. Cell. Microbiol. **1:** 101-117.

Sjöbring, U. (1992) Isolation and molecular characterisation of a novel albumin-binding protein from group G streptococci. Infect. Immun. **60:** 3601-3608.

Smeltzer, M.S., Gillaspy, A.F., Pratt Jr, F.L., Thames, M.D., and Iandolo, J.J. (1997) Prevalence and chromosomal map location of *Staphylococcus aureus* adhesin genes. Gene **196:** 249-259.

Snodgrass, J.L., Mohamed, N., Ross, J.M., Sau, S., Lee, C.Y., and Smeltzer, M.S. (1999) Functional analysis of the *Staphylococcus aureus* collagen adhesin B domain. Infect. Immun. **67:** 3952-3959.

Sottile, J., Schwarzbauer, J., Selegue, J., and Mosher, D.F. (1991) Five type I modules of fibronectin forma functional unit that binds to fibroblasts and *Staphylococcus aureus*. J. Biol. Chem. **266:** 12840-12843.

Speziale, P., Job, D., Visai, L., Bozzini, S., House-Pompeo, K., Lindberg, M., and Höök, M. (1996) A monoclonal antibody enhances ligand binding of fibronectin MSCRAMM (adhesin) from *Streptococcus dysgalactiae*. J. Biol. Chem. **271:** 1371-1378.

Starovasnik, M.A., Skelton, N.J., O'Connell, M.P., Kelley, R.F., Reilly, D., and Fairbrother, W.J. (1996) Solution structure of the E-domain of staphylococcal protein A. Biochemistry **35:** 1558-1569.

Stock, J.B., Ninfa, A.J., and Stock, A.M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:** 450-490.

Strong, D.D., Laudano, A.P., Hawiger, J., and Doolittle, R.F. (1982) Isolation, characterisation, and synthesis of peptides from human fibrinogen that block the staphylococcal clumping reaction and construction of a synthetic clumping particle. Biochemistry **21:** 1414-1420.

Supersac, G., Piémont, Y., Kubina, M., Prévost, G., and Foster, T.J. (1997) Assessment of the role of gamma toxin in experimental endophthalmitis using a *hlg*-deficient mutant of *Staphylococcus aureus*. Microb. Path. **24:** 241-251.

Switalski, L.M., Patti, J.M., Butcher, W., Gristina, A.G., Speziale, P., and Höök, M. (1993) A collagen receptor on *Staphylococcus aureus* strains isolated from patients with septic arthritis mediates adhesion to cartilage. Mol. Microbiol. **7:** 99-107.

Symersky, J., Patti, J.M., Carson, M., House-Pompeo, K., Teale, M., Moore, D., Jin, L., Schneider, A., DeLucas, L.J., Höök, M. and Narayana, S.V.L. (1997) Structure of the collagen-binding domain from a *Staphylococcus aureus* adhesin. **4:** 833-838.

Tegmark, K., Karlsson, A., and Arvidson, S. (2000) Identification and characterisation of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **37:** 398-409.

Tenover, F.C., and Gaynes, R.P. (2000) The epidemiology of *Staphylococcus* infection. *In* Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A. and Rood, J.I. (ed.), Gram-Positive Pathogens. ASM Press, Washington D.C. pp. 414-421.

Tremaine, M., Brockman, D.K., Betley M.J. (1993) Staphylococcal enterotoxin A gene (*sea*) expression is not affected by the accessory gene regulator (*agr*). Infect. Immun. **61:** 356-359.

Tung, H.S., Guss, B., Hellman, U., Persson, L., Rubin, K., Ryden, C. (2000) A bone sialoprotein-binding protein from *Staphylococcus aureus*: a member of the staphylococcal Sdr family. Biochem. J. **345:** 611-9

Uhlén, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L., and Lindberg, M. (1984). Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. J. Biol. Chem. **259:** 1695-1702.

Van Dyke, M., Sirito, M., and Sawadogo, M. (1992) Single-step purification of bacterially expressed polypeptides containing an oligo-histidine domain. Gene **111:** 99-104.

Vandenesch, F., Kornblum, J., and Novick R.P. (1991) A temporal signal, independent of *agr*, is required for *hla* but not *spa* transcription in *Staphylococcus aureus*. J. Bacteriol. **173:** 6313-6320.

Varga, Z., Jacob, M.P., Robert, L., and Fülöp, jr T. (1989) Identification and signal transduction mechanism of elastin peptide receptor in human leukocytes. FEBS Lett. **258:** 5-8.

Vaudaux, P., Pittet, D., Haeberli, A., Huggler, E., Nydegger, U.E., Lew, D.P., and Waldvogel, F.A. (1989) Host factors selectively increase staphylococcal adherence on inserted catheters: a role for fibronectin and fibrinogen or fibrin. J. Infect. Dis. **160:** 865-875.

Vaudaux, P., Pittet, D., Haeberli, A., Lerch, P.G., Morgenthaler, J.-J., Proctor, R.A., Waldvogel, F.A., and Lew, D.P. (1993) Fibronectin is more active than fibrin or fibrinogen in promoting *Staphylococcus aureus* adherence to inserted intravascular catheters. J. Infect. Dis. **167:** 633-641.

Vaudaux, P.E., Francois, P., Proctor, R.A., McDevitt, D., Foster, T.J., Albrecht, R.M., Lew, D.P., Wabers, H., and Cooper, S.L. (1995) Use of adhesion defective mutants of *Staphylococcus aureus* to define the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. Infect. Immun. **63:** 585-590.

Vaudaux, P.E., Monzillo, V., Francois, P., Lew, D.P., Foster, T.J., and Berger-Bachi, B. (1998) Introduction of the *mec* element (methicillin resistance) into *Staphylococcus aureus* alters in vitro functional activities of fibrinogen and fibronectin adhesins. Antimicrob. Agents. Chemother. **42:** 564-570.

Verdrengh, M., and Tarkowski, A. (1997) Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus*. Infect. Immun. 65: 2517-2521.

Verhoef, J. (1997) Host defense against infection. *In* K.B. Crossley and G.L. Archer (ed.), The Staphylococci in Human Disease. Churchill Livingstone, New York, N.Y. pp. 213-232.

Verhoef, J., Peterson, P.K., Verburgh, H.A. (1979) Host-parasite relationship in staphylococcal infections: the role of the staphylococcal cell wall during the process of phagocytosis. Antonie Van Leeuwenhoek **45:** 49-.

Völker, U., Dufour, A., and Haldenwang, W.G. (1995a) The *Bacillus subtilis rsbU* gene product is necessary for RsbX-dependent regulation of σ^B. J. Bacteriol. **177:** 114-122.

Völker, U., Völker, A., Maul, B., Hecker, M., Dufour, A., and Haldenwang, W.G. (1995b) Separate mechanisms activate σ^B of *Bacillus subtilis* in response to environmental and metabolic stresses. J. Bacteriol. **177:** 3771-3780.

Vrhovski, B., and Weiss, A.S. (1998) Biochemistry of tropoelastin. Eur. J. Biochem. **258:** 1-18.

Wann, E.R., Fehringer, A.P., Ezepchuk, Y.V., Schlievert, P.M., Bina, P., Reiser, R.F., Höök, M.M., and Leung, D.Y.M. (1999) *Staphylococcus aureus* isolates from patients with kawasaki disease expresses high levels of protein A. Infect. Immun. **67:** 4737-4743.

Wann, E.R., Gurusiddappa, S., and Höök, M. (2000) The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. J. Biol. Chem. **275:** 13863-13871.

Weiss, J., Bayer, A.S., and Yeaman, M. (2000) Cellular and extracellular defenses against staphylococcal infections. *In* Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A. and Rood, J.I. (ed.), Gram-Positive Pathogens. ASM Press, Washington D.C. pp. 431-441.

Wolz, C., McDevitt, D., Foster, T.J., and Cheung, A.L. (1996) Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. Infect. Immun. **64:** 3142-3147.

Wolz, C., Pöhlmann-Dietze, P., Steinhuber, A., Chien, Y-T., Manna, A., van Wamel, W., and Cheung, A. (2000) *Agr*-independent regulation of fibronectin-binding protein(s) by the regulatory locus *sar* in *Staphylococcus aureus*. Mol. Microbiol. **36:** 230-243.

Wu, S., de Lencastre, H., and Tomasz, A. (1996) Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J. Bacteriol. **178:** 6036-6042.

Xie, G., Brettin, T.S., Bonner, C.A., and Jensen, R.A. (1999) Mixed-function supraoperons that exhibit overall conservation, albeit shuffled gene organisation, across wide intergenomic distances within eubacteria. Microb. Comp. Genomics. **4:** 5-28.

Yeaman, M.R., Sullam, P.M. Dazin, P.F. Norman, D.C., and Bayer, A.S. (1992) Characterisation of *Staphylococcus aureus*-platelet binding by quantitative flow cytometric analysis. J. Infect. Dis. **166:** 65-73.

Yeh, H., Anderson, N., Ornstein-Goldstein, N., Bashir, M.M., Rosenbloom, J.C., Abrams, W., Indik, Z., Yoon, K., Parks, W., Mecham, R., and Rosenbloom, J. (1989) Structure of bovine elastin gene and S1 muclease analysis of alternative splicing of elastin mRNA in the bovine nuchal ligament. Biochemistry **28:** 2365-2370.

Youngman, P. (1987) Plasmid vectors for recovering and exploiting Tn917 transpositions in Bacillus and other Gram-positive bacteria. Ch. 4 in: Plasmids, a practical approach. Hardy, K.G. (ed.). IRL Press, Oxford, pp.79-104.

Zhang, L., Jacobsson, K., Vasi, J., Lindberg, M., and Frykberg, L. (1998) A second IgG-binding protein in *Staphylococcus aureus*. Microbiology **144:** 985-991.