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Studies on Clumping Factor A of *Staphylococcus aureus*

A thesis submitted for the degree of Doctor of Philosophy

by

Judy Higgins

Department of Microbiology
Trinity College, Dublin

August 2005

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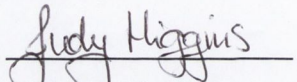

Judy Higgins

Summary

Clumping factor A is a fibrinogen-binding protein of *S. aureus.* It is a cell wall-associated protein consisting of an N-terminal ligand-binding region A, an unusual SD dipeptide repeat region, which acts as a stalk to project region A away from the cell surface, and a C-terminal wall-spanning and anchoring domain. It is an important virulence factor in a variety of animal models of infection.

Region A of clumping factor A (ClfA) can be subdivided into three domains – N1, N2 and N3. Incubation of recombinant ClfA region A with purified proteases showed that domain N1 can be cleaved from region A. Domain N1 was cleaved by *S. aureus* enzymes (metalloprotease and serine protease) and by human enzymes (neutrophil serine proteases). Using both recombinant proteins and ClfA expressed on the surface of *S. aureus*, it was demonstrated that the removal of domain N1 of the ligand-binding region did not influence fibrinogen-binding activity.

ClfA expressed on the surface of *S. aureus* was compared to protein A for its ability to inhibit opsonophagocytosis by human polymorphonuclear leukocytes (PMNL). Both ClfA and protein A, a known antiphagocytic factor in *S. aureus*, were shown to inhibit phagocytic uptake by PMNL. The activity of ClfA was partially dependent on binding to fibrinogen, but residual antiphagocytic activity was observed even in the absence of fibrinogen. In addition, a mutant of *clfA* was generated that expressed a ClfA protein that could not bind fibrinogen. The antiphagocytic activity of this protein, both in the absence and presence of fibrinogen, was similar to that of native ClfA in the absence of fibrinogen. The antiphagocytic activities of several staphylococcal cell wall-associated proteins were tested in the surrogate Gram-positive host *Lactococcus lactis*. The fibrinogen-binding proteins ClfB, SdrG and Fbl, as well as the non-fibrinogen-binding SdrD, were shown to have antiphagocytic properties.

New methods were developed to facilitate the generation of mutations in *S. aureus* that are unmarked by antibiotic resistance determinants. Two temperature-sensitive plasmids were constructed that allow allelic replacement without the introduction of resistance markers to the chromosome. This method was used to generate an unmarked *clfA*-null mutation that could be combined with several other mutations in a single strain.

The regulation of *clfA* was investigated using a transcriptional reporter fusion and Northern hybridisation. *clfA* was shown to be upregulated in stationary phase

by the alternative σ-factor, SigB. *clfA* was also expressed transiently in early exponential phase. Two promoters were identified upstream of *clfA* by primer extension analysis. A SigB-dependent promoter, active in stationary phase, allowed initiation of transcription 31 bp upstream of the *clfA* start codon. A SigA-dependent promoter, active in early exponential phase, had a transcription start site 1222 bp upstream of the *clfA* start codon. Using rifampicin treatment it was determined that *clfA* is transcribed strongly even in late stationary phase.

This study has revealed properties of ClfA that are likely to influence its role in virulence and that might explain the importance of ClfA in animal models of infection and why targeting ClfA in immunotherapy and vaccination might be successful in the treatment and prevention of *S. aureus* disease.

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Contents

Declaration ii
Summary iii
Acknowledgements v
Publications vi
List of Tables xiii
List of figures xiv
Key to abbreviations xvii
Dedication xxi

Chapter 1 Introduction 1
1.1 Biology of the staphylococci 2
1.1.1 Classification and identification 2
1.1.2 The *S. aureus* cell wall 2
1.1.3 Anchoring of surface proteins in the *S. aureus* cell wall 4
1.2 Staphylococcal diseases 5
1.3 *S. aureus* virulence factors 6
1.3.1 Host defences against infection 6
1.3.2 Inhibition of neutrophil chemotaxis 8
1.3.3 Pore-forming cytolytic toxins 8
1.3.4 Resistance to phagocytosis 9
1.3.4.1 Immunoglobulin-binding protein A 10
1.3.4.2 Capsular polysaccharide 11
1.3.4.3 Complement-binding protein 12
1.3.5 Resistance to antimicrobial peptides 12
1.3.5.1 Surface modifications 13
1.3.5.2 Defensin-binding protein 13
1.3.5.3 Proteases 14
1.3.6 Survival inside human cells 14
1.3.6.1 Polymorphonuclear leukocytes 14
1.3.6.2 Epithelial and endothelial cells 15
1.3.7 Immunomodulatory molecules 16
1.3.7.1 Immunoglobulin-binding protein A 16
1.3.7.2 Major histocompatibility complex class II-analog protein. 16

1.3.7.3 Toxin superantigens 17
1.3.8 Modulation of blood coagulation 17
1.3.8.1 Platelet activation 18
1.3.8.2 Coagulase 19
1.3.8.3 Extracellular fibrinogen-binding protein 20
1.3.9 Surface proteins containing LPXTG motifs 21
1.3.9.1 Clumping factor A 21
1.3.9.1.1 Structure of clumping factor A 21
1.3.9.1.2 Role in pathogenesis 23
1.3.9.2 Clumping factor B 24
1.3.9.3 SD-repeat proteins and Sas proteins 25
1.3.9.4 Collagen-binding protein, fibronectin-binding proteins and protein A 27
1.3.9.5 Iron-regulated adhesin 28
1.3.10 Extracellular proteases 29
1.3.10.1 Metalloprotease 29
1.3.10.2 Serine protease 30
1.3.10.3 Cysteine proteases and staphopains 31
1.3.10.4 Serine protease-like operon 32
1.3.10.5 Activation and regulation of proteases 32
1.3.11 Iron uptake systems 33
1.3.12 Biofilm formation 36
1.4 Vaccines and novel therapeutic approaches to *S. aureus* infection 37
1.4.1 Bivalent capsular polysaccharide conjugate vaccine: 'StaphVAX' 38
1.4.2 Passive immunisation against ClfA: 'Veronate' 39
1.4.3 Passive immunisation with humanised monoclonal antibody: 'Aurexis' 40
1.5 Regulation of virulence factors 41
1.5.1 Two component regulatory systems 41
1.5.1.1 The accessory gene regulator 42
1.5.1.2 Other two-component regulatory systems 43
1.5.2 Transcription factors 45
1.5.2.1 Single domain proteins 45
1.5.2.2 Two-domain proteins 47

1.5.2.3 MarR-like proteins 48
1.5.3 Sigma Factors 49
1.5.3.1 Sigma factor A 49
1.5.3.2 Sigma factor B 50
1.5.3.3 Sigma factor H 52
1.5.4 Summary of interactions between staphylococcal regulators 52
1.6 Rationale for this study 53

Chapter 2 Materials and Methods 56
2.1 Bacterial strains and growth conditions 57
2.2 Plasmids 57
2.3 DNA manipulations 58
2.4 Polymerase chain reaction (PCR) 58
2.5 Southern hybridisation 59
2.6 DNA sequencing and sequence alignment 59
2.7 Isolation of solublised cell wall proteins 60
2.8 SDS-PAGE and Western immunoblotting 60
2.9 Preparation of polyclonal antisera 61
2.10 Recombinant protein purification 61
2.11 Preparation of concentrated *S. aureus* supernatants and incubation with recombinant protein 62
2.12 Protease treatment of recombinant protein 62
2.13 Protease treatment of whole *S. aureus* cells 63
2.14 Fibrinogen adherence assays 63
2.15 Transformation 64
2.16 Transduction 65
2.17 Construction of temperature-sensitive shuttle plasmids 65
2.18 Allelic replacement mutagenesis 66
2.19 Assay for coagulase 68
2.20 Fibrinogen clumping test 68
2.21 Overlap primer PCR mutagenesis 68
2.22 Construction of *clfA::lacZ* fusion 69
2.23 Construction of Newman strains by transduction 70
2.24 β-galactosidase assay 71
2.25 Polymorphonuclear leukocyte (PMNL) isolation 71

2.26 Phagocytosis assay 72
2.27 Whole cell dot immunoblotting 73
2.28 RNA isolation 73
2.29 Northern hybridisation and densitometric analysis 74
2.30 Primer extension analysis 75

Chapter 3 Proteolytic degradation of region A of ClfA 76
3.1 Introduction 77
3.2 Results 79
3.2.1 Degradation of recombinant ClfA by supernatants of *S. aureus* 79
3.2.2 Degradation of recombinant ClfA by purified aureolysin 81
3.2.3 Degradation of recombinant ClfA by purified SspA serine protease 81
3.2.4 Degradation of recombinant ClfA by human cathepsin G and human neutrophil elastase 82
3.2.5 Binding of $rClfA_{(40-559)}$ and $rClfA_{(220-559)}$ to fibrinogen 83
3.2.6 Cleavage of ClfA on the *S. aureus* cell surface by purified aureolysin and SspA serine protease 84
3.2.7 Fibrinogen binding of full length and proteolytically cleaved ClfA on *S. aureus* cells 85
3.2.8 Multiple sequence alignment of domain N1 of ClfA 86
3.3 Discussion 88

Chapter 4 Genetic manipulation of *clfA* 92
4.1 Introduction 93
4.1.1 Genetic manipulation in *S. aureus* 93
4.1.2 Transposon mutagenesis 94
4.1.3 Allelic replacement mutagenesis 94
4.1.3.1 Plasmid incompatibility 94
4.1.3.2 Directed plasmid integration 95
4.1.3.3 Allelic replacement using temperature-sensitive plasmids 97
4.1.4 Temperature-sensitive plasmids 98
4.1.5 Site-directed mutagenesis of *clfA* 99

4.1.6 Summary 99
4.2 Results 100
4.2.1 Construction and validation of Newman *clfA* and *spa* mutants 100
4.2.2 Construction and validation of pTS*ermC* and pTS*tetK* 101
4.2.3 Allelic replacement mutagenesis of *clfA* 103
4.2.3.1 Construction of pBlueΔ*clfA* 103
4.2.3.2 Construction of pJH1 104
4.2.3.3 Allelic replacement in *S. aureus* 105
4.2.3.4 Identification of *clfA*-null (*clfA5*) mutants in *S. aureus* ... 105
4.2.4 Isolation and validation of non-fibrinogen-binding mutant of ClfA 107
4.2.5 Construction of *clfA::lacZ* transcriptional fusion 108
4.2.5.1 Validation of *clfA::lacZ* transcriptional fusion 109
4.3 Discussion 111

Chapter 5 Antiphagocytic activity of ClfA 114
5.1 Introduction 115
5.2 Results 119
5.2.1 Antiphagocytic activity of ClfA and protein A in *S. aureus* Newman 119
5.2.2 Antiphagocytic activity of ClfA in *S. aureus* 8325-4 120
5.2.3 Antiphagocytic activity of ClfA in *Lactococcus lactis* 122
5.2.4 Effect of fibrinogen on the antiphagocytic effect of ClfA 123
5.2.5 Antiphagocytic properties of other surface proteins 124
5.3 Discussion 126

Chapter 6 Regulation of expression of *clfA* 133
6.1 Introduction 134
6.2 Results 137
6.2.1 Expression of clumping factor A on *S. aureus* cell surface 137
6.2.2 Measurement of *clfA* promoter activity using a β-galactosidase reporter 139
6.2.3 Effect of SarA and Agr on *clfA* promoter activity 141
6.2.4 Effect of salicylic acid on *clfA* promoter activity 142

6.2.5 Measurement of *clfA* transcription by Northern hybridisation. 142
6.2.6 Mapping of *clfA* transcripts by Northern hybridisation 143
6.2.7 Identification of transcriptional start sites by primer extension 146
6.2.8 Measurement of *clfA* transcript half-life in stationary phase .. 148
6.3 Discussion 149

Chapter 7 General discussion 154

References 158

List of Tables

Following page

Table 2.1 Bacterial strains....75

Table 2.2 Plasmids....75

Table 2.3 Primers....75

List of Figures

Following page

Figure 1.1 Structure of *S. aureus* peptidoglycan 2
Figure 1.2 Staphylococcal surface proteins 4
Figure 1.3 Surface protein anchoring in *S. aureus* 4
Figure 1.4 Cell envelope modifications in *S. aureus* 14
Figure 1.5 Diagrammatic representation of fibrinogen structure 18
Figure 1.6 Topology of the DEv-IgG fold 22
Figure 1.7 Apo structures of domains N23 of ClfA, ClfB and SdrG and SdrG-peptide complex 22
Figure 1.8 Protease operons and protease activation in *S. aureus* 30
Figure 1.9 Two-component regulatory systems in *S. aureus* 42
Figure 1.10 Genetic organisation of *B. subtilis* and *S. aureus sigB* operons 50
Figure 1.11 Summary of interactions between regulatory systems in *S. aureus* 52
Figure 3.1 Proteolysis of $rClfA_{(40-559)}$ by *S. aureus* supernatants 79
Figure 3.2 Cleavage events in domain N1 of $rClfA_{(40-559)}$ 80
Figure 3.3 Amino acid sequence of ClfA from *S. aureus* strain Newman 80
Figure 3.4 Degradation of $rClfA_{(40-559)}$ by aureolysin 81
Figure 3.5 Degradation of $rClfA_{(40-559)}$ by SspA serine protease 83
Figure 3.6 Degradation of $rClfA_{(40-559)}$ by cathepsin G and elastase 83
Figure 3.7 Adherence of $rClfA_{(40-559)}$ and $rClfA_{(220-559)}$ to immobilised fibrinogen 83
Figure 3.8 Degradation of ClfA on *S. aureus* cell surface by aureolysin and SspA 85
Figure 3.9 Adherence of protease-treated SH1000 cells to immobilised fibrinogen 85
Figure 3.10 Multiple alignment of amino acid sequences of domain N1 of ClfA . 87
Figure 3.11 Model of ClfA on the bacterial cell surface in complex with fibrinogen γ-chain peptide 91
Figure 4.1 Suicide integration 95
Figure 4.2 Transcriptional fusion to *lacZ* 95
Figure 4.3 Plasmid integration and excision 97

Figure 4.4 Overlap primer PCR 99
Figure 4.5 Southern hybridisation of Newman strains 101
Figure 4.6 Western immunoblotting of Newman strains 101
Figure 4.7 Construction of pTS*ermC* and pTS*tetK* 103
Figure 4.8 Maps of pTS*ermC* and pTS*tetK* plasmids 103
Figure 4.9 Construction of *clfA5* null mutation 103
Figure 4.10 Construction of pBlueΔ*clfA* 103
Figure 4.11 Construction of pJH1 105
Figure 4.12 Allelic replacement mutagenesis with pJH1 105
Figure 4.13 Validation of Newman *spa clfA5* and 8325-4 *spa clfA5* strains 107
Figure 4.14 Overlap primer PCR mutagenesis of *clfA* in pCF77 107
Figure 4.15 Construction of pCF77PY 107
Figure 4.16 Validation of pCF77PY in 8325-4 *spa clfA5* 109
Figure 4.17 pAZ106 and integration strategy 109
Figure 4.18 Construction of pAZ106F12R12 and pAZ106F13R12 109
Figure 4.19 Southern hybridisation of *clfA::lacZ* strains 109
Figure 4.20 β-galactosidase activity of Newman strains bearing *lacZ* fusions 109
Figure 5.1 Phagocytosis of *S. aureus* strains by human PMNL 119
Figure 5.2 Phagocytosis of Newman strains by human PMNL 119
Figure 5.3 Phagocytosis of 8325-4 *spa clfA5* strains expressing ClfA derivatives 121
Figure 5.4 Phagocytosis of *L. lactis* strains by human PMNL 123
Figure 5.5 Dot immunoblotting of *L. lactis* strains 123
Figure 5.6 Phagocytosis of *L. lactis* ClfAPY strains by human PMNL 123
Figure 5.7 Phagocytosis of *L. lactis* expressing fibrinogen-binding proteins 125
Figure 5.8 Phagocytosis of *L. lactis* strains expressing surface proteins 125
Figure 5.9 Schematic diagram of possible interactions between *S. aureus* and human PMNL 131
Figure 6.1 Western immunoblotting of solublised cell wall-associated proteins of *S. aureus* strains 138
Figure 6.2 Whole cell dot immunoblotting of *S. aureus* strains 140
Figure 6.3 β-galactosidase activity of *S. aureus* strains bearing *clfA::lacZ* 140
Figure 6.4 β-galactosidase activity of *agr* and *sarA* strains bearing *clfA::lacZ* 142
Figure 6.5 β-galactosidase activity of *S. aureus* Newman in salicylic acid 142

Figure 6.6 Northern hybridisation of *S. aureus* strains 142
Figure 6.7 Localisation of 5' and 3' ends of *clfA* mRNAs by Northern hybridisation 144
Figure 6.8 Nucleotide sequence 5' to *clfA* 144
Figure 6.9 Nucleotide sequence 3' to *clfA* 144
Figure 6.10 Primer extension analysis of SigB-dependent *clfA* transcription start site by autoradiography 146
Figure 6.11 Primer extension analysis of SigB-dependent *clfA* transcription start site by phosphorimaging 148
Figure 6.12 Primer extension analysis of SigB-independent *clfA* transcription start site 148
Figure 6.13 *clfA* mRNA levels in stationary phase following rifampicin treatment 148

Key to abbreviations

Single-letter amino acid code

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

Three-letter amino acid code

Ala	alanine
Gln	glutamine
His	histidine
Lys	lysine

Nucleotide bases

A	adenine
C	cytosine
T	thymine
G	guanine
N	any base

Key to abbreviations (contd.)

Antibiotics

Amp	ampicillin
Cam	chloramphenicol
Erm	erythromycin
Tet	tetracycline
Kan	kanamycin

Other abbreviations

µF	microfaraday(s)
µg	microgramme(s)
µl	microlitre(s)
20 x SSC	3 M NaCl, 0.3 M Na Citrate
4-MU	4-methylumbelliferone
aa	amino acid residue(s)
AIP	autoinducing peptide
BHI	brain heart infusion
bp	base pair(s)
BSA	bovine serum albumin
ca.	circa
cDNA	copy DNA
contd.	continued
CP5	serotype 5 capsular polysaccharide
CP8	serotype 8 capsular polysaccharide
dATP	dideoxyadenosinetriphosphate
DIG	digoxygenin
DNA	deoxyribonucleic acid
dNTP	dideoxynucleotidetriphosphate
dUTP	dideoxyuridinetriphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fcγ	Constant region of IgG
FITC	fluorescein isothiocyanate
FnBPs	FnBPA and FnBPB
FSB	final sample buffer

Key to abbreviations (contd.)

GlcNAc	N-acetlyglucosamine
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IC_{50}	50% inhibitory concentration
IgG	immunoglobulin G
IgM	immunoglobulin M
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase pair(s)
kDa	kilodalton(s)
L	litre(s)
M	molar
mA	milliamp(s)
MAC	membrane attack complex
MHC	major histocompatibility compex
min	minute(s)
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
MRSA	methicillin resistant *S. aureus*
MSCRAMM	microbial surface component recognising adhesive matrix molecules
MSSA	methicillin sensitive *S. aureus*
MU	Miller Unit(s)
MUG	4-methylumbelliferyl β-D-galactopyranoside
MurNAc	N-acetlymuramic acid
N1	domain N1 of region A
N123	region A
N2	domain N2 of region A
N23	domains N2 and N3 of region A
N3	domain N3 of region A
NEAT	near transporters

Key to abbreviations (contd.)

ng	nanogramme(s)
NHS	normal human serum
nm	nanometre(s)
nM	nanomolar
nt	nucleotide(s)
OD_{600}	optical density at 600 nm
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIA	polysaccharide intercellular adhesin
PMNL	polymorphonuclear leukocyte(s)
pmol	picomole(s)
PVDF	polyvinylidene difluoride
rClfA	recombinant ClfA region A
rClfB	recombinant ClfB region A
RNA	ribonucleic acid
rpm	revolution(s) per minute
rRNA	ribosomal RNA
rSdrG	recombinant SdrG region A
RT	reverse transcriptase
SA-IVIG	*S. aureus* intravenous immunoglobulin preparation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE	40 mM Tris-Acetate, 1 mM EDTA
TIGR	The Institute of Genomic Research
TS	10 mM Tris-HCl, 0.9% NaCl, pH 7.4
TSA	trypticase soy agar
TSB	trypticase soy broth
U	unit(s)
UV	ultraviolet
V	volt(s)
X-Gal	5-bromo-4-chloro-3-inolyl β-D-galactopyranoside
Ω	ohm(s)

For Michael

Chapter 1

Introduction

1.1 Biology of the staphylococci

1.1.1 Classification and identification

Bacteria of the genus *Staphylococcus* are Gram-positive organisms that characteristically divide in more than one plane to form grape-like clusters. Molecular typing and genetic analyses have shown that the staphylococci form a well-defined genus consisting of at least 26 species, 13 of which are associated with humans (Schleifer and Kroppenstedt, 1990) and have placed the staphylococci in the *Bacillus-Lactobacillus-Streptococcus* cluster of the *Micrococcaceae* (Ludwig *et al.*, 1985; Stackebrandt and Teuber, 1988). The staphylococci are most closely related to *Enterococcus*, *Bacillus* and *Listeria*. The G+C content of staphylococcal DNA is low, in the range 30-38%, with the G+C content of *S. aureus* DNA at 33% (Kuroda *et al.*, 2001). The staphylococci are extremely halotolerant (growing at up to 3.5 M NaCl) and are resistant to desiccation.

Staphylococcus aureus is a non-motile, non-sporulating, catalase positive coccus that forms smooth, entire, raised colonies. It is distinguished from other staphylococci by the presence of a golden pigment, the secretion of coagulase and a thermostable DNase, and the ability to ferment mannitol. Coagulase is a secreted protein that binds fibrinogen and also activates prothrombin to initiate the host blood coagulation pathway (see Section 1.3.8.2). Coagulase has been shown to be a virulence factor in several animal models of staphylococcal infection (Hasegawa and San Clemente, 1978; Sawai *et al.*, 1997; Jonsson *et al.*, 1985), and coagulase production is a marker for virulence that separates *S. aureus* from the other staphylococci, which are generally less pathogenic (Phonimdaeng *et al.*, 1990).

1.1.2 The *S. aureus* cell wall

The cell wall envelope functions as a physical barrier, protecting bacteria from the environment and acting as a rigid exoskeleton that prevents cell lysis in environments of low osmolarity. The glycan strands of all bacterial peptidoglycan consist of repeating disaccharide units, N-acetylglucosamine–(β1→4)-N-acetylmuramic acid (GlcNAc–MurNAc) (Figure 1.1; Ghuysen and Strominger,

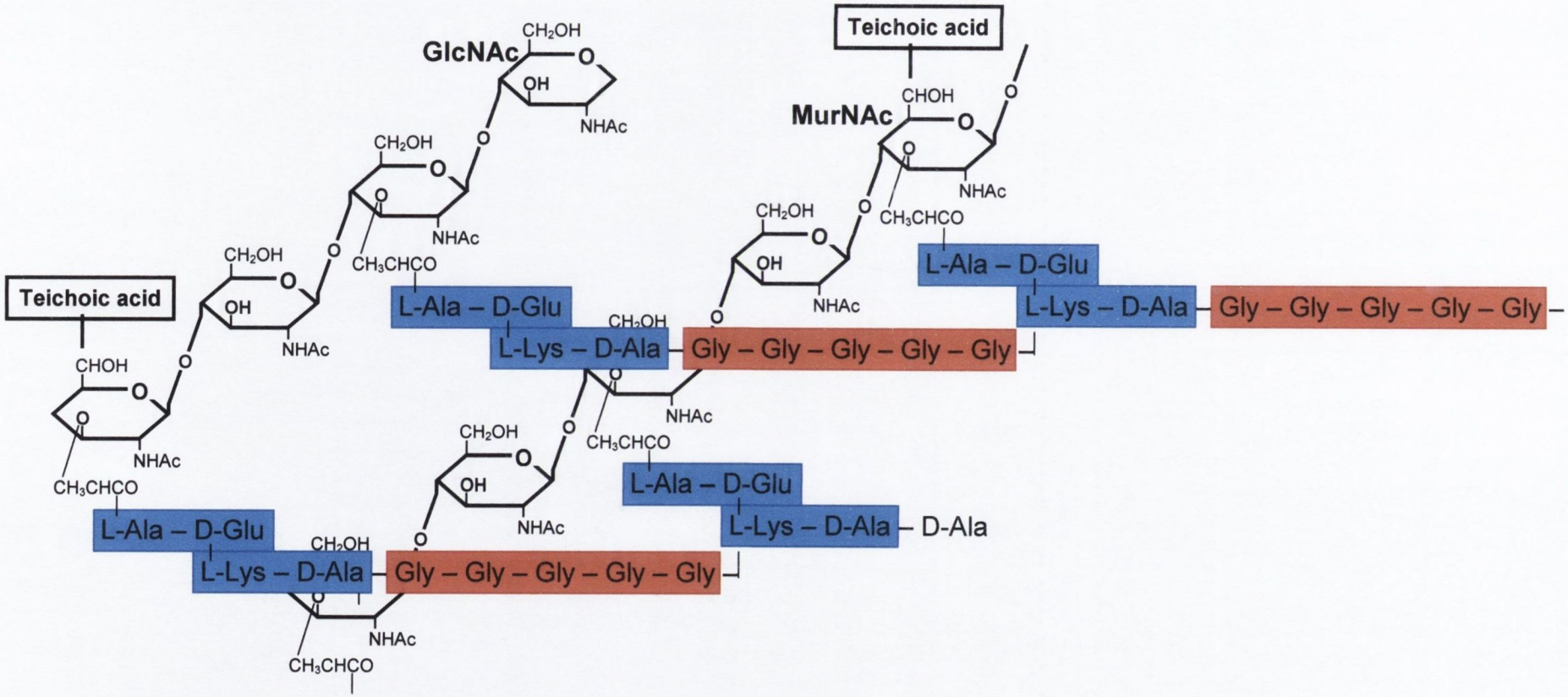


Fig 1.1 Structure of *S. aureus* peptidoglycan

β(1→4)-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) form the murein backbone. Cell wall peptides (tetrapeptides; coloured blue) are amide-linked to MurNAc. Pentaglycine bridging peptides (red) link the cell wall peptides of neighbouring chains. Not all cell wall peptides are involved in cross-bridging. Those that do not undergo transpeptidation retain an additional C-terminal D-Ala. Teichoic acids are linked at C6 of MurNAc.

1963). Glycan chains are cross-linked by short cell wall peptides (tetrapeptides), generating a three-dimensional molecular network that maintains the integrity of the bacterium (Ghuysen *et al.*, 1965; Tipper and Strominger, 1965). *S. aureus* has a unique cell wall peptidoglycan characterised by a pentaglycine bridging peptide that makes it susceptible to lysostaphin (Schleifer and Kandler, 1972) and lysine as the diamino acid in the cell wall peptide (Figure 1.1). The cell wall is approximately 50% peptidoglycan by weight and also contains ribitol teichoic acid, which is substituted with N-acetyl glucosamine and D-alanine (Ward *et al.*, 1981; Endl *et al.*, 1983; Collins *et al.*, 2002). This is linked to peptidoglycan at C6 of MurNAc (Figure 1.1). Embedded in the cell membrane is lipoteichoic acid that is composed of poly(glycerol phosphate) substituted with D-alanine (Fischer *et al.*, 1988). Substitutions on teichoic acid and lipoteichoic acid confer defensin resistance (Collins *et al.*, 2002; Peschel, 2002), and are discussed in Section 1.3.5.1.

Peptidoglycan biosynthesis occurs in the bacterial cytoplasm, membrane and extracellular cell wall compartments (Navarre and Schneewind, 1999; Strominger *et al.*, 1967). After synthesis in the cytoplasm, a MurNAc precursor forms a phosophodiester bond with an undecaprenol carrier in the cytoplasmic membrane (lipid I), followed by attachment of GlcNAc, to generate lipid II (Strominger *et al.*, 1967). Cell wall peptides (NH_2 - L-Ala - D-Gln - L-Lys - D-Ala - D-Ala - COOH) are synthesised on the lipid II unit in the cytoplasm, amide linked through the lactic acid moiety of MurNAc (Figure 1.1; Tipper *et al.*, 1967). MurNAc-GlcNAc units are transferred from lipid II to peptidoglycan extracellularly via transglycosylation, which is carried out by penicillin-binding proteins. Cell wall peptides of newly synthesised units are linked to cell wall peptides of neighbouring MurNAc-GlcNAc chains through pentaglycine cross-bridges (Figure 1.1; Strominger *et al.*, 1967). Cross-linking of cell wall peptides (transpeptidation) is also carried out by penicillin-binding proteins. This process removes the terminal D-Ala from the cell wall peptide, yielding a mature tetrapeptide (Navarre and Schneewind, 1999). Transglycosylation and transpeptidation are the targets of β-lactam antibiotics. Cell wall-associated proteins are joined to un-crosslinked cell wall peptides.

1.1.3 Anchoring of surface proteins in the *S. aureus* cell wall

S. aureus has the potential to express up to 20 proteins on its surface that are covalently anchored to cell wall peptidoglycan by a process called 'sorting'. These proteins typically contain an N-terminal secretory signal sequence and a C-terminal cell wall sorting signal, consisting of an LPXTG motif followed by a membrane-spanning domain and a positively charged tail (Fischetti *et al.*, 1990; Schneewind *et al.*, 1992). Some cell wall-associated proteins and their features are depicted in Figure 1.2. Many cell wall-associated proteins contain ligand-binding domains (termed region A) close to their N-termini and were initially characterised by function (e.g. the fibrinogen-binding protein clumping factor A (ClfA; McDevitt *et al.*, 1994)). Additional cell wall-associated proteins have more recently been identified by genetic and genomic analyses (see Section 1.3.9.3; Josefsson *et al.*, 1998; Roche *et al.*, 2003).

Cell wall-associated proteins of *S. aureus* are covalently linked to peptidoglycan by sortase (SrtA). Proteins synthesised in the cytoplasm are secreted via the Sec-dependent pathway, which leads to removal of the secretory signal sequence (Figure 1.3). The presence of a C-terminal membrane-spanning domain, followed by positively charged residues, leads to retention of the C-terminus of the protein in the Sec machinery (Schneewind *et al.*, 1993).

Sortase, an extracellular, membrane-bound enzyme, cleaves the protein between T and G of the LPXTG motif and transfers the N-terminal portion to G in the pentaglycine bridge of the lipid-linked peptidoglycan precursor in the membrane (Figure 1.3; Navarre and Schneewind, 1994; Perry *et al.,* 2002). The peptidoglycan precursor, with the attached protein, is then linked to the peptidoglycan by transglycosylation (Ton-That *et al.*, 1997; Navarre *et al.*, 1998; Ton-That and Schneewind, 1999).

Sortase A anchors the fibrinogen-binding protein ClfA (Section 1.3.9.1), the immunoglobulin-binding protein A (Spa; Sections 1.3.4.1, 1.3.7.1 and 1.3.9.4), the fibronectin-binding proteins (FnBPA and FnBPB; Sections 1.3.6.2 and 1.3.9.4), and all other secreted proteins containing an LPXTG sorting motif, to the cell wall. Analysis of the *S. aureus* genome sequence identified a second sortase gene (*srtB*). This gene is part of an iron-regulated operon that includes surface protein genes that are involved in iron acquisition (see Section 1.3.11; Mazmanian *et al.*, 2002; 2003). One of these proteins, IsdC, is synthesized as a precursor with an

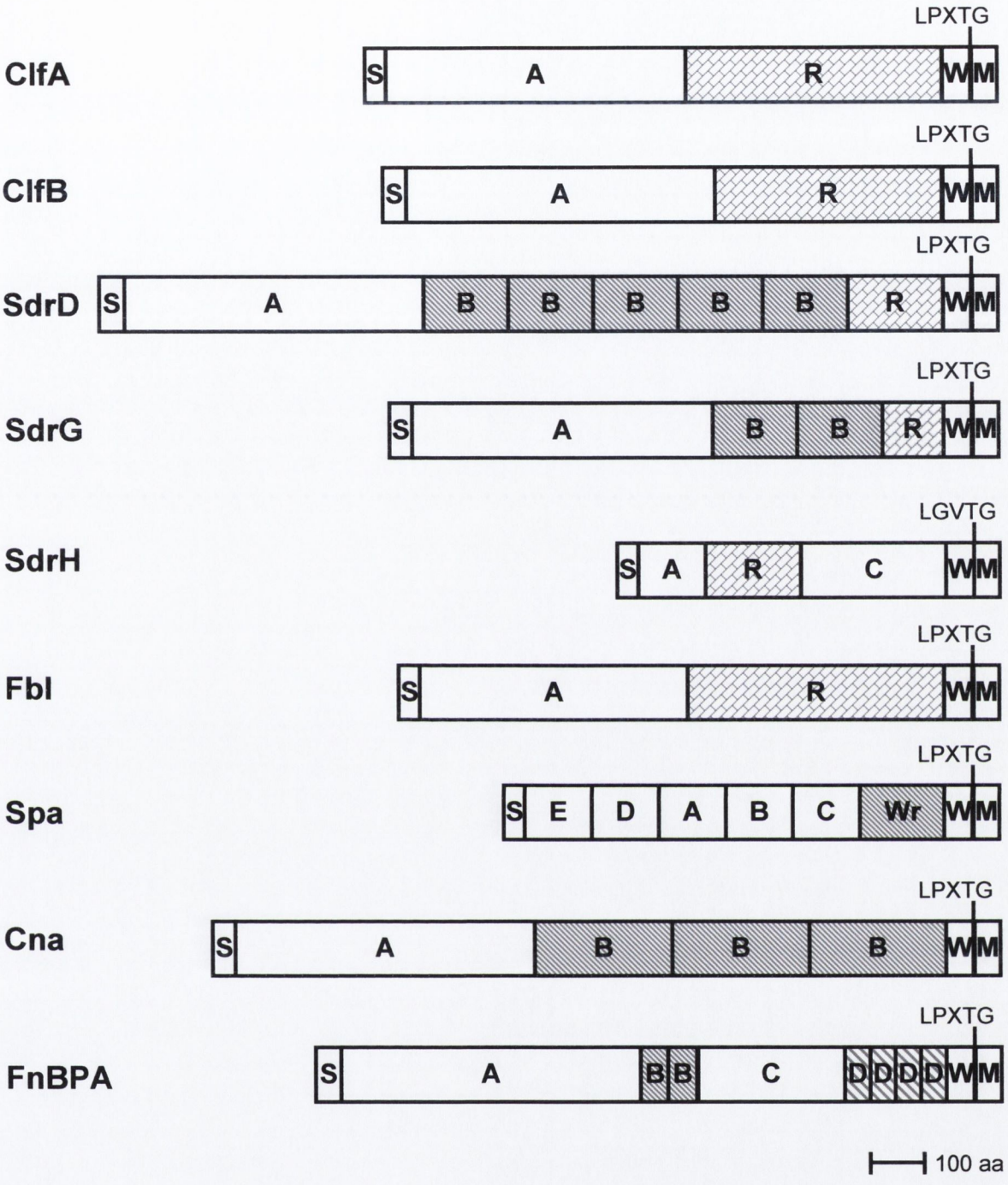


Fig 1.2 Staphylococcal surface proteins

Schematic representation of surface proteins from *S. aureus* (ClfA, ClfB, SdrD, Spa, Cna and FnBPA), *S. epidermidis* (SdrG and SdrH) and *S. lugdunensis* (Fbl). The relative sizes of the signal sequence (S), region A (A), B-repeat region (B), SD-repeat region (R), C-region (C), D-repeat region (D) and wall/membrane spanning regions (WM) are shown. LPXTG motifs are indicated. Spa domains E, D, A, B and C are homologous ligand-binding repeats.

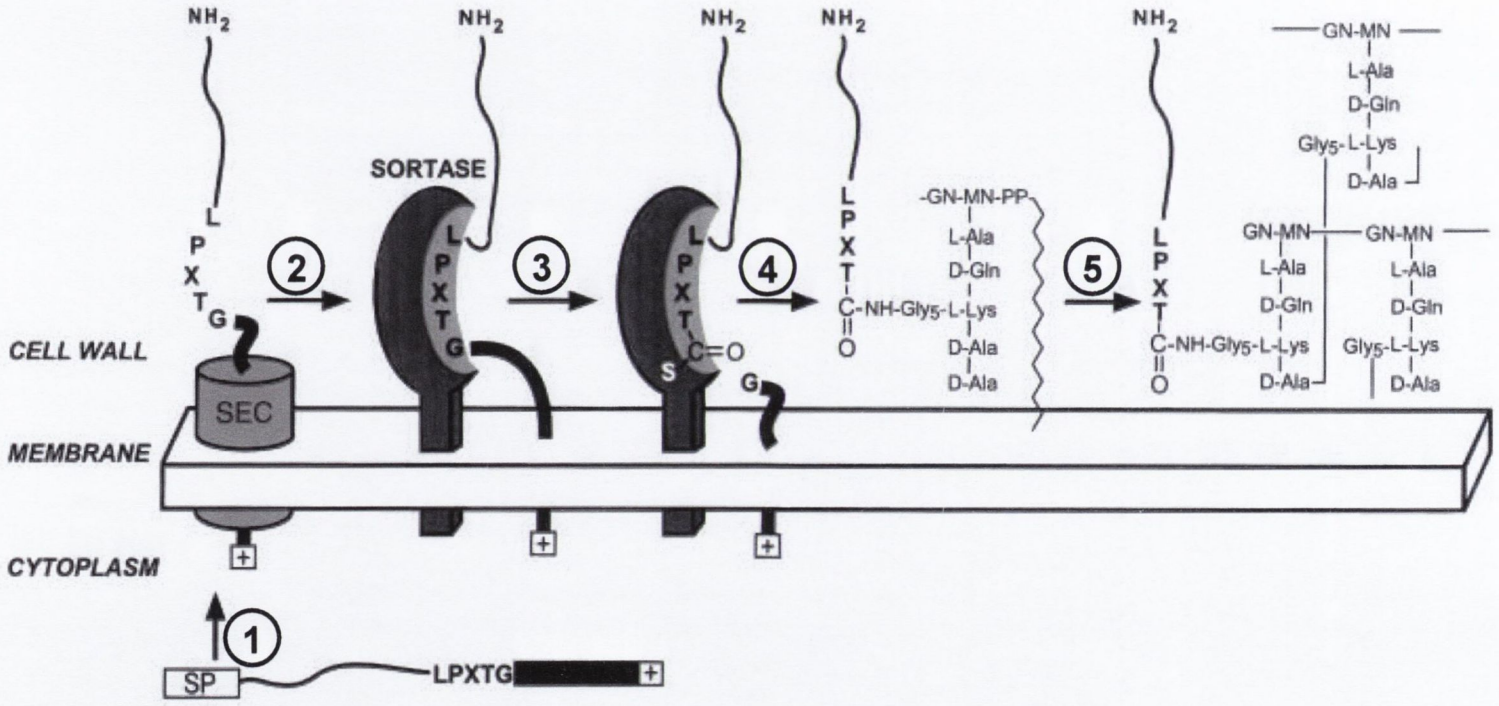


Fig 1.3 Surface protein anchoring in *S. aureus*

(1) Export: precursor proteins with an N-terminal signal peptide (SP) pass through the secretory (Sec) pathway, and the signal peptide is removed. (2) Retention: the C-terminal sorting signal retains polypeptides within the secretory pathway. (3) Cleavage: sortase cleaves between the T and the G of the LPXTG motif, resulting in the formation of a thioester intermediate at the active-site cysteine. (4) Linkage: the acyl-enzyme intermediate is resolved, leading to the synthesis of an amide bond between the surface protein and the uncross-linked pentaglycine bridge on lipid II. (5) Cell wall incorporation: lipid-linked surface protein is incorporated into the cell wall via transglycosylation. The murein pentapeptide subunit with the attached surface protein is then cross-linked to other cell wall peptides, generating the mature murein tetrapeptide. Adapted from Mazmanian *et al.*, 2001.

NPQTN C-terminal sorting signal, which is anchored in the cell wall by sortase B. *srtA* mutants fail to display numerous surface proteins and are attenuated in a murine renal abscess model of infection (Mazmanian *et al.*, 2000), whereas *srtB* mutants are not attenuated (Mazmanian *et al.*, 2002).

1.2 Staphylococcal diseases

S. aureus permanently colonises the moist squamous epithelium of the anterior nares of approximately 20% of the population. A further 60% are transiently colonised, and approximately 20% are permanent non-carriers (Peacock *et al.*, 2001). Additional carriage sites include the hairline, wrists, axillae and perineum (Lowy, 1998). Colonisation is known to be a risk factor for the development of *S. aureus* disease in both the hospital and the community (Wertheim *et al.*, 2004; von Eiff *et al.*, 2001). The most common types of *S. aureus* infection are superficial skin lesions such as boils, impetigo and abscesses. In the case of localised infections such as these, the host inflammatory response contains the infection, which is dealt with by neutrophils. If the organism gains access to the bloodstream (bacteraemia) it can infect internal tissues such as bone (osteomyelitis), the joints (septic arthritis), kidneys (nephritis) and lungs (pneumonia) (Lowy, 1998). In soft tissues purulent collections of bacteria can serve as reservoirs for the initiation of recurrent infection (Musher *et al.*, 1994). *S. aureus* is a leading cause of infective endocarditis, causing both native valve endocarditis, affecting intravenous drug users, the elderly and those in hospital, and prosthetic valve endocarditis (Lowy, 1998).

S. aureus also causes toxin-mediated diseases such as toxic shock syndrome, (associated with production of Toxic Shock Syndrome Toxin-1 and enterotoxins), severe necrotising pneumonia, (associated with Panton-Valentine leukocidin-producing strains), scalded skin syndrome (associated with production of epidermolytic toxins) and food poisoning (caused by ingestion of enterotoxins).

The treatment of *S. aureus* disease is complicated by the emergence of resistance to antimicrobial agents. *S. aureus* strains have developed resistance to all of the commonly used antibiotics, including those introduced to combat nosocomial infection. This began in the late 1940s with resistance to penicillin, followed by resistance to methicillin shortly after its introduction in the 1960s (Eickhoff, 1972). Although methicillin has been superseded in therapeutic use by

other β-lactam antibiotics, methicillin-resistant *S. aureus* (MRSA) remain resistant to all β-lactams, third-generation cephalosporins and cephamycins. For this reason the term MRSA is synonymous with multiple resistance. Resistance to aminoglycoside antibiotics in *S. aureus* is widespread and resistance to fluoroquinolones has been shown to develop rapidly (Woodford, 2005; Blumberg *et al.*, 1991). In addition, isolated cases of high-level resistance to the glycopeptide antibiotic vancomycin have recently emerged (Weigel *et al.*, 2003; Flannagan *et al.*, 2003).

1.3 *S. aureus* virulence factors

S. aureus can express a variety of potential virulence factors. These can be categorised according to their likely roles in pathogenesis, and include adhesins, evasins and toxins. Adhesins are involved in bacterial adherence to surfaces, evasins are involved in evasion of the host immune response and toxins have toxic effects on the host. In some cases (e.g. protein A (Sections 1.3.4.1, 1.3.7.1 and 1.3.9.4)) a single factor can have functions in more than one of these categories. The majority of *S. aureus* virulence factors are proteins. They may be secreted, anchored in the cell wall (Section 1.1.3) or non-covalently associated with the cell wall (e.g. Eap/Map (Sections 1.3.2 and 1.3.7.2) and coagulase (Section 1.3.8.2)). In this Section virulence factors will be introduced in the context of their interactions with the host and with host defences.

1.3.1 Host defences against infection

The skin and mucous membranes form an effective physical barrier against staphylococci. If this barrier is broken due to injury or surgery, *S. aureus* can gain entry to the body, where it is confronted by both the innate and acquired immune responses of the host. *S. aureus* infection of the skin induces a strong inflammatory response that involves the migration of leukocytes (neutrophils and macrophages) to the site of infection. These phagocytic cells attempt to engulf and dispose of the bacteria, with the help of available antibodies and complement.

Complement is a family of proteins, and fragments derived from them by proteolysis, with many roles in both innate and acquired immunity, including the direct killing of foreign cells and the regulation of other effectors of the immune

response (Moore, 2004). In combating bacteria such as *S. aureus* the role of complement is to recruit effector molecules that label the bacteria and target them for destruction by immune effector cells such as neutrophils. This process of complement fixation occurs by three pathways; the alternative and mannose-binding lectin pathways are part of the innate immune system, whereas the classical pathway is part of the acquired immune response, as it involves specific interaction with antibody bound to antigens on target cells. One outcome of complement activation is the assembly of a 'membrane attack complex' (MAC) on the membrane of invading cells, which can lead to pore formation and cell lysis. However, Gram-positive bacteria are resistant to MAC-mediated lysis, presumably due to inhibition of MAC deposition by peptidoglycan (Cooper, 1999). One of the main purposes of complement fixation on Gram-positive bacteria is opsonisation, to promote phagocytosis by professional phagocytes (neutrophils and macrophages). Initially professional phagocytes are attracted to the site of infection by chemoattractant molecules such as small peptide fragments released during complement activation (C3a and C5a) and by formylated peptides released by growing bacteria. The membranes of professional phagocytes contain specific receptors for complement fragments and N-formylated peptides that enhance the efficiency of phagocytosis. Neutrophils also carry specific receptors that recognise the Fc region of immunoglobulin G and complement proteins that are bound to the bacterial surface (Fcγ receptors and complement receptors, respectively), which facilitate efficient bacterial uptake and killing.

At the initial stage of *S. aureus* infection, bacteria and their products are additionally taken up by macrophages and other antigen-presenting cells and transported to the lymph nodes where the stimulation of acquired immunity begins. B cells in the lymph nodes are stimulated to differentiate and secrete antibodies that neutralise toxins and recognise bacterial targets, promoting more efficient phagocytosis of bacterial cells. However, it is clear that this system does not function efficiently against *S. aureus*, as antibodies to *S. aureus* antigens are present in all humans but appear insufficient to prevent subsequent infection (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005) This is discussed in Section 5.1.

S. aureus is a successful pathogen as it uses multiple mechanisms involving a variety of virulence factors to adhere to surfaces within the body, to invade and damage tissues and to inhibit human innate and acquired immunity.

1.3.2 Inhibition of neutrophil chemotaxis

Leukocyte migration is a key event in host defence against invading pathogens. Chemoattractants are generated by *S. aureus* by the secretion of N-formylated peptides (a by-product of bacterial translation) or complement activation (cleavage of C5 to release C5a), and subsequently by the activation of leukocytes (Schiffmann *et al.*, 1975). The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14.1-kD secreted protein encoded by bacteriophages that occur in about 60% of clinical isolates (de Haas *et al.*, 2004; Veldkamp *et al.*, 2000). Two separate active sites on CHIPS are involved in binding to the receptors for complement fragment C5a and the formylated peptide receptors on neutrophils and monocytes, causing potent, specific inhibition of chemotaxis (Haas *et al.*, 2004; Postma *et al.*, 2004; 2005). It is hypothesized that CHIPS plays a major role early in infection and is an important virulence factor (de Haas *et al.*, 2004).

The extracellular adherence protein, Eap (also called Map; see Section 1.3.7.2), is an anti-inflammatory factor that inhibits neutrophil recruitment. Eap occurs predominantly in culture supernatants (Palma *et al.*, 1999). It is not covalently linked to the cell wall, but some molecules bind to the bacterial surface following secretion (Hussain *et al.*, 2002). Eap interacts with intercellular adhesion molecule 1 (ICAM-1) on the surface of endothelial cells and disrupts receptor-mediated leukocyte adhesion to endothelial cells. This process is vital to the recruitment of inflammatory cells to the site of infection. Thus, expression of Eap by *S. aureus* reduced neutrophil recruitment to the peritoneal cavity of infected mice (Chavakis *et al.*, 2002). The inhibitory activities of CHIPS and Eap give *S. aureus* novel mechanisms to inhibit leukocyte migration and may potentially be exploited as anti-inflammatory therapeutic compounds.

1.3.3 Pore-forming cytolytic toxins

One of the principal features of *S. aureus* is its ability to secrete toxins that damage the membranes of host cells. The expression of cytolytic toxins that damage leukocytes contributes to the development of abscesses by killing neutrophils that are attempting to engulf and kill bacteria. Leukotoxins are the major cause of pyogenicity which is the hallmark of many *S. aureus* infections.

Cytolytic toxins that form β-barrel pores in the cytoplasmic membranes of target cells cause leakage and ultimately cell lysis. The archetypal toxin of this class is α-haemolysin, which is secreted as a monomer that associates into a heptamer in the host membrane with β-strands from each monomer assembling into a 14-stranded β-barrel pore (Montoya and Gouaux, 2003). α-haemolysin (encoded by *hla*) lyses mammalian erythrocytes and is an important virulence factor in staphylococcal infection (Bhakdi and Tranum-Jensen, 1991; Hildebrand *et al.*, 1991).

The bicomponent leukotoxins of *S. aureus* (including γ-haemolysin, Panton-Valentine leukocidin (PVL), leukocidin E/D and leukocidin M/F-PV-like) comprise two subunits that are secreted separately and assemble on the leukocyte membrane, beginning with binding of monomers, followed by oligomerisation to form a single heptameric pore, culminating in the formation of clusters of pores (Kaneko and Kamio, 2004). γ-haemolysin lyses both erythrocytes and leukocytes, whereas PVL is toxic only to leukocytes (Tomita and Kamio, 1997; Menestrina *et al.*, 2003).

The genes encoding γ-haemolysin (*hlg*) were found to be present in the genomes of over 90% of randomly selected *S. aureus* strains, whereas the *pvl* genes (encoded on a lysogenic bacteriophage) were present in less than 2% of strains (Peacock *et al.*, 2002; Prévost *et al.*, 1995a; Holmes *et al.*, 2005). There is a strong association between PVL expression and severe necrotic skin infections and pneumonia (Prévost *et al.*, 1995b; Lina *et al.*, 1999), suggesting that PVL enhances virulence in these infections. Recently, community-acquired MRSA strains have emerged that produce PVL and cause severe necrotic haemorrhagic pneumonia and contagious severe skin infections in previously healthy individuals (Saïd-Salim *et al.*, 2005; Gillet *et al.*, 2002).

1.3.4 Resistance to phagocytosis

A characteristic feature of *S. aureus* disease is abscess formation (Lowy, 1998). Polymorphonuclear leukocytes (PMNL; neutrophils) are the primary line of defence against staphylococci (Verdrengh and Tarkowski, 1997), and their accumulation at a site of infection causes the formation of an abscess. The cytokine-mediated migration of PMNL to the site of infection is triggered by bacteria and tissue-based macrophages (Yao *et al.*, 1997). CHIPS, Eap and PVL, discussed above, are

capable of inhibiting this migration. *S. aureus* also has mechanisms to avoid opsonophagocytosis and killing by PMNL.

Phagocytosis is a receptor-mediated process that uses actin rearrangements to internalise particles. Professional phagocytes (e.g. PMNL and macrophages) express receptors that recognise, bind and trigger the internalisation of pathogens. Opsonin-independent phagocytosis occurs independently of antibody and complement and is triggered by the recognition of molecular motifs on the surface of pathogens by mannose receptors and integrins. Opsonophagocytosis involves Fcγ receptors or complement receptors (CR1, CR3 and CR4), which bind particles coated with specific immunoglobulin G (IgG) or complement, respectively (Celli and Finlay, 2002). Fcγ receptor-mediated phagocytosis results in the extension of F-actin-rich lamellopodia, which engulf the bacteria, and activation of the oxidative burst and pro-inflammatory cascade (Cox *et al.*, 1999; Celli and Finlay, 2002). Complement-mediated phagocytosis results in engulfment of the bacteria without lamellopodia extension, and does not result in cell activation (Aderem and Underhill, 1999). Following uptake, the bacteria reside in a phagosome, which fuses with intracellular granules and lysosomes to become a phagolysosome. In the phagolysosome bacteria are killed by reactive oxygen species (oxygen-dependent killing), antimicrobial peptides and enzymes (oxygen-independent killing) (Mayer-Scholl *et al.*, 2004). *S. aureus* expresses several factors that directly inhibit opsonophagocytosis by PMNL.

1.3.4.1 Immunoglobulin-binding protein A

Protein A is an immunoglobulin-binding protein that is expressed on the surface of over 95% of *S. aureus* strains (Forsgren and Nordstrom, 1974). It is maximally expressed during the exponential phase of growth and is down-regulated post-exponentially by *agr* and *sarA*, two global regulators of virulence genes in *S. aureus* (see Section 1.5; Fournier and Klier, 2004). It comprises four or five homologous 58-62 amino acid repeats designated E, D, A, B and C (Figure 1.2). Each domain is capable of binding the Fc region of IgG (Fcγ; Hillson *et al.*, 1993).

Protein A is a potent inhibitor of PMNL phagocytosis *in vitro* (Gemmell *et al.*, 1991). The ability of protein A to bind IgG leads to inhibition of opsono-phagocytosis through the prevention of productive interactions between IgG and Fcγ receptors on the PMNL surface (Gemmell *et al.*, 1991). Because IgG binds to

protein A via the Fc portion, Fcγ receptors on PMNL cannot recognise the bound antibody. This leads to impaired phagocytosis, even in the presence of specific antibody recognising protein A or other surface antigens. The abundance of protein A on the *S. aureus* cell surface leads to coating of the cells with IgG, which may protect the cells from recognition by other opsonins (Massey *et al.*, 2002). Protein A can also bind to the Fab region of V_H3-class immunoglobulins, in particular a subset of IgM molecules. This gives the protein immunosuppressive activity, as discussed in Section 1.3.7.1. Protein A also has adhesive properties; its ligands include tumour necrosis factor receptor (TNFR1) on epithelial cells and von Willebrand factor in serum, and are discussed in Section 1.3.9.4. Protein A has multiple functions in thwarting the host immune response and is a virulence factor in murine infection models of subcutaneous infection, sepsis and septic arthritis and staphylococcal pneumonia (Patel *et al.*, 1987; Palmqvist *et al.*, 2002; Gomez *et al.*, 2004).

1.3.4.2 Capsular polysaccharide

Immunity to staphylococcal disease also involves complement deposition by the classical, alternative and mannose-binding lectin (MBL) pathways (Wilkinson *et al.*, 1978; Verbrugh *et al.*, 1979; Neth *et al.*, 2002). Binding of complement to the bacterial surface and to opsonising antibodies leads to complement activation and highly efficient opsonophagocytosis by PMNL (Wilkinson *et al.*, 1978; Cunnion *et al.*, 2001).

The majority of clinical isolates of *S. aureus* produce serotype 5 or serotype 8 capsular polysaccharide (Karakawa and Vann, 1982; Sompolinsky *et al.*, 1985; Na'was *et al.*, 1998). This prevents access to the cell surface by soluble proteins and serum components. Capsular polysaccharide has antiphagocytic properties due to inhibition of binding of opsonising antibodies that recognise proteins, teichoic acids and peptidoglycan on the cell surface (Thakker *et al.*, 1998). This is further discussed in Section 5.1. Capsular polysaccharide also plays an important role in the inhibition of complement fixation, as it prevents access of complement proteins to the *S. aureus* cell surface (Cunnion *et al.*, 2003).

Capsular polysaccharide is expressed only in stationary phase cells *in vitro*, though *in vivo* expression in endocardial vegetations has been demonstrated (Lee *et al.*, 1993). Reduced O-acetlylation of the capsular polysaccharide decreases its

antiphagocytic activity. This is believed to be due to increased penetration by antibodies that recognise the cell surface (Bhasin *et al.*, 1998). Through the use of capsule-deficient mutants it was shown that capsular polysaccharide is a virulence factor in a murine model of staphylococcal bacteraemia (Thakker *et al.*, 1998). The inhibition of opsonophagocytosis by capsular polysaccharide contributes to virulence by preventing bacterial clearance by PMNL in the bloodstream, liver and spleen (Bhasin *et al.*, 1998; Luong and Lee, 2002).

1.3.4.3 Complement-binding protein

In addition to the anti-opsonic properties of capsular polysaccharide, *S. aureus* avoids complement deposition by secreting Efb, an extracellular fibrinogen-binding protein that also binds to complement protein C3 (Lee *et al.*, 2004). Component C3 is the common linking feature of all three complement activation pathways, allowing Efb to inhibit all pathways of complement activation and deposition. Efb inhibits complement-mediated opsonophagocytosis, either by blocking deposition of C3 on the bacterial cell surface or preventing further complement activation beyond C3b (Lee *et al.*, 2004). Efb also has fibrinogen-binding activity (see Section 1.3.8.3) and is a virulence factor in a rat wound infection model (Palma *et al.*, 1996).

1.3.5 Resistance to antimicrobial peptides

In addition to its ability to inhibit opsonophagocytosis by PMNL, *S. aureus* has mechanisms to inhibit bacterial killing by defensins and other antimicrobial peptides once it becomes phagocytosed. Antimicrobial peptides are an important feature of innate immunity. They are expressed by epithelial and hematopoietic cells and are present in large amounts in the azurophil granules of PMNL. They are present on mucosal surfaces, in the airway and on skin. Antimicrobial peptides include small anionic peptides (e.g. dermicidin), small cationic peptides (e.g. cathelicidin LL-37), anionic and cationic peptides that form disulphide bonds (e.g. α-defensins and β-defensins) and some small peptides derived from larger proteins (e.g. lactoferricin from lactoferrin) (Brogden, 2005). The azurophil granules of PMNL contain high concentrations of defensins, which are released into the phagolysosome upon particle ingestion. Defensins and cationic

antimicrobial peptides interact with anionic phospholipids in the bacterial membrane, leading to permeabilisation of the membrane and pore formation (Zasloff, 2002).

1.3.5.1 Surface modifications

S. aureus is insensitive to defensins and other antimicrobial peptides and is poorly killed by human PMNL (Mullarky *et al.*, 2001; Gresham *et al.*, 2000). Mechanisms of resistance to killing include modifications of molecules on the cell surface that change the surface charge of the bacteria, inhibiting cationic antimicrobial peptides.

Modification of phosphatidylglycerol in the cell membrane with L-lysine reduces the negative charge at the membrane surface, as the amino group of the L-lysine is free in solution (Figure 1.4). This leads to repulsion of cationic peptides. The enzyme MprF is responsible for this modification, and an *mprF* mutant was attenuated in a murine model of sepsis and septic arthritis (Peschel *et al.*, 2001).

D-Alanine modifications of teichoic acid in the cell wall and lipoteichoic acid in the membrane also confer defensin resistance (Collins *et al.*, 2002; Peschel, 2002). Deletion of the *dlt* operon, which is responsible for these modifications, led to increased killing of *S. aureus* by defensin peptides and reduced virulence in the murine model of sepsis and septic arthritis (Collins *et al.*, 2002). This mechanism of defensin resistance is also based on changes in the charge on the cell surface, as the amino group of D-alanine is free in solution, decreasing the negative charge on the molecules (Figure 1.4; Peschel *et al.*, 1999).

1.3.5.2 Defensin-binding protein

S. aureus secretes staphylokinase, a 136-amino acid protein that binds and inactivates human defensins (Jin *et al.*, 2004). Staphylokinase is encoded by lysogenic bacteriophages, and was first recognised for its plasminogen-activating activity (Sakharov *et al.*, 1996; Collen, 1998). It was shown to be produced by 67% of strains tested (Jin *et al.*, 2004). Staphylokinase induces the release of defensins from the granules of PMNL and effectively neutralises them. Staphylokinase binds defensins in a >1:1 molar ratio; in fact one staphylokinase molecule appears capable of binding and inactivating 6 defensin molecules. At

least one of the defensin-binding sites overlaps with the plasminogen-binding site, reducing plasminogen activation in the presence of defensins (Jin *et al.*, 2004).

1.3.5.3 Proteases

S. aureus produces numerous extracellular proteases that are believed to mediate tissue damage during infection (discussed in Section 1.3.10; Shaw *et al.*, 2004; Potempa *et al.*, 1988). The metalloprotease aureolysin (Section 1.3.10.1), has recently been shown to inactivate cathelicidin LL-37, a human antimicrobial peptide. Aureolysin cleaves cathelicidin LL-37, abolishing its antimicrobial activity (Sieprawska-Lupa *et al.*, 2004). Strains producing aureolysin were more resistant to killing by LL-37 than isogenic aureolysin mutants. As secretions on the skin contain antimicrobial peptides in order to kill bacteria, proteolytic inactivation of human antimicrobial peptides may be an important mechanism in the colonisation of the skin by *S. aureus* (Sieprawska-Lupa *et al.*, 2004).

1.3.6 Survival inside human cells

It has recently become clear that *S. aureus* can survive inside a variety of cell types, where it is protected from the host immune response. These include PMNL, which are professional phagocytes, and epithelial and endothelial cells, which are non-professional phagocytes. Invasion of cells allows passage out of the bloodstream into tissues, whereas intracellular survival can facilitate the spread of infection and leads to recurrent and persistent infection.

1.3.6.1 Polymorphonuclear leukocytes

Bacterial survival inside the polymorphonuclear leukocyte (PMNL) phago-lysosome following phagocytic uptake depends on resistance to killing by antimicrobial peptides (Section 1.3.5) and to inhibition of their activation. Activated PMNL undergo an oxidative burst, which is mediated by O_2^- production by the NADPH oxidase complex and results in changes in vacuolar pH and the activation of antimicrobial peptides inside the phagocytic vacuole (Segal, 2005). Myelo-peroxidase is also involved in *S. aureus* killing, possibly through the production of HOCl and iodination of bacterial proteins, or by protecting digestive enzymes in

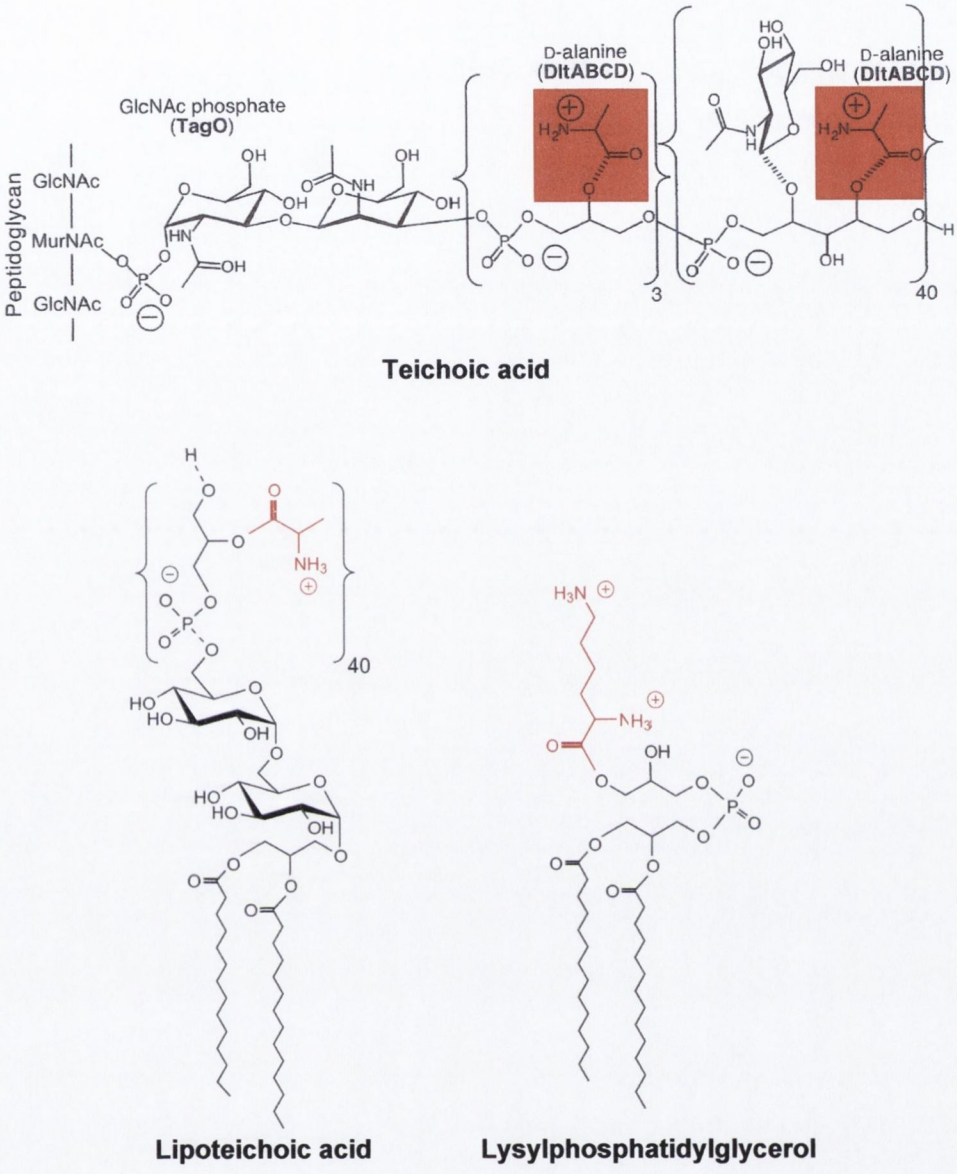


Fig 1.4 Cell envelope modifications in *S. aureus*

Modification of the bacterial cell envelope components involved in resistance to cationic antimicrobial peptides. Anionic cell envelope components are shown in black and cationic substituents are shown or highlighted in red. Free amino groups on the cationic D-alanine and L-lysine substituents neutralize negative charges contributed by phosphate groups. Modified from Peschel, 2002 and Weidenmayer *et al.*, 2004.

the vacuole from oxidative denaturation through the removal of excess H_2O_2 (Hampton *et al.*, 1998; Chapman *et al.*, 2002; Segal, 2005). Sufferers of chronic granulomatous disease, a range of inherited disorders of PMNL granule function, frequently present with recurrent *S. aureus* pneumonia and abscesses. This is generally as a result of defects in the NADPH oxidase pathway that affect the ability to form reactive-oxygen species and thus activate antimicrobial peptides in the phagocytic vacuole (Liese *et al.*, 1996; Segal, 2005).

The global regulator *sarA* controls the survival of *S. aureus* in PMNL by influencing the intracellular location of the bacteria (Gresham *et al.*, 2000). Electron microscopy showed that bacteria were present inside PMNL in 'spacious phagosomes', 'tight phagosomes' or were free in the cytoplasm. Bacteria in tight phagosomes (predominantly *sarA*$^-$ strains) were more likely to be killed, whereas *sarA*$^+$ strains tended to be located in spacious phagosomes (which resemble those formed by macropinocytic uptake), in spacious phagosomes whose membranes were deteriorating, or were free in the cytoplasm (Gresham *et al.*, 2000). The spacious phagosomes observed may be incompetent for fusion with granules, thus allowing bacteria to avoid contact with antimicrobial peptides (Gresham *et al.*, 2000). The involvement of *sarA* in survival inside PMNL suggests that the bacteria respond to the intracellular environment by altering the expression of virulence factors. Growing bacteria secrete cytolytic toxins (see Section 1.3.3) that destroy the membrane and promote escape of the bacteria into the cytoplasm. PMNL taken from a site of infection in mice were able to establish infection when administered to healthy animals, suggesting that bacterial survival inside PMNL allows persistence and possible recurrence of *S. aureus* infection (Gresham *et al.*, 2000).

1.3.6.2 Epithelial and endothelial cells

S. aureus can promote invasion into a range of non-professional phagocytic cells, including epithelial and endothelial cells (Massey *et al.*, 2001; Dziewanowska *et al.*, 1999; Lammers *et al.*, 1999; Fowler *et al.*, 2000; Sinha *et al.*, 1999). The mechanism of uptake involves a fibronectin bridge between the fibronectin-binding proteins FnBPA and FnBPB (see Section 1.3.9.4; Jett and Gilmore, 2002; Dziewanowska *et al.*, 1999; Peacock *et al.*, 1999) and the host cell fibronectin receptor, integrin $\alpha_5\beta_1$ (Sinha *et al.*, 1999; Massey *et al.*, 2001; Kintarak *et al.*,

2004). Activation of the integrin receptor causes cell signalling events that culminate in actin rearrangements and the phagocytic uptake of the bacteria into the host cell.

Bacterial uptake into epithelial and endothelial cells can lead to severe tissue damage. This can be caused by lysis of the phagocytic vacuole by pore-forming toxins, bacterial multiplication in the cytosol and necrotic death of the cells (Mempel *et al.*, 2002). *S. aureus* is also capable of survival inside the cells in a slow-growing semi-dormant state (called small colony variants). Intracellular survival in this state allows avoidance of the host immune response and antibiotics and leads to persistence. Interactions with epithelial cells may also be important in passing into and out of the circulatory system, allowing the bacteria to move from blood vessels into internal organs (e.g. bone, joints, muscle tissue) (Massey *et al.*, 2001; Mempel *et al.*, 2002).

1.3.7 Immunomodulatory molecules

In addition to avoidance of phagocytic uptake and resistance to killing, *S. aureus* produces factors that allow it to influence the host immune response. These include surface proteins and secreted toxins.

1.3.7.1 Immunoglobulin-binding protein A

As well as its antiopsonic properties (see Section 1.3.4.1) protein A of *S. aureus* is also immunosuppressive. Its ability to bind to B-cells bearing IgM (via the invariant portion of the Fab region of V_H3-class immunoglobulins) causes their activation, proliferation and subsequent apoptotic depletion (Sasano *et al.*, 1993; Sasso *et al.*, 1989). This can remove a significant proportion of the repertoire of potential antibody-secreting cells from the spleen and bone marrow (Goodyear and Silverman, 2004), giving protein A potent immunosuppressive activity.

1.3.7.2 Major histocompatibility complex class II-analog protein

The major histocompatibility complex (MHC) class II-analog protein, Map, also known as Eap (anti-inflammatory factor, see Section 1.3.2) is not covalently linked to the cell wall, but has the ability to bind to the bacterial surface following

secretion (Hussain *et al.*, 2002). Approximately 70% of Map is found in culture supernatants (Palma *et al.*, 1999). Map has 6 repeated domains, within each of which lies a 31-amino acid motif with strong homology to the MHC class II β-chain (Jonsson *et al.*, 1995). This motif allows Map to bind to T-cell receptors, inhibiting antigen presentation and resulting in the alteration of T-cell function. It reduces T-cell proliferation, causing a reduction in the delayed-type hypersensitivity (cell-mediated, Th1) response (Lee *et al.*, 2002). Map inhibits clearance from abscesses in internal organs in chronically infected mice (Lee *et al.*, 2002). By pushing the immune system towards a Th2-type response, Map may decrease phagocytic uptake of bacteria and enhance the intracellular survival of *S. aureus* (Harraghy *et al.*, 2003).

1.3.7.3 Toxin superantigens

Most *S. aureus* strains secrete several superantigen toxins that are associated with food poisoning and toxic-shock syndrome (Fraser *et al.*, 2000; Michie and Cohen, 1998). Superantigens bind directly to invariant regions of MHC class II molecules on antigen-presenting cells and to T-cell receptors. When expressed at high levels, superantigen toxins cause the unrestricted expansion of T-cells and massive release of cytokines from macrophages and T-cells. This cytokine release mediates toxic shock, causing tissue damage and multiorgan dysfunction (Marrack and Kappler, 1990; Bone, 1994). Superantigen-mediated T-cell activation is antigen-independent and the ensuing inflammation does not serve to fight staphylococcal infection, but rather overwhelms the host immune system, leading to toxic shock, multiple organ system failure and death. Low-level expression of superantigen toxins can cause immune suppression by the local depletion of T-cells. Enterotoxins also cause staphylococcal food poisoning if ingested (Bohach *et al.*, 1990).

1.3.8 Modulation of blood coagulation

The host response to tissue damage or bacterial infection is exploited by *S. aureus* to promote infection. This includes interference with blood clotting and wound healing, and use of the wound healing process to establish a focus of infection. Fibrinogen is an essential factor in blood clotting and wound healing,

due to its dual roles in the formation of fibrin clots and in its interactions with platelets (Herrick *et al.*, 1999). It is a 340-kDa glycoprotein composed of six polypeptide chains, two Aα, two Bβ and two γ-chains, arranged in a symmetrical dimeric structure (Figure 1.5). It circulates in the bloodstream at a concentration of approximately 3 mg/ml (9 μM). At sites of tissue injury fibrinogen is converted to fibrin by thrombin, with the release of fibrinopeptides from the N-terminal ends of the Aα and Bβ chains (Figure 1.5). Fibrin monomers polymerise, with the aid of additional factors (e.g. Factor XIIIa), and undergo intermolecular cross-linking to form a stable fibrin clot (Mosesson, 1997). Fibrin(ogen) is degraded by plasmin and other host proteases. Fibrinogen acts both as an adhesive protein, essential for platelet aggregation at sites of tissue injury, and in the formation of insoluble fibrin clots as part of the blood coagulation cascade.

1.3.8.1 Platelet activation

Platelets are important for blood coagulation and haemostasis as they aid in the repair of breaches in the walls of blood vessels. Platelet activation leads to thrombus formation. *S. aureus* induces platelet activation by a number of mechanisms. The fibrinogen-binding protein ClfA, the fibronectin-binding proteins FnBPA and FnBPB and protein A have been implicated in this process (O'Brien *et al.*, 2002a; Loughman *et al.*, 2005; Fitzgerald *et al.*, 2006). Platelets are believed to play an important role in the development of endocardial lesions and infective endocarditis, by acting as a surface that facilitates bacterial adherence (Scheld *et al.*, 1978).

ClfA is an important virulence factor in experimental infective endocarditis (Sullam *et al.*, 1996; Siboo *et al.*, 2001; Moreillon *et al.*, 1995; Que *et al.*, 2001). ClfA is the dominant surface protein responsible for rapid platelet activation in *S. aureus* cells from the stationary phase of growth (O'Brien *et al.*, 2002a). This process requires fibrinogen and ClfA-specific IgG. In the absence of ClfA fibrinogen-binding activity, specific IgG and complement promote slow activation of platelets (Loughman *et al.*, 2005). The fibronectin-binding proteins FnBPA and FnBPB can also bind fibrinogen and can stimulate platelet aggregation in *S. aureus* cells grown to exponential phase, in a manner similar to ClfA. They can also trigger platelet activation in a second mechanism, by creating a fibronectin bridge between *S. aureus* and platelets (Fitzgerald *et al.*, 2006).

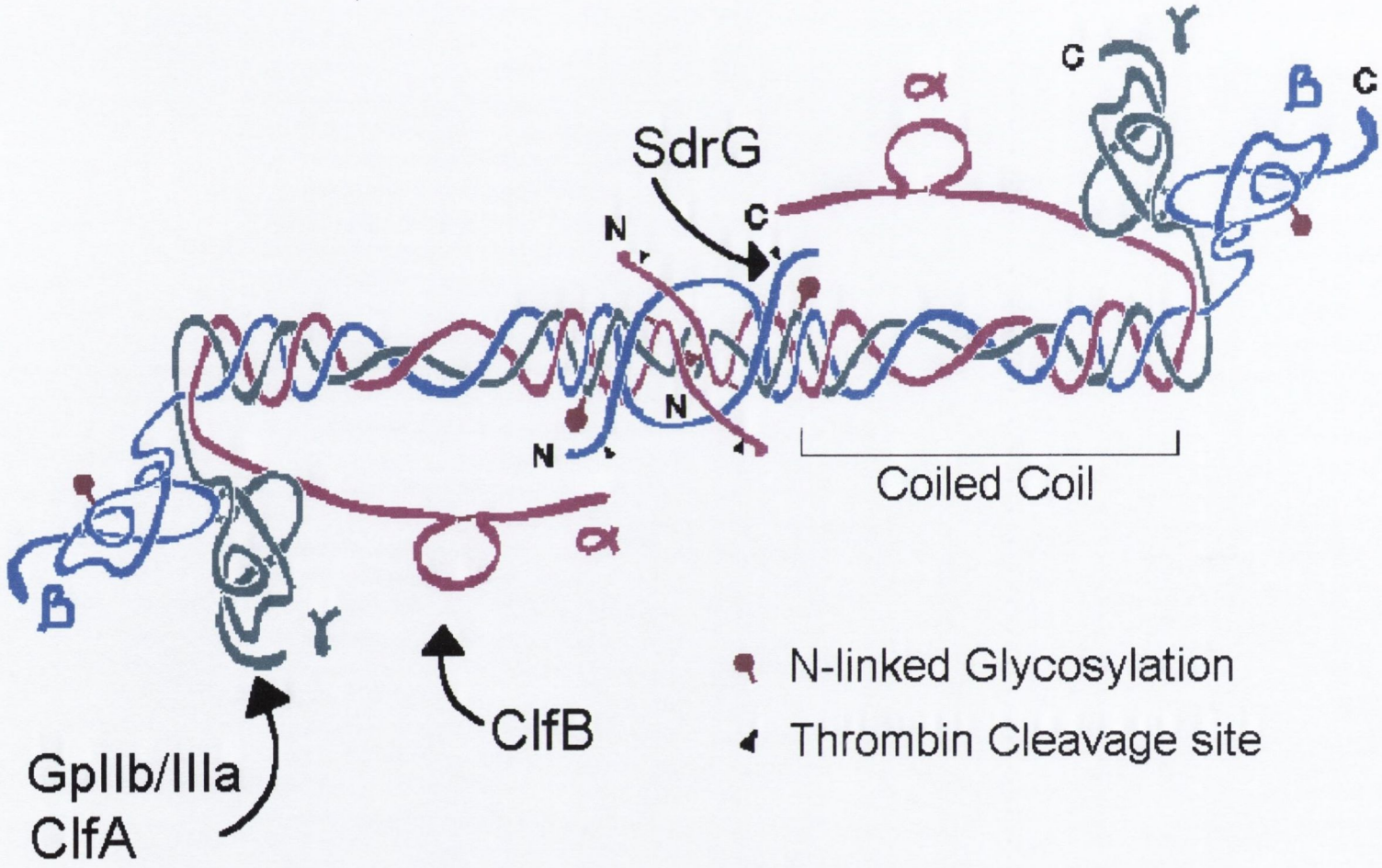


Fig 1.5 Diagrammatic representation of the structure of fibrinogen

The thrombin cleavage sites are indicated by black triangles and sites of N-linked glycosylation are indicated with a red tag. The positions of the binding sites of ClfA, ClfB, SdrG and the platelet glycoprotein GpIIb/IIIa are indicated. Adapted from www-biology.ucsd.edu/faculty/doolittle.

It appears that bacterial cells equipped with sufficient surface-bound fibrinogen molecules (via ClfA, FnBPA or FnBPB) can engage the low affinity platelet glycoprotein GpIIb/IIIa, aided by bound IgG molecules that promote clustering of FcγRIIa IgG receptors (Loughman *et al.*, 2005). This triggers activation, and subsequent platelet aggregation. Interaction between specific IgG directed against the staphylococcal surface proteins and the platelet receptor FcγRIIa is necessary for platelet activation to occur. In order to achieve platelet activation, a second interaction must occur, either between fibrinogen or fibronectin bound to the bacterial cell and GpIIb/IIIa or between complement proteins deposited on the bacteria and complement receptors.

It is possible that other staphylococcal mechanisms of interacting with platelets occur. Protein A may be involved in both direct binding to complement receptor gC1qR/p33 on platelets and indirect binding to the platelet glycoprotein GpIb, mediated via a bridge between protein A and von Willebrand factor (O'Brien *et al.*, 2002a; Pawar *et al.*, 2004; Nguyen *et al.*, 2000). It is not clear whether platelet activation is triggered by these interactions. Whether any such activation is dependent on the IgG-FcγRIIa interaction, as is the case with ClfA and the FnBPs, is unknown.

These mechanisms of platelet activation are likely to be important in the development of infective endocarditis. Through this mechanism, *S. aureus* exploits the presence of specific IgG in host serum to promote endocardial disease.

1.3.8.2 Coagulase

During physiological blood coagulation, prothrombin is converted to thrombin through a specific cleavage by trypsin in response to tissue damage. Cleavage generates a new N-terminus that triggers folding of the thrombin molecule into an active conformation. Thrombin cleaves fibrinogen to yield fibrin, which creates the basis of a blood clot at the site of tissue damage.

Secretion of coagulase is one of the hallmarks of *S. aureus*. It activates the host blood coagulation pathway independently of tissue damage by complexing with and activating prothrombin (Hemker *et al.*, 1975; Kawabata *et al.*, 1986). Conformational changes occur in prothrombin upon coagulase binding that allow it to become active without undergoing proteolytic cleavage (Friedrich *et al.*, 2003).

The coagulase molecule consists of two independently folded α-helical N-terminal domains, a C-terminal repeat region including between 6 and 9 27-amino acid repeats, and a highly conserved central domain. The N-terminal domains contain the prothrombin-binding and activation sites and the C-terminal repeat regions can bind fibrinogen (Friedrich *et al.*, 2003; Boden and Flock, 1992). The 2-3 residues at the extreme N-terminus of coagulase insert into the trypsin activation pocket in prothrombin. This causes conformational changes which trigger the folding of the prothrombin activation domain. This mechanism has been defined as the prototype for a novel mechanism of zymogen activation called 'molecular sexuality' (Friedrich *et al.*, 2003). The ability to bind both prothrombin and fibrinogen simultaneously may be involved in bringing coagulase-activated prothrombin into close proximity with fibrinogen, allowing the formation of fibrin.

It might be predicted that the ability to create a fibrin clot around bacterial cells hinders phagocytosis. Although coagulase has been shown to be a virulence factor in animal models of pulmonary infection and murine mastitis (Sawai *et al.*, 1997; Jonsson *et al.*, 1985) it is perhaps surprising that it did not contribute to the pathogenesis of *S. aureus* in a rat endocarditis model (Baddour *et al.*, 1994; Moreillon *et al.*, 1995).

1.3.8.3 Extracellular fibrinogen-binding protein

The extracellular fibrinogen-binding protein Efb is a secreted 15.8-kDa protein that binds the α-chain of fibrinogen (Palma *et al.*, 1998; 2001). The N-terminal domain of Efb contains two repeated regions that bear structural similarity to the fibrinogen-binding C-terminal domain of coagulase (Section 1.3.8.2; Boden and Flock, 1994; Watanabe *et al.*, 2005). These regions of Efb and coagulase compete for the same binding domain on fibrinogen, which is close to an RGD sequence recognised by platelet receptor GpIIb/IIIa (Palma *et al.*, 2001). Efb also has antiopsonic activity, due to its ability to bind complement component C3 at the C-terminal A domain (Section 1.3.4.3).

Using a site-specific mutant it was shown that Efb plays an important role in the prevention of wound healing in rats (Palma *et al.*, 1996). Efb can bind to fibrinogen that is bound to activated platelets, inhibiting platelet aggregation (Palma *et al.*, 2001). Pre-treatment of mice with Efb has been shown to prolong

bleeding time, and Efb can prevent death of animals following the administration of platelet agonists sufficient to stimulate thrombosis (Shannon *et al.*, 2005).

The ability of *S. aureus* to secrete a molecule that inhibits platelet activation and aggregation is the likely explanation for the observed delay in healing of an infected wound in the skin of rats or mice. The apparently paradoxical behaviour of *S. aureus* in both promoting and inhibiting platelet activation can be explained. Early in infection (e.g. in bacteraemia) bacteria cause activation and aggregation in order to create conditions that promote endovascular infection. Later in infection, when bacteria have gained a foothold in a lesion, delaying the healing process might be advantageous to the long-term survival and proliferation of the bacteria.

1.3.9 Surface proteins containing LPXTG motifs

Secreted *S. aureus* proteins that contain an LPXTG sorting motif towards the C-terminus are covalently attached to the cell wall peptidoglycan by sortase A (see Section 1.1.3). Some of these proteins are depicted in Figure 1.2, and many have multiple functions (e.g. protein A, clumping factor B). One of the important features of many LPXTG-containing surface proteins of *S. aureus* is adhesion to components of the host extracellular matrix. As a result, they have been ascribed the acronym MSCRAMMs (microbial surface components recognising adhesive matrix molecules) (Patti and Höök, 1994). These proteins allow binding to molecules of the host extracellular matrix and many contribute to virulence (Foster and Höök, 1998).

1.3.9.1 Clumping factor A

1.3.9.1.1 Structure of clumping factor A

Clumping factor A (ClfA) is a fibrinogen-binding surface protein of *S. aureus*. It contains an N-terminal Sec-dependent secretory signal peptide, followed by a 520-residue region A that contains the ligand binding domain, region R consisting primarily of SD dipeptide repeats and a C-terminal domain that allows anchoring to the cell wall peptidoglycan by sortase A (Figures 1.2 and 3.3; McDevitt *et al.*, 1994; 1995; Mazmanian *et al.*, 2001). Region R of ClfA acts as a stalk that allows

the functional projection of the ligand-binding domain away from the cell surface. Reduction of the length of region R has deleterious effects on fibrinogen-binding activity (Hartford *et al.*, 1997).

ClfA binds to the extreme C-terminus of the γ-chain of fibrinogen, the same site as the human platelet integrin GpIIb/IIIa (Figure 1.5; McDevitt *et al.*, 1997; Medved *et al.*, 1997). The minimum ligand-binding domain of ClfA comprises residues 220-559 (McDevitt *et al.*, 1995) and was designated domains N2 and N3 (N23) based on the domain organisation of the related protein clumping factor B (ClfB) (Perkins *et al.*, 2001; see Figure 3.2 for domain organisation of region A of ClfA).

The crystal structure of ClfA domains N2 and N3 ($rClfA_{(221-559)}$) has been solved. This revealed that the individual domains N2 and N3 show a novel variation of the immunoglobulin fold, designated the DEv-IgG fold (Deivanayagam *et al.*, 2002). The full-length region A of ClfA (residues 40-559) has not been crystallised (Deivanayagam *et al.*, 1999). Domain N1 is predicted to be composed mainly of β-sheet structure, and may be organised into more than one sub-domain (Deivanayagam *et al.*, 2002). The DEv-IgG fold of domains N2 and N3 resembles the folding pattern of the globular domains of IgG, with additional β-strands (D' and D") between strands D and E (Figure 1.6). This fold is also found in the ligand-binding domains of the collagen-binding adhesin CNA, clumping factor B, FnBPA and FnBPB (Deivanayagam *et al.*, 2002). The additional structural elements in the variant IgG folds do not disturb the core region of the IgG fold. Three metal ions with octahedral coordination geometry, which may represent Ca^{2+} or Mg^{2+} ions, were co-crystallised in the $rClfA_{(221-559)}$ structure, and partial occupancy of these sites may occur (Figure 1.7; Deivanayagam *et al.*, 2002). The observation of metal ions associated with N23 may be related to the inhibition of fibrinogen-binding activity in ClfA by the presence of millimolar concentrations of Ca^{2+} (O'Connell *et al.*, 1998).

Computer docking studies simulated the structure of $rClfA_{(221-559)}$ in complex with the C-terminal 17 amino acids of the fibrinogen γ-chain (γ-chain peptide). The most energetically favourable solutions resembled the crystal structure of a related fibrinogen-binding protein from *S. epidermidis*, SdrG, which has been crystallised with a bound ligand (Figures 1.2 and 1.7; Deivanayagam *et al.*, 2002; Ponnuraj *et al.*, 2003). SdrG uses a dynamic 'dock, lock and latch' mechanism to bind the Bβ-chain of fibrinogen, whereby the C-terminal end of domain N3 forms a latch that folds over the loosely docked ligand in the binding pocket, locking it into place via

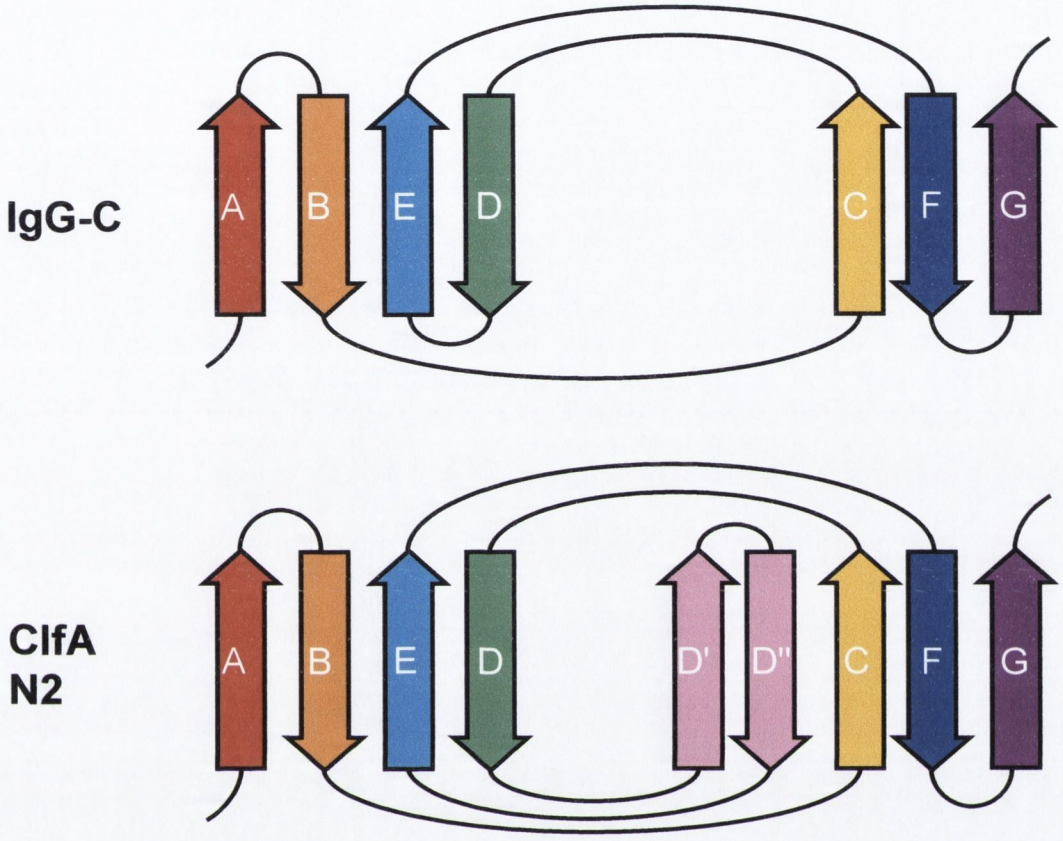


Fig 1.6 Topology of the DEv-IgG fold

Individual MSCRAMM domains show a novel variation of the immunoglobulin fold, designated the DEv-IgG fold. The N2 domain of ClfA region A is shown to indicate this novel fold. Compared to the IgG-C fold (top), the DEv-IgG fold contains additional β-strands, D' and D" (coloured pink). Adapted from Deivanayagam *et al.*, 2002.

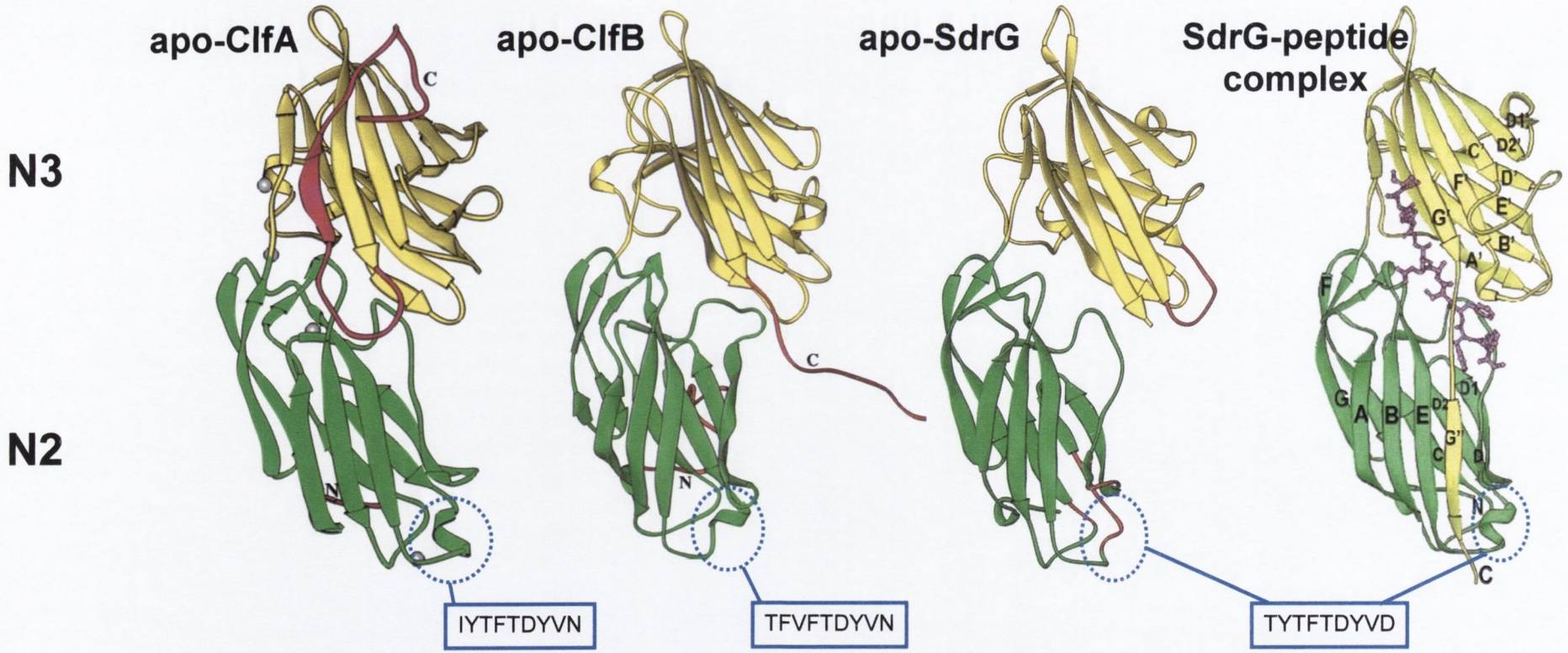


Fig 1.7 Apo structures of domains N23 of ClfA, ClfB and SdrG and SdrG-peptide complex

Ribbon representations of $rClfA_{(221-559)}$, $rClfB_{(282-542)}$ and $rSdrG_{(276-597)}$. Regions of poor resolution are shown in red and metal ions in silver. The C-terminus of $rClfA_{(221-559)}$ (latching peptide; red) loops back and folds into the N3 domain, partially blocking the proposed ligand-binding cleft. In $rClfB_{(282-542)}$ this peptide is located in the latching cleft of another rClfB molecule. In apo-SdrG the peptide (G'') is free in solution but interacts with N2 in the SdrG-peptide complex. Domains N2, N3 and TYTFTDYVD-like motifs are indicated. Fibrinogen β-chain peptide analogue in complex with SdrG is shown in ball and stick form. Strand designations correspond to DEv-IgG fold (Fig 1.6). SdrG-peptide complex is taken from Ponnuraj *et al.*, 2003.

β-strand complementation with N2 (Figure 1.7; Ponnuraj *et al.*, 2003). The C-terminal end of domain N3 (the latching peptide) forms a regular β-strand (G") that inserts between two strands (E and D) of N2 (the latching cleft). A conserved TYTFTDYVD-like motif forms the back of the latching cleft in rSdrG, and is found in a similar location in the structures of rClfA and rClfB. This motif is likely to be involved in binding of the latching peptide to the latching cleft in domain N2 (Figure 1.7; Ponnuraj *et al.*, 2003). The β-strand complementation that occurs in SdrG between the latching peptide (G" strand of N3) and domain N2 is also likely to occur in both ClfA and ClfB (Ponnuraj *et al.*, 2003).

The crystal structure of ClfA showed that the putative latching peptide in the apo form (coloured red in Figure 1.7) was bound to domain N3, where it partially occluded the peptide-binding trench. This is likely to be an artefact of crystallisation, as computer docking studies using the γ-chain peptide allowed the prediction of several residues in the occluded binding trench that are likely to be involved in ligand binding (Deivanayagam *et al.*, 2002). ClfA is likely to use the 'dock, lock and latch' mechanism to bind the γ-chain peptide (Ponnuraj *et al.*, 2003).

1.3.9.1.2 Role in pathogenesis

ClfA has been shown to be an important virulence factor in several infection models. It promotes adherence of *S. aureus* cells to *ex vivo* canine arteriovenous shunts, a process that involves fibrinogen, fibronectin and platelets (Vaudaux *et al.*, 1995). Clumping factor was first identified as a staphylococcal virulence factor in experimental endocarditis in rats, where a Newman *clfA* mutant produced 50% less frequent infections than the wild type strain (Moreillon *et al.*, 1995). The role of ClfA in this model was shown definitively by transfer of *clfA* to *Streptococcus gordonii* and to *Lactococcus lactis*. Initial work in *Strep. gordonii* attributed a definite role to ClfA in rat endocarditis, but the pathogenicity of the surrogate host in this model meant that the increase in infectivity was only clear within a narrow window of inocula (Stutzmann Meier *et al.*, 2001). To clarify this issue, the same group transferred *clfA* to *L. lactis*, a close relative of the streptococci that is poorly infective in this model. It was clearly shown that expression of ClfA on the lactococcal cell surface was necessary and sufficient to cause endocarditis in rats (Que *et al.*, 2001). ClfA was identified as a critical adhesin capable of mediating

endovascular infection on its own. The use of a surrogate host eliminated the problem of functional redundancy of staphylococcal factors, as only one specified factor was expressed.

ClfA has also been found to be a virulence factor in a murine model of sepsis and septic arthritis. Newman *clfA* caused significantly less severe arthritis than the isogenic wild-type strain or a complemented mutant (Josefsson *et al.*, 2001). In addition, the wild-type strain caused higher mortality than the *clfA* mutant. This showed clearly that ClfA is an important virulence factor in this model of systemic and invasive infection. The same strains were injected directly into the knee joints, by-passing the systemic and invasive phases of septic arthritis. The ClfA-producing strain caused significantly more inflammation within the joint tissue. This indicated that ClfA itself has pro-inflammatory properties. In the same study it was shown that active immunisation against ClfA (using $rClfA_{(40\text{-}559)}$) gave protection against arthritis and that passive immunisation with human IgG with high titre against ClfA protected mice from septic death (Josefsson *et al.*, 2001). The contribution of ClfA to the virulence of *S. aureus* is assumed to be related to its fibrinogen-binding activity.

Anti-ClfA antibodies have been detected in both 'normal' and convalescent sera, though a correlation between staphylococcal disease and antibody production has not been proven (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005). The presence of anti-ClfA antibodies in both normal and convalescent sera implies that normal exposure to *S. aureus* as a commensal of the skin causes the formation of anti-ClfA antibodies, but that the antibodies in normal sera are not protective against invasive *S. aureus* disease. This may be a result of immune evasion and immunosuppression by *S. aureus* (see Sections 1.3.2-1.3.4, 1.3.6 and 1.3.7) and allows the exploitation of low-level antibodies found in sera to enhance virulence, as discussed in Section 1.3.8.1 (Loughman *et al.*, 2005).

1.3.9.2 Clumping factor B

Clumping factor B (ClfB) is a fibrinogen-binding protein that has a very similar structural organisation to ClfA, sharing 93% amino acid identity in region R and 26% identity in region A (Figures 1.2 and 1.7; Ní Eidhin *et al.*, 1998). However, ClfB binds to a different domain of fibrinogen compared to ClfA (Figure 1.5; Perkins *et al.*, 2001) and can additionally bind to cytokeratin 10 (O'Brien *et al.*,

2002b). Whether the regions of ClfB that bind fibrinogen (via the α-chain) and cytokeratin 10 (via the C-terminal 'tail' region; Walsh *et al.*, 2004) are the same is not clear.

ClfB is expressed in the early exponential phase of growth *in vitro*. Transcription of *clfB* is activated by Rot (see Section 1.5.2.1) and ceases towards the end of exponential growth due to downregulation of Rot (McAleese *et al.*, 2001; Saïd-Salim *et al.*, 2003). Loss of fibrinogen-binding activity by ClfB in stationary phase is due to (i) cleavage of region A by a metalloprotease (aureolysin, see Section 1.3.10.1) which destroys ligand-binding activity and (ii) dilution of the existing protein on cells as they continue to divide (McAleese *et al.*, 2001).

The ability of ClfB to bind cytokeratin 10 on the surface of desquamated nasal epithelial cells suggests that the protein has an important function in adherence to the squamous epithelial surface (O'Brien *et al.*, 2002b). The possible importance of this interaction in nasal colonisation by *S. aureus* is emphasised by the presence of higher titre antibodies recognising ClfB in the sera of individuals who do not carry *S. aureus* in the nose (non-carriers) than of those who do (carriers) (Dryla *et al.*, 2005). Both intranasal and systemic immunisation with recombinant ClfB were recently shown to be protective in a murine model of nasal colonisation (A. Schaffer *et al.*, manuscript in preparation). Protection correlated with serum antibody levels to ClfB. In addition, a monoclonal antibody directed against ClfB inhibited *S. aureus* binding to mouse cytokeratin 10 and protected against colonisation in naïve mice. ClfB may prove to be a suitable candidate antigen for vaccination against nasal colonisation by *S. aureus*.

1.3.9.3 SD-repeat proteins and Sas proteins

Three additional genes containing the SD dipeptide repeat region R have been identified in *S. aureus*. The closely linked and tandemly arrayed genes encode members of the Clf-SD repeat (Sdr) protein family SdrC, SdrD and SdrE (Josefsson *et al.*, 1998). Each protein has similar structure to ClfA and ClfB, but includes additional repeat sequences (B repeats) between region A and region R (see Figure 1.2 for SdrD). Within the A regions of each protein the TYTFTDYVD-like motif, which may be involved in 'dock, lock and latch' ligand binding, is conserved (Josefsson *et al.*, 1998; Ponnuraj *et al.*, 2003). SdrE is not present in

all *S. aureus* strains. It bears significant homology to the bone sialoprotein-binding protein Bbp, and the two genes (*sdrE* and *bbp*) may be allelic variants (Tung *et al.*, 2000; M.P. Brennan, unpublished results). SdrE can stimulate platelet activation, probably by a complement- and IgG-dependent process (O'Brien *et al.*, 2001a; M.P. Brennan, unpublished results). The importance of SdrE in pathogenesis is suggested by the observation that *sdrE* is over-represented in strains associated with invasive disease (Peacock *et al.*, 2002). The ligands or biological activities of SdrC and SdrD are unknown. *sdrC* and *sdrD* are highly conserved and present in all strains tested (Josefsson *et al.*, 1998).

Analysis of the complete genome sequences of *S. aureus* revealed an additional 10 putative proteins containing LPXTG sorting motifs (Roche *et al.*, 2003). Several of the novel cell wall-associated proteins, called *S. aureus* surface proteins (SasA, SasC-K), share similar structural organisation with the Clf-Sdr protein family. Some have repeat regions (B repeats), rather than SD dipeptide repeats. The presence of two of these genes (*sasG* and *sasH*) is associated with invasive disease, and antibodies to the Sas proteins have been recovered from convalescent sera, indicating that these proteins are expressed *in vivo* during infection (Roche *et al.*, 2003).

SasG is homologous to the plasmin-sensitive cell wall-associated protein Pls of *S. aureus* and the accumulation-associated protein Aap of *S. epidermidis*. Aap is involved in biofilm formation (see Section 1.3.12) whereas Pls has been shown to have anti-adhesive properties in *S. aureus* (Savolainen *et al.*, 2001). SasG, Pls and Aap have been shown to promote bacterial adherence to nasal epithelial cells, most likely through the same receptor (Roche *et al.*, 2003). These interactions may be important in nasal colonisation. In addition, expression of SasG, like Pls, reduced adherence of *S. aureus* to fibronectin and fibrinogen (Savolainen *et al.*, 2001; Roche *et al.*, 2003).

SasA is a high molecular weight protein with extensive serine-rich repeats linking the N-terminal A domain to the cell wall. The *sasA* gene is linked to several genes encoding glycosyltransferases and Sec homologs. Proteins with similar features to SasA are expressed from loci with the same genetic organisation in streptococci (Hsa and GspB from *Strep. gordonii* and SrpA from *Strep. sanguis*) (Takahashi *et al.*, 2004; Stinson *et al.*, 2003; Takamatsu *et al.*, 2005; Plummer *et al.*, 2005). Glycosylation of these proteins is required for solubility and secretion (Takamatsu *et al.*, 2004). SasA (also called SraP), like Hsa, GspB and SrpA,

promotes adhesion to platelets and is a virulence factor in an animal model of endocarditis (Siboo *et al.*, 2005).

1.3.9.4 Collagen-binding protein, fibronectin-binding proteins and protein A

Other cell wall-associated adhesins of *S. aureus* include the collagen-binding protein CNA, the fibronectin-binding proteins FnBPA and FnBPB and protein A. The collagen-binding protein CNA contains a ligand-binding A domain, part of which has a single globular domain with the DEv-IgG fold structure similar to that of ClfA domains N2 and N3 (Patti *et al.*, 1994; Deivanayagam *et al.*, 2002). It binds to collagen and may be involved in the progression of infections of the bone such as arthritis and osteomyelitis. It is a virulence factor in animal models of septic arthritis, endocarditis, osteomyelitis and keratitis, and vaccination against CNA is protective in septic arthritis (Patti *et al.*, 1994; Hienz *et al.*, 1996; Elasri *et al.*, 2002; Rhem *et al.*, 2000; Nilsson *et al.*, 1998).

cna is not present in all *S. aureus* strains, and although two reports have indicated that the *cna* gene is associated more frequently with isolates from patients than with isolates from healthy adults in the UK (Peacock *et al.*, 2002; Booth *et al.*, 2001), *cna* was found to be underrepresented in human invasive *S. aureus* strains compared to human carriage strains in the Netherlands (van Leeuven *et al.*, 2005). In another study, 46% of *S. aureus* strains isolated from surgical patients were found to be cna^+ (Arciola *et al.*, 2005). Therefore, the correlation between *cna* and invasive *S. aureus* disease is currently uncertain.

The fibronectin-binding proteins FnBPA and FnBPB are multi-functional proteins containing fibronectin-, fibrinogen- and elastin-binding domains. The fibrinogen- and elastin-binding domain is localised within the A region (see Figure 1.2 for FnBPA; Wann *et al.*, 2000; Roche *et al.*, 2004), which is composed of domains N1, N2 and N3. Domains N2 and N3 possess the DEv-IgG fold structure of ClfA domains N2 and N3 (Deivanayagam *et al.*, 2002). The fibronectin-binding domains are localised within repeated sequences in the B, C and D domains. This is an unfolded region of the protein, which forms contacts with repeated F1 modules in fibronectin by β-strand complementation (Massey *et al.*, 2001; Schwarz-Linek *et al.*, 2003). Interactions between antiparallel β-strands in the repeats of FnBPs and repeated F1 modules in fibronectin generate a tandem β-

zipper mechanism of binding. This allows bacterial binding with high specificity and affinity to fibronectin, which is involved in invasion of mammalian epithelial and endothelial cells via integrin $\alpha_5\beta_1$ (see Section 1.3.6.2; Schwarz-Linek *et al.*, 2003).

Although the FnBPs can bind fibrinogen, they have a different role from ClfA in septic arthritis, contributing to the induction of systemic inflammation rather than the development of arthritis (Palmqvist *et al.*, 2005). This may be due to protease sensitivity of the fibrinogen-binding A region (McGavin *et al.*, 1997; Karlsson *et al.*, 2001).

Protein A (Spa) is an immunoglobulin-binding protein that mediates *S. aureus* binding to the Fc region of IgG and the constant region of Fab from V_H3-class immunoglobulins. This allows interaction with some classes of IgM. These binding abilities give protein A antiopsonic and immunosuppressive qualities (see Sections 1.3.4.1 and 1.3.7.1). Protein A contains 4 or 5 immunoglobulin-binding repeat regions (E-C; see Figure 1.2), followed by a short repeat region of variable length and a cell-wall sorting signal. In addition to immunoglobulins, protein A can bind to and activate tumour necrosis factor-α receptor 1 (TNFR1) on epithelial cells. This may be involved in the initiation of infection and the induction of inflammation during *S. aureus* pneumonia (Gomez *et al.*, 2004). Protein A can also bind to von Willebrand factor, a large, multimeric glycoprotein that mediates platelet adhesion at sites of endothelial damage and is important in haemostasis. This adhesive interaction with von Willebrand factor may be involved in the initiation of endovascular infection (Hartlieb *et al.*, 2000; M. O'Seaghdha, unpublished results). In addition, protein A is capable of binding to platelets directly, via the multi-functional receptor gC1qR/p33 and indirectly, under high-shear conditions, via von Willebrand factor and its platelet receptor GpIb (see Section 1.3.8.1; Pawar *et al.*, 2004). These interactions may be important for binding of *S. aureus* cells to platelet-fibrin clots in the body and for platelet activation (Nguyen *et al.*, 2000; O'Brien *et al.*, 2002a).

1.3.9.5 Iron-regulated adhesin

The expression of a subset of staphylococcal genes is induced under iron-limiting conditions, which are likely to resemble growth conditions in serum and within the host during infection. Iron regulation in *S. aureus* involves Fur, an iron-

responsive transcriptional regulator. This repressor prevents transcription of a specific set of genes in the presence of iron. In the absence of iron, the repression is alleviated and transcription occurs. These genes and their products are for the most part involved in iron acquisition (see Section 1.3.11).

isdA (iron-regulated surface determinant) is under the control of Fur, and is upregulated under iron-limiting conditions. IsdA (also called StbA, Seg7 and FrpA) binds to fibrinogen, fibronectin, transferrin, hemin, fetuin and haemoglobin, but not haptoglobin (Taylor and Heinrichs, 2002; Mazmanian *et al.*, 2002; 2003; Wiltshire and Foster, 2001; Morrisey *et al.*, 2002; Clarke *et al.*, 2004). IsdA is involved in the uptake of iron across the cell envelope (see Section 1.3.11; Mazmanian *et al.*, 2003). It is also a physiologically relevant adhesin for fibronectin and fibrinogen (Clarke *et al.*, 2004). Antibodies to IsdA have been found in convalescent sera, indicating that it is expressed *in vivo* (Morrisey *et al.*, 2002; Clarke *et al.*, 2004).

The ligand-binding domain of IsdA is a NEAT (near transporters) domain (Clarke *et al.*, 2004). These structural domains are found in proteins encoded by genes close to siderophore genes in Gram-positive bacteria, and are involved in binding to iron-containing molecules (see Section 1.3.11). It is likely that IsdA can function as a fibrinogen and fibronectin adhesin under iron-limiting growth conditions. The differential regulation of adhesins in response to the environment may be an important adaptation of opportunistic human pathogens.

1.3.10 Extracellular proteases

S. aureus secretes an array of proteases that facilitate tissue destruction, provide defence against the host immune response, allow interception of bacterial and host enzymes and alteration of surface proteins. Three catalytic classes of protease are secreted from *S. aureus* cells: metalloprotease, serine proteases and cysteine proteases.

1.3.10.1 Metalloprotease

Aureolysin is a calcium-dependent zinc metalloprotease produced by *S. aureus* in post-exponential phase. It is produced as a proenzyme and is activated by post-translational processing (Chan and Foster, 1998). The specific mechanism of aureolysin maturation is unknown, but it is not autocatalytic (Shaw *et al.*, 2004). It

does not involve SspA, SspB or ScpA (Sections 1.3.10.2 and 1.3.10.3), but may involve proteases of the *spl* operon (Section 1.3.10.4) (Reed *et al.*, 2001; Shaw *et al.*, 2004). *aur*, the gene encoding aureolysin, occurs in two allelic forms and is highly conserved, implying that it has important functions in the cell, including the processing of other protease precursors (Sabat *et al.*, 2000).

Aureolysin cleaves the glutamyl endopeptidase (V8 serine protease, SspA) from its precursor form (Drapeau, 1978; Shaw *et al.*, 2004) and cleaves lipase (Rollof and Normark, 1992), the major autolysin of *S. aureus* (Foster, 1995), ClfB (see Section 1.3.9.2; McAleese *et al.*, 2001) and the human antibacterial peptide cathelicidin LL-37 (see Section 1.3.5.3; Sieprawska-Lupa *et al.*, 2004). Aureolysin also affects the stimulation of B and T lymphocytes and inhibits immunoglobulin production (Prokesova *et al.*, 1991). However, despite numerous functions that influence staphylococcal virulence factors, mutation of *aur* does not lead to significant attenuation of *S. aureus* in animal models of infection (Shaw *et al.*, 2004; Calander *et al.*, 2004).

1.3.10.2 Serine protease

The serine glutamyl endopeptidase SspA (V8 protease) is secreted as a proenzyme that is activated by proteolytic cleavage by aureolysin (Drapeau, 1978). In the absence of aureolysin, pro-SspA is activated at a low level by a mechanism other than aureolysin, SspB, ScpA or the Spl proteases (Shaw *et al.*, 2004; Reed *et al.*, 2001). *sspA* forms an operon with *sspB*, encoding a cysteine protease, and *sspC*, encoding an inhibitor of SspB (Figure 1.8; Section 1.3.10.3).

SspA is produced in post-exponential phase (Shaw *et al.*, 2004) and has narrow substrate specificity. It cleaves the staphylococcal surface proteins FnBPA and protein A (McGavin *et al.*, 1997; Karlsson *et al.*, 2001). This may facilitate detachment from ligand-coated surfaces or immune evasion by the removal of epitopes. SspA is also capable of cleaving the heavy chains of human immunoglobulins *in vitro* (Prokesova *et al.*, 1992), the human α_1-protease inhibitor (Potempa *et al.*, 1986) and is involved in the activation of other staphylococcal proteases, as discussed below. *sspA* forms part of an operon, and polar mutation of *sspA* led to decreased virulence in a murine tissue abscess model, murine bacteraemia and wound infection, but not in murine septic arthritis (Shaw *et al.*, 2004; Coulter *et al.*, 1998; Calander *et al.*, 2004). However, the use of a non-polar

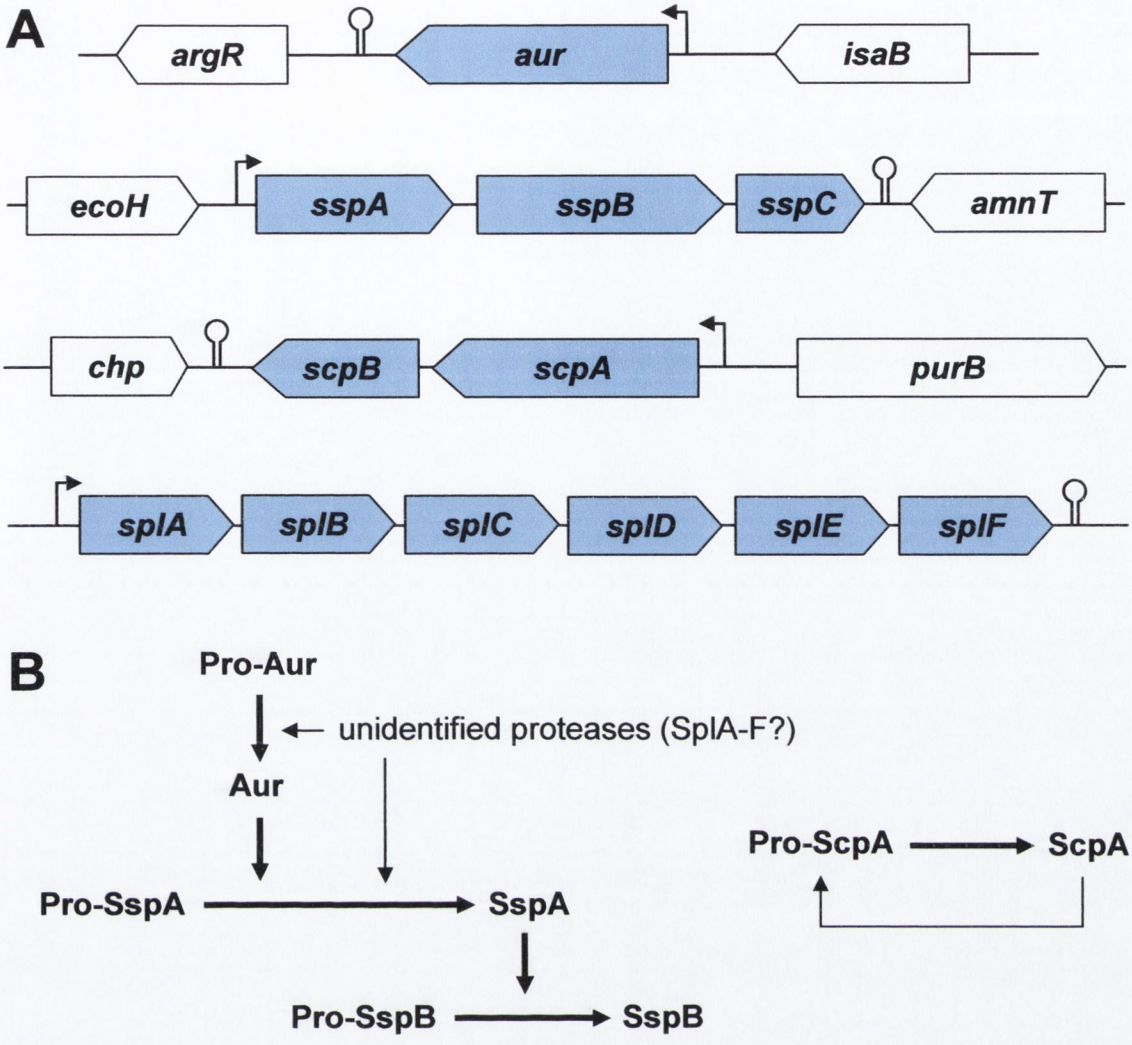


Fig 1.8 Protease operons and protease activation in *S. aureus*

A. Schematic representation of the aureolysin gene (*aur*), the *ssp* (serine protease), *scp* (cysteine protease) and *spl* (serine-protease-like) operons. Protease genes are coloured blue, arrows indicate promoter positions and stem loops indicate transcription terminator positions.

B. Aureolysin is activated from a pro-form by an unidentified protease. Aureolysin cleaves pro-SspA to the serine protease SspA. In the absence of aureolysin this activation occurs at a very low level, carried out by an unidentified protease. SspA cleaves the cysteine protease SspB from its pro-form. The cysteine protease ScpA is cleaved from its pro-form, possibly by an auto-catalytic mechanism. The position of the Spl proteases in this cascade is uncertain. They may be involved in activation of aureolysin. Adapted from Shaw *et al.*, 2004.

mutation of *sspA* showed no attenuation in the murine tissue abscess model (Rice *et al.*, 2001), implying that inactivation of *sspB* or *sspC* was responsible for attenuation in the polar *sspA* mutant (Shaw *et al.*, 2004).

1.3.10.3 Cysteine proteases and staphopains

Staphopain A (ScpA) is a papain-like cysteine protease that is produced during the post-exponential phase of growth as a pre-form and undergoes proteolytic cleavage. Its maturation is independent of aureolysin, SspA, SspB and the Spl proteases. It has a very broad substrate range, relatively little specificity in cleavage site, and may undergo autocatalytic activation (Shaw *et al.*, 2004). Staphopain A has elastinolytic activity and may be involved in tissue destruction (Potempa *et al.*, 1988). *scpA* forms part of a bi-cistronic operon including *scpB* (Figure 1.8), which encodes a specific inhibitor of staphopain A, named staphostatin A (ScpB) (Rzychon *et al.*, 2003). Staphostatins have high substrate specificity and form non-covalent 1:1 complexes with their corresponding enzymes or proenzymes, abolishing their cysteine protease activity (Dubin, 2003). Recombinant staphopain A is toxic to *E. coli* cells unless it is co-expressed with fully-functional staphostatin A (Wladyka *et al.*, 2005).

The cysteine protease staphopain B (SspB) is encoded in an operon with the serine protease SspA (Rice *et al.*, 2001) and staphostatin B (SspC; Massimi *et al.*, 2002). The organisations of the *scp* and *ssp* operons are similar, where *scpA* and *scpB* are homologous to *sspB* and *sspC* (Figure 1.8). SspB is cleaved from a secreted proenzyme by SspA (Chan and Foster, 1998; Rice *et al.*, 2001), although the uncleaved proenzyme has protease activity (Massimi *et al.*, 2002). Staphostatin B (SspC) is an intracellular inhibitor of staphopain B (SspB), and *sspC* mutants display a profound pleiotropic phenotype due to the degradation of several cytoplasmic proteins by SspB (Shaw *et al.*, 2005). Staphostatins represent a novel element of post-translational protease control, exhibiting marked specificity and no cross-inhibition (Rzychon *et al.*, 2003). They appear to prevent intracellular toxicity of the unsecreted cysteine proteases that possess enzymatic activity even as pre-proenzymes (Rzychon *et al.*, 2003; Wladyka *et al.*, 2005; Shaw *et al.*, 2005).

Deletion of *sspB* caused attenuation of virulence in a murine abscess model of infection (Shaw *et al.*, 2004), whereas non-polar deletion of *sspA* had no influence

on virulence (Rice *et al.*, 2001). Mutation of *sspC* caused a growth defect and changes in the production of secreted proteins, due to the degredation of cytoplasmic proteins, presumably by SspB (Shaw *et al.*, 2005), implying that SspB is the protease that is most important for virulence in this operon.

1.3.10.4 Serine protease-like operon

Six putative serine protease-like genes (*splABCDEF*) were identified in the *spl* operon. The predicted products share significant homology with each other, and with the SspA serine protease (Reed *et al.*, 2001). They contain secretory signal sequences but no profragments, implying that they are secreted but do not require extracellular activation. All 6 proteins contain the catalytic triad of trypsin-like serine proteases, and at least two, SplB and SplC, exhibit serine protease activity (Reed *et al.*, 2001). The operon is maximally expressed in post-exponential phase. The operon was present in only 64% of a small sample of *S. aureus* strains tested, although one additional strain contained part of the operon. Deletion of the *spl* operon had no effect on the virulence of strain RN6390 in a rat systemic infection model (Reed *et al.*, 2001). Though their importance in virulence may be limited, the lack of a requirement for activators means that these proteases may be responsible for the activation of other proteases in the cascade (Shaw *et al.*, 2004). Perhaps the presence of any one of these homologous proteases is sufficient for such an activation function.

1.3.10.5 Activation and regulation of proteases

The extracellular proteases of *S. aureus* are activated in a cascade that features aureolysin close to the top (Figure 1.8). Aureolysin is activated from a pro-form by an unidentified protease (possibly one of the Spl proteases). Activated aureolysin cleaves pro-SspA (V8 serine protease) to its active form. In the absence of aureolysin this activation occurs at a very low level, implying that another unidentified protease is capable of this cleavage. SspA cleaves the cysteine protease SspB from its pro-form, although the pro-form itself has enzymatic activity, which is inhibited intracellularly by staphostatin B. The cysteine protease ScpA is cleaved from its pro-form, which also has enzymatic activity and is inhibited intracellularly by staphostatin A. This cleavage may be auto-catalytic

(Shaw *et al.*, 2004; 2005). The position of the Spl proteases in this cascade is uncertain, and though they may be involved in the initial steps of aureolysin activation, no evidence for this has been presented. Their placement in the cascade is based on the observation that the Spl proteases appear not to require activation, and may therefore play a role in the first activation steps of the cascade.

All *S. aureus* extracellular proteases are produced in post-exponential phase and are upregulated by the cell density-sensing mechanism *agr* (Section 1.5.1.1; Shaw *et al.*, 2004; Reed *et al.*, 2001). With the exception of *spl*, which is upregulated by SarA, the extracellular protease operons of *S. aureus* are down-regulated by SarA (Section 1.5.2.1; Dunman *et al.*, 2001; Shaw *et al.*, 2004). The influence of the alternative sigma factor, SigB, on the regulation of these proteases is likely to be mediated through *agr* or *sarA* (Section 1.5.3.2; Bischoff *et al.*, 2004; Shaw *et al.*, 2004). *sarA* and *agr* may control proteases in response to the environment, allowing rapid turnover of specific products in response to particular stimuli (Karlsson *et al.*, 2001; Lindsay and Foster, 1999; McAleese *et al.*, 2001).

1.3.11 Iron uptake systems

The ability to sequester iron in the body is a primary host defence mechanism against bacterial infection. Bacteria require iron for biosynthesis, as a structural component of cytochromes (as haem) and as a cofactor for redox-active proteins in energy metabolism. Bacterial iron uptake systems specific for host iron-binding molecules, such as transferrin, lactoferrin and hemin, combine to allow uptake of this vital nutrient (Braun, 2001). Iron uptake can involve secreted siderophores that bind free iron and are recognized and transported into the bacterial cytoplasm. *S. aureus* iron uptake systems comprise siderophores, siderophore- or haem-binding molecules on the cell surface and ATP-dependent permeases for transport across the cytoplasmic membrane. Genomic analysis has revealed that *S. aureus* contains seven loci with homology to iron transport systems (Skaar *et al.*, 2004a). Of these, five have been characterised to some degree.

S. aureus produces at least four siderophores including staphyloferrin A, staphyloferrin B, aureochelin and staphylobactin (Drechsel *et al.*, 1993; Haag *et al.*, 1994; Konetschny-Rapp *et al.*, 1990; Courcol *et al.*, 1997; Dale *et al.*, 2004a). The genes responsible for the production of these siderophores are unknown, with

the exception of staphylobactin (encoded by the nine-gene *sbn* operon; Dale *et al.*, 2004a). *S. aureus* can transport these and other (non-staphylococcal) siderophores into the cell, where they are used as an iron source.

The *sirABC* operon encodes an iron transport system essential for staphylobactin uptake. It is divergently transcribed from the *sbn* operon and regulated by Fur, the iron-responsive transcriptional regulator (Heinrichs *et al.*, 1999; Dale *et al.*, 2004b). The system comprises a lipoprotein, SirA, and an ATP-binding cassette (ABC) transporter made up of SirB and SirC. ABC transporter systems couple ATP-hydrolysis to the transport process, typically containing an ATP-binding domain and two transmembrane domains (forming a permease) that facilitate transport of specific solutes across the membrane (Higgins, 2001). *sbn*, *sirA* and *sirB* mutants display growth deficiency compared to wild type *S. aureus* in iron-restricted growth media, indicating that this iron-uptake system is important for *S. aureus* growth (Dale *et al.*, 2004a; 2004b).

The iron-regulated locus *sstABCD* also encodes a putative siderophore transporter homologous to siderophore-transport systems in other bacteria. SstA and SstB are believed to form the membrane permease of an ABC transporter, of which SstC is the ATPase. SstD is a lipoprotein (Morrissey *et al.*, 2000). Reduction of SstD levels using antisense RNA did not affect the growth of *S. aureus* under iron-limiting conditions, indicating that alternative iron-uptake systems can compensate for loss of SstD (Morrissey *et al.*, 2000).

A third siderophore transport system, encoded by the *fhu* (ferric hydroxamate uptake) operon, allows the scavenging of hydroxamate siderophores (Sebulsky and Heinrichs, 2001; Sebulsky *et al.*, 2000; 2003). This system involves two lipoproteins on the outer surface of the cytoplasmic membrane (FhuD1 and FhuD2) that direct the iron-containing siderophore to an ABC transporter system consitsting of the FhuC ATPase and a membrane-embedded permease, made up of FhuB and FhuG (Sebulsky *et al.*, 2003).

In addition to the siderophore-transport systems, *S. aureus* possesses at least two haem-iron-uptake systems. One of these is *hts* (haem transport system), encompassing *htsABC*. HtsB and HtsC form an ABC transporter permease. The ATP-binding moiety of this system is unknown. HtsA is likely to be under the control of Fur (Skaar *et al.*, 2004a). Disruption of the *hts* locus caused decreased virulence of *S. aureus* in intravenously infected mice. This haem transport system

may be involved in the scavenging of haem-iron in the liver and other tissues during infection (Skaar *et al.*, 2004a).

The *isd* locus is also involved in the transport of haem-iron across the cell envelope of *S. aureus*. The *isd* locus comprises five transcriptional units (*isdA*, *isdB*, *isdCDEFsrtBisdG*, *isdH* and *isdI*). The iron-regulated system includes cell wall-anchored surface proteins (IsdABCH), a membrane transporter (IsdDEF), a sortase (SrtB) and two cytoplasmic proteins (IsdGI) (Skaar and Schneewind, 2004). IsdC has an atypical cell-wall sorting signal (NPQTN) and is a substrate for sortase B (SrtB), encoded within the same operon. IsdA, IsdB and IsdH contain LPXTG sorting signals and are substrates for sortase A (see Section 1.1.3; Mazmanian *et al.*, 2003).

The cell wall-anchored protein IsdA binds transferrin, hemin, fetuin and haemoglobin. IsdA also has high affinity for fibrinogen and fibronectin (see Section 1.3.9.5; Clarke *et al.*, 2004). IsdB binds haemoglobin and IsdH binds haptoglobin (Dryla *et al.*, 2003; Mazmanian *et al.*, 2003). IsdC can bind hemin and may be involved in trafficking haem-iron through the cell wall peptidoglycan to the cytoplasmic membrane, where IsdD, IsdE and IsdF form an ABC transporter that is necessary for passage of the haem-iron into the cytoplasm (Taylor and Heinrichs, 2002; Mazmanian *et al.*, 2002; 2003; Skaar and Schneewind, 2004). Inside the cell, IsdG and IsdI bind the haem-iron with high affinity and degrade it, releasing biliverdin and free iron, which is used as a nutrient source (Skaar *et al.*, 2004b). Inactivation of *srtA*, *srtB* or individual components of the *isd* system does not abolish the ability of *S. aureus* to utilise haem-iron as a nutrient source, indicating that other pathways (likely including *hts*, above) are also involved. In addition, the growth rate of *S. aureus* cells was not affected by *isdA* mutation, indicating that other iron acquisition systems are capable of substituting for IsdA (Clarke *et al.*, 2004).

Both the *fhu* and *isd* iron-uptake systems contain multiple extracellular receptors for iron-containing compounds and a single ABC transporter. Each of these iron-uptake systems is induced under low-iron conditions, and *S. aureus* may use them to access iron from multiple sources in different environments within the host. For example, early in systemic infection staphylococci predominantly utilise haem-iron, and later in infection, when bacteria have occupied niches in tissues that are devoid of haem-iron, siderophores assume a vital function for the acquisition of iron from non-haem sources (Skaar *et al.*, 2004a). The presence of

multiple iron-uptake systems underlines the importance of iron acquisition in the progression of *S. aureus* disease.

1.3.12 Biofilm formation

During the past 20 years *Staphylococcus epidermidis* has emerged as a leading cause of nosocomial infections (von Eiff *et al.*, 2002). It is normally a harmless commensal of the skin, lacking the multiple virulence factors that allow *S. aureus* to invade the host and thwart the immune system (Gill *et al.*, 2005). However, it has come to prominence as a pathogen due to its ability to colonise implanted medical devices, where biofilm formation is a crucial factor (von Eiff *et al.*, 2002).

The multi-layered high-density structure of biofilm protects the bacteria from antibiotics and the immune system (Costerton *et al.*, 1999). Formation of a biofilm is initiated when bacteria adhere to biomaterial surface, a process that is mediated by surface-associated proteins such as the major autolysin AtlE and the fibrinogen-binding protein SdrG (also called Fbe), which is homologous to ClfA of *S. aureus* (see Section 1.3.9.1; Heilmann *et al.*, 1997; Nilsson *et al.*,1998; Hartford *et al.*, 2001). The majority of cells in the biofilm are not in contact with the surface but are held together by the polysaccharide intercellular adhesin (PIA), a charged homopolymer consisting of β(1→6)-linked N-acetylglucosamine. As well as providing the scaffolding that holds the multilayered cell complex together, PIA has recently been shown to contribute directly to the avoidance of innate immunity by promoting resistance to antimicrobial peptides (Vuong *et al.*, 2004).

Some clinical isolates from device-related infection form biofilm *in vitro* but do not express PIA (Rohde *et al.*, 2005). This PIA-independent biofilm formation requires expression of the surface-located accumulation-associated protein Aap. The intact, full length protein is cleaved by a serine protease, which removes the N-terminal A domain, exposing a repeated region that promotes intercellular interactions (Rohde *et al.*, 2005).

The ability to form biofilm is also likely to be important in the pathogenesis of *S. aureus* infection. *S. aureus* carries genes homologous to the *ica* genes necessary for PIA expression in *S. epidermidis*, and some *S. aureus* strains have been shown to express PIA, particularly when grown under anaerobic conditions (Fluckiger *et al.*, 2005; Cramton *et al.*, 2001) and *in vivo* (McKenney *et al.*, 1999). However, the role of PIA in the avoidance of innate immunity by *S. aureus* has not

been investigated, although the important role of PIA in *S. epidermidis* suggests that it is likely to be a virulence factor in *S. aureus*.

1.4 Vaccines and novel therapeutic approaches to *S. aureus* infection

S. aureus possesses a wide range of virulence factors that exacerbate infection and thwart the host immune system. The treatment of *S. aureus* disease is complicated by the emergence of resistance to antimicrobial agents. Some *S. aureus* strains have developed resistance to all of the commonly used antibiotics, including those used to combat nosocomial infection, e.g. vancomycin (see Section 1.2). Although antibiotic-resistant strains are no more virulent than susceptible strains, they are more difficult to eradicate, particularly in immunocompromised or elderly patients (Lowy, 1998).

Traditional antimicrobial chemotherapy targets bacterial metabolism and biosynthesis, leading either to cell lysis or to inhibition of bacterial growth, which assists the host immune system in clearing infections. Active vaccination, on the other hand, primes the host immune system in advance, allowing it to recognise specific bacterial pathogens and to develop immunological memory. Passive immunotherapy uses antibodies raised in other hosts, administered to aid in the clearance of bacteria, through blocking the functions of target molecules and enhancement of PMNL phagocytosis, in naïve or immunocompromised individuals. Vaccination must be undertaken in advance and delivers long-term immunity, whereas antimicrobial chemotherapy and passive immunisation can be used both prophylactically and to treat pre-existing infection, but give only short-term protection.

New methods have recently been developed to identify potential *S. aureus* targets for antimicrobial drugs and vaccines. Potential vaccine antigens include those that are expressed exclusively or at elevated levels during growth in the infected host. Convalescent human sera have been used to screen for immunoreactive proteins that are expressed *in vivo*. This approach has been used to screen random short peptides from the *S. aureus* genome (Weichhart *et al.*, 2003; Etz *et al.*, 2002), cell lysates and extracts using two-dimensional electrophoresis (Vytvytska *et al.*, 2002) and to test the responses to individual purified recombinant surface proteins (Roche *et al.*, 2003; Dryla *et al.*, 2005).

Genes that are expressed at high levels during infection in animals were identified by *in vivo* expression technology (IVET; Lowe *et al.*, 1998), and genes that were required for survival of bacteria *in vivo* were identified by signature-tagged mutagenesis (STM; Mei *et al.*, 1997; Coulter *et al.*, 1998). Genes required for full virulence in a murine model of systemic infection were identified by DNA size marker identification technology (SMIT; Benton *et al.*, 2004). Several biosynthetic, catabolic, regulatory and transport genes were identified, as well as known virulence factors and genes that were previously unknown, demonstrating that there are likely to be many genes with potential as targets. However, these technologies have been disappointing in their ability to identify new or novel virulence factors.

To date several approaches have been taken to develop vaccines and immunotherapy to combat staphylococcal infections.

1.4.1 Bivalent capsular polysaccharide conjugate vaccine: 'StaphVAX'

Capsular polysaccharides are poorly immunogenic, and a booster response to them is not observed after repeated immunisations (O'Riordan and Lee, 2004). Anti-capsular polysaccharide antibodies are, however, strongly pro-opsonic (Thakker *et al.*, 1998). Capsular polysaccharide coupled to a protein carrier molecule is more immunogenic, produces increased T-cell-dependent responses compared to the native polysaccharide and generates a booster response upon repeated immunisation. *S. aureus* capsular polysaccharide of serotypes 5 (CP5) and 8 (CP8) (representing 85% of clinical *S. aureus* strains) conjugated to the non-toxic recombinant exotoxin A from *Pseudomonas aeruginosa* gave rise to serum antibodies in mice after two injections. The third immunisation generated a booster response (Fattom *et al.*, 1993).

StaphVAX is a bivalent anti-capsular polysaccharide vaccine consisting of conjugates of CP5 and CP8 with *P. aeruginosa* exotoxin A and has been developed by NABI, Inc. (Florida, USA). It has undergone phase I, II and III clinical trials (Fattom *et al.*, 1993; Welch *et al.*, 1996; Shinefield *et al.*, 2002). The vaccine was administered in two doses (6 weeks apart) to patients with end-stage renal disease, who are particularly susceptible to *S. aureus* infection. The vaccine resulted in more than four-fold increases in antibody titre in approximately 75% of patients and demonstrated some efficacy in the four weeks following the second

injection. Antibody levels and the level of protection against *S. aureus* declined after 40 weeks (Shinefield *et al.*, 2002). A second phase III clinical trial of this vaccine is underway to try to improve its efficacy. A second-generation vaccine is being developed that also includes capsular polysaccharide from strain 336, resulting in greater coverage of clinically relevant *S. aureus* strains. Perhaps the conjugation of capsular polysaccharides to a staphylococcal protein antigen (rather than a *P. aeruginosa* toxoid) would improve immunogenicity.

1.4.2 Passive immunisation against ClfA: 'Veronate'

Antibodies against surface proteins have two important biological properties. Firstly, like those raised against capsular polysaccharide, they are pro-opsonic, enhancing phagocytosis and bacterial clearance (Rozalska *et al.*, 1993; Nilsson *et al.*, 1998). Secondly, antibodies against adhesins have the potential to block adherence of *S. aureus* to host ligands, preventing adherence of bacteria to surfaces (McDevitt *et al.*, 1995; Mohamed *et al.*, 1999; Rennermalm *et al.*, 2001).

The gene encoding clumping factor A, *clfA* (see Section 1.3.9.1), is present in 100% of invasive isolates and 98% of carriage isolates of *S. aureus* (Peacock *et al.*, 2002). It is expressed during infection (Etz *et al.*, 2002; Weichhart *et al.*, 2003) and isogenic *clfA* mutants are attenuated in animal models of *S. aureus* infection (Moreillon *et al.*, 1995; Que *et al.*, 2000; Stutzmann Meier *et al.*, 2001; Josefsson *et al.*, 2001). ClfA therefore represents a good vaccine candidate, and studies in animal models have shown that immunisation against ClfA or prophylaxis with anti-ClfA antibodies gives protection (Josefsson *et al.*, 2001; Brouillette *et al.*, 2002).

Passive immunisation is the administration of polyclonal or monoclonal antibodies, produced exogenously (intravenous immunoglobulin, or IVIG), in order to prevent or treat disease. One rationale for the use of IVIG is to replace a broad spectrum of missing antibodies in an immunocompromised host, thereby providing protection against common pathogens (Sacher, 2001). IVIG also has the potential to be used prophylactically following exposure to pathogens, including viruses, or to combat infection (Sawyer, 2000; Snydman, 2001).

Josefsson *et al.* (2001) showed that both human and rat antibody preparations containing high titres of anti-ClfA-specific IgG could be used prophylactically to decrease mortality in a murine model of *S. aureus* sepsis. Inhibitex Inc. (Georgia, USA) have developed a hyperimmunoglobulin, SA-IGIV, derived from plasma

donors with high titres of anti-ClfA IgG, for the prevention of staphylococcal infections in very low birthweight infants (Vernachio *et al.*, 2003). This IgG preparation recognised the *S. aureus* cell surface, specifically inhibited bacterial adherence to immobilised fibrinogen, and acted as an efficient opsonin in an *in vitro* phagocytosis assay. When used in conjunction with vancomycin, SA-IVIG was therapeutically effective in a rabbit model of catheter-induced infective endocarditis caused by an MRSA strain (Vernachio *et al.*, 2003). A clinical trial of SA-IVIG is currently underway to evaluate its safety and efficacy.

1.4.3 Passive immunisation with humanised monoclonal antibody: 'Aurexis'

In addition to the SA-IVIG product, Inhibitex Inc. have developed a humanised monoclonal antibody product, Aurexis. A murine monoclonal antibody was raised against the ligand-binding region A of ClfA and can both inhibit ClfA-mediated adherence to fibrinogen and promote phagocytosis. Humanisation of this antibody removed potentially antigenic murine epitopes in the molecule and replaced parts of the Fc region that differed from human IgG. The antibody efficiently protected against intravenous challenge (Hall *et al.*, 2003) and significantly enhanced the therapeutic efficacy of vancomycin in a model of established infectious endocarditis (Patti, 2004). Aurexis is currently undergoing phase II clinical trials aimed at use in the therapy of *S. aureus* bacteraemia, in conjunction with conventional antimicrobial therapies (Patti, 2004).

Inundating an infected host with highly specific anti-staphylococcal antibodies aids in efficient opsonophagocytosis (Patti, 2004), and aids in complement deposition and the development of protective immunity. Antibodies have also been observed to penetrate biofilms (Maira-Litran *et al.*, 2002) and have been observed to detach bound cells from immobilised substrates (Visai *et al.*, 2000). Planktonic bacteria would likely become more susceptible both to antibiotics and to the host immune system. The use of protective antibody preparations in conjunction with conventional antimicrobial chemotherapy is a potentially new method of treatment for acute infectious diseases (Patti, 2004). More rapid elimination of bacteria from the body may additionally aid in reducing the development of antimicrobial resistance.

In addition, a DNA vaccination approach has been used to some effect in heifers. Immunisation with DNA encoding the fibrinogen-binding domain of ClfA and the fibronectin-binding domain of FnBPA was partially protective when followed by a booster injection of the recombinant proteins (Brouillette *et al.*, 2002; Shkreta *et al.*, 2004). DNA vaccination against surface proteins may be a viable approach towards protecting animals against *S. aureus* infection.

1.5 Regulation of virulence factors

The production of exoproteins *in vitro* by *S. aureus* follows a temporal programme. In general, adhesins are produced before toxins and enzymes (Chan *et al.*, 1998; Novick, 2003; Cheung *et al.*, 2004). This may also be the case *in vivo* (Novick, 2003). It is believed that the expression of adhesins at the beginning of the infectious process allows the bacteria to establish themselves at a focus of infection. Once established, *S. aureus* secretes exoproteins that damage host tissue and thwart the immune response (see Section 1.3). These exoproteins also facilitate detachment and spreading throughout the body (McAleese *et al.*, 2001; McGavin *et al.*, 1997). Although it may not be strictly true, this is a useful paradigm for the study of surface proteins and secreted virulence factors. The bi-phasic pattern of expression is controlled by a density-sensing mechanism encoded by the *agr* locus, other two-component regulatory systems, transcription factors and alternative sigma factors.

1.5.1 Two component regulatory systems

Two-component regulatory systems generally consist of a sensor, which is often an integral membrane protein, and a response regulator, which is cytoplasmic and is often a transcription factor. Stimulation of the sensor leads to activation of an enzymatic domain that activates the response regulator via phosphorylation. The response regulator acts on target genes, either directly or indirectly regulating their expression (Stock *et al.*, 1989). This allows the expression of specific genes or subsets of genes in response to particular stimuli.

1.5.1.1 The accessory gene regulator

The Agr (accessory gene regulator) system is a two-component population density-sensing system that is activated in late-exponential phase, upregulating many secreted proteins and downregulating several surface proteins (Novick and Jiang, 2003; Dunman *et al.*, 2001). *agr* induction causes downregulation of protein A and upregulation of α-haemolysin and DNA gyrase in post-exponential phase (Dunman *et al.*, 2001; Novick, 2003).

The *agr* locus consists of two divergent transcription units, driven by promoters P2 and P3. The P2 operon encodes AgrACDB, the components of the sensing system, and the P3 transcript is RNAIII, the effector molecule (Figure 1.9). AgrC is a transmembrane signal receptor (Lina *et al.*, 1998). The *agr*-activating ligand is the post-translationally modified auto-inducing peptide (AIP), which is processed from AgrD, presumably by AgrB (Zhang *et al.*, 2002), though an additional membrane protein, SvrA, may also be involved (Garvis *et al.*, 2002). AIP is secreted from the cell and binds to the extracellular N-terminal domain of AgrC (Ji *et al.*, 1995), leading to activation of the response regulator, AgrA, via a phosphorylation event (Koenig *et al.*, 2004). Activated AgrA then binds to the P2 and P3 promoters, inducing further production of AIP, AgrC and AgrA from P2 and transcription of RNAIII from P3 (Koenig *et al.*, 2004).

The downstream effects of *agr* activation are mediated not through AgrA, which binds only to the P2-P3 region of the *agr* locus, but by RNAIII (Koenig *et al.*, 2004). RNAIII, which also encodes δ-haemolysin, has a complex secondary structure that gives it stability (Benito *et al.*, 2000). The mechanisms by which RNAIII regulates transcription are uncertain. It may act by binding the RNA encoding target proteins, interfering with their translation, or by binding regulatory proteins, titrating them away from binding sites at target genes (Novick, 2003). Binding of *hla* mRNA (encoding α-haemolysin) by complementary sequences in the 5' end of RNAIII prevents folding into an untranslatable configuration, causing upregulation of *hla* at the translational level (Novick, 2003). Conversely, translation of *spa* mRNA is blocked by binding of the 3' end of RNAIII to the translation initiation site via complementary sequences (Novick, 2003). In this way, *agr* may selectively upregulate or downregulate specific target genes.

When cell density rises the concentration of extracellular AIP increases, causing induction of *agr*. Fibrinogen-mediated clumping has been observed to cause

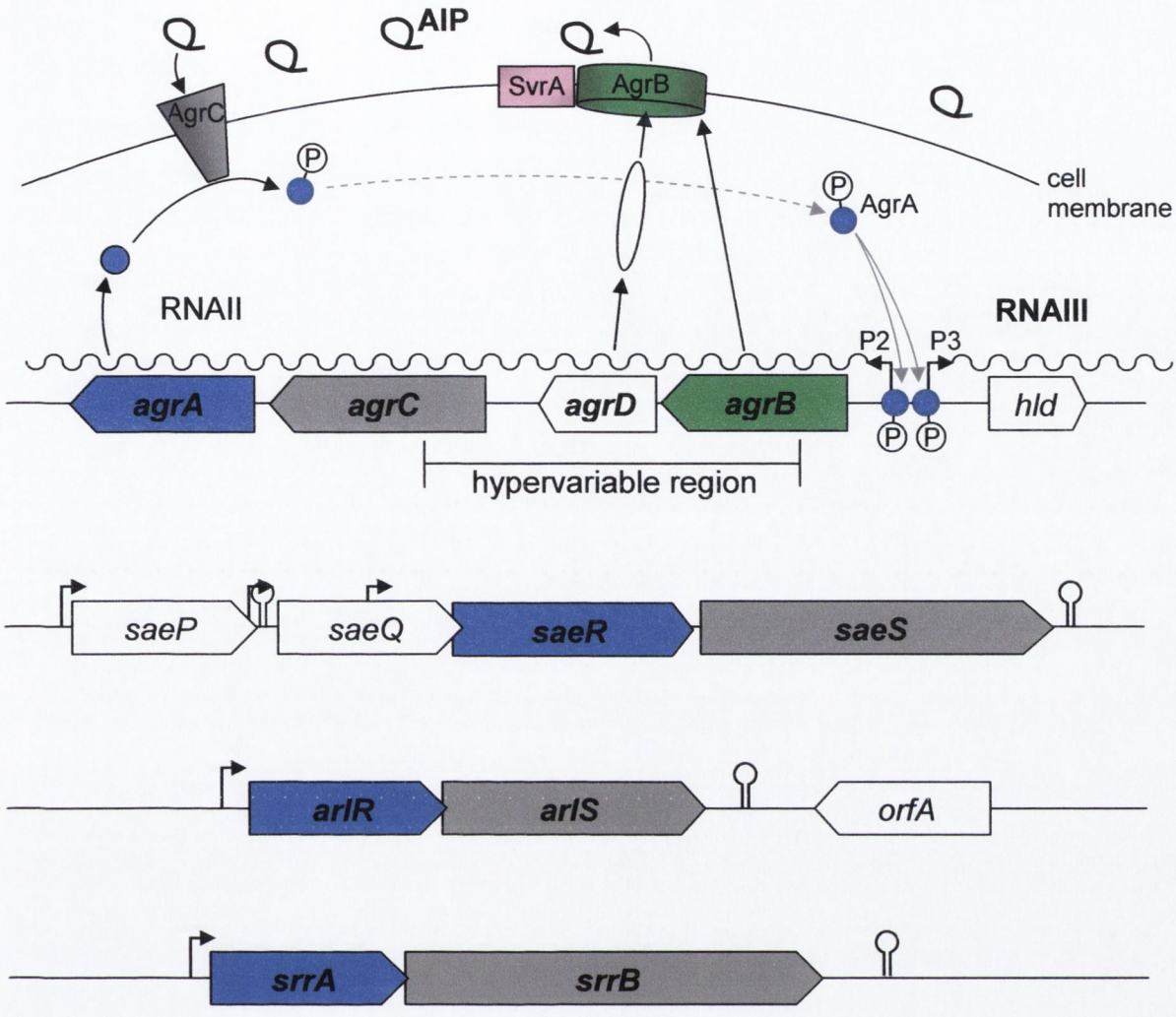


Fig 1.9 Two component regulatory systems in *S. aureus*

agrC, *saeS*, *arlS* and *srrB* (grey) encode sensor proteins, while *agrA*, *saeR*, *arlR* and *srrA* (blue) encode response regulators. Components labelled in bold form part of the defined regulatory system. Arrows and hairpin loops indicate promoters and transcription terminators, respectively. In the *agr* system AgrD is processed by AgrB (and SvrA) to form the secreted auto-inducing peptide, AIP. Binding of AIP to the sensor AgrC allows phosphorylation of AgrA, which can then bind to the P2 and P3 promoter regions causing auto-induction of the system. AgrA binds only to the P2-P3 region. The effector molecule of *agr* is RNAIII, produced from P3. SaeR, ArlR and SrrA are the effectors of their respective regulatory systems, regulating the expression of specific target genes in response to the stimuli transmitted through their sensor proteins. The putative genes *saePQ* may also be involved in the *sae* response, but their roles have not been defined.

induction of *agr* (Rothfork *et al.*, 2003). Although this is likely to be relevant to quorum sensing *in vivo*, a feedback effect on ClfA is unlikely, as ClfA is Agr-independent (Wolz *et al.*, 1996; Dunman *et al.*, 2001).

The *agr* system is conserved in all staphylococcal species, and has numerous subgroups based on variations in the amino acid sequences of AIP and the sensor region of AgrC, encoded by a hypervariable region within *agrBDC* (Figure 1.9; Ji *et al.*, 1997). *S. aureus* has 4 *agr* specificity groups. The AIP of each group can block autoinduction and thus virulence gene expression in other groups. This novel type of interference between staphylococci may serve to isolate groups in a mixed infection (Novick, 2003). *agr* has been shown to be a virulence factor in several animal infection models (Abdelnour *et al.*, 1993; Gillaspy *et al.*, 1995). In addition, mutations in *agr* are associated with increased biofilm formation and persistence *in vivo* (Yarwood and Schlievert, 2003). The role of *agr* in virulence is likely to be due to its regulatory influence on other virulence factors, rather than any direct interaction with the host.

1.5.1.2 Other two-component regulatory systems

Seven other two-component regulatory systems have so far been characterised to some degree in *S. aureus*. They are encoded by *saeRS*, *arlRS*, *ssrAB* (Figure 1.9), *lytRS*, *yycGF*, *vraSR* and *yhcSR*.

The *saeRS* (*S. aureus* exoprotein expression) locus encodes a classical two-component regulatory system that allows response to several environmental stimuli, including high salt, low pH, glucose and subinhibitory concentrations of antibiotics (Novick, 2003). *sae* is involved in the upregulation of α- and β-haemolysins, DNase, coagulase and fibronectin binding protein A at the transcriptional level (Giraudo *et al.*, 1997; Steinhuber *et al.*, 2003), and downregulates capsular polysaccharide production in strain Newman (Steinhuber *et al.*, 2003). The Sae response does not influence *agr* or act via RNAIII (Giraudo *et al.*, 1997). Although it can be transcribed in the absence of *agr*, *sae* is upregulated by *agr* (Giraudo *et al.*, 2003). *sae* may therefore form a link between cell density signalling and environmental signalling in *S. aureus* (Novick and Jiang, 2003).

ArlRS (autolysis-related locus) was identified by transposon insertion as a two-component regulator of autolysis and the NorA multidrug efflux pump (Fournier

and Hooper, 2000). This system either directly or indirectly decreases the production of α- and β-haemolysins, lipase, coagulase, SspA serine protease and particularly protein A. The effects of *arl* on virulence factors are mediated mainly through SarA and Agr (Fournier *et al.*, 2001). It has recently been shown that *arl* indirectly regulates NorA via MgrA (Kaatz *et al.*, 2005) and that the effect of ArlRS on *spa* transcription is mediated through an indirect effect on DNA supercoiling (Fournier and Klier, 2004). The stimulus to which *arl* responds is not known.

lytRS is a two-component regulatory system that is also involved in autolysis. LytS is predicted to contain 6 transmembrane domains at its N-terminus, while other sensor proteins in *S. aureus* contain only two. The structure is similar to transport proteins, implying that the response of LytRS involves transmembrane transport, though the stimulus is unknown. *lyt* upregulates *lrgA* and *lrgB* (directly downstream of *lytRS*), which inhibit the extracellular activity of murein hydrolases (Brunskill and Bayles, 1996). Inactivation of these autolysins prevents cell growth by restricting addition to the peptidoglycan layer of the cell wall and also confers penicillin tolerance (Groicher *et al.*, 2000).

The *srrAB* (staphylococcal respiratory response) locus (also known as *srhSR*) is involved in anaerobic respiration. Although capable of normal growth in aerobic conditions, *srr* mutants are growth-defective in anaerobic conditions (Yarwood *et al.*, 2001; Throup *et al.*, 2001). *srr* is involved in regulation of energy metabolism, and may be activated by menaquinone. It inhibits the expression of *agr* RNAIII and may itself be directly repressed by *agr*, forming an autorepression circuit. In this way, *ssr* may form a link between energy metabolism and cell density signalling (Yarwood and Schlievert, 2003).

The *yycGF* locus of *S. aureus* is homologous to *yycGF* of *B. subtilis* and is specific to and essential for growth in low-G+C-content Gram-positive bacteria. A consensus YycF binding site has been identified, and direct binding to three promoter regions (*ssaA*, *isaA* and *lytM*) has been demonstrated *in vitro* (Dubrak and Msadek, 2004). 12 *S. aureus* genes possess the consensus YycF-binding site, including staphylokinase (Section 1.3.5.2) and the surface protein SdrD (Section 1.3.9.3). Genes controlled by the *yycFG* two-component regulatory system include putative or proven virulence factors (e.g. *sdrD*, *sak*, *IsaA*) or are involved in cell wall metabolism (e.g. the glycylclycine endopeptidase, *lytM*). The *yycFG* system may be essential in low-G+C-content Gram positive bacteria because of its involvement in cell wall metabolism (Dubrak and Msadek, 2004).

The *vraSR* (vancomycin resistance-associated sensor/regulator) locus is involved in S. aureus resistance to vancomycin and is upregulated in hetero-vancomycin resistant *S. aureus* strains (Kuroda *et al.*, 2001). It is not essential for growth, but regulates cell wall biosynthesis through the control of murein monomer biosynthesis and peptidoglycan polymerisation (Kuroda *et al.*, 2003). The VraSR system is induced in response to the presence of glycopeptide antibiotics, downregulating cell wall biosynthesis, resulting in increased antibiotic resistance.

The recently identified two-component regulatory system encoded by the *yhcSR* locus is essential for *S. aureus* growth *in vitro*. In addition, the induction of *yhcSR* increases resistance to cell-wall active antibiotics. YchSR may regulate the expression of genes essential for *S. aureus* growth, including cell wall biosynthetic pathways (Sun *et al.*, 2005).

These two-component regulatory systems respond to specific extracellular stimuli and modulate the expression of target genes. Analysis of the complete genome sequence of *S. aureus* identified up to 16 potential two-component regulatory systems, including those described above. Additional, uncharacterised systems may allow response to additional environmental stimuli, particularly those encountered *in vivo* during infection.

1.5.2 Transcription factors

Transcription factors interact with DNA to increase or decrease the rate of transcription of target genes. Apart from the response regulators associated with two-component regulatory systems, the transcription factors known in *S. aureus* bear significant sequence and structural homology to *sarA*. SarA and its homologs have a conserved winged-helix structure that is common in eukaryotic DNA-binding proteins (Cheung *et al.*, 2004). The proteins in this family can be categorised on the basis of structure: single-domain proteins that occur as homodimers and two-domain proteins that occur as monomers.

1.5.2.1 Single domain proteins

SarA (staphylococcal accessory regulator) is a global regulator of *S. aureus* virulence factors. The expression of *sarA* reaches its peak in late exponential phase (Manna and Cheung, 2001), and coincides with activation of *agr*, possibly

due to direct interaction between SarA and the *agr* promoters (Chien and Cheung, 1998). SarA is a small (14.5 kDa) basic protein encoded on three overlapping transcripts, produced from the promoters P1, P2 and P3. P3 is dependent on the stationary phase accessory sigma factor, SigB (Section 1.5.3.2), while P1 and P2 are SigA-dependent (Section 1.5.3.1). This may allow expression of *sarA* in a variety of niches within the host and regulation in response to at least three independent stimuli.

The crystal structure of SarA revealed that it is a single-domain protein that occurs as homodimers, with each monomer containing a four-helix core region and an 'inducible region' comprising a β-hairpin turn and a carboxy-terminal loop (Shumacher *et al.*, 2001). Upon DNA binding, conformational changes occur that allow the inducible regions to encase the DNA. SarA binds as a dimer to an AT-rich sequence in the promoter regions of target genes (Chien *et al.*, 1999; Rechtin *et al.*, 1999). A shorter consensus SarA binding site has been identified as ATTTTAT (Sterba *et al.*, 2003).

SarA activates its own expression and is downregulated by SarR (Bayer *et al.*, 1996; Manna and Cheung, 2001). SarA upregulates *agr* by binding to the region between P2 and P3 (Morfeldt *et al.*, 1996; Dunman *et al.*, 2001). Phenotypically *sarA* promotes the synthesis of the fibronectin-binding proteins FnBPA and FnBPB, the fibrinogen-binding protein ClfB and haemolysins, and inhibits the production of protein A, aureolysin and the cysteine protease SspB (Dunman *et al.*, 2001). Mutation of *sarA* also reduces the capacity of *S. aureus* to form biofilm *in vitro* and *in vivo* (Valle *et al.*, 2003; Beenken *et al.*, 2003; 2004). Many of these effects are likely to be mediated indirectly by other regulators affected by SarA, such as *agr* (Section 1.5.1.1) and *sarT*, below.

SarR is a 13.6-kDa regulatory protein that binds to the *sarA* promoters and downregulates *sarA* expression. Its expression peaks during the post-exponential phase of growth, allowing repression of *sarA* transcription in late exponential and stationary phase (Manna and Cheung, 2001). Its dimeric structure has two winged-helix domains and a central α-helical core. The winged-helix domains each consist of a helix-turn-helix motif and a β-hairpin turn, which are both DNA binding domains (Liu *et al.*, 2001). The helix-turn-helix domain may interact with the major groove of DNA while the β-hairpin turn interacts with the minor groove (Cheung *et al.*, 2004). The centre of the dimer is a canyon-like structure with a concave DNA-binding surface with many exposed positively-charged residues,

which are conserved amongst the SarA homologs (Cheung *et al.*, 2004). The activation domain on the opposite side of the molecule contains some patches of negatively charged residues, which are not conserved in the other SarA homologs.

SarT (formerly *sarH2*) is a 16.1-kDa basic protein that is downregulated by *sarA* or *agr* (Schmidt *et al.*, 2001). *sarT* represses α-haemolysin. Downregulation of *sarT* by *sarA* causes upregulation of *hla* transcription, explaining the indirect regulation of α-haemolysin production by *sarA* (Cheung *et al.*, 2004). *sarT* activates the transcription of *sarS* (Schmidt *et al.*, 2003) and represses the transcription of *sarU* (Manna and Cheung, 2003). *agr* may downregulate *sarT* in a pathway that also involves *saeRS* and *rot* (Cheung *et al.*, 2004). SarT acts as a regulatory link between other SarA homologs.

Rot (repressor of toxins) is larger than the other one-domain SarA homologs and has an acidic pH. Rot positively regulates *sarS* and the adhesins *spa* and *clfB,* and negatively regulates transcription of lipase, α- and β-haemolysins and the *spl* and *ssp* protease operons (Saïd-Salim *et al.*, 2003). By upregulating adhesins and downregulating toxins, *rot* may aid in the initiation of infection. Rot is likely to be inhibited post-transcriptionally by RNAIII of *agr* (Saïd-Salim *et al.*, 2003).

sarV and *sarX* were identified from the *S. aureus* genome as *sarA* homologs. SarV has been shown to be involved in the regulation of autolysis and is repressed by the binding of SarA or MgrA to the *sarV* promoter (Manna *et al.*, 2004). *sarV* may be part of the common pathway used by MgrA and SarA to control autolysis in *S. aureus*.

1.5.2.2 Two-domain proteins

The two-domain proteins of the SarA family contain two homologous domains, with each single domain sharing homology with the smaller, single domain proteins described above. The crystal structure of SarS (formerly *sarH1*) revealed that it is a monomeric winged-helix protein with two homologous halves (Li *et al.*, 2003). The structure of the SarS monomer is similar to the SarR dimer, containing two typical DNA-binding motifs separated by a well ordered loop. Extensive and conserved interactions between the two domains occur and the concave DNA binding surface is highly positively charged. The activation domain on the opposite side of the molecule contains negatively charged patches. SarS is

repressed by *agr* (probably via SarT or Rot) and activates protein A by binding directly to the *spa* promoter (Tegmark *et al.*, 2000).

SarU (formerly *sarH3*) is a 29.3-kDa protein with two homologous domains that is an activator of RNAIII and is repressed by SarT (Manna and Cheung, 2003). SarU, SarT and *agr* may from part of a feedback circuit that allows expression of *agr* at low cell density *in vivo*. *agr* downregulates *sarT* (in a pathway that also involves *saeRS* and *rot*), derepressing *sarU* and allowing activation of RNAIII. This may explain why RNAIII has been observed to be activated *in vivo* in *agrACDB* mutants (Xiong *et al.*, 2002). *sarY* was identified as a two-domain *sarA* homolog by sequence analysis but has not yet been characterised (Cheung *et al.*, 2004).

The structural data suggest that the SarA homologs interact with DNA in three ways. It appears that DNA needs to be foreshortened (possibly through local decreases in negative supercoiling) to fit into the central canyon-like structure before binding to the concave DNA-binding surface. The proteins may be involved in 'holding' promoter sequences in optimal or sub-optimal positions, thus increasing or decreasing expression (Cheung *et al.*, 2004). Additionally, multiple dimers may bind to promoter sequences, bending the DNA into a closed configuration that encircles the Sar proteins, preventing transcription. This mechanism may function in a similar way to mammalian histones. Thirdly, heterodimer formation may occur between the one-domain SarA homologs, interfering with the functions of homodimers, causing interference or new patterns of expression under certain growth conditions (Cheung *et al.*, 2004).

1.5.2.3 MarR-like proteins

MgrA (multiple gene regulator), formerly known as Rat (regulator of autolytic activity), is structurally more similar to MarR of *E. coli* than to single-domain or two-domain SarA homologs (Luong *et al.*, 2003). It has a dimeric winged-helix structure with extensive coiled-coil interactions between two unique α-6 helices from each monomer (Cheung *et al.*, 2004). *mgrA* is transcribed optimally from two promoters and positively regulates its own expression (Ingavale *et al.*, 2003). It is involved in autolysis and is an activator of capsular polysaccharide and nuclease production and a repressor of α-haemolysin, coagulase, proteases and protein A (Ingavale *et al.*, 2003; Luong *et al.*, 2003). MgrA affects the transcription of *hla*

and *spa* in a SarA-independent manner (Ingavale *et al.*, 2005) and regulates target genes in both an *agr*-independent pathway, involving direct binding to target promoters, and an *agr*-dependent pathway that does not involve SarT (Luong *et al.*, 2003). It is a specific repessor of the NorA efflux pump (Kaatz *et al.*, 2005). MgrA appears to be an important regulator of virulence determinants in *S. aureus*, although its place in the overall regulatory cascade has not been well defined. SarZ and two additional homologs of MgrA were discovered by homology searching of the *S. aureus* genome sequence, but have not been characterised (Cheung *et al.*, 2004).

1.5.3 Sigma Factors

Sigma factors interact with the RNA polymerase holoenzyme to allow initiation of transcription at specific promoter sequences. The promoter sequences recognised by different sigma (σ) factors differ, allowing specific subsets of genes to be transcribed when a particular σ-factor is available. *S. aureus* has three known σ-factors, SigA, SigB and SigH (Morikawa *et al.*, 2003).

1.5.3.1 Sigma factor A

The primary σ-factor in *S. aureus* is SigA (formerly PlaC; Deora and Misra, 1996). It bears significant homology to the *B. subtilis* and *E. coli* primary σ-factors (σ^A and σ^{70}, respectively) and, when reconstituted with *E. coli* RNA polymerase holoenzyme, can initiate transcription from both *S. aureus* and *E. coli* promoters *in vitro* (Deora and Misra, 1996). No strict consensus SigA binding site was identifiable upstream of *S. aureus* genes because of the AT-rich nature of the genome (Wang *et al.*, 2004). However, sequences similar to the *B. subtilis* consensus appear to promote transcription initiation in *S. aureus* (Deora and Misra, 1996). The SigA vegetative σ-factor is responsible for transcription from the majority of *S. aureus* promoters (Wang *et al.*, 2004). Disruption of the gene encoding SigA with an antibiotic resistance cassette was only possible in cells bearing the wild-type gene on a plasmid, indicating that the factor is necessary for cell growth (Nesin *et al.*, 1990).

1.5.3.2 Sigma factor B

The main accessory σ-factor in *S. aureus* is SigB. It bears homology to SigB of *B. subtilis*. SigB is one of 16 alternative σ-factors in *B. subtilis* and is involved in response to environmental stress (Hecker *et al.*, 1996). In *S. aureus* SigB is also involved in the stress response, allowing transcription of a specific subset of genes including virulence genes (Kullik *et al.*, 1998; Gertz *et al.*, 2000; Bischoff *et al.*, 2004). A consensus SigB-binding site (GttTaa – N_{12-15} – gGGTAt) has been identified in *S. aureus* (Gertz *et al.*, 2000; Homerova *et al.*, 2004).

The *sigB* loci in *B. subtilis* and *S. aureus* encode post-translational regulators of SigB (Figure 1.10). Under normal conditions in *B. subtilis*, when SigB is inactive, it is bound tightly by the anti-σ-factor RsbW (Benson and Haldenwang, 1993). Dephosphorylated RsbV can release SigB from RsbW, allowing transcription from SigB-dependent promoters. The phosphorylation status of RsbV is controlled by RsbU and RsbP (Wise and Price, 1995; Vijay *et al.*, 2000), which respond to environmental signals transduced through the RsbRST sensing module (Scott *et al.*, 2000). Homologs of *rsbRST* and *rsbP* are not present in the *S. aureus* genome, although the other protein components of the system appear to work in a similar fashion to their *B. subtilis* homologs, despite some differences in their regulation (Kullik and Giachino, 1997; Palma and Cheung, 2001).

In *S. aureus* at least four transcripts are responsible for the production of SigB under different growth conditions (Figure 1.10; Kullik and Giachino, 1997). The longest of these, encoding the entire *rsbUVWsigB* operon, ensures basal expression of *sigB* during exponential growth (Kullik and Giachino, 1997). In *S. aureus* RsbW is an anti-σ-factor that binds and sequesters SigB (Miyazaki *et al.*, 1999). A transcript encompassing *rsbWsigB* is expressed constitutively, allowing maintenance of a high level of SigB sequestered by RsbW in the cell. In stationary phase cells a short transcript encoding only *sigB* is induced at high levels, raising the concentration of free SigB in the cells (Kullik and Giachino, 1997). A transcript encompassing *rsbVWsigB* is produced in response to heat shock (and possibly other stress) and is important for the induction of *sigB* in stationary phase (Kullik and Giachino, 1997; Palma and Cheung, 2001). RsbU is also necessary for induction of *sigB* in response to stress, at least *in vitro* (Giachino *et al.*, 2001). It appears likely that RsbU and RsbV interact with the RsbW/SigB complex to free

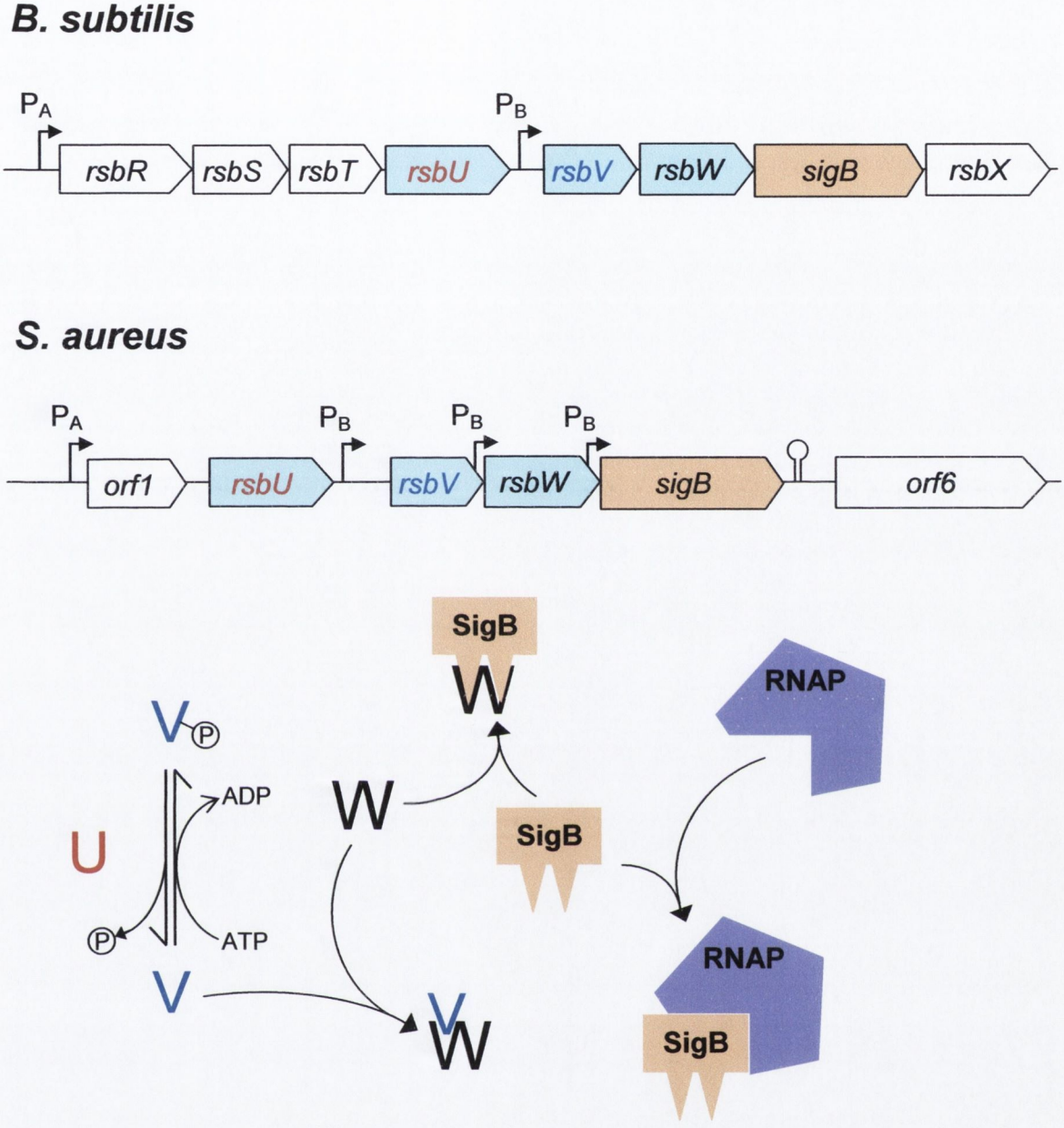


Fig 1.10 Genetic organisation of *B. subtilis* and *S. aureus sigB* operons

In *B. subtilis* RsbRSTU form a sensory mechanism that, apart from RsbU, is absent in *S. aureus*. The mechanism of signal transduction to RsbU in *S. aureus* is unknown, but an *rsbU*-independent mechanism can also dephosphorylate RsbV. RsbW is an anti-σ-factor that inhibits SigB interactions with RNA polymerase (RNAP). Dephosphorylated RsbV binds to RsbW releasing SigB.

In the upper panel, arrows and hairpin loops indicate promoters and transcription terminators, respectively. P_A and P_B indicate SigA-dependent and SigB-dependent promoters, respectively. Adapted from Voelker *et al.*, 1995; Kullik and Giachino, 1997; Knobloch *et al.*, 2004.

SigB, allowing transcription to occur from SigB-dependent promoters (Palma and Cheung, 2001).

SigB influences gene products that are putatively involved in cell envelope biosynthesis and turnover, intermediary metabolism and signalling pathways, as well as virulence factors. The majority of genes upregulated by SigB possess a strong consensus SigB-binding site in their promoter regions, whereas those downregulated by SigB do not (Bischoff *et al.*, 2004). This implies indirect downregulation, possibly through *agr*, other two-component regulatory systems or transcription factors of the SarA family. The *sarA* P3 promoter is dependent on SigB for transcription (Bayer *et al.*, 1996). SigB upregulates *sarA*, *sarS* and *arlRS* in stationary phase (Bischoff *et al.*, 2001; Bischoff *et al.*, 2004), although it has been reported not to influence *sarA* activity in SH1000 (Horsburgh *et al.*, 2002). Conflicting reports also exist of the influence of SigB on *agr* (Bischoff *et al.*, 2001; Bischoff *et al.*, 2004; Horsburgh *et al.*, 2002). It appears that choice of strain and experimental approach have influenced the results of these investigations, leaving the role of SigB in the regulation of these regulators uncertain.

Strain differences in relation to SigB are mainly caused by the presence of an 11-base-pair (bp) deletion in *rsbU* in strains of the 8325/RN6390 lineage. This mutation causes a frameshift and premature termination of the RsbU polypeptide (Kullik *et al.*, 1998). This influences the response of these laboratory strains to cellular stress and affects pigmentation, protease and haemolysin activity (Giachino *et al.*, 2001; Horsburgh *et al.*, 2002).

Mutation of *rsbU* or *sigB* caused attenuation of virulence in the murine model of sepsis and septic arthritis (Jonsson *et al.*, 2004). Despite an influence on adhesin production, *sigB* had no significant influence on the outcome of experimental rat endocarditis (Entenza *et al.*, 2005). The availability of SigB in the cell has an effect on internalisation of *S. aureus* by osteoblasts, although additional SigB-independent factors are also involved (Nair *et al.*, 2003). The presence of functional RsbU in strain 8325-4 had no significant influence on the outcome in a murine subcutaneous skin abscess model (Horsburgh *et al.*, 2002), although this may have been due to a lack of additional virulence factors such as capsular polysaccharide, which is also defective in this strain (O'Riordan and Lee, 2004). Other studies have investigated the role of *sigB* in additional animal models of infection using 8325 or RN6390 strains and have found no significant differences from wild-type (Nicholas *et al.*, 1999; Chan *et al.*, 1998).

It appears that *sigB* is important for virulence in *S. aureus*, at least in systemic infection and septic arthritis (Jonsson *et al.*, 2004), but that strain choice and culture conditions are vital to studies of this regulator (Bischoff *et al.*, 2004).

1.5.3.3 Sigma factor H

Analysis of the genome sequence of *S. aureus* revealed only one putative σ-factor gene in addition to *sigA* and *sigB*. This was designated *sigH* because of its similarity to *sigH* of *B. subtilis* (Morikawa *et al.*, 2003). SigH is one of the 16 accessory σ-factors in *B. subtilis*, many of which are involved in sporulation. The loci in *S. aureus* corresponding to the 14 additional *B. subtilis* σ-factors contain deletions of the genes encoding the σ-factors, indicating a smaller number of stress-related functions in *S. aureus*, compared to *B. subtilis*. SigH of *B. subtilis* is involved in sporulation and the regulation of the development of natural genetic competence (Grossman, 1995). *sigH* was found not to regulate genes in *S. aureus* that are homologous to *B. subtilis* sporulation-associated genes. Because of its regulation of *comE* and *comG*, which are homologous to competence-related genes in *B. subtilis*, it has been suggested that SigH is a regulator of the development of natural DNA competence in *S. aureus* (Morikawa *et al.*, 2003).

1.5.4 Summary of interactions between staphylococcal regulators

A summary of the interactions between staphylococcal regulators is depicted in Figure 1.11. The two central regulators appear to be *agr* RNAIII and SarA. Deletion of either of these regulators has pleiotropic effects on virulence gene expression. This is likely to be due to the interruption of normal regulation of virulence-associated genes themselves, and of their regulators.

agr responds to increased local cell density and the induced RNAIII has direct and indirect effects on virulence genes (e.g. α-haemolysin, protein A) and regulatory genes (e.g. *rot*, *sarT*). This activation occurs before the onset of stationary phase. The regulation of target genes by RNAIII may be through RNA-RNA interactions, enhancing or disrupting translation, or by the titration of activators of target gene transcription (Novick, 2003).

SarA upregulates *agr*, feeding into this regulatory pathway, but is repressed in stationary phase by SarR, limiting its activation of *agr*. SarA also has direct and

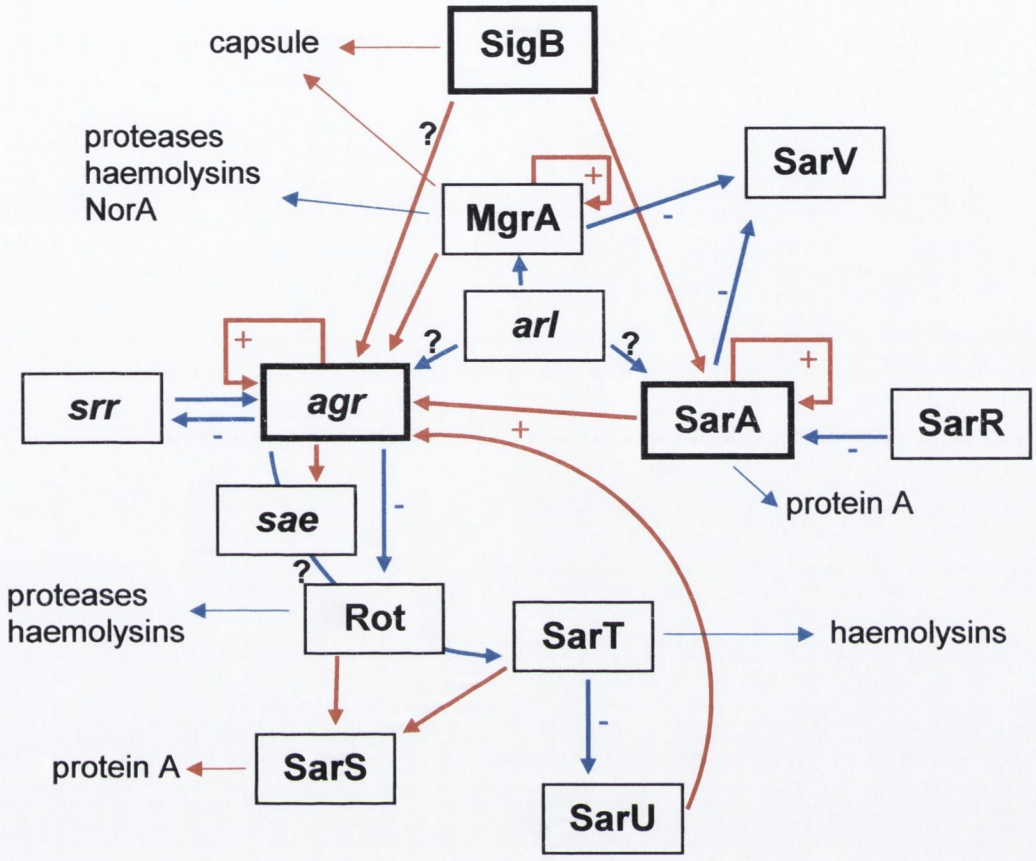


Fig 1.11 Summary of interactions between regulators in *S. aureus*

agr and SarA play central roles in the regulation of virulence determinants in *S. aureus*. Two-component regulatory systems, SarA homologs and the alternative σ-factor SigB exert many of their regulatory effects through *agr* and SarA, or are themselves regulated by *agr* or SarA. Red arrows indicate positive regulation, blue arrows indicate negative regulation. Question marks (?) denote uncertain interactions. Two-component regulatory systems *srr*, *sae* and *arl* respond to extracellular stimuli. *agr* responds to increased local cell density. SigB responds to numerous types of cellular stress. The integrated network of regulation allows the generation of an appropriate response to signals and stimuli, depending on the environment.

indirect effects on target genes. It binds to the P2-P3 promoter region of *agr* (causing activation of transcription) and to the promoter region of protein A (causing repression of transcription) (Morfeldt *et al.*, 1996; Dunman *et al.*, 2001; Chien *et al.*, 1999). The ability to directly both activate and repress transcription implies that the effects of SarA (and its homologs) are exerted via changes in the degree of DNA supercoiling in promoter regions (Fournier and Klier, 2004).

The alternative σ-factor, SigB, and the two-component regulatory systems allow response to stress and to extracellular environmental signals. A complex integrated network of regulators is required, as the appropriate response within the cell depends on the environment and the status of all sensory systems. *agr* and SarA play central roles in responding to environmental signals in *S. aureus*, exerting their effects both directly and indirectly, via the SarA homologs.

The regulatory network in *S. aureus* is complex and depends on the interplay of sigma factors, transcription factors and signal transduction events via two-component regulatory systems. There is also evidence of strain-to-strain variation. The interactions between regulators are likely to differ during infection *in vivo* from what is seen *in vitro*, and to vary between different niches within the host, and even between individual bacteria in a single population *in vivo* (Cheung *et al.*, 1998; Xiong *et al.*, 2005; van Wamel *et al.*, 2002). Regulators of virulence gene expression are possible therapeutic targets. However an understanding of their precise roles during infection is vital.

1.6 Rationale for this study

Clumping factor A (ClfA) is an important virulence factor in several animal models of infection, including experimental endocarditis in rats (Moreillon *et al.*, 1995; Stutzmann Meier *et al.*, 2001; Que *et al.*, 2001) and a murine model of sepsis and septic arthritis (Josefsson *et al.*, 2001). Active immunisation against ClfA gave protection against arthritis, and passive immunisation with human IgG with high titre against ClfA protected mice from septic death (Josefsson *et al.*, 2001). In addition, passive immunisation with monoclonal function-blocking anti-ClfA antibody is protective in infectious endocarditis (Patti, 2004).

The presence of anti-ClfA antibodies in both 'normal' and convalescent sera (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005) implies that normal exposure to *S. aureus* as a commensal of the skin causes the formation of anti-ClfA antibodies,

but that the low-level anti-ClfA antibodies in such sera are not protective against invasive *S. aureus* disease. Some rare individuals with unusually high titre antibodies are presumably protected (Vernachio *et al.*, 2003).

The effect of ClfA in the virulence of *S. aureus* is assumed to be related to its fibrinogen-binding activity, though this has not been investigated. The aim of this study was to give insight into the reason why ClfA is such an important virulence factor for *S. aureus*, and why anti-ClfA antibodies in many human sera are not protective, although passive immunisation with IVIG or monoclonal antibodies appears to be. This investigation combined three approaches.

Firstly, it was necessary to determine whether ClfA, like ClfB, is subject to proteolytic degradation, and whether proteolysis had an effect on fibrinogen binding. Removal of the fibrinogen-binding activity of ClfA by protease degradation could function in allowing *S. aureus* to detach from fibrinogen-coated surfaces. It is important to know whether region A of ClfA is subject to proteolytic degradation, as protease degradation may also affect the immunogenicity of the protein. The susceptibility of region A of ClfA to proteolysis is dealt with in Chapter 3.

Secondly, the importance of ClfA in the virulence of *S. aureus* in a variety of animal models suggests that it may have a role in the evasion of the immune response. Therefore, the possibility that ClfA is an antiphagocytic factor was investigated. The possibility that fibrinogen binding may affect the antiphagocytic properties of ClfA was also investigated. High-titre antibodies directed against capsular polysaccharide, which is an antiphagocytic factor, aid in the clearance of *S. aureus* by opsonophagocytosis. A role in the inhibition of opsonophagocytosis might explain the importance of ClfA in virulence, and also why high-titre anti-ClfA antibodies aid in clearance of bacteria although normal levels of antibody in serum do not. The antiphagocytic activity of ClfA is investigated in Chapter 5.

Finally, the regulation of expression of ClfA was investigated. ClfA is present predominantly on *S. aureus* cells in the stationary phase of growth (O'Brien *et al.*, 2002a; McDevitt *et al.*, 1994; 1995), unlike the other *S. aureus* fibrinogen-binding surface proteins ClfB, FnBPA and FnBPB, which are mainly found on cells from the exponential phase of growth (McAleese *et al.*, 2001; McGavin *et al.*, 1997). Knowledge of the expression of ClfA and the regulators that control its expression may give clues to the role of ClfA in virulence. Differential expression of these fibrinogen-binding proteins factors may be important in the pathogenesis of *S.*

aureus infection, possibly allowing binding to and detachment from fibrinogen-coated surfaces at different stages of infection. The regulation of expression of ClfA *in vitro* is investigated in Chapter 6.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 2.1. *S. aureus* strains were routinely cultured on trypticase soy agar (TSA, Oxoid) or broth (TSB) with shaking at 200 rpm for liquid cultures. For expression studies, strains were grown in brain heart infusion broth (BHI, Difco) at 37 °C with shaking at 200 rpm. These cultures were routinely grown in 50 ml broth in 250 ml Erlenmyer flasks, or 500 ml in 2.5 L Erlenmyer flasks. *E. coli* strains were cultured in L broth or agar (Difco) at 37 °C, with shaking at 200 rpm for liquid cultures. *L. lactis* strains were cultured in M17 medium (Difco) containing 0.5% (v/v) glucose without shaking at 30 °C. Snap-frozen stocks of all strains were maintained at –70 °C in L broth, TSB or M17 broth containing 10% (v/v) glycerol. Antibiotics were incorporated in media where appropriate at the following concentrations: 2 µg/ml or 10 µg/ml tetracycline, 10 µg/ml chloramphenicol, 100 µg/ml ampicillin, 5 µg/ml erythromycin, 10 µg/ml kanamycin and 5 µg/ml minocycline. Antibiotics were purchased from Sigma Chemical Co.

2.2 Plasmids

Plasmids used in this study are listed in Table 2.2. Plasmids were isolated using Promega Wizard SV Plus MiniPrep Plasmid Isolation Kit or Qiagen HiSpeed Midi Plasmid Isolation Kit, according to manufacturers' instructions, with the following modifications for Gram-positive hosts: for *S. aureus* plasmid isolations 2 µg/ml lysostaphin (AMBI, New York) was added to the resuspension buffer, and for *L. lactis* plasmid isolations 400 µg/ml mutanolysin (Sigma) and 500 µg/ml lysozyme (Sigma) was added to the resuspension buffer, and incubated at 37 °C for 10 min to facilitate cell lysis. Plasmids to be used for electroporation or cloning were ethanol precipitated to concentrate DNA and remove salts. Briefly, one third volume of 3 M sodium acetate (pH 5.2) and an equal volume of absolute ethanol were added to plasmid preparations, mixed thoroughly, and incubated at –70 °C for at least 30 min. Precipitated DNA was pelleted at 4°C, washed with 70% (v/v) ethanol, air-dried and dissolved in water (Analar).

2.3 DNA manipulations

DNA manipulations were performed, unless indicated, according to standard methods (Sambrook and Russell, 2000). PCR products and restriction-digested fragments were routinely purified or gel purified (from 1% (w/v) agarose gels in TAE buffer (40 mM Tris-Acetate, 1 mM ethylenediaminetetraacetic acid (EDTA))) using the HiPure PCR Product purification kit (Roche) according to manufacturer's instructions. Enzymes were purchased from Sigma Chemical Co., Roche, New England Biolabs, Stratagene or Promega and were used according to manufacturers' instructions. Other chemicals were purchased from Sigma Chemical Co. or BDH.

2.4 Polymerase chain reaction (PCR)

Primers were purchased from Sigma-Genosys and are listed in Table 2.3. Where possible, plasmid DNA (approximately 10 ng) was used as template. Otherwise, approximately 100 ng of chromosomal DNA was used. Reaction mixtures (100 µl) typically contained 100 pm each of the relevant forward and reverse primers, 2.5 nM dNTPs, 1.5 mM $MgCl_2$, 10-100 ng template DNA and 1.25 U *Taq* DNA polymerase, in a standard *Taq* reaction buffer (Promega). Amplifications were performed in a Hybaid Omnigene thermocycler with initial denaturation at 94 °C, followed by 30 cycles of denaturation (30-60 seconds at 94 °C), annealing (1 min at 50-68 °C, depending on specific primers) and elongation [1-5 min at 72 °C, depending on product length (1 min/kb)], and a final elongation step of 10 min at 72 °C. Digoxygenin (DIG)-labelled probes were synthesised by PCR as described, including 2.5 mM DIG-dUTP mix (Roche) in place of dNTPs and using gel-purified PCR product as template.

DNA fragments to be used for cloning were amplified using the high fidelity *Pfu* polymerase (Promega). Reaction mixtures (100 µl) typically contained 100 pm each of the relevant forward and reverse primers, 2.5 nM dNTPs, 2 mM $MgSO_4$, 10-100 ng template DNA and 5 U *Pfu* DNA polymerase, in a standard *Pfu* reaction buffer (Promega). Amplification was carried out with an initial 1 min denaturation step before addition of the enzyme, followed by 30 cycles of denaturation (30-60 seconds at 94 °C), annealing (1 min at 50-68 °C, depending on specific primers) and elongation (1-18 min at 72 °C, depending on product length (2 min/kb)), and a

final elongation step of 10 min at 72 °C. Products were visualised with UV light after electrophoresis through 1% (w/v) agarose gels in TAE buffer and ethidium bromide staining. PCR products used in cloning or as template for DIG-labelled probes were purified on the basis of size from 1% (w/v) agarose gels.

2.5 Southern hybridisation

DNA-DNA hybridisations were carried out using the DIG system (Roche) according to manufacturer's instructions. Genomic DNA was prepared from stationary phase cultures of *S. aureus* using the Genomic DNA purification kit from Edge BioSystems. 2 µg/ml lysostaphin was included in the sphaeroplast buffer and incubated at 37 °C for 10 min prior to cell lysis. Approximately 7 µg genomic DNA was digested with *Hind*III for 16 hours at 37 °C before separation on 1% (w/v) agarose gels. DNA was depurinated, fragmented, denatured and transferred to positively charged membranes (Roche) by capillary transfer in 20 x SSC (3 M NaCl, 0.3 M NaCitrate) and immobilised by baking at 80 °C for 2 hours. Membranes were prehybridised at 68 °C in standard prehybridisation solution [5 x SSC, 0.1% (w/v) N-laurylsarcosine, 0.02% (w/v) sodium dodecyl sulphate, 1 x blocking reagent (Roche)] for 2-4 hours and hybridised in the same solution containing 0.5-1 µg/ml DIG-labelled DNA probe.

Probes were made in two rounds of PCR, as described in Section 2.4, in order to minimise background. The product of the first round was gel-purified as described in Section 2.3 and used as template in the second step, which incorporated DIG-dUTP. Probes were heated to 95 °C and placed on ice to allow denaturation before hybridisation on membranes for 16 hours at 68 °C. Membranes were washed and developed according to the instructions of the DIG system, using CSPD (Roche) as chemiluminescent substrate. Membranes were exposed to X-ray film (X-Omat, Kodak) and film was developed in an automated developing machine (Kodak X-Omat 1000 Processor).

2.6 DNA sequencing and sequence alignment

Confirmatory sequencing of DNA constructs was performed by Lark Technologies. Manual DNA sequencing performed alongside primer extension analysis is described in Section 2.30. Alignment of DNA sequences was carried

out using the ClustalW service on the website of the European Bioinformatics Institute. Sequences came from strains COL (TIGR), Newman (McDevitt *et al.*, 1994), 8325-4 (University of Oklahoma), MRSA 252 and MSSA 476 (Sanger Institute), MW2, N315 and Mu50 (Japanese consortia) and were freely available. Transcriptional terminator prediction was carried out using TransTerm, which is freely available from TIGR.

2.7 Isolation of solublised cell wall proteins

S. aureus cells were washed twice with phosphate buffered saline (PBS) and resuspended at an optical density at 600 nm (OD_{600}) of 40.0 per ml in 30% (w/v) raffinose (Sigma) in 50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$. Protease inhibitors (1 x complete mini, Roche) and 200 µg/ml lysostaphin were added and cells were incubated at 37 °C for 10 min. Cells were placed briefly on ice before pelleting of protoplasts at 10,000 x g for 10 min. The supernatant containing solublised cell wall proteins was aspirated and mixed with 2 x final sample buffer (FSB, Laemmli system, Sigma) before separation of proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% (w/v) acrylamide gels and Western immunoblotting as described in Section 2.8.

2.8 SDS-PAGE and Western immunoblotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Laemmli buffer system in 7.5% (w/v) or 10% (w/v) acrylamide separating gels with 4.5% stacking gels. Samples were mixed with equal volumes of 2 x FSB (Sigma) and heated at 95-100 °C for 5 min, before brief centrifugation to pellet debris. In general, 20 µl of each sample was separated at 25-30 mA for 2-3 hours and gels were either stained in Coomassie brilliant blue and destained in 45% (v/v) methanol, 10% (v/v) acetic acid or transferred to polyvinylidene difluoride (PVDF) membranes (Roche) by wet transfer in 20 mM Tris, 150 mM glycine, 20% (v/v) methanol. Electrophoresed alongside samples, as size standards, were prestained protein markers (New England Biolabs), including protein bands of the following sizes: 175 kDa, 83 kDa, 62 kDa, 47.5 kDa, 32.5 kDa, 25 kDa, 16.5 kDa and 6.5 kDa. Non-specific binding to membranes was blocked by incubation for 2-16 hours in 10% (w/v) skimmed milk powder (Marvel,

Cadbury Ltd.) in TS buffer (10 mM Tris-HCl, 0.9% (w/v) NaCl, pH 7.4). Membranes were incubated with primary and secondary antibodies for 1 hour each in 10% (w/v) Marvel in TS buffer, with three 10 min washes after each incubation with TS buffer containing 0.05% (v/v) Tween 20 (TS Tween). Unless otherwise stated, primary antibodies were polyclonal rabbit anti-ClfA region A (see Section 2.9) used at 1/5,000-1/20,000 dilution and secondary antibodies were either horseradish peroxidase-conjugated goat anti-rabbit IgG (at 1/2000 dilution, AMBI) or peroxidase-conjugated protein A (at 1/2000 dilution, Sigma). Chemiluminescent substrate (Lumiglo, New England Biolabs) was used according to manufacturer's instructions and detected by exposure to X-ray film and development as described in Section 2.5. Approximate molecular weights of bands were calculated by comparison to molecular weight markers using Rf measurements.

2.9 Preparation of polyclonal antisera

Two young New Zealand White rabbits, whose pre-immune sera showed no reactivity with *S. aureus* cell wall proteins, were injected with 25 µg $rClfA_{(40-559)}$ (see Section 2.10) in PBS, emulsified with an equal volume Freund's complete adjuvant. The total volume was 1 ml. Three subsequent injections of 25 µg $rClfA_{(40-559)}$, in 1 ml total volume using Freund's incomplete adjuvant, were given at 21 day intervals. 21 days after the final boost, the rabbits were bled, serum recovered, and the immunoglobulin fraction was purified by the method of Owen (1985). Antisera were absorbed against *E. coli* lysate (Pierce) to remove antibodies that reacted with *E. coli* antigens.

2.10 Recombinant protein purification

Full-length recombinant region A of ClfA ($rClfA_{(40-559)}$, N123) was purified from a 2 L culture of *E. coli* Topp 3 containing plasmid pCF40 (O'Connell *et al.*, 1998). $rClfA_{(220-559)}$ (N23) was purified from *E. coli* Topp 3 containing plasmid pCF41 (O'Connell *et al.*, 1998). Expression was induced by addition of 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG) to exponentially growing cells. After 4 hours of induction, cells were harvested by centrifugation and resuspended in 30 ml PBS containing protease inhibitors (Complete EDTA-free, Roche). Cells were lysed in

a French pressure cell, and the lysate was cleared by high-speed centrifugation, followed by filtration. The His-tagged recombinant proteins were affinity purified on a nickel-charged column (HiTrap Chelating, Pharmacia), followed by dialysis twice in 5 L of 25 mM Tris-HCl, pH 7.9 buffer containing 10 mM EDTA and anion exchange chromatography on a HiTrap Mono Q column (Pharmacia) using a NaCl gradient for elution. This was followed by dialysis in 5 L of 4 mM Tris-HCl, pH 7.5, 100 mM NaCl (without EDTA) and a second nickel affinity purification and dialysis twice in 5 L PBS. Both purified proteins were stable for 24 hours at 37 °C.

2.11 Preparation of concentrated *S. aureus* supernatants and incubation with recombinant protein

S. aureus strains 8325-4 *sarA*, 8325-4 *sarA aur* and 8325-4 *sspA* were grown for 16-18 hours in TSB and cells pelleted. Culture supernatant (20 ml) was aspirated and concentrated 10 x in a Centricon YM-10,000 centrifugal concentrating device (Amicon). $rClfA_{(40-559)}$ was incubated in PBS with the concentrated supernatant at 1 x concentration with or without 25 mM o-phenanthroline (Sigma) for 60 min at 37 °C. Samples were taken at intervals and protease reactions stopped by addition of equal volumes of 2 x FSB. 20 µl samples were heated at 95 °C and electrophoresed through 10% (w/v) acrylamide gels, which were stained with Coomassie brilliant blue and destained as described in Section 2.8. Selected samples were transferred to nitrocellulose (Protran; Schleicher and Schuell) by wet transfer, stained on membranes with amido black and excised for N-terminal sequence determination by the PNAC facility, Cambridge, UK.

2.12 Protease treatment of recombinant protein

$rClfA_{(40-559)}$ was incubated in PBS with purified aureolysin, human cathepsin G, human neutrophil elastase (all kindly supplied by Jan Potempa, Krakow) or SspA (V8 protease, glutamylendopeptidase, Sigma) at a range of concentrations (20-1000 ng/ml), for various times at 37 °C. The inhibitors o-phenanthroline, EDTA and α_2-macroglobulin and α-1-antitrypsin were added to some samples. Reactions were stopped by addition of equal volumes of 2 x FSB and placing on ice. Samples were heated at 95 °C and electrophoresed through 10% (w/v)

acrylamide gels, which were stained with Coomassie brilliant blue and destained as in Section 2.8.

2.13 Protease treatment of whole *S. aureus* cells

S. aureus strain 8325-4 SH1000 was grown in BHI broth without antibiotic to mid-exponential phase and diluted 1/100 to fresh, prewarmed broth. Cells were harvested after 2.5 hours of growth, corresponding to mid-exponential phase, washed twice in PBS and resuspended at OD_{600} 10.0 per ml in PBS. Aureolysin (2 µg/ml) or SspA serine protease (2 µg/ml), with or without the inhibitors EDTA (10 µM), o-phenanthroline (20 µM) or α_2-macroglobulin (0.75 U/ml) were added and incubated at 37 °C for 45 min. Cells were incubated briefly on ice, then pelleted and washed three times in PBS. Following protease treatment, samples were split and cells were used in fibrinogen adherence assays (Section 2.14) and for preparation of solublised cell wall proteins by lysostaphin digestion, followed by SDS-PAGE and Western immunoblotting (Sections 2.7 and 2.8).

2.14 Fibrinogen adherence assays

Wells of 96-well microtitre dishes (Sarstedt) were coated for 16 hours at 4 °C with 100 µl of 0-40 µg/ml human fibrinogen (Calbiochem) in PBS. Wells were washed 3 times with PBS and blocked to eliminate non-specific binding by incubation for 2 hours at 37 °C with filtered 5% (w/v) bovine serum albumin (BSA) in PBS.

For the bacterial adherence assay bacteria were grown to the desired growth phase, harvested and washed twice in PBS, before adjustment to OD_{600} 1.0 per ml or 4.0 per ml, as appropriate. In some cases, serial dilutions were then made. 100 µl of cell suspension was applied to each well and incubated at room temperature for 90 min with gentle shaking. Cells were then washed twice with PBS and adherent bacteria were fixed with 25% (v/v) formaldehyde, before washing and staining with 0.5% (w/v) crystal violet. Wells were then washed four times with PBS, and 50 µl 5% (v/v) acetic acid was added to each well and the stain released by gentle shaking. Absorbance at 570 nm was measured in an enzyme-linked immuno-sorbent assay (ELISA) plate reader.

For the ELISA-type assay recombinant proteins were quantitated using the BCA Assay kit (Pierce) and diluted in PBS containing 2% (w/v) BSA. A variety of concentrations of recombinant protein were incubated in fibrinogen-coated wells (5 µg/ml) that had been blocked with BSA as above for one hour. Wells were washed and bound proteins were detected by incubation with rabbit polyclonal anti-ClfA region A antibodies (at 1/10,000 dilution; Section 2.9) for 1 hour. After washing, secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG (at 1/2000 dilution, AMBI)) was added for 1 hour, washed and detected by the addition of 100 µl of a chromogenic substrate solution (1 mg/ml tetramethyl-benzidine and 0.006% (v/v) H_2O_2 in 0.05 M phosphate citrate buffer, pH 5.0). Plates were developed for 10 min in the dark. The reaction was stopped by the addition of 2 M H_2SO_4 (50 µl/well), and plates were read at 450 nm in an ELISA plate reader.

2.15 Transformation

Plasmids were introduced into *E. coli* strains XL1-Blue or Topp 3 made competent by $CaCl_2$ treatment (Sambrook and Russell, 2000), and plated on selective media. Transformants were initially screened for the presence of chimeric plasmids by the rapid colony method of Le Gouill and Dery (1991). This method allows the screening of a large number of transformants (up to 40 at one time) on the basis of plasmid size and helps to identify candidates for further analysis by facilitating the rapid elimination of colonies bearing incorrectly-sized plasmids.

Shuttle and suicide vectors were introduced to *S. aureus* strain RN4220 (Kreiswirth *et al.*, 1983) by electroporation. Briefly, RN4220 cells were grown to mid-exponential phase in TSB and harvested at 4 °C, washed twice in 0.5 M sucrose and resuspended in 1/50 the original volume. These cells were used fresh or snap-frozen and stored at –70 °C. 200 µl cell suspension was mixed with plasmid DNA and electroporated at 20 °C in a cuvette with 0.2 mm electrode gap at 25 V, with 200 Ω resistance and 25 µF capacitance. Plasmids used for electroporation were ethanol precipitated and dissolved in water to concentrate DNA and minimise salt carryover. Cells were transferred to 300 µl TSB and incubated at 37 °C with shaking for 60-100 min before plating on antibiotic-containing agar plates.

Transformants were single colony-purified and the antibiotic resistance phenotype confirmed before screening by PCR, plasmid restriction, Southern hybridisation and Western immunoblotting, as appropriate.

2.16 Transduction

Plasmids and chromosomal insertions bearing resistance markers were moved between *S. aureus* strains by generalised bacteriophage Φ85-mediated transduction (Foster, 1998). Bacteriophages were propagated on the donor strain and used to infect the recipient strain, with antibiotic selection for the required marker. Colonies were single-colony purified twice on TSA plates containing the appropriate antibiotic and 0.05% (w/v) sodium citrate to eliminate contaminating phage particles. Transductants were screened, as appropriate, by PCR, plasmid restriction, Southern hybridisation or Western immunoblotting.

2.17 Construction of temperature-sensitive shuttle plasmids

Two temperature-sensitive shuttle plasmids (pTS*ermC* and pTS*tetK*) were constructed, to retain the multiple cloning site, replication and copy control mechanisms of pTS2 (Greene *et al.*, 1995), and to replace the weak chloramphenicol resistance marker (*cat*) with the strong inducible erythromycin resistance determinant of pE194 (*ermC*) or the tetracycline resistance determinant of pT181 (*tetK*). A 2.9 kilobase (kb) fragment of the pTS2 backbone was PCR-amplified using *Pfu* polymerase (Promega) and primer pair pTSF2*Sac*I/pTSR2*Nhe*I (see Table 2.3 for primers), incorporating *Sac*I and *Nhe*I restriction sites, respectively, to exclude the chloramphenicol resistance determinant. The *ermC* insert was amplified from pE194 with primer pair *ermC*F1*Sac*I/*ermC*R1*Nhe*I to yield a product of ca. 1050 base pairs (bp). The *tetK* insert was amplified from pT181 using primer pair *tetK*F1*Sac*I/*tetK*R1*Nhe*I, the latter of which contains a natural *Nhe*I site. This product was ca. 1650 bp in length.

Fragments were extracted from 1% (w/v) agarose gels before cleavage with *Sac*I and *Nhe*I for 16 hours. Four samples, each containing 1100 ng *tetK* or *ermC* insert and 300 ng plasmid backbone in 30 µl ligase buffer, were incubated with 1.5 U T4 DNA ligase (Roche) for 16 hours at 16 ºC. Ligations were pooled, ethanol

precipitated and dissolved in 15 µl water before electroporation to *S. aureus* RN4220, as described in Section 2.15.

Transformants were selected on TSA plates containing 10 µg/ml erythromycin, 2 µg/ml tetracycline or 10 µg/ml chloramphenicol and incubated at 28 °C. The pTS-*tetK* ligation yielded 113 colonies and the pTS-*ermC* ligation yielded 60 colonies. 8 transformants of each were single colony purified on the appropriate selective antibiotics. Colonies were grown in 5 or 50 ml TSB at 28 °C with antibiotic selection and plasmids prepared with the Wizard SV Plus MiniPrep or the Qiagen HiSpeed Midi Plasmid Isolation Kits for restriction analysis. The temperature-sensitive phenotype of each construct was verified by spreading dilutions of overnight broth cultures (grown with antibiotic selection at 28 °C) on duplicate TSA plates containing antibiotics and incubation at 28 °C and 42 °C.

2.18 Allelic replacement mutagenesis

Bases 110-114 of the *clfA* coding sequence were replaced with a *Hind*III restriction site (AAGCTT). This introduced a +1 frameshift that caused premature termination of translation of the polypeptide, creating a *clfA* null mutant bearing no resistance marker (*clfA5*).

Upstream (890 bp) and downstream (1562 bp) fragments were amplified by PCR from 8325-4 genomic DNA using Pfu polymerase (Promega) and primer pairs F1*Eco*RI/R6*Hind*III and F7*Hind*III/R7*Bam*HI, respectively (see Table 2.3 for primers). These primers were designed to introduce a +1 frameshift when cleaved with *Hind*III and ligated together. Products were purified from 1% (w/v) agarose gels and cleaved with *Hind*III and either *Eco*RI or *Bam*HI, as appropriate, for 2 hours at 37 °C. DNA was purified using the HiPure PCR Product purification kit (Roche) and 200 ng of the upstream fragment and 110 ng of the downstream fragment were ligated in a 20 µl volume for 1 hour.

Plasmid pBluescript II SK was cleaved with *Eco*RI and *Bam*HI, gel-purified and 40 ng was added to the PCR fragments in a final volume of 30 µl ligase buffer and 1.5 U T4 DNA ligase added. Transformants were selected on L agar plates containing 100 µg/ml ampicillin and 5-bromo-4-chloro-3-inolyl β-D-galacto-pyranoside (X-gal) to identify those with a disrupted *lacZα* gene. Plasmid DNA was analysed by digestion with restriction enzymes and the *clfA5* mutation verified by DNA sequencing. The plasmid was designated pBlueΔ*clfA*.

pBlueΔ*clfA* and pTS*ermC* plasmid DNA was prepared using the Qiagen HiSpeed Midi Plasmid Isolation Kit, ethanol precipitated and dissolved in water. Each plasmid was cut with *Bam*HI. To minimise background due to vector relegation, *Bam*HI-cut pBlueΔ*clfA* was treated with shrimp alkaline phosphatase (Roche) according to manufacturer's instructions. Duplicate ligations (30 µl volumes) were prepared containing 150 ng vector (pBlueΔ*clfA*) and 200 ng insert (pTS*ermC*) DNA. Control ligation contained no insert. After ligation for 2 hours, reactions were pooled, transformed into *E. coli* XL1-Blue and plated on L agar plates containing 100 µg/ml ampicillin. Cells transformed with the ligation mixture yielded 192 colonies, whilst the control yielded 60 colonies. Additionally, transformants from the pTS*ermC* ligation were streaked on L plates containing 100 µg/ml erythromycin, because *ermC* is known to be expressed sufficiently to overcome the high intrinsic resistance of *E. coli* K12. 10 of the 12 colonies that grew well on 100 µg/ml erythromycin contained the desired chimeric plasmid (pJH1). Because ligation of pBlueΔ*clfA* and pTS*ermC* was at a single common restriction site, two orientations of the chimeric plasmid were possible. Because this was not important for the purpose of allelic replacement, pJH1 was chosen arbitrarily. Plasmid constructs were verified by restriction analysis.

pJH1 was prepared from *E. coli* XL1-Blue using the Qiagen HiSpeed Midi Plasmid Isolation Kit, ethanol precipitated, and 1 µg was electroporated into *S. aureus* RN4220 and transformants selected on TSA plates containing 10 µg/ml erythromycin at 28 ºC. Temperature sensitivity was verified (see Section 2.17). The plasmid was transferred into Newman *spa* and 8325-4 *spa* by Φ85-mediated transduction (see Section 2.16), selecting on TSA plates containing 10 µg/ml erythromycin at 28 ºC.

Cultures containing pJH1 were grown on TSB broth containing 10 µg/ml erythromycin at 28 ºC. Serial dilutions were plated in duplicate on TSA containing 10 µg/ml erythromycin and one set of plates incubated at 28 ºC and the other at 42 ºC. The number of colonies growing at 28 ºC was 10^3-fold higher than those at 42 ºC. Putative integrants were single colony purified at 42 ºC. Colonies were inoculated into drug-free broth and incubated at 28 ºC to encourage plasmid replication and excision. The cultures were diluted 1/100 into drug-free broth and grown at 42 ºC for 8 hours. Under these conditions excised plasmids would not replicate and would be rapidly lost due to the lack of selective pressure. A proportion of plasmids (ca. 10^{-3}) may re-integrate at this temperature. The culture

was diluted 1/100 and grown for 16 hours at 28 ºC without erythromycin. This procedure was repeated 3 times, cultures were diluted, plated for single colonies on drug-free TSA plates and single colonies screened for sensitivity to erythromycin.

A diagnostic PCR screen with primers F1*Eco*RI and R7*Bam*HI, followed by digestion of the product with *Hind*III, demonstrated the introduction of the desired frameshift mutation.

Newman *spa clfA5* and 8325-4 *spa clfA5* were compared to their parental strains to ensure coagulase and haemolytic activity had not been affected by the mutagenesis. This was done to ensure that spontaneous *agr* mutations had not occurred. Coagulase activity was tested as described in Section 2.19. Titres of all strains were >64. Haemolytic activity was tested by streaking colonies on rabbit and sheep blood plates. All colonies retained the haemolytic profile of their parental strain.

2.19 Assay for coagulase

Coagulase activity was measured by mixing of equal volumes of serial dilutions of stationary phase *S. aureus* culture supernatants with rabbit plasma (diluted 1/3 in PBS) in a test tube. The formation of a clot after incubation at 37 ºC for 2 hours was the endpoint. The coagulase titre was the reciprocal of the highest dilution showing clot formation.

2.20 Fibrinogen clumping test

Bacterial cells taken from stationary phase cultures or agar plates were emulsified in 10 µl saline on a glass microscope slide. 10 µl human fibrinogen (1 mg/ml; Calbiochem) was added and mixed thoroughly by swirling. Appropriate positive and negative controls were included. Visible clumping was the endpoint.

2.21 Overlap primer PCR mutagenesis

Amino acid substitutions $P_{336}S$ and $Y_{338}A$ were shown to severely reduce the affinity of $rClfA_{(220\text{-}559)}$ for fibrinogen (Deivanayagam *et al.*, 2002). Mutations were introduced to *clfA* in plasmid pCF77 (Hartford *et al.*, 1997) by overlap primer PCR

mutagenesis. In the first step the mutation was introduced to two overlapping PCR products (1400 bp and 700 bp) using primer pairs F10*Kpn*I/$RP_{336}SY_{338}$A and $FP_{336}SY_{338}$A/R7*Bam*HI, pCF77 as template and *Pfu* polymerase (Promega). The primers $FP_{336}SY_{338}$A and $RP_{336}SY_{338}$A are complementary. These PCR products were gel-purified and combined as template for the second PCR step, which used the flanking primers F10*Kpn*I and R7*Bam*HI. An additional 30 second template annealing step at 60 °C was included after denaturation in this second PCR. The 2.1 kb product of the second PCR step contained mutations in the codons for P_{336} and Y_{338} (*clfA*PY) consisting of 3 nucleotide mismatches: the P_{336} codon (CCC) was substituted with S (TCC) and the Y_{338} codon (TAT) with A (GCT). The product was gel purified before digestion for 16 hours with *Kpn*I and *Bam*HI.

pCF77 was prepared from *E. coli* XL1-Blue using the Promega Wizard SV Plus MiniPrep Plasmid Isolation Kit and digested for 16 hours with *Kpn*I and *Bam*HI. The larger (approximately 7 kb) fragment was gel-purified and 20 ng ligated to 180 ng of the purified *Kpn*I/*Bam*HI-cut *clfA*PY fragment for 16 hours at 16 °C. Colonies were initially screened by the rapid colony method. Putative pCF77PY plasmids were prepared using the Promega Wizard SV Plus MiniPrep Plasmid Isolation Kit and analysed by cleavage with restriction enzymes. The *clfA* mutations in one plasmid were verified by DNA sequencing. This plasmid was electroporated to *S. aureus* RN4220, single colony purified and transduced into 8325-4 *spa clfA5.*

2.22 Construction of a *clfA::lacZ* fusion

A transcriptional reporter fusion to the *clfA* promoter and upstream elements was constructed using the suicide vector pAZ106 (Kemp *et al.*, 1991). An internal *clfA* region A fragment (nucleotides 43 to 1677) was amplified by PCR with *Pfu* polymerase from plasmid pCF77 (Hartford *et al.*, 1997) using primers F13*Bam*HI and R12*Eco*RI. A longer fragment containing upstream sequences (nucleotides -781 to 1677) was prepared in the same way using primers F12*Bam*HI and R12*Eco*RI. The products were gel-purified and digested with *Eco*RI and *Bam*HI for 2 hours at 37 °C. Plasmid pAZ106 was prepared from *E. coli* XL1-Blue using the Promega Wizard SV Plus MiniPrep Plasmid Isolation Kit and digested with *Eco*RI and *Bam*HI as above, followed by gel purification. 100 ng of vector and 300 ng of individual inserts were ligated at 4 °C for 16 hours using T4 DNA ligase, and

transformed to *E. coli* XL1-Blue, with selection on L agar plates containing 100 µg/ml ampicillin.

Large plasmids were isolated using the Promega Wizard SV Plus MiniPrep Plasmid Isolation Kit, and restriction analysis identified correct constructs. One representative plasmid for each construct (pAZ106F12R12 and pAZ106F13R12) was prepared in larger scale using the Qiagen HiSpeed Midi Plasmid Isolation Kit. Three preparations were ethanol precipitated together and dissolved in 500 µl water before electroporation to *S. aureus* RN4220 at 20 °C, as described in Section 2.15. In order to obtain transformant colonies of pAZ106 derivatives, plasmid DNA must become integrated into the chromosome by a single-crossover event upon entry into the cell. To increase the probability of this occurrence, larger amounts of plasmid DNA than usual were used in these experiments. A range of concentrations of DNA was used to obtain optimal transformation. 12 µg of plasmid pAZ106F13R12 DNA or 4 µg of plasmid pAZ106F12R12 DNA gave optimal transformation efficiency, yielding approximately 30 colonies on TSA containing 10 µg/ml erythromycin. Colonies were single colony purified on TSA plates containing 10 µg/ml erythromycin and formed blue colonies on X-gal plates due to expression of β-galactosidase enzyme from *lacZ*.

Genomic DNA from representative colonies was isolated using the Genomic DNA Purification Kit (Edge BioSystems) and used as template in a PCR screen using primers F13*Bam*HI and R14*lacZ*. Presence of a 2.6 kb PCR product indicated correct insertion of the plasmid.

2.23 Construction of strains by transduction

The *clfA::lacZ* element was transduced (see Section 2.16) using selection for the erythromycin resistance marker of pAZ106 to the following *S. aureus* strains: Newman, Newman *agr*, Newman *sarA*, Newman *sigB rsbU*, Newman *sigB rsbU sarA,* Newman *sigB rsbU agr,* 8325-4 and 8325-4 SH1000.

Newman *sigB rsbU* was constructed by transduction of *sigB::*Tet^R from 8325-4 *sigB* (Chan *et al.*, 1998) and screened by the method of Kullik (1997) to reveal that the adjacent *rsbU* defect of 8325-4 had been co-transduced. Newman *sarA* and Newman *sigB rsbU sarA* were constructed by transduction of the *sarA::*Kan^R mutation from 8325-4 *sarA* (Chan and Foster, 1998) and selection on 200 µg/ml kanamycin. Newman *sigB rsbU agr* was constructed by transduction of the

*agr::*Tmn^R mutation from Newman *agr* (McAleese *et al.*, 2001) and selection on 5 µg/ml minocycline.

Each construct was checked carefully by comparing expression of haemolysins on blood agar and for pigmentation. Strains Newman, 8325-4 and SH1000 have characteristic expression of haemolytic activity on rabbit blood agar plates. SH1000 and Newman show the characteristic golden pigmentation of *S. aureus*, whereas 8325-4 does not. The pigmentation of SH1000 is particularly strong. This is useful for checking and validating strains. In addition, resistance to erythromycin, tetracycline and kanamycin was monitored in all strains. Newman *clfA::lacZ* formed deep blue colonies on X-gal plates. Strain constructions were confirmed by Southern hybridisation, as described in Section 2.5, using a *clfA*-specific probe made with primers F13*Bam*HI and R12*Eco*RI, and a *lacZ*-specific probe made with primers R14*lacZ* and F14*lacZ*. Activity of the construct was validated by comparison with Newman *clfB::lacZ* (McAleese *et al.*, 2003), *spa*$^+$ *spa::lacZ* and *hla*$^+$ *hla::lacZ* (Chan and Foster, 1998) in a β-galactosidase assay (see Section 2.24).

2.24 β-galactosidase assay

Cells were assayed for β-galactosidase production using 4-methyl-umbelliferyl β-D-galactopyranoside (MUG) as substrate, according to the method of Chan and Foster (1998). Briefly, bacterial cells (100-2500 µl culture) were harvested by centrifugation in microfuge tubes, the supernatant aspirated and cell pellets snap frozen at –70 °C. Samples were thawed and resuspended in 500 µl AB buffer (100 mM NaCl, 60 mM K_2HPO_4, 40 mM KH_2PO_4) before addition of 50 µl MUG (4 mg/ml) and incubation at 22-25 °C for 60 min. Reactions were stopped by addition of 500 µl 2 M $NaHPO_4$ and fluorescence was measured using a Perkin Elmer LS50B luminescence spectrometer, with excitation at 365 nm and emission at 455 nm. β-galactosidase activity was calculated using a range of concentrations of the end-product, 4-methylumbelliferone (4-MU), as standards. Miller units of β-galactosidase activity (pmol 4-MU produced per minute per ml of cells at OD_{600} 1.0) were calculated according to the formula:

Miller Units= pmol 4-MU produced/(dilution factor x time x OD_{600}).

2.25 Polymorphonuclear leukocyte (PMNL) isolation

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

2.26 Phagocytosis assay

Whole bacterial cells grown to stationary phase were washed twice in PBS and labelled with 30 µg/ml fluorescein isothiocyanate (FITC; Sigma) in PBS for 1 hour at 37 °C with shaking. Cells were washed three times in PBS and counted in a haemocytometer (Neubauer), adjusted to 1x10^9 cfu/ml in RPMI and frozen at -20 °C. FITC-labelling of bacteria did not affect their ability to bind fibrinogen (data not shown). Bacteria were thawed on ice and diluted to 5 x 10^7 cfu/ml in RPMI. Pooled human serum (kindly provided by Jos van Strijp, Utrecht) was diluted in RPMI and fibrinogen (Calbiochem) was added to serum where necessary. Bacteria (50 µl) were opsonised in 10 µl diluted serum (with or without fibrinogen) for 10 min at 37 °C, followed by addition of 50 µl pre-warmed PMNL (isolated as in Section 2.23) and incubation at 37 °C with vigorous shaking. The final concentration of fibrinogen (where present) was the same percentage as the serum concentration, calculated as 3 mg/ml = 100%. The final bacteria:PMNL ratio was 10:1. Reactions were stopped after 5-15 min by addition of 100 µl ice-cold 1% (w/v) paraformaldehyde in PBS. The percentage of PMNL bearing FITC-

labelled bacteria was determined by flow cytometric analysis of 5,000 cells using a FACScan flow cytometer (Becton Dickinson). Fluorescence quenching with trypan blue showed that >85% bacteria associated with PMNL were internalised (data not shown). Microscopic analysis of samples showed no gross extracellular clumping of bacteria (data not shown). Statistical analyses were performed using the student *t* test for paired data in Kaleidagraph (Synergy software). *P* values < 0.01 were considered significant.

2.27 Whole cell dot immunoblotting

Bacteria were grown to the appropriate growth phase and washed twice with PBS before adjustment to OD_{600} 1.0-4.0 per ml, as appropriate. Doubling dilution series were made and 5 µl of each dilution was dropped onto a nitrocellulose membrane (Protran, Schleicher and Schuell). After drying for at least 30 min membranes were blocked with 10% (w/v) Marvel in TS buffer for 2 hours with shaking, as described in Section 2.8. Membranes were incubated with anti-ClfA region A primary antibody at room temperature with shaking for 1 hour, washed 3 times in TS Tween, incubated with protein A peroxidase secondary antibody and again washed 3 times with TS Tween, as described in Section 2.8. Chemiluminescent substrate (Lumiglo, New England Biolabs) was used according to manufacturer's instructions and chemiluminescence detected by exposure of X-ray film and its subsequent development, as described in Section 2.5.

2.28 RNA isolation

S. aureus cells were grown for 12 hours in BHI broth, diluted 1:100 in fresh broth and grown to early exponential phase (2 hours) to eliminate carry-over from stationary phase, then diluted again 1:50 in fresh broth. Samples were taken from these cultures at intervals, cells were harvested by centrifugation, supernatants aspirated and pellets were snap-frozen and stored at –70 °C. Samples were gently thawed on ice and RNA was isolated using the RNA Pro Blue kit (QBiogene) according to manufacturer's instructions. RNA was quantified by UV spectroscopy and frozen at –70 °C in aliquots for future use.

2.29 Northern hybridisation and densitometric analysis

Northern hybridisation was carried out using the DIG system (Roche) according to manufacturer's instructions. RNA (5 µg) was electrophoresed through 1% (w/v) agarose, 2% (v/v) formaldehyde gels in 4-mopholinepropanesulfonic acid (MOPS) buffer and transferred to positively charged membranes (Boehringer Mannheim) in 20 X SSC by capillary transfer and immobilised as in Section 2.5, in RNase-free conditions and solutions. Membranes were prehybridised for 1-2 hours at 50 °C in DIG Easy-Hyb solution (Roche) and hybridised in the same solution containing 50-500 ng/ml specific DIG-labelled DNA probes for 16 hours at 50 °C. Subsequent washing and developing steps were carried out according to manufacturer's instructions for RNA-DNA hybridisation, using CSPD as chemiluminescent substrate, and developed as for Southern hybridisation (see Section 2.5).

Probes were amplified by PCR to incorporate DIG-dUTP, as described in Section 2.5. Primer pair F6*Hind*III/R7*Bam*HI was used to amplify *clfA* region A. Primer pairs F33/R33 and F33/R34 were used to amplify probes 1 and 2, respectively. Primer pairs F21/R21 and F22/R22 were used to amplify probes 3 and 4, respectively. The *clfA* region A probe and probes 1 to 4 were amplified from pCF77 plasmid DNA. Primer pair F*vwb*/R*vwb* was used to amplify the *vwb* region A probe from 8325-4 genomic DNA. Primers are listed in Table 2.3.

Cells were grown for 20 hours before treatment with 200 µg/ml rifampicin. Samples were taken at intervals, RNA was isolated and Northern hybridisation carried out as described above using a probe for *clfA* region A. Duplicate membranes were prepared and probed for 16S rRNA. This was included as a control for lane loading that allowed calculation of the relative amounts of *clfA* transcript in each sample. Densitometric analysis was carried out using Quantity One software (BioRad). Band volumes (intensity x area) were measured. To normalise for sample loading the values for *clfA* band volumes were divided by those for 16S rRNA for each sample. The percentage of mRNA remaining at each time point was calculated, assigning the highest value 100%. Percentage mRNA remaining was plotted against time on a semi-logarithmic scale.

2.30 Primer extension analysis

Mapping of the 5' end of *clfA*-specific transcripts was carried out by primer extension analysis using components of the AMV Primer Extension System (Promega) and substituting Superscript III Reverse Transcriptase (RT) and its buffers (Stratagene) for AMV RT (Promega). Superscript III RT has higher fidelity and decreased exonuclease activity compared with AMV RT. Primers Pex1 and R35 were used, with pCF77 as template. Primers were labelled with [α-^{32}P]dATP (Amersham Biosciences) and annealed to denatured template according to the instructions of the AMV Primer Extension Kit. Extension reaction was carried out with Superscript III RT according to manufacturer's instructions, and the product was ethanol precipitated and loaded on a 6% (w/v) polyacrylamide, 7 M urea gel. Manual DNA sequencing was carried out with the ^{T7}Sequencing Kit (Amersham Biosciences) according to manufacturer's instructions, using the same, unlabelled, primers and incorporating [α-^{35}S]dATP. Sequencing reactions were run alongside primer extension reactions on the same gel to allow determination of the location of the 5' end of the transcript. Gels were dried on a vacuum gel dryer, exposed to X-ray film and developed as described in Section 2.5.

The primer extensions carried out in the laboratory of our collaborators used the same method with the following exceptions. [α-^{32}P]dATP labelling was used for both primer extension and sequencing reactions, and AMV RT (Promega) was used for the primer extension reaction. Total RNA was isolated from Newman or Newman *clfA2* cells grown to OD_{600} 1.0 per ml and used as template for cDNA synthesis using Pex2 (see Table 2.3 for primers). A size ladder was derived from sequencing of the *norA* gene with a *norA*-specific primer, to allow determination of product length. The image was captured digitally using a phosphorimaging system (Storm 860; Molecular Dynamics, Sunnyvale, California).

Table 2.1 Bacterial strains

Strain	Relevant properties[a]	Reference
S. aureus		
8325-4	NCTC 8325 cured of prophages	Novick, 1967
RN4220	Restriction-deficient derivative of 8325-4	Kreiswirth *et al.*, 1983
8325-4 *sarA*	*sarA*::Kan^R	Chan and Foster, 1998
8325-4 *sarA aur*	*sarA*::Kan^R *aur*::Erm^R	Karlsson *et al.*, 2001
8325-4 *sspA*	*sspA*::Erm^R	Karlsson *et al.*, 2001
8325-4 SH1000	*rsbU*$^+$ derivative of 8325-4	Horsburgh *et al.*, 2002
8325-4 SH1000 *clfA::lacZ*	*clfA::lacZ* derivative of SH1000 [Erm^R]	This study
8325-4 *clfA::lacZ*	*clfA::lacZ* derivative of 8325-4 [Erm^R]	This study
8325-4 *spa*	*spa*::Kan^R	Patel *et al.*, 1987
8325-4 *spa clfA2*	*spa*::Kan^R *clfA*::Erm^R	This study
8325-4 *spa clfA5*	*spa*::Kan^R *clfA5*	This study
8325-4 *sigB*	*sigB*::Tet^R	Chan *et al.*, 1998
8325-*4 sarA*	*sarA*::Kan^R	Chan and Foster, 1998
Newman	NCTC 8178	Duthie and Lorenz, 1952
Newman *spa*	*spa*::Kan^R	This study
Newman *clfA2*	*clfA*::Erm^R	McDevitt *et al.*, 1994
Newman *spa clfA2*	*spa*::Kan^R *clfA*::Erm^R	This study
Newman *spa clfA5*	*spa*::Kan^R *clfA5*	This study
Newman *agr*	*agr*::Tmn^R	McAleese *et al.*, 2001
Newman *sarA*	*sarA*::Kan^R	This study
Newman *sigB rsbU*	*sigB*::Tet^R *rsbU*	This study
Newman *sigB rsbU sarA*	*sigB*::Tet^R *rsbU sarA*::Kan^R	This study
Newman *sigB rsbU agr*	*sigB*::Tet^R *rsbU agr*::Tmn^R	This study
Newman *clfA::lacZ*	*clfA::lacZ* transduced into Newman [Erm^R]	This study
Newman *agr clfA::lacZ*	*clfA::lacZ* transduced into Newman *agr* [Tmn^R, Erm^R]	This study
Newman *sarA clfA::lacZ*	*clfA::lacZ* transduced into Newman *sarA* [Kan^R, Erm^R]	This study
Newman *sigB rsbU clfA::lacZ*	*clfA::lacZ* transduced into Newman *sigB rsbU* [Tet^R, Erm^R]	This study
Newman *sigB rsbU sarA clfA::lacZ*	*clfA::lacZ* transduced into Newman *sigB rsbU sarA* [Tet^R, Kan^R, Erm^R]	This study
Newman *sigB rsbU agr clfA::lacZ*	*clfA::lacZ* transduced into Newman *sigB rsbU agr* [Tet^R, Tmn^R, Erm^R]	This study

Continued...

Table 2.1 (contd.) **Bacterial strains**

Strain	Relevant properties[a]	Reference
Newman *clfB::lacZ*	*clfB::lacZ* [Erm^R]	McAleese *et al.*, 2003
Newman *spa*$^+$ *spa::lacZ*	*spa*$^+$ *spa::lacZ* [Erm^R]	McAleese *et al.*, 2003
Newman *hla*$^+$ *hla::lacZ*	*hla*$^+$ *hla::lacZ* [Erm^R]	McAleese *et al.*, 2003
E. coli		
XL1-Blue	Highly transformable strain *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*(F' *proAB lacI*^{q}*ZΔM15* Tn*10* [Tet^R])	Stratagene
Topp 3	Protease-deficient strain Rif^R (F' *proAB lacI*^{q}*ZΔM15* Tn*10* [Tet^R] [Kan^R])	Stratagene
L. lactis		
MG1363	Plasmid-free derivative of NCD 0712	Gasson, 1983
NZ9800	MG1363 bearing nisin resistance plasmid [Cam^R]	Kuipers *et al.*, 1993

a) abbreviations: Kan^R, kanamycin resistance; Tet^R, tetracycline resistance; Erm^R, erythromycin resistance; Cam^R, chloramphenicol resistance; Tmn^R, tetracycline/minocycline resistance

Table 2.2 Plasmids

Plasmid	Relevant features[a]	Reference
pBluescript II SK	*E. coli* cloning vector [Amp^R]	Stratagene
pCF40	pQE-30 containing DNA encoding *clfA* region A [Amp^R]	O'Connell *et al.*, 1998
pTS2	*S. aureus* plasmid containing temperature-sensitive replicon [Cam^R]	Greene *et al.*, 1995
pE194	*S. aureus* plasmid [Erm^R]	Horinouchi and Weisblum, 1982b
pT181	*S. aureus* plasmid [Tet^R]	Khan and Novick, 1983
pTS*ermC*	*S. aureus* plasmid containing temperature-sensitive replicon [Erm^R]	This study
pTS*tetK*	*S. aureus* plasmid containing temperature-sensitive replicon [Tet^R]	This study
pBlueΔ*clfA*	pBluescript containing *clfA* DNA, incorporating frameshift mutation [Amp^R]	This study
pJH1	Shuttle plasmid containing pBlueΔ*clfA* and pTS*ermC* [Amp^R, Erm^R]	This study
pCU1	Shuttle plasmid derived from pC194 and pUC19, maintaining high copy number in *E. coli* [Amp^R] and *S. aureus* [Cam^R]	Augustin *et al.*, 1992
pCF77	pCU1 containing 4.15 kb *clfA* insert [Amp^R, Cam^R]	Hartford *et al.*, 1997
pCF77EV	pCF77 incorporating EV mutations in region A [Amp^R, Cam^R]	Hartford *et al.*, 2001a
pCF77PY	pCF77 incorporating PY mutations in region A [Amp^R, Cam^R]	This study
pAZ106	*S. aureus lacZ*-expression vector [Erm^R]	Kemp *et al.*, 1991
pAZ106F12R12	$clfA^+$ *clfA::lacZ*-expression vector [Erm^R]	This study
pAZ106F13R12	*clfA::lacZ*-expression vector [Erm^R]	This study
pKS80	*L. lactis* expression vector [Erm^R]	Wells *et al.*, 1993
pKS80*clfA*	pKS80 containing *clfA* coding sequence [Erm^R]	O'Brien *et al.*, 2002a
pKS80*spa*	pKS80 containing *spa* coding sequence [Erm^R]	O'Brien *et al.*, 2002a
pKS80*clfB*	pKS80 containing *clfB* coding sequence [Erm^R]	O'Brien *et al.*, 2002a
pKS80*sdrG*	pKS80 containing *sdrG* coding sequence [Erm^R]	Hartford *et al.*, 2001b
pKS80*fbl*	pKS80 containing *fbl* coding sequence [Erm^R]	Mitchell *et al.*, 2004
pKS80*sdrD*	pKS80 containing *sdrD* coding sequence [Erm^R]	O'Brien *et al.*, 2002a
pKS80*sdrH*	pKS80 containing *sdrH* coding sequence [Erm^R]	Mitchell, 2004

Continued...

Table 2.2 (contd.) **Plasmids**

Plasmid	Relevant features[a]	Reference
pNZ8037	Nisin-inducible *L. lactis* expression vector [Cam^R]	De Ruyter *et al.*, 1996
pNZ8037*clfA*	pNZ8037 containing *clfA* coding sequence [Cam^R]	Loughman *et al.*, 2005
pNZ8037*clfA*PY	pNZ8037 containing *clfA* coding sequence, incorporating mutations [Cam^R]	Loughman *et al.*, 2005

a) abbreviations: Amp^R, ampicillin resistance; Kan^R, kanamycin resistance; Tet^R, tetracycline resistance; Erm^R, erythromycin resistance; Cam^R, chloramphenicol resistance

Table 2.3 Primers

Primer	Sequence[a]
pTSF2*Sac*I	TCC GAG CTC CCC GAT TTA AGC ACA CCC
pTSR2*Nhe*I	CTA GCT AGC GCC ACC TAA AAA GGA GCG
*ermC*F1*Sac*I	TCC GAG CTC GTG CTA TAA TTA TAC
*ermC*R1*Nhe*I	CTA GCT AGC GGT GTC ACA AGA CAC TCT
*tetK*F1*Sac*I	TCC GAG CTC CGA TTT TTG GCA ACA TGA
*tetK*R1*Nhe*I	CTA GCT AGC CAC TCA TAG TTC
*spa*F1	CTT TAC AAA TAC ATA CAG GGG
*spa*R1	CGC CAG TTT CTG GTA ATG C
F1*Eco*RI	GCC GAA TTC CAA ATA CAT CAA CAT GAC TTT GC
R6*Hind*III	GGC AAG CTT GCT TCT TTA CTG CTG AGT AGT C
F7*Hind*III	GCC AAG CTT GCA AGT GAA AAT AGT GTT ACG
R7*Bam*HI	CGC GGA TCC CTC TGG AAT TGG TTC AAT TTC
F10*Kpn*I	CCG GTA CCA TAA ATT ACA CAT C
$FP_{336}SY_{338}A$	GAC CAT G**T**C CGC T**GC** TAT TGA CC
$RP_{336}SY_{338}A$	GGT CAA TA**G** **C**AG CGG **A**CA TGG TC
F12*Bam*HI	CGC GGA TCC CAA ATA CAT CAA CAT GAC TTT GC
R12*Eco*RI	CCG GAA TTC CCT CTG GAA TTG GTT CAA TTT C
F13*Bam*HI	CGC GGA TCC TCG ATT GGC GTG GCT TCA GT
F14*lacZ*	AAC CCT GGC GTT ACC C
R14*lacZ*	CAC AGC GGC TGG TTC GG
16SF1	GCT CAG GAT GAA CGC TGG C
16SR1	GGT GTG TAC AAG ACC CGG G
F21	CAC CTA AAA ATG GTA CTA ATG C
R21	CCT ATT GAT GCT AAT AAT CCC C
F22	CAT TGG GAG CAT TAT GTG TAT C
R22	GTC AGT TTC AAC GAC TAG ATA C
F33	AGT TCA ACT TTA TCA AAT GAA AAT
R33	TGA TGA TGC TCT TCT GTT GC
R34	TCT ATT GCA ATT TTT TTA GCA G
R35	TTA CTA GGT AAA ATA TGC ACG
Pex1	CAC GCC AAT CGA TTT TTT CCG
Pex2	CTT CTT TAC TGC TGA GTA GTC CAA AAC C
F*vwb*	CCG GAA TTC CAG TGG TTT CTG GGG AGA AG
R*vwb*	CCG CCA TGG GCC ATG CAT TAA TTA TTT GCC A

a) introduced restriction endonuclease cleavage sites are underlined. Mismatches with *clfA* coding sequence are highlighted in bold.

Chapter 3

Proteolytic Degradation of Region A of Clumping Factor A

3.1 Introduction:

The predicted amino acid sequence of ClfA from strain Newman consists of 933 amino acids. It comprises an N-terminal Sec-dependent secretory signal peptide (residues 1-39), followed by a 520-residue region A that contains the ligand binding domain, a 308-residue region R consisting primarily of SD dipeptide repeats and C-terminal domains allowing anchoring to the cell wall peptidoglycan by sortase A (Figures 1.2 and 3.3; McDevitt *et al.*, 1995; Mazmanian *et al.*, 2001). The length of the repeat region R varies from 308 to 404 residues between different strains.

ClfA binds to the extreme C-terminus of the γ-chain of fibrinogen, the same site as human platelet integrin GPIIb/IIIa (Figure 1.5; McDevitt *et al.*, 1997; Medved *et al.*, 1997). The minimum ligand-binding domain of ClfA comprises residues 220-559 (McDevitt *et al.*, 1995) and has been designated domains N2 and N3 (N23) based on the crystal structure and domain organisation of ClfB N23 (Perkins *et al.*, 2001). The crystal structure of ClfA domains N23 ($rClfA_{(221-559)}$) has been solved. The individual globular domains show a novel variation of the immunoglobulin fold, designated the DEv-IgG fold that is shared by the ligand-binding domains of CNA, ClfB, FnBPA and FnBPB (Section 1.3.9.1.1; Deivanayagam *et al.*, 2002).

ClfA has been reported at a variety of apparent molecular weights in Western immunoblotting of *S. aureus* extracts with anti-ClfA region A antibodies (Hartford *et al.*, 1997; Hartford *et al.*, 1999; O'Brien *et al.*, 2002a; Siboo *et al.*, 2001). ClfA and recombinant region A ($rClfA_{(40-559)}$) migrate in SDS-PAGE with apparent molecular weights far higher than predicted, including forms at >200 kDa, and have been reported to be susceptible to proteolytic breakdown (Hartford *et al.*, 1997; Deivanayagam *et al.*, 1999). Attempts to crystallise the full length region A of ClfA ($rClfA_{(40-559)}$) were unsuccessful. The crystals formed contained only domains N2 and N3, having lost N1 due to proteolysis by traces of protease remaining in material purified from *E. coli* (Deivanayagam *et al.*, 1999). The aberrant migration of the region A of ClfA appears to be attributable to domain N1. In addition, region R makes a substantial contribution to the aberrant migration of ClfA molecules that are released from the cell surface (Hartford *et al.*, 1997). Partly because of difficulties arising from the aberrant migration of ClfA and its breakdown products, the nature of proteolytic sensitivity of region A has not previously been investigated.

ClfB has a very similar structural organisation to ClfA, sharing 93% amino acid identity in region R and 26% identity in region A (Ní Eidhin *et al.*, 1998). ClfB is sensitive to proteolysis by aureolysin, the metalloprotease of *S. aureus* (McAleese *et al.*, 2001). Cleavage at the SLAVA motif between residues 197-201 caused a loss of fibrinogen-binding activity in ClfB (Section 1.3.9.2; McAleese *et al.*, 2001).

Aureolysin is expressed by *S. aureus* cells in the post-exponential phase of growth. It activates SspA (V8 serine protease) from its precursor form, which in turn activates staphopain B (SspB), a cysteine protease that is co-expressed from the same operon as SspA (see Section 1.3.10 and Figure 1.8; Shaw *et al.*, 2004; Rice *et al.*, 2001). In the context of human infection, staphylococcal proteases may aid tissue destruction and invasion, may modulate the activity of bacterial factors (e.g. ClfB) and may also influence the activity of host proteins and proteases. Human cathepsin G and human neutrophil elastase are two serine proteases secreted from azurophil granules into the phagolysosome of human PMNL to aid in the destruction of invading pathogens (Berton, 1999). These and other human proteases may contribute to the protease activation cascade and modulation of surface components of *S. aureus*.

Domain N1 (residues 40-220) of ClfA has not been crystallised and little is known about its properties because of the protease sensitivity of the region (Deivanayagam *et al.*, 1999). Domain N1 is predicted to be composed mainly of β-sheet structure, and may be organised into more than one subdomain (Deivanayagam *et al.*, 2002). N1 is not part of the minimum ligand-binding truncate of ClfA that spans residues 220-559, and it serves no known function. At the junction of domains N1 and N2, ClfA contains the sequence SLAAVA (residues 217-222), similar to the SLAVA aureolysin cleavage site of ClfB. This study investigated whether ClfA can be cleaved by aureolysin at this site, whether other proteases are capable of cleavage within region A, and whether any such cleavage event removes or reduces fibrinogen-binding activity in ClfA.

3.2 Results:

3.2.1 Degradation of recombinant ClfA by supernatants of *S. aureus*

Recombinant, His-tagged region A of ClfA from strain Newman ($rClfA_{(40-559)}$) was purified from *E. coli* as described in Section 2.10. Purification of full-length region A has proved difficult in the past (Deivanayagam *et al.*, 1999). Purification on a nickel-charged column caused metalloproteases and other nickel-chelating proteins from *E. coli* initially to co-purify with the His-tagged protein. These were removed by dialysis in EDTA-containing buffer (EDTA inhibited proteolytic activity by removing chelated metal ions from metalloproteases) followed by anion exchange chromatography to purify $rClfA_{(40-559)}$ from contaminants. After anion exchange chromatography $rClfA_{(40-559)}$ and its breakdown products were present, and a second round of nickel-affinity chromatography was used to remove breakdown products, leaving only pure, full-length $rClfA_{(40-559)}$, as seen in Figure 3.1, lane 1 in each panel. This protein has a predicted molecular weight of 57 kDa, but an apparent molecular weight (by SDS-PAGE) of circa 80 kDa.

$rClfA_{(40-559)}$ was incubated with supernatants of *S. aureus* strains 8325-4 *sarA*, *sarA aur* and *sspA* as described in Section 2.11. 8325-4 *sarA* produces large amounts of secreted proteases due to upregulation of protease operons in the *sarA* background (Shaw *et al.*, 2004). 8325-4 *sarA aur* also produces large amounts of proteases, but not the metalloprotease aureolysin, which is an activator of SspA (V8 serine protease). 8325-4 *sspA* produces normal amounts of proteases, but not SspA. This strain is also defective in production of SspB and SspC, due to polar effects of the *sspA*::Erm^R insertion mutation on the *ssp* operon (Shaw *et al.*, 2004), as well as lacking normal activation of any proteases that rely on SspA or SspB. SspC is an inhibitor of SspB (see Section 1.3.10.3; Shaw *et al.*, 2005).

Incubation of $rClfA_{(40-559)}$ with supernatant from 8325-4 *sarA* caused rapid degradation of the circa (ca.) 80-kDa protein (Figure 3.1, panel A). After only two minutes of incubation, all $rClfA_{(40-559)}$ had been totally degraded (Figure 3.1, panel A, lane 2). Three main bands appeared in its place: one of ca. 42 kDa, one of ca. 40 kDa, and a third of ca. 30 kDa, revealed by N-terminal sequencing to be the activated form of SspA (N-terminus: VIPLN). The N-terminal sequence of the 40-kDa band could not be determined, but that of the 42-kDa band was VAADA. This

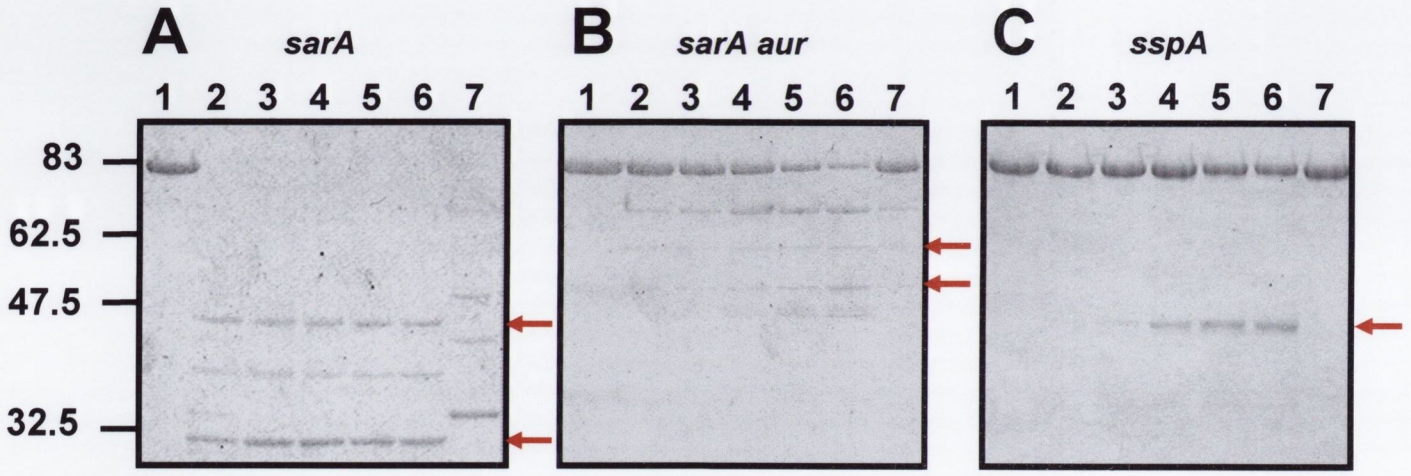


Fig 3.1 Proteolysis of rClfA$_{(40-559)}$ by *S. aureus* supernatants

rClfA$_{(40-559)}$ was incubated with 10 x concentrated supernatants of 8325-4 *sarA* (panel A), 8325-4 *sarA aur* (panel B), and 8325-4 *sspA* (panel C) at 37 °C, separated on 10% (w/v) acrylamide gels and stained with Coomassie brilliant blue. In each panel: lane 1, rClfA$_{(40-559)}$ only; lanes 2-6, rClfA$_{(40-559)}$ + supernatant after 2, 5, 15, 30 and 60 min, respectively; lane 7, rClfA$_{(40-559)}$ + supernatant + 20 mM o-phenanthroline. Red arrows indicate bands, the N-termini of which were subsequently sequenced. Sizes are in kDa.

corresponded to cleavage of $rClfA_{(40-559)}$ before V_{221}, within the putative aureolysin cleavage site, SLAA↑VA. It appeared likely that this cleavage was carried out by aureolysin metalloprotease in the supernatant. Addition of 20 mM o-phenanthroline, a specific potent inhibitor of aureolysin, did not inhibit proteolysis, but resulted in an increase in the sizes of all three products, possibly indicating that all three rely on aureolysin for cleavage (Figure 3.1, panel A, lane 7). The 40-kDa band may represent a protease that is over-expressed in 8325-4 *sarA*, possibly aureolysin itself, which migrates at ca. 40 kDa on SDS-PAGE (Shaw *et al.*, 2004).

To investigate the role of aureolysin, $rClfA_{(40-559)}$ was incubated with supernatant of 8325-4 *sarA aur*, which does not produce the metalloprotease. This resulted in slower breakdown (Figure 3.1, panel B). Due to the lack of aureolysin, this strain does not produce as much active SspA as 8325-4 *sarA*. However, even in the absence of aureolysin, SspA is activated at a slow rate by another, undefined mechanism (Shaw *et al.*, 2004). Proteolysis by this aureolysin-deficient supernatant resulted in formation of 4 main breakdown products of ca. 70 kDa, 58 kDa, 49 kDa and 45 kDa. The N-terminal sequences of two of these bands were determined. The band of 58 kDa had the N-terminal sequence TTSND and the band of 49 kDa had the N-terminal sequence SAPQS. These fragments represented two cleavage events in $rClfA_{(40-559)}$ within domain N1, before residues T_{149} and S_{189} (see Figures 3.2 and 3.3). The amino acid sequences at these sites represented typical glutamyl endopeptidase cleavage sites (NE↑T and NE↑S). Additional putative glutamyl endopeptidase cleavage sites were identified at residues S_{54} (NE↑S) and T_{98} (QE↑T) (see Figure 3.3). Cleavage at the latter site may have been responsible for the 70-kDa band. The 45-kDa band may have been produced by cleavage at D_{200} (KD↑V) or D_{195} (TD↑A). Indeed, on close inspection, the 45-kDa band appeared to be two bands, perhaps representing partial cleavage at both sites. SspA serine protease (V8 protease) is a glutamyl endopeptidase, specifically cleaving peptide bonds on the carboxyl side of aspartic and glutamic acid residues. Addition of 20 mM o-phenanthroline did not inhibit this pattern of cleavage (Figure 3.1, panel B, lane 7).

To investigate whether V8 serine protease was indeed responsible for the cleavage of $rClfA_{(40-559)}$, the protein was incubated with supernatant of 8325-4 *sspA*. This strain produces aureolysin at normal levels (less than 8325-4 *sarA*), but no SspA serine protease or SspB cysteine protease. It may also be defective in proteases downstream in the cascade of activation that rely on SspA or SspB.

rClfA$_{(40-559)}$ was slowly broken down from ca. 80 kDa to 42 kDa (Figure 3.1, panel C). Addition of 20 mM o-phenanthroline to this reaction completely inhibited breakdown (Figure 3.1, panel C, lane 7). It appeared, therefore, that the 42-kDa band was the product of aureolysin cleavage of rClfA$_{(40-559)}$, but that additional proteases, including SspA, were capable of degradation of rClfA$_{(40-559)}$ at numerous sites within domain N1. The C-terminus of rClfA$_{(40-559)}$ was not protected in these assays, and the possibility cannot be ruled out that some proteolysis occurred at the C-terminus of domain N3, which was not detectable by SDS-PAGE. Mass spectrometric analysis would have been necessary to determine the precise molecular weights of products of proteolysis. However, the major proteolytic event on rClfA$_{(40-559)}$ in staphylococcal supernatants appeared to be the rapid and complete cleavage to a 42-kDa band, apparently by aureolysin. Only in the absence of aureolysin were slower cleavage events observed.

3.2.2 Degradation of recombinant ClfA by purified aureolysin

To demonstrate definitively that aureolysin caused the cleavage described above, rClfA$_{(40-559)}$ was incubated for 30 min with varying amounts of purified aureolysin (purified from 8325-4 *sarA* culture supernatants, a kind gift of Prof. Jan Potempa, Krakow) and analysed by SDS-PAGE, as described in Section 2.12. The amounts of aureolysin used in this experiment were insufficient to be visualised by Coomassie staining (Figure 3.4, lane 2). rClfA$_{(40-559)}$ was degraded over 30 min by increasing concentrations of aureolysin, up to 1 µg/ml final concentration. As was observed with the 8325-4 *sspA* culture supernatant, a single cleavage event occurred (Figure 3.4, lanes 3-10), reducing rClfA$_{(40-559)}$ from ca. 80 kDa to 42 kDa. This band corresponded in size to that with N-terminal residue V_{221} (Section 3.2.1). Addition of 20 mM o-phenanthroline completely inhibited cleavage by aureolysin (Figure 3.4, lane 11). This showed that aureolysin is capable of the concentration-dependent cleavage of rClfA$_{(40-559)}$ from 80 kDa to a single 42-kDa product.

3.2.3 Degradation of recombinant ClfA by purified SspA serine protease

To demonstrate definitively that SspA was causing the aureolysin-independent cleavage described above, rClfA$_{(40-559)}$ was incubated with purified SspA

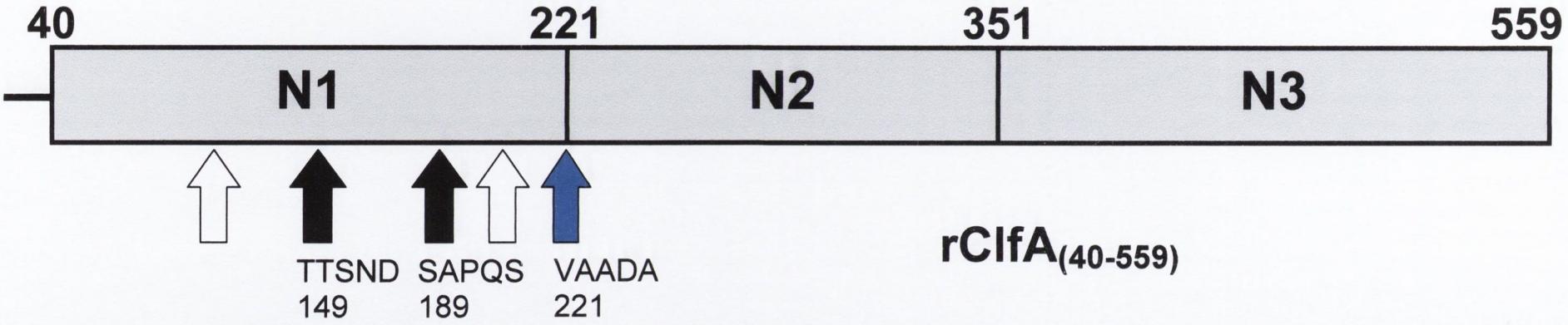


Fig 3.2 Cleavage events in domain N1 of rClfA$_{(40-559)}$

Schematic representation of region A indicating cleavage sites. Blue arrow indicates aureolysin cleavage site. Black arrows indicate two SspA cleavage sites, the N-termini of which were sequenced. White arrows indicate approximate positions of two additional putative SspA cleavage sites identified from the amino acid sequence of ClfA at these positions.

Fig 3.3 Amino acid sequence of ClfA from *S. aureus* strain Newman

Residues 1-39 constitute the signal peptide. Residues 40-559 constitute region A containing domains N1, N2 and N3. Residues 560-863 make up region R. Residues from 864-933 constitute regions W and M. The LPDTG sorting motif (amino acids 896-900) is underlined. Regions outside region A are coloured grey. SLAAVA motif is coloured red. Residues revealed in N-terminal sequencing are highlighted yellow. N-terminal sequencing revealed residue 151 as S, rather than F, illustrated here in the Newman sequence. Putative SspA cleavage sites are coloured blue. Identified cleavage sites and putative cleavage sites are marked with arrows.

(endoproteinase Glu-C, Sigma) and analysed by SDS-PAGE and Coomassie staining. Active SspA itself runs at about 30 kDa on SDS-PAGE (Section 3.2.1; Shaw *et al.*, 2004). Insufficient purified protease was used in this experiment to be visible on stained gels (Figure 3.5, lane 2). Incubation with SspA resulted in a similar pattern of bands to those observed with supernatant of 8325-4 *sarA aur*. Increasing concentrations of the protease caused increased degradation of $rClfA_{(40\text{-}559)}$ from ca. 80 kDa to major bands of ca. 70 and 45 kDa and minor bands (appearing only at higher concentrations of protease) of ca. 77 and 58 kDa (Figure 3.5). Again, the lowest band (45 kDa) appeared to comprise two fragments. The 77-kDa band might have resulted from cleavage at or near S_{54} (NE↑S) (see Figure 3.3), and was not observed in the supernatant experiment (Section 3.2.1). Cleavage at or near T_{98} (QE↑T) might have been responsible for the 70-kDa band. The 58-kDa band corresponded in apparent molecular weight to that with N-terminal sequence TTSND resulting from cleavage at T_{149} (NE↑T) in Section 3.2.1. The two bands at ca. 45 kDa might have been produced by cleavage at or near D_{200} (KD↑V) and D_{195} (TD↑A). The band at 49 kDa, observed after degradation by *S. aureus* 8325-4 *sarA aur* supernatant (in Section 3.2.1) did not appear in this experiment, although the N-terminal sequence of that fragment, SAPQS, was consistent with cleavage by a glutamyl endopeptidase (S_{189}, NE↑S).

α_2-macroglobulin is a general inhibitor of proteases, forming complexes with them at a 1:1 stoichiometry. It is not a potent inhibitor of SspA serine protease, but gives partial inhibition of activity at high concentrations. Partial inhibition of degradation of $rClfA_{(40\text{-}559)}$ by SspA was seen in the presence of a high concentration of α_2-macroglobulin (Figure 3.5, lane 11).

It can be concluded, therefore, that SspA serine protease was responsible for most of the cleavage of $rClfA_{(40\text{-}559)}$ observed in aureolysin-deficient supernatant in Section 3.2.1, and that degradation was concentration-dependent.

3.2.4 Degradation of recombinant ClfA by human cathepsin G and human neutrophil elastase

To investigate whether human serine proteases present in the azurophil granules of neutrophils were capable of the degradation of ClfA, $rClfA_{(40\text{-}559)}$ was incubated with purified human cathepsin G and human neutrophil elastase (kind gifts of Prof. Jan Potempa, Krakow). Incubation with both human cathepsin G and

human neutrophil elastase resulted in cleavage of $rClfA_{(40-559)}$ into fragments of ca. 53 kDa and 45 kDa (Figure 3.6). The 53-kDa band was apparent only at low protease concentrations. A faint band migrating slightly more slowly than the major 45-kDa band appeared at lower concentrations of elastase (Figure 3.6, panel B, lanes 3-6). The ca. 45-kDa band corresponded to the shortest N-terminal truncate of $rClfA_{(40-559)}$ produced by the staphylococcal SspA serine protease (Section 3.2.3). Both cathepsin G and elastase are inhibited by α-1-antitrypsin. Complete inhibition of the degradation of $rClfA_{(40-559)}$ by these proteases was observed in the presence of excess α-1-antitrypsin (Figure 3.6, panels A and B, lane 11). Although cleavage close to the C-terminus of $rClfA_{(40-559)}$ could not be ruled out, it appeared that human serine proteases were capable of the almost complete removal of domain N1 from recombinant region A of ClfA. The cleavage was concentration-dependent. Mass spectroscopy would be necessary to show the precise molecular weights of these products. The possibility that cleavage of region A occurred close to the C-terminus was addressed in experiments where the full-length protein was displayed on the surface of *S. aureus* cells (see Section 3.2.6).

3.2.5 Binding of $rClfA_{(40-559)}$ and $rClfA_{(220-559)}$ to fibrinogen

The cleavage of region A from N123 (represented by $rClfA_{(40-559)}$) to N23 by proteases might affect the binding of ClfA to fibrinogen. In order to address this issue, $rClfA_{(220-559)}$ was purified from *E. coli* cells using the same procedure as for $rClfA_{(40-559)}$ (Section 2.10). $rClfA_{(220-559)}$ represents N23 and includes an N-terminal 6 x His tag. An ELISA-type assay was used to measure the binding of the recombinant proteins to fibrinogen immobilised in the wells of microtitre dishes (Section 2.14).

The kinetics of binding of $rClfA_{(40-559)}$ and $rClfA_{(220-559)}$ to 5 μg/ml immobilised fibrinogen were similar between 0.002 μM and 2 μM protein (Figure 3.7, panel A), although higher protein concentrations were needed for saturation to be reached and apparent K_D values calculated. Following incubation in microtitre dishes, excess unbound proteins were aspirated from the wells and samples analysed by SDS-PAGE and Western immunoblotting. Comparison of fresh protein and protein incubated at room temperature for 1 hour in the adherence assay showed that no significant proteolysis of either recombinant protein had occurred during

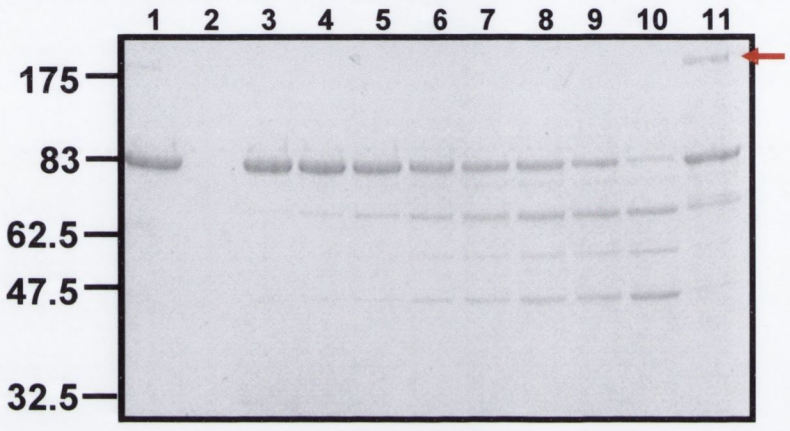


Fig 3.5 Degradation of rClfA$_{(40-559)}$ by SspA serine protease

rClfA$_{(40-559)}$ was incubated with purified SspA for 30 min at 37 °C, separated on a 10% (w/v) acrylamide gel and stained with Coomassie brilliant blue. Lane 1, rClfA$_{(40-559)}$; lane 2, SspA (1 µg/ml); lanes 3-10, rClfA$_{(40-559)}$ + SspA at increasing concentrations (20 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml, 500 ng/ml, 1 µg/ml); lane 11, rClfA$_{(40-559)}$ + SspA (300 ng/ml) + α_2-macroglobulin (0.125 U/ml). Arrow indicates α_2-macroglobulin. Sizes are in kDa.

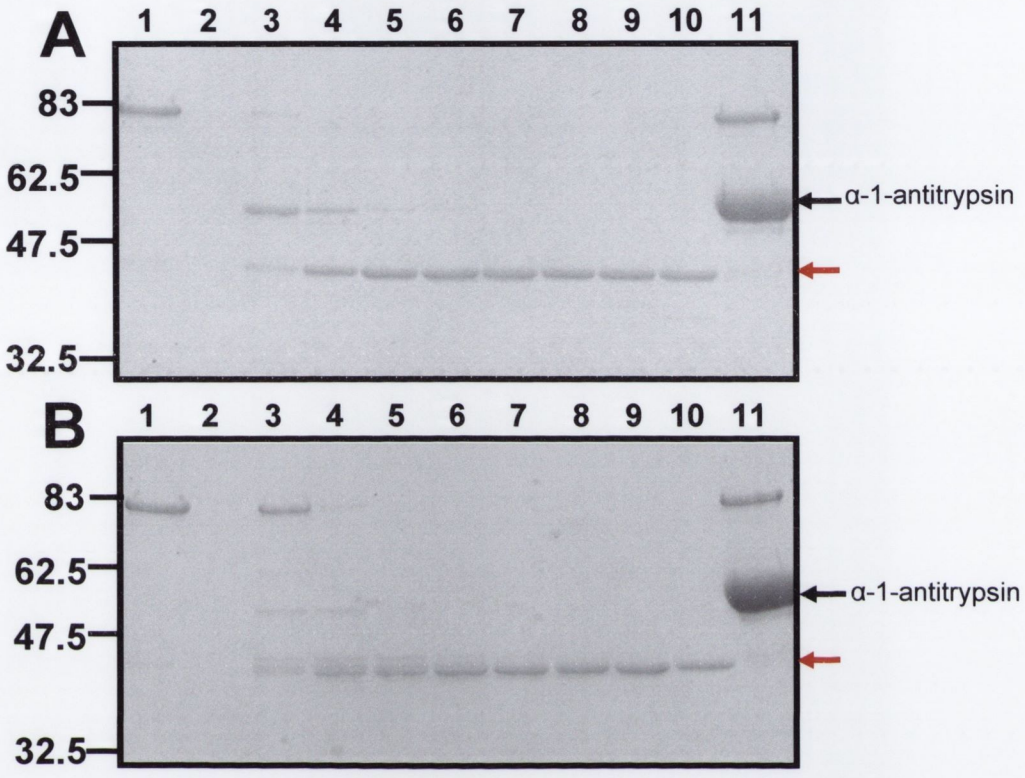
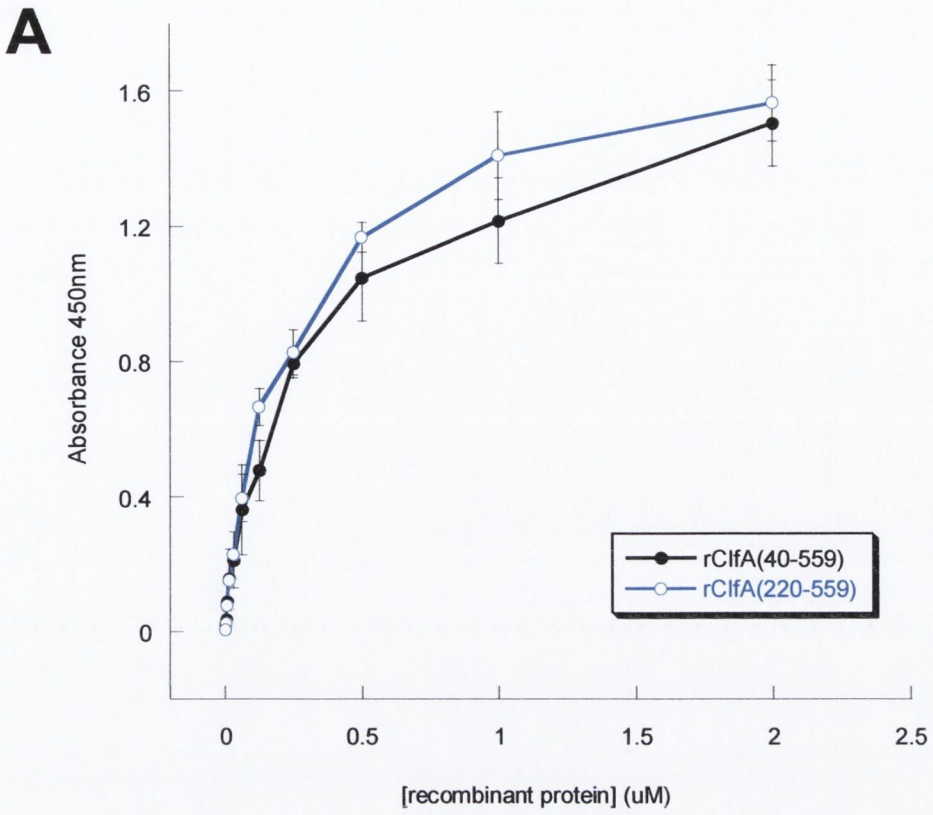


Fig 3.6 Degradation of rClfA$_{(40-559)}$ by cathepsin G and elastase

rClfA$_{(40-559)}$ was incubated with purified human neutrophil cathepsin G (panel A) or purified human neutrophil elastase (panel B) for 30 min at 37 °C, separated on 10% (w/v) acrylamide gels, and stained with Coomassie brilliant blue. Lane 1, rClfA$_{(40-559)}$; lane 2, protease (1 µg/ml); lanes 3-10, rClfA$_{(40-559)}$ + protease at increasing concentrations (20 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml, 500 ng/ml, 1 µg/ml); lane 11, rClfA$_{(40-559)}$ + protease (300 ng/ml) + α-1-antitrypsin (1 mg/ml). Lowest migrating products in each panel are marked with a red arrow. Sizes are in kDa.



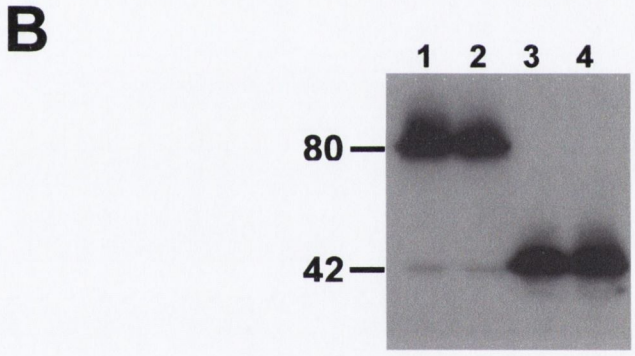


Fig 3.7 Adherence of rClfA$_{(40-559)}$ and rClfA$_{(220-559)}$ to immobilised fibrinogen

A. Recombinant proteins were incubated in wells coated with 5 µg/ml fibrinogen and detected with polyclonal rabbit anti-ClfA region A (rClfA$_{(40-559)}$) antibodies. rClfA$_{(40-559)}$ represents N123 and rClfA$_{(220-559)}$ represents N23.

B. Following incubation with fibrinogen, excess recombinant proteins were separated by SDS-PAGE, transferred to PVDF membrane and Western immunoblotting using anti-ClfA antibodies allowed visualisation of the integrity of the proteins. Lane 1, rClfA$_{(40-559)}$ before incubation; lane 2, rClfA$_{(40-559)}$ following incubation; lane 3, rClfA$_{(220-559)}$ before incubation; lane 4, rClfA$_{(220-559)}$ following incubation.

incubation (Figure 3.7, panel B). These data indicate that domain N1 of ClfA is not involved in fibrinogen binding. Although this result has previously been described, the protease-sensitivity of the full-length protein had not been considered in those studies (McDevitt *et al.*, 1995; 1997; O'Connell *et al.*, 1998).

3.2.6 Cleavage of ClfA on the *S. aureus* cell surface by purified aureolysin and SspA serine protease

In order to determine whether ClfA on the surface of *S. aureus* cells is subject to proteolysis by aureolysin and SspA, whole bacterial cells were incubated with proteases, followed by extraction of cell wall-associated proteins, which were visualised by Western immunoblotting. Strain SH1000 was used for these experiments. This strain is a derivative of 8235-4 in which the defective *rsbU* gene has been replaced with the wild-type gene of strain COL, resulting in wild-type activity of SigB (see Sections 1.5.3.2 and 6.1; Horsburgh *et al.*, 2002). The consequences of this are that SH1000 produces low levels of exoproteases including aureolysin and SspA serine protease (Horsburgh *et al.*, 2002; Shaw *et al.*, 2004) and high levels of ClfA, making it a more suitable strain for use in these studies than 8325-4 or Newman (see Chapter 6).

S. aureus cells with undegraded ClfA on the surface were obtained by growing SH1000 cells to mid-exponential phase, at which point little ClfA was present on the cell surface (see Chapter 6), and diluting into fresh broth. These cells were grown to early exponential phase, where some ClfA was present (see Chapter 6), but aureolysin and other proteases were not yet active (Shaw *et al.*, 2004; Horsburgh *et al.*, 2002), washed extensively and incubated with purified aureolysin or SspA, as described in Section 2.13. After additional washing, cell wall-associated proteins were solublised by lysostaphin digestion and SDS-PAGE and Western immunoblotting was carried out using anti-ClfA region A antibodies, as described in Sections 2.7 and 2.8.

Following incubation without proteases, ClfA was observed mainly as a single band of approximately 185 kDa. A faint band appeared at ca. 150 kDa (Figure 3.8, lane 1). After incubation with aureolysin, the 185-kDa band had disappeared and was replaced by a 150-kDa band (Figure 3.8, lane 2), indicating complete proteolysis to a single, smaller truncate. The nature of the method of protein isolation (lysostaphin digestion) ensured that the only proteins observed were

those that had been cleaved at the N-terminus, their C-termini being specifically liberated from cell wall peptidoglycan by lysostaphin after protease treatment and washing. Inclusion of EDTA or o-phenanthroline with aureolysin inhibited proteolysis (Figure 3.8, lanes 3 and 4). Following incubation with SspA serine protease, the larger 185-kDa protein was degraded to a single band of ca. 155 kDa (Figure 3.8, lane 5).

As had been observed with recombinant protein, the band produced by SspA serine protease degradation migrated slightly more slowly than that produced by aureolysin degradation. α_2-macroglobulin only partially inhibited proteolysis by SspA serine protease. N-terminal sequencing of these products was not possible, as cell wall extracts are complex mixtures of high molecular weight proteins and individual protein yields are low. The difference observed between full-length and aureolysin-cleaved ClfA on the cell surface (ca. 35 kDa) was very close to that observed between full-length (N123) and aureolysin-cleaved rClfA$_{(40\text{-}559)}$ (N23), above (ca. 38 kDa).

It would be reasonable to argue that the ca. 185-kDa band observed as the highest molecular weight form of ClfA in cell wall extracts corresponds to full length ClfA, and that the ca. 150-kDa band produced by aureolysin treatment corresponds to ClfA devoid of domain N1 following cleavage at or close to the SLAAVA motif. These data clearly show that native staphylococcal proteases are capable of the degradation of ClfA on the cell surface of *S. aureus*.

3.2.7 Fibrinogen binding of full length and proteolytically cleaved ClfA on *S. aureus* cells

The fibrinogen-binding activity of ClfB on the surface of *S. aureus* cells is lost upon proteolytic removal of domain N1 of region A (McAleese *et al.*, 2001). However, rClfB N23 binds to fibrinogen, despite the absence of domain N1 (Perkins *et al.*, 2001). The presence of the N-terminal 6 x His tag on rClfB N23 is sufficient to allow fibrinogen binding by this molecule, indicating that only the C-terminal residues of N1 are required for fibrinogen binding (Perkins *et al.*, 2001). In order to study whether this was also the case in rClfA$_{(220\text{-}559)}$ (i.e. to determine the effect of proteolytic cleavage on fibrinogen-binding by ClfA) fibrinogen-binding experiments in which the full-length protein or protease-cleaved protein was displayed on the surface of *S. aureus* cells were carried out.

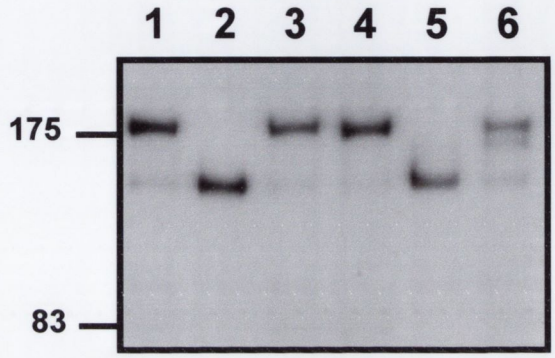


Fig 3.8 Degradation of ClfA on *S. aureus* cell surface by aureolysin and SspA

Cells were grown from an exponential phase starter culture to early exponential phase, washed in PBS and resuspended at OD_{600} 10.0 per ml in PBS. Proteases and/or protease inhibitors were added and incubated for 45 min at 37 °C. Cells were placed briefly on ice before being harvested and washed 3 times in PBS. Cell wall-associated proteins were solublised by lysostaphin digestion and protoplasts pelleted. The supernatant was aspirated and equal volunes of 2 x FSB were added to each sample, before being heated at 95 °C and electrophoresed through a 7.5% (w/v) polyacrylamide gel. Proteins were transferred to PVDF membrane and Western immunoblotting was carried out. Polyclonal rabbit anti-ClfA region A antibody was used as primary antibody with protein A peroxidase as the detection system. Lane 1, SH1000 + PBS; lane 2, SH1000 + aureolysin (2 µg/ml); lane 3, SH1000 + aureolysin (2 µg/ml) + 20 mM o-phenanthroline; lane 4, SH1000 + aureolysin (2 µg/ml) + 10 mM EDTA; lane 5, SH1000 + SspA (2 µg/ml); lane 6, SH1000 + SspA (2 µg/ml) + α_2-macroglobulin (0.5 U/ml).

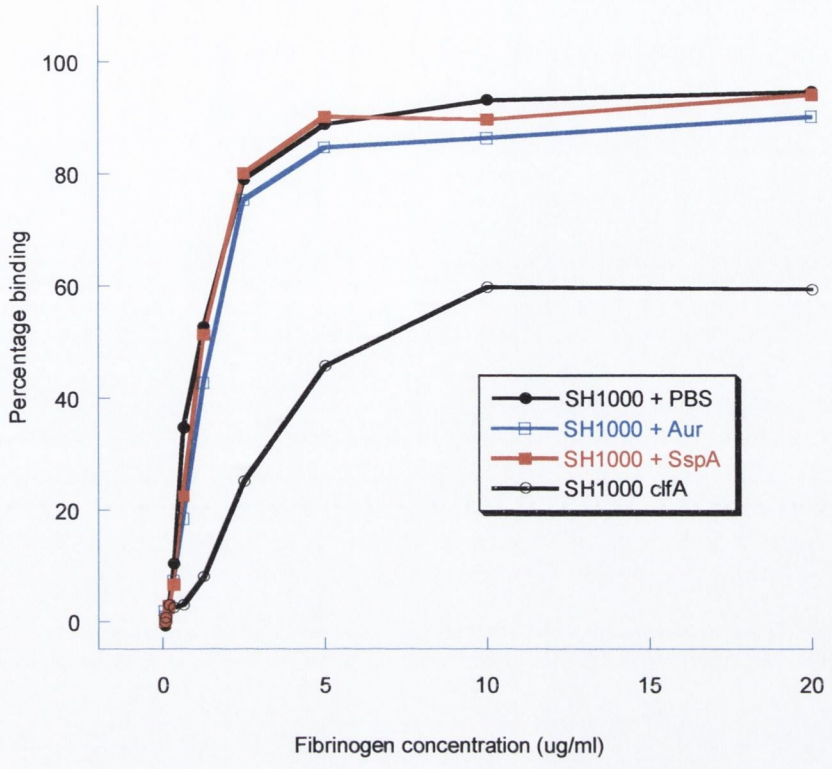


Fig 3.9 Adherence of protease-treated SH1000 cells to immobilised fibrinogen

Cells were grown from an exponential phase starter culture to early exponential phase, washed in PBS and resuspended at OD_{600} 10.0 per ml in PBS. Proteases and/or protease inhibitors were added and incubated for 45 min at 37 °C. Cells were placed briefly on ice before being harvested, and washed 3 times in PBS. Cells were adjusted to OD_{600} 1.0 per ml and incubated on the wells of a microtitre plate (Sarstedt) that had been coated overnight with dilutions of human fibrinogen and blocked with 5% (w/v) BSA. Wells were washed with PBS and adherent bacteria fixed with 25% (v/v) formaldehyde. Adherent bacteria were stained with 5% (w/v) crystal violet. After washing, the stain was released to solution in 5% (v/v) acetic acid and absorbance at 570 nm was measured in an ELISA plate reader. Binding is expressed as percentage of the positive control (SH1000 + PBS; 100% = 0.3535 ± 0.003). SH1000 + PBS, SH1000 + Aur and SH1000 + SspA correspond to lanes 1,2 and 5 in Figure 3.8, respectively.

The same protease-treated samples that were used for Western immunoblotting in Section 3.2.6 were also tested in a bacterial adherence assay, as described in Section 2.14. No significant difference in adherence to fibrinogen was observed between SH1000 cells incubated in PBS alone, with aureolysin or with SspA (Figure 3.9). SH1000 *clfA* was included as a control, because ClfB, FnBPA and FnBPB are expressed during the exponential phase of growth. However, all three of these fibrinogen-binding proteins lose their fibrinogen-binding capacity as a result of proteolytic degradation (McAleese *et al.*, 2001; McGavin *et al.*, 1997; Wann *et al.*, 2000). The contribution of other fibrinogen-binding proteins is shown in the SH1000 *clfA* mutant. However, loss of ClfB and FnBP fibrinogen-binding activity by protease cleavage would likely not be detected in this assay if ClfA activity is unaffected. ClfA is clearly the dominant fibrinogen adhesin under these conditions. This result indicated that the loss of domain N1 of ClfA had no influence on the ability of the cells to bind immobilised fibrinogen.

3.2.8 Multiple sequence alignment of domain N1 of ClfA

The amino acid sequences of ClfA of 8 *S. aureus* strains were aligned using the ClustalW service on the website of the European Bioinformatics Institute. Sequences came from strains COL (sequenced by TIGR), Newman (McDevitt *et al.*, 1994), 8325 (University of Oklahoma), MRSA 252 and MSSA 476 (Sanger Institute), MW2, N315 and Mu50 (Japanese consortia).

Of 181 residues in domain N1, 153 were identical in all 8 strains (Figure 3.10). 19 of the 28 substitutions occurred in only one of the 8 strains. 18 of these occurred only in strain MRSA 252, and are depicted in bold in Figure 3.10. One additional substitution was found within the signal sequence in this strain. 15 of the 18 substitutions in domain N1 that are unique to this strain occurred N-terminal to the first substitution observed in any other strain (at residue 103). These substitutions may be due to sequencing errors or reflect genuine differences.

The other substitution within domain N1 that was unique to strain Newman is underlined in Figure 3.10. The DNA used to make plasmid pCF40, from which $rClfA_{(40-559)}$ was expressed in *E. coli*, was cloned from strain Newman (O'Connell *et al.*, 1998). As described in Section 3.2.1., N-terminal sequencing of one of the products of proteolysis of $rClfA_{(40-559)}$ revealed the amino acid sequence TTSND. This sequence was not present in the published Newman sequence. As can be

seen from the multiple sequence alignment (Figure 3.10), all other strains contain the sequence TTSND at that position, where Newman has TTFND. Residue F_{151} in Newman *clfA* is therefore likely to be the result of a sequencing error.

Apart from F_{151}, the sequence of strain Newman *clfA* N1 was identical to those of strains COL and 8325. The sequences of strains MW2 and MSSA 476 were identical, as were the sequences of strains N315 and Mu50. The sequence of strain MRSA 252 *clfA* N1 was unique and was placed between the Newman/ COL/8325 and MW2/MSSA 476 groups. Substitutions closer to the C-terminal end of domain N1 were common to these groupings. T_{120} was deleted in the MW2/MSSA 476 group. Substitutions at residues 102, 121 and 230 were common to strains MRSA 252 and MW2/MSSA 476. Sequences at residues 103, 120 and 218 were common to MRSA 252 and the Newman/COL/8325 group. Sequences at residues 193, 203, 208 and 210 distinguished the N315/Mu50 group from all other strains, while the MW2/MSSA 476 group alone had a substitution at residue 219.

The substitution at residue 219 was within the SLAA↑VA sequence that, in strain Newman, was cleaved by aureolysin (Figure 3.10). The putative SspA serine protease cleavage sites identified in Section 3.2.1, shown in blue in Figure 3.10, were conserved between all 8 strains.

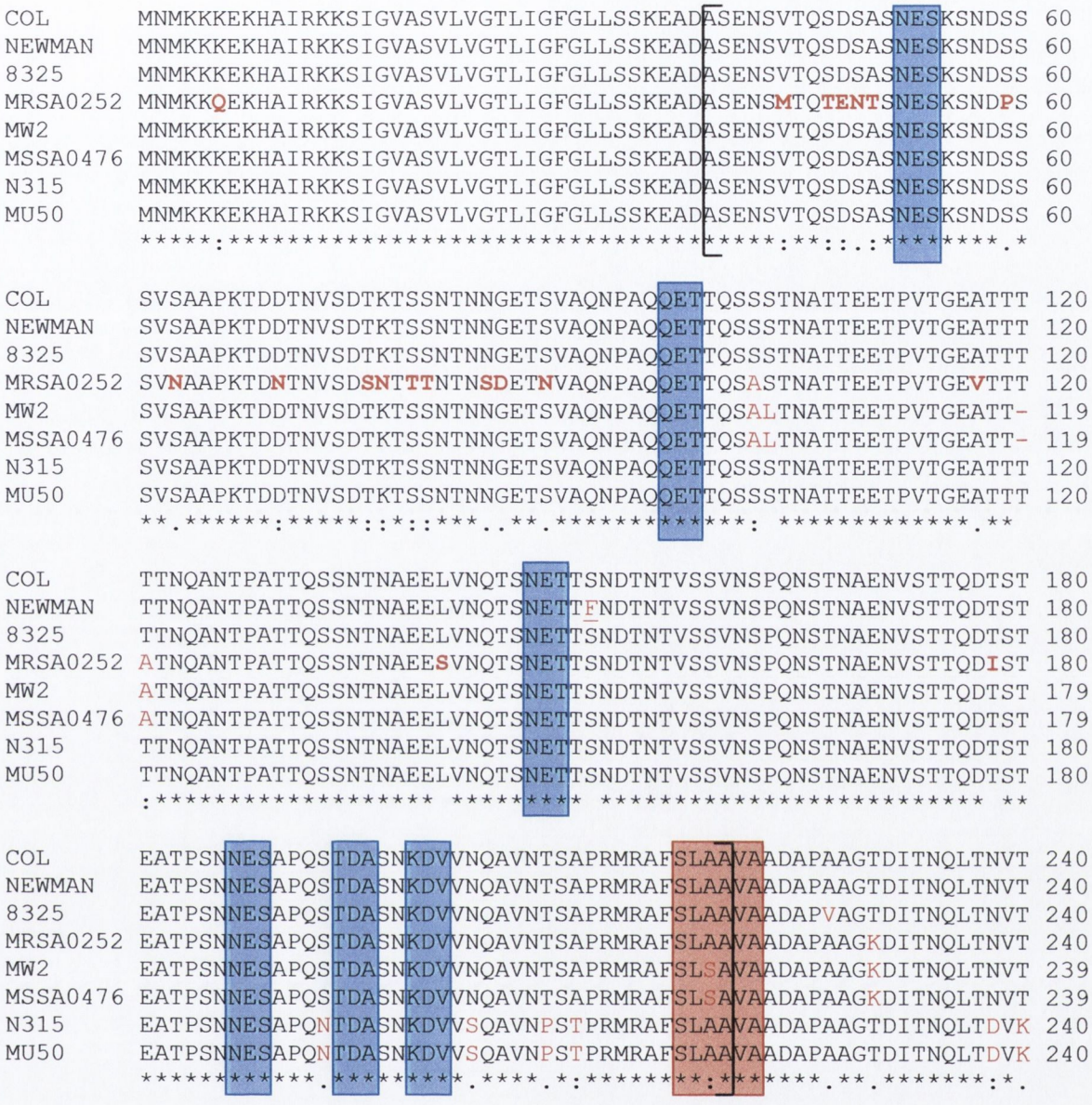


Fig 3.10 Multiple alignment of amino acid sequences of domain N1 of ClfA

All available amino acid sequences of ClfA were aligned using ClustalW. The first 240 residues are shown. The extremities of domain N1 (amino acids 40-220) are marked with black brackets. Strains are indicated on the left of each row. Conserved residues are marked beneath the alignment with an asterisk. Variant residues are indicated as . (non-conservative substitution) or : (conservative substitution) and are coloured red. Red and blue boxes show putative aureolysin and SspA cleavage sites, respectively. Substitutions seen only in strain MRSA 252 are shown in bold. Residue F_{151} in strain Newman is underlined.

3.3 Discussion

The data presented in this Chapter show that *S. aureus* produces at least two proteases that are capable of the removal of domain N1 from region A of ClfA. Supernatants of strain 8325-4 *sarA* caused rapid proteolysis of recombinant region A ($rClfA_{(40-559)}$). Supernatants of isogenic protease mutants of 8325-4, as well as purified staphylococcal proteases, showed that the staphylococcal metalloprotease (aureolysin) and the SspA (V8) serine protease were responsible for the degradation. The actions of purified aureolysin and SspA on $rClfA_{(40-559)}$ were both concentration- and time-dependent. Human neutrophil endosomal serine proteases were also shown to be capable of proteolysis of $rClfA_{(40-559)}$, giving similar sized fragments to SspA.

Purified aureolysin and SspA serine protease cleaved full-length ClfA on the staphylococcal cell surface to a form that corresponds to ClfA devoid of domain N1 of region A. Domains N2 and N3 of region A appeared to be stable and refractory to proteolysis. This is likely to be due to the globular DEv-IgG fold structure displayed by these domains (Deivanayagam *et al.*, 2002). In addition, the stability of the globular domains N2 and N3 in native ClfA on the staphylococcal surface implies that they would be largely stable in $rClfA_{(40-559)}$, except perhaps for a short stretch at the extreme C-terminus, that might not be exposed in the native molecule. This reasoning allows the conclusion that all discernible truncation of $rClfA_{(40-559)}$ occurred N-terminal to domains N2 and N3.

The predicted molecular weight of region A of ClfA (N123, $rClfA_{(40-559)}$) is ca. 57 kDa, but it has an apparent molecular weight of ca. 80 kDa on SDS-PAGE. N23 has a molecular weight of 37 kDa (Deivanayagam *et al.*, 1999) and migrates at 42 kDa after being cleaved by aureolysin. N1 is 180 amino acids in length, while N2 comprises 149 residues, and N3 is 191. Despite this, domain N1 contributes almost half of the apparent molecular weight of N123. On *S. aureus* cells a similar difference in apparent molecular weight between full-length and aureolysin-cleaved ClfA was observed. A protein band of ca. 185 kDa was reduced to ca. 150 kDa by cleavage of domain N1.

An extended, loosely folded structure for domain N1 would explain both its major contribution to the aberrant migration of region A, and its susceptibility to proteolysis. Perhaps the improved purification method described here and inhibition of zinc metalloproteases and serine proteases during purification might

allow crystals of full-length ClfA with domain N1 to be obtained and the structure solved.

Forms of ClfA with apparent molecular weight >200 kDa (described by Hartford *et al.*, 1997) were not observed in this study. This is likely to be as a result of the inclusion of $MgCl_2$ in the lysostaphin digestion buffer, which helps to stabilise protoplasts and prevent their lysis. The >200-kDa form of ClfA observed by Hartford *et al.* (1997) is likely to represent protein that had not been sorted to the cell wall peptidoglycan, retained the membrane-spanning C-terminal domain and that was released from lysed protoplasts during protein isolation. The overexpression of ClfA from plasmids may have contributed to the large amounts of the unsorted form observed in that study.

It is possible that additional staphylococcal proteases are capable of cleavage within domain N1 of region A. This may explain the absence after degradation with purified SspA serine protease of a band of ca. 49 kDa that was observed after degradation by *S. aureus* 8325-4 *sarA aur* supernatant. This band may be the product of another staphylococcal protease in the supernatant, for example SspB or one of the Spl proteases (Section 1.3.10.4; Rice *et al.*, 2001). Alternatively, the production of shorter N-terminal truncates by purified SspA may have eliminated the ca. 49-kDa band. Nevertheless, the truncation of $rClfA_{(40\text{-}559)}$ to N23 by aureolysin appears to be the final cleavage event in ClfA, and cleavage within N1 may be irrelevant to the surface-expressed protein. This goes some way to explaining the absence of N-terminal products of degradation of ClfA. Following cleavage at the SLAAVA motif by aureolysin, domain N1 is presumably degraded by other proteases and the loss of epitopes makes the products undetectable with antibodies. A similar observation has been made with ClfB (McAleese *et al.*, 2001).

The amino acid sequence of domain N1 is highly conserved between strains of *S. aureus*, with the exception of strain MRSA 252. Amino acid substitutions within domain N1 in this strain may reflect a different function for N1 or a reduced importance of the function of N1 in the virulence of this strain. Interestingly, strain COL, which produces Pls and does not bind to fibrinogen (Savolainen *et al.*, 2001) has an identical domain N1 to Newman and 8325. 8325 (and therefore its derivatives 8325-4 and SH1000) has the same amino acid sequence within N1 as Newman. This observation correlated well with the similarity of the patterns of

proteolysis between recombinant protein of Newman origin and cell-surface exposed ClfA from SH1000.

The putative and identified SspA cleavage sites were conserved between all 8 strains. The SLAAVA motif is less highly conserved, strains MSSA 476 and MW2 carrying the sequence SLSAVA. The corresponding aureolysin cleavage site in ClfB is SLAVA. The $A_{219}S$ substitution in the metalloprotease cleavage site of ClfA in these strains may not impact significantly on cleavage, as the minimum consensus sequence for aureolysin cleavage has not been defined. The conservation of these protease cleavage sites may reflect the importance of proteolytic breakdown of domain N1 of ClfA in the pathogenesis of staphylococcal infection.

The fibrinogen-binding ability of ClfA displayed on the cell surface of SH1000 was not affected by the removal of domain N1. This is in contrast to ClfB, which lost the ability to bind fibrinogen following cleavage by aureolysin. This was demonstrated both for rClfB N123 and ClfB that was expressed on the cell surface (McAleese *et al.*, 2001; Perkins *et al.*, 2001). In addition, the fibrinogen-binding activities of $rClfA_{(40\text{-}559)}$ (N123) and $rClfA_{(220\text{-}559)}$ (N23) were similar. This indicates that in ClfA domain N1 is not involved in fibrinogen binding. Although this has been reported (McDevitt *et al.*, 1995; 1997; O'Connell *et al.*, 1998), previous studies did not consider proteolysis of domain N1, and did not take steps to prevent it.

The fibronectin-binding proteins FnBPA and FnBPB lost the ability to bind fibronectin following cleavage by SspA (McGavin *et al.*, 1997). It has since been shown that the A regions of FnBPs bind to fibrinogen. The fibrinogen-binding site is N-terminal to the sites of SspA cleavage. However, binding studies with whole cells were not carried out (Wann *et al.*, 2000). Loss of ligand-binding activity by these MSCRAMMs could allow detachment of bacteria bound to a ligand-coated surface, contributing to the spread of infection. For ClfA, this is not the case, as loss of domain N1 did not cause loss of fibrinogen binding activity.

The 'dock, lock and latch' model of fibrinogen binding (Ponnuraj *et al.*, 2003) suggests that when the fibrinogen γ-chain peptide ligand is bound, N2 and N3 lie folded back with respect to region R instead of being projected in the linear sequence N1-N2-N3-R (see Figure 3.11). Perhaps in this conformation, domain N1 has different properties to those observed with recombinant protein or in the absence of ligand. Domain N1 may interact with region R or even be protected

from proteolysis when ligand is bound. These possibilities were not investigated in this study.

Enzymatic removal of domain N1 may protect *S. aureus* cells during the infectious process from host antibodies by removal of immunodominant epitopes. Domain N1 of ClfA may also be involved in the binding of additional ligands. Domain N1 or specific peptides of N1 may have biological activity in the infected host (e.g. immunomodulation similar to formylated peptides). Bacterial proteins (e.g. CHIPS, formylated peptides) have been shown to influence phagocyte recruitment to sites of infection. The products of proteolytic breakdown of ClfA may also have an immunomodulatory effect. Further study of the role of domain N1 may reveal novel aspects of this protein.

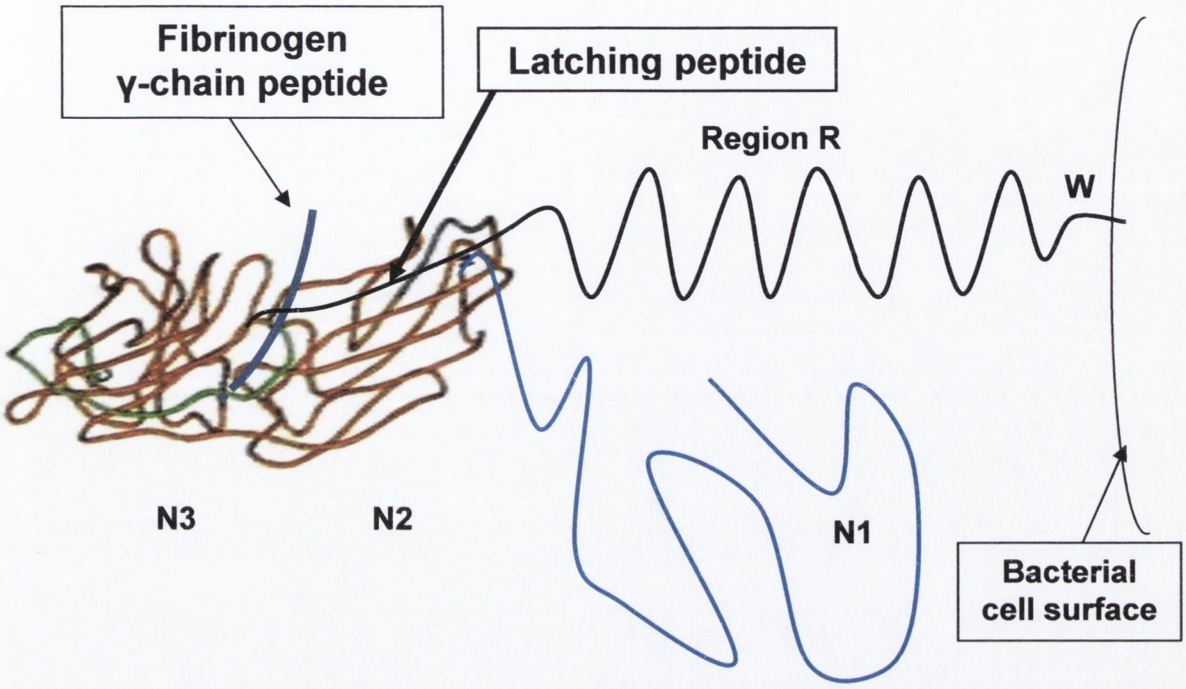


Fig 3.11 Model of ClfA on the bacterial cell surface in complex with the fibrinogen γ-chain peptide

Region W is believed to span the cell wall. Region R acts as a stalk for the functional projection of region A at the cell surface. Domains N2 and N3 have a globular structure. Domain N1 may be elongated and may or may not make contacts with other parts of the protein. The γ-chain peptide of fibrinogen binds in the pocket between domains N2 and N3, as shown. After docking of the fibrinogen ligand the latching peptide folds in between β-sheets of domain N3, reversing the orientation of domains N1, N2 and N3 with respect to the cell surface. Modified from Ponnuraj *et al.*, 2003.

Chapter 4

Genetic Manipulation of *clfA*

4.1 Introduction:

4.1.1 Genetic manipulation in *S. aureus*

Engineered genetic mutations designed to eliminate virulence factors in *S. aureus* usually integrate a selectable marker that can be used to identify, purify and maintain specific mutants in the laboratory. Genes encoding antibiotic resistance are commonly used for this purpose. However, the small number of useful antibiotic resistance markers available in *S. aureus* has made it difficult to eliminate multiple factors in a single strain. The isolation of mutants defective in four fibrinogen-binding proteins (ClfA, ClfB, FnBPA and FnBPB) or in factors suspected of playing a role in platelet activation (ClfA, ClfB, SdrE and protein A) has required a novel approach.

Drug resistance markers used in molecular genetics of *S. aureus* include erythromycin (*ermC*: O'Reilly *et al.*, 1986; Phonimdaeng *et al.*, 1990), tetracycline (*tetK*: O'Connell *et al.*, 1993; Greene *et al.*, 1995), tetracycline and minocycline (*tetM*: Sloane *et al.*, 1991; Novick *et al.*, 1993), chloramphenicol (*cat*: Shaw *et al.*, 1985; Horinouchi and Weisblum, 1982a), kanamycin-neomycin (Patel *et al.*, 1989), gentamycin-kanamycin (Patti *et al.*, 1994), spectinomycin (Murphy *et al.*, 1985) and ethidium bromide (Patel *et al.*, 1987). Some resistance determinants are of limited use because resistant colonies are difficult to select when the marker is present at a single copy per cell (e.g. the chloramphenicol resistance marker associated with pTV1*ts* and pTS1, and the ethidium bromide marker used by Patel *et al.* to inactivate the protein A gene). Aminoglycoside resistance markers are difficult to transduce because spontaneous mutants arise at high frequency in staphylococci, making transductants difficult to identify. Erythromycin and tetracycline resistance determinants have been found to be very reliable (Foster, 1998). In addition, most natural isolates express high levels of resistance to β-lactam antibiotics, further narrowing the range of drugs that are useful against staphylococci. These difficulties have made it important to develop new strategies to isolate mutations such as frameshifts and deletions which do not involve the insertion of antibiotic resistance markers.

4.1.2 Transposon mutagenesis

Null mutations can be generated by transposon insertion, directed plasmid integration or allelic replacement. Transposon mutagenesis requires no knowledge of the targeted gene; only a phenotypic screen for mutants is required. In principle transposon insertion mutagenesis relies upon the lack of site-specificity of the transposable element. However, most naturally occurring transposons exhibit insertion site preferences, creating 'hot spots' for insertion and leaving some regions devoid of insertions. This problem was overcome recently by use of the *bursa aurealis* element, which exhibited no sequence preference in the genome of *S. aureus* Newman (Bae *et al.*, 2004). Saturation transposon mutagenesis of key strains creates an important resource for screening for new virulence factors and regulators. However, since the availability of genome sequence data for *S. aureus*, transposon mutagenesis has become less important as site-specific mutants in all genes can now be designed.

4.1.3 Allelic replacement mutagenesis

Allelic replacement is used to introduce site-specific mutations and is carried out in two steps. The first step involves the design and assembly of the mutation. This is usually carried out in plasmids that can replicate in *Escherichia coli*. The second step involves the delivery of the construct into *S. aureus* and recombination with the chromosomal locus. This requires use of one of three approaches: incompatible plasmids, suicide plasmids or temperature-sensitive plasmids.

4.1.3.1 Plasmid incompatibility

In plasmid incompatibility the mutated gene is introduced in *S. aureus* on a shuttle plasmid. A second, incompatible, plasmid is introduced to the same strain. Selection for the incompatible plasmid causes elimination of the shuttle plasmid, requiring growth for 100-200 generations. Replacement mutants can then be detected by screening for a loss of resistance associated with the shuttle plasmid and a gain of mutant phenotype (Patel *et al.*, 1987). Plasmid incompatibility is

time-consuming and the extensive serial dilution under selection involved may lead to the introduction of point mutations in unrelated loci.

4.1.3.2 Directed plasmid integration

Suicide plasmids are plasmids lacking replication functions in *S. aureus*. Upon introduction by electroporation they must integrate directly into the chromosome by homologous recombination or be rapidly lost. Suicide plasmids can be used in directed integration to interrupt genes and for allelic replacement.

Directed integration involves the introduction of a portion of the target gene to a suicide plasmid and integration in the *S. aureus* chromosome by homologous recombination (Figure 4.1). A single cross-over event leads to duplication of the inserted sequence and disruption of the gene by the entire plasmid. Suicide integration occurs upon introduction of the plasmid to *S. aureus* strain RN4220 (Kreiswirth *et al.*, 1983), a restriction-deficient strain that is used as an intermediate host for genetic manipulations involving plasmids. The plasmid-associated resistance marker can be used for phage-mediated transduction of the mutation to other strains. An advantage of directed plasmid integration for gene disruption is that mutations are relatively easy to isolate, compared with allelic replacement. However, mutations generated in this manner are generally polar, disrupting downstream genes within an operon. Another disadvantage is that duplication of portions of the gene, which is necessary for recombination, can result in residual gene function, particularly in short genes. Additionally, duplication of parts of the chromosome by suicide integration can, in the absence of selection, allow reversion to wild-type by plasmid excision. This is an important consideration if strains are to be used in animal models of infection, where continued antibiotic selection is difficult.

An example of directed plasmid integration is the generation of transcriptional fusions using pAZ106 (Figure 4.2; Kemp *et al.*, 1991). Plasmid pAZ106 has been used to generate transcriptional reporter fusions to *clfB*, *spa* and haemolysin A (*hla*) in *S. aureus* (McAleese *et al.*, 2001; Chan and Foster, 1998). The plasmid bears no *S. aureus* replication functions, but contains a promoterless Gram-positive β-galactosidase gene (*lacZ*) following a multiple cloning site. Inserted DNA becomes the only region of homology to the *S. aureus* chromosome and integration occurs in that region, fusing the chromosomal (wild-type) copy of the

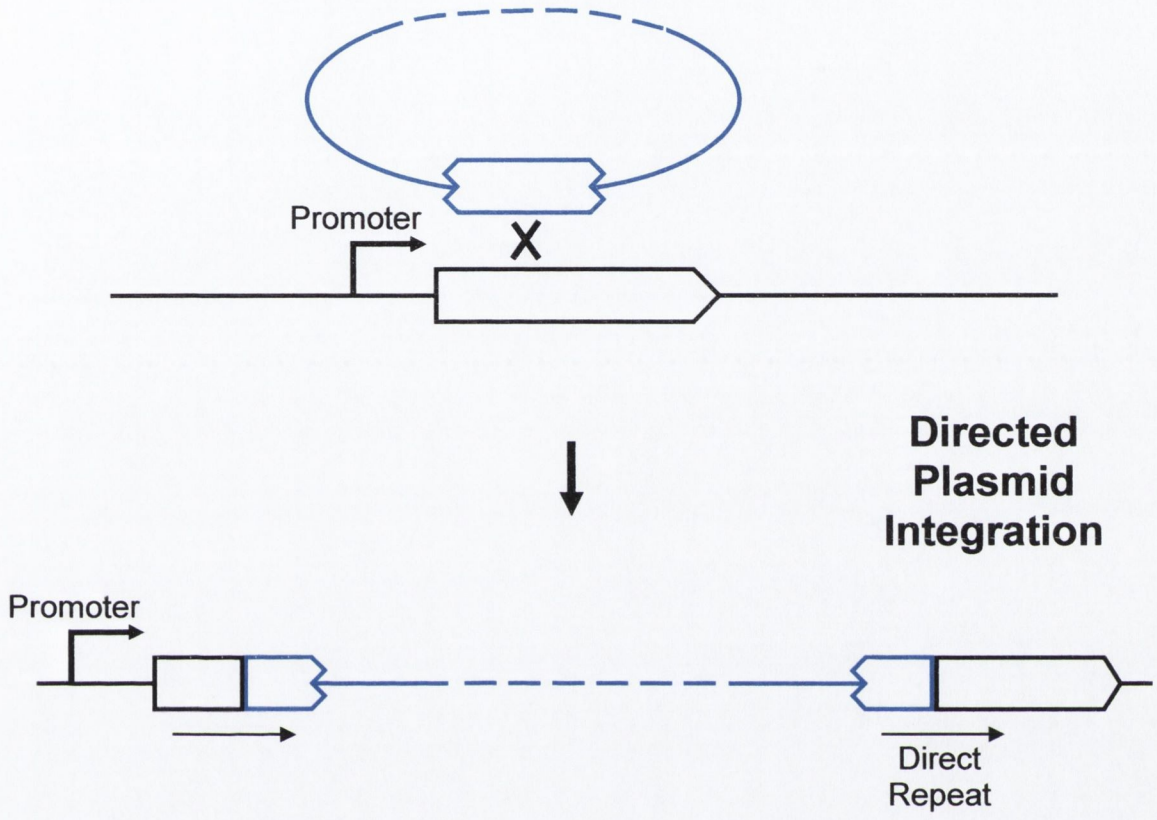


Fig 4.1 Suicide integration

Plasmid integration at the chromosomal locus by homologous recombination (single cross-over event) causes duplication of the inserted sequence, creating direct repeats. The suicide vector (blue) has no replication functions for this organism and is stably integrated. The sequence inserted in the plasmid determines the site of integration.

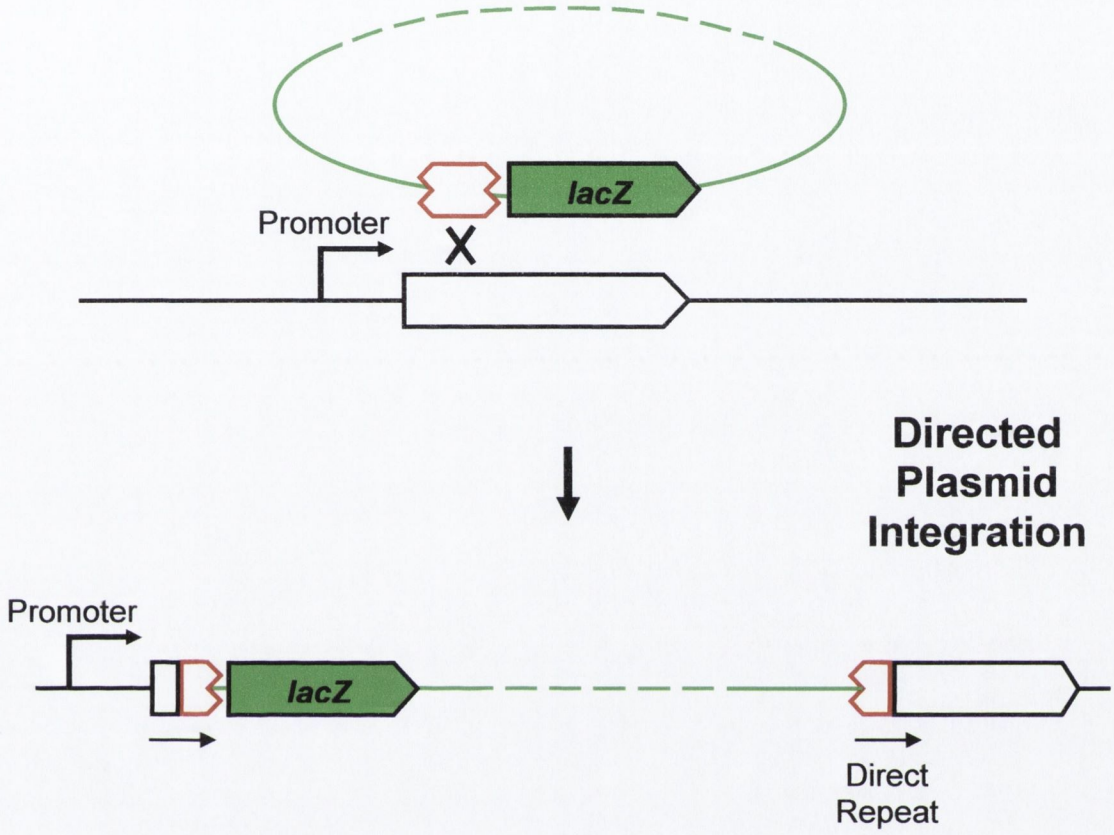


Fig 4.2 Transcriptional fusion to *lacZ*

The 5' end of the gene is inserted upstream of the *lacZ* gene in pAZ106. Plasmid integration at the chromosomal locus occurs by homologous recombination (single cross-over event). This causes duplication of the inserted sequence and fusion of the 5' end of the chromosomal copy of the gene, and upstream sequences, to *lacZ*. The *lacZ* element is promoterless but carries a ribosome-binding site. The promoter of the gene drives transcription of the 5' end of the gene and *lacZ* as a single transcript. The polypeptides are translated independently.

inserted DNA to the β-galactosidase gene (Figure 4.2). The inserted copy of the DNA becomes fused to the 3' end of the gene. Transcription occurs from the promoter but drives β-galactosidase production in the place of the natural gene product. The amount of β-galactosidase produced is measured quantitatively, and is a reporter of promoter activity. If a copy of the promoter of the gene in question is included in the plasmid, duplication occurs and the gene is not inactivated (e.g. *hla*$^{+}$ *hla::lacZ*; Chan and Foster, 1998). This is particularly useful if the gene is essential. If the promoter is omitted a null mutation is generated (e.g. *clfB::lacZ*; McAleese *et al.*, 2001). A minimum of 500-700 bp of homology is required for suicide plasmid integration. If the 5' end of the gene contains residues essential for function and the region is duplicated, a null mutation will in fact not be generated, as the 5' end of the gene will be expressed as normal. The *clfB::lacZ* mutation generated a functionally null mutation as the N-terminal ClfB product of the fusion was secreted into the medium and not anchored in the cell wall as is the wild-type protein. A deletion in the 3' end of a gene may not result in a loss of function if a large N-terminal truncate is biologically active. The isolation of null mutations by directed integration becomes more difficult with shorter genes because of the need to include 500-700 bp of DNA to provide sufficient homologous sequence to allow recombination.

Directed plasmid integration can also be used for allelic replacement. Integration of the suicide plasmid occurs at the introduced region of homology directly upon introduction to *S. aureus*. Single cross-over integration leads to duplication of flanking regions of DNA (Figure 4.3, A). If selection for a mutation-associated (rather than plasmid-associated) resistance marker is employed, a double cross-over event can occur via an intermediate (Figure 4.3, B), at low frequency, and result in direct replacement of the wild-type allele (Figure 4.3, C). Transductional resolution of single cross-over integrants can be used to increase the yield of double cross-over replacement mutants (McDevitt *et al.*, 1992). The isolation of stable replacement mutants requires the use of an antibiotic resistance determinant associated with the mutation. As with directed plasmid integration using suicide plasmids, above, integration occurs in strain RN4220. A mutation-associated resistance marker is again necessary for phage-mediated transduction of the mutation to other strains, including the transductional resolution of integrants.

4.1.3.3 Allelic replacement using temperature-sensitive plasmids

Allelic replacement by recombination in *S. aureus* can also be carried out using plasmids with a thermosensitive replicon derived from pE194 (Horinuchi and Weisblum, 1982b) or pT181 (Khan and Novick, 1983). Such temperature-sensitive plasmids can replicate in *S. aureus* at 30 °C, but not at 42 °C. Growth at restrictive temperatures causes loss of the plasmid and reveals variants in which the plasmid has integrated into the chromosome. Selection for plasmid-associated resistance at the restrictive temperature selects for single cross-over integration (Figure 4.3, panel A). Selection for mutation-associated resistance can result in either single or double cross-over recombination. Growth of single cross-over integrants at the temperature permissive for replication induces plasmid excision (Biswas *et al.*, 1993). In the absence of selection, a further temperature shift to the restrictive temperature results in loss of the excised plasmid. Excision can result in the removal or retention of the mutation (Figure 4.3, C). A proportion of the cells retain the wild-type gene in the chromosome and the remainder retain the mutation. Temperature-sensitive plasmids can be transferred between strains at the permissive temperature, allowing allelic replacement to be performed by temperature shifting in the desired strain. This method has been successfully used to generate individual mutations in the *fnbA* and *fnbB* genes in *S. aureus* (Greene *et al.*, 1995).

An example of directed integration allowing gene interruption or subsequent allelic replacement is the use of pG$^+$host temperature sensitive plasmids from lactococci (Maguin *et al.*, 1996). pG$^+$host plasmids have successfully been used for gene inactivation by directed plasmid integration in *S. aureus* (Supersac *et al.*, 1998; O'Brien *et al.*, 2002a), *S. epidermidis* (Hartford *et al.*, 2001b), as well as *Streptococcus pyogenes* (Perez-Casal *et al.*, 1995), *Strep. equi* (M. Meehan, personal communication), *Strep. thermophilus, Lactococcus lactis* and *Enterococcus faecalis* (Maguin *et al.*, 1996). One advantage of pG$^+$host is that the plasmid can be maintained as a stable integrant at 37 °C without antibiotic selection (Hartford *et al.*, 2001b). A second advantage of this vector system is that single copy integration can be achieved in the strain of choice, instead of integration in RN4220 followed by transduction. pG$^+$host vectors can be used both for gene disruption and for allelic replacement mutagenesis by inducing excision and loss of the integrated plasmid by temperature shifting (Supersac *et al.*, 1998).

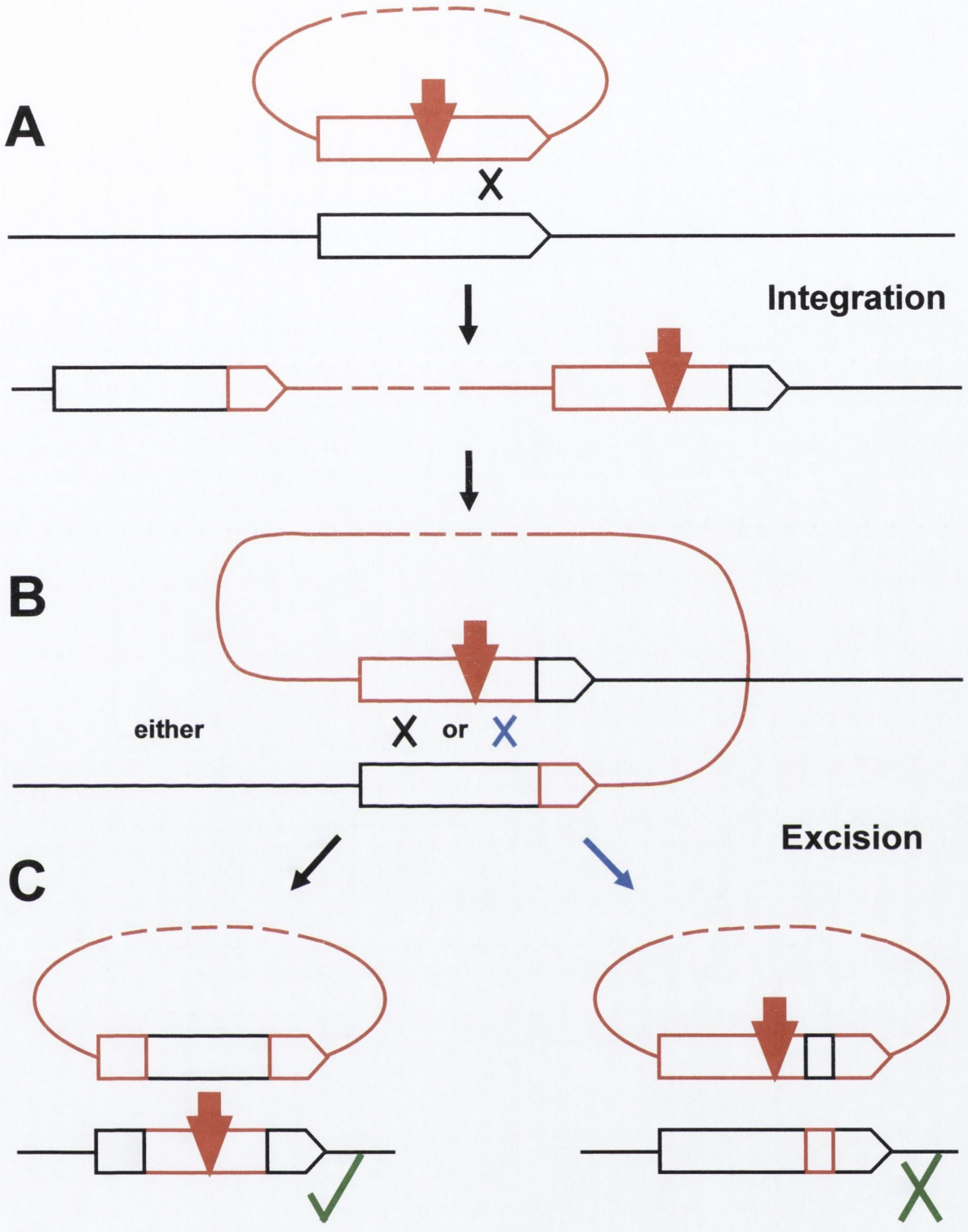


Fig 4.3 Plasmid integration and excision

A: Plasmid integration at the chromosomal locus by single cross-over event on one side of the mutation causes duplication of the inserted sequence.

B: A second recombination event allows plasmid excision via an intermediate.

C: A second cross-over on the same side of the mutation (in blue) as the first causes removal of the mutation to the plasmid. A second cross-over on the opposite side (in black) to the first causes removal of the wild-type gene, resulting in successful allelic replacement.

Allelic replacement mutagenesis using temperature-sensitive plasmids is more flexible than directed plasmid integration, as it allows the possibility of allelic replacement without the requirement of a mutation-associated resistance marker. This allows the construction of non-polar mutations by the generation of in-frame deletions (Sau *et al.*, 1997) or insertions (Rice *et al.*, 2001). Allelic replacement allows single-base pair substitutions in chromosomal loci, facilitating study of gene regulation (e.g. changes to operators, promoters and other DNA motifs; Kaatz *et al.*, 2005). In the absence of a mutation-associated marker a simple screen for successful replacement is desirable.

4.1.4 Temperature-sensitive plasmids

pTS2 is a useful temperature-sensitive plasmid in *S. aureus* (Greene *et al.*, 1995). It contains the replication functions and copy control mechanism of pTV1*ts* (derived from pE194*ts*), the multiple cloning site of pBluescript SK and the chloramphenicol resistance determinant of pC221 (Shaw *et al.*, 1985). A shuttle plasmid, pCL10, incorporated the ampicillin resistance gene and origin of replication of pBR322 combined with the chloramphenicol resistance marker and origin of replication of pTV1*ts* (Sau *et al.*, 1997). The weakness of these plasmids is the chloramphenicol resistance determinant. This determinant has been widely used to introduce mutations that are associated with a second resistance determinant (e.g. erythromycin or tetracycline), using selection for the mutation-associated resistance determinant. Using the chloramphenicol determinant alone it is impossible to select integrants, as the concentration of chloramphenicol allowing growth of integrants does not sufficiently suppress background growth of cells that retain the unintegrated plasmid. It is possible to screen for presence or absence of chloramphenicol resistance only by testing single colonies grown in the absence of antibiotic. New temperature-sensitive plasmids with alternative resistance determinants are required to overcome these technical difficulties and allow faster, more versatile and easier allelic replacement mutagenesis.

4.1.5 Site-directed mutagenesis of *clfA*

Site-directed mutagenesis of plasmids can easily be carried out using overlap primer PCR (O'Connell *et al.*, 1998). This method allows the direct introduction of point mutations or deletions in plasmids (Figure 4.4).

In order to test the role of fibrinogen binding in the possible antiphagocytic effect of ClfA, a ClfA derivative that cannot bind fibrinogen needed to be expressed on the cell surface. The crystal structure of ClfA domains N23 revealed residues that were necessary for fibrinogen-binding activity in the recombinant protein ($rClfA_{(221-559)}$; Deivanayagam *et al.*, 2002). However, the substitutions were not introduced to ClfA expressed on the *S. aureus* cell surface. Overlap primer PCR was used to introduce two amino acid substitutions at positions $P_{336}S$ and $Y_{338}A$ in ClfA expressed from a multicopy plasmid in *S. aureus*.

4.1.6 Summary

This Chapter describes the construction and validation of mutant strains and plasmids used in this study. These include strains constructed by generalised transduction of null mutations in surface protein genes in order to study their relative role in resistance to phagocytosis by *S. aureus*. The construction of two temperature sensitive plasmids based on pTS2, but containing alternative antibiotic resistance genes, is described. These plasmids were designed to facilitate more efficient allelic replacement in *S. aureus*. The use of one of these plasmids to generate a novel *clfA*-null mutation, introduced by allelic replacement to the *S. aureus* chromosome, is also described. In addition, the establishment of a *clfA::lacZ* transcriptional reporter fusion using pAZ106, to allow analysis of the regulation of expression of *clfA* is reported. The generation of a non-fibrinogen-binding derivative of ClfA expressed from a multicopy plasmid on the *S. aureus* cell surface is also described. This derivative was produced by mutation of specific nucleotides by overlap primer PCR to yield a protein with amino acid substitutions in the fibrinogen-binding site.

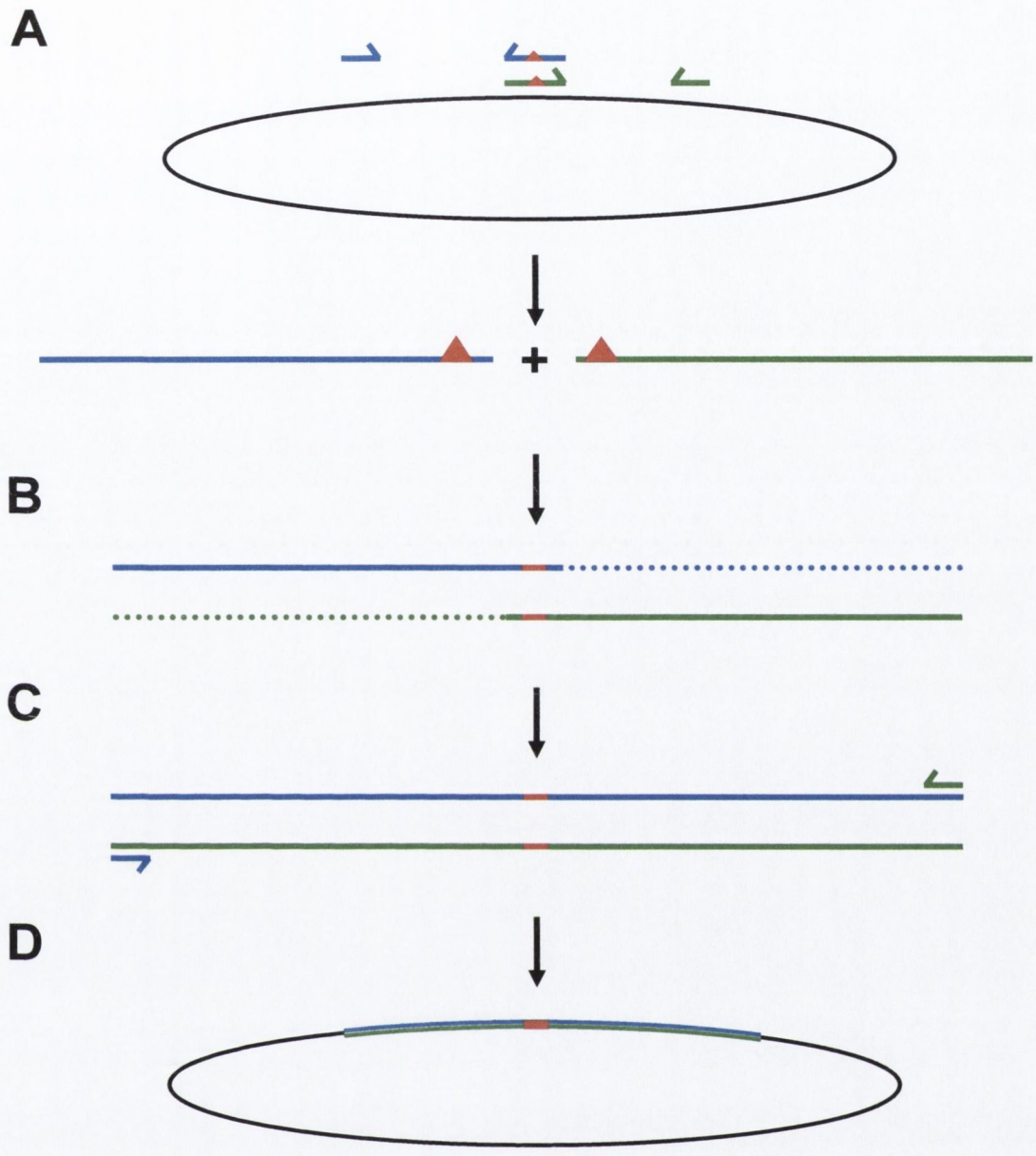


Fig 4.4 Overlap primer PCR

Central, overlapping mutagenic primers contain mismatches (red triangles). Two PCR amplimers are produced independently (A) and combined as templates in a second PCR step (B). The amplimers anneal at the region of overlap and self-prime, forming full-length products containing the mutations that were introduced as mismatches. Inclusion of the non-mutagenic flanking primers in this second PCR allows amplification of the full-length mutated products (C). Incorporation of restriction endonuclease cleavage sites in the flanking primers allows cloning into a plasmid vector (D).

4.2 Results:

4.2.1 Construction and validation of Newman *clfA* and *spa* mutants

In order to assess the possible antiphagocytic functions of clumping factor A and protein A it was necessary to construct a strain lacking both factors. This was achieved by combining previously isolated mutations by bacteriophage-mediated generalised transduction. The mutation to be transduced was delivered into the recipient cell where it replaced the wild-type copy by homologous recombination. Southern hybridisation and Western immunoblotting were used to determine that the constructed strains had the appropriate genotype and phenotype.

The *clfA2* mutation (McDevitt *et al.*, 1994) was generated in strain Newman by integration of the transposable element Tn*917*, which encodes erythromycin resistance. This mutation was found to combine insertion of the transposable element with a 4 kb deletion of part of the coding sequence and 5' sequences (strain DU5876; McDevitt *et al.*, 1994). A mutation in the protein A gene (*spa*) bearing a kanamycin-neomycin resistance marker (Patel *et al.*, 1989) was transduced from an 8325-4 background (strain DU5819, Patel *et al.*, 1989) into strains Newman wild-type and Newman *clfA2* using bacteriophage 85, as described in Section 2.16.

Genomic DNA was prepared from the strains, digested with *Hind*III overnight and Southern hybridisation was used to confirm insertions in *clfA* and *spa*, as described in Section 2.5 (Figure 4.5). DIG-labelled probes were synthesised by PCR using the primer sets F7*Hind*III/R7*Bam*HI (*clfA*) and *spa*F1/*spa*R1 (*spa*) (Table 2.3). Regions spanned by probes are depicted in Figure 4.5, panel C.

The *clfA* probe was 1.5 kb in size and spanned the entire length of the DNA encoding the unique region A. In *clfA2* mutants this region is entirely missing (Figure 4.5, panel C; McDevitt *et al.*, 1994). In Southern hybridisation the single 6.8 kb *clfA*-reactive *Hind*III fragment present in wild-type strains was absent in *clfA2* strains (Figure 4.5, panel A).

The *spa* probe was 1.4 kb in length and spanned part of the DNA encoding the unique ligand-binding domains of protein A. A *Hind*III site was present within this sequence. Wild type strains contained *Hind*III fragments of 4.1 kb and 2.7 kb that hybridised with the *spa* probe (Figure 4.5, panel B, lanes 1 and 3). The *spa::*Kan^R mutation is a deletion-insertion. A 1.2 kb *Bcl*I fragment internal to *spa* was

replaced with a 2 kb fragment carrying an ethidium bromide resistance marker. This resistance marker was subsequently disrupted by the insertion of a kanamycin-neomycin resistance determinant in the place of an internal *Hind*III fragment (Figure 4.5, panel C; Patel *et al.*, 1989). The *spa* probe hybridised with a single band of 3.8 kb in the *spa*::Kan^R strains (Figure 4.5, panel B). The DNA complementary to the 3' end of the *spa* probe is missing in these mutants.

These data confirmed the fidelity of the transductants. Expression of ClfA and protein A was tested. Western immunoblotting of solubilised cell wall-associated proteins (described in Sections 2.7 and 2.8) was used to show that the *clfA2* insertion had eliminated ClfA production (Figure 4.6). Full length ClfA migrates with an apparent molecular weight of 185 kDa in SDS-PAGE (see Chapter 3). Significant breakdown of ClfA was observed in Newman cells grown to stationary phase (Figure 4.6; see Chapter 6). Strains that were wild-type with respect to *clfA* produced a band of 185 kDa that reacted with rabbit polyclonal anti-ClfA region A antibodies (Figure 4.6, red arrow), whereas *clfA2* mutant strains did not. In addition, strains that were wild-type with respect to *spa* expressed a ca. 47 kDa protein (Figure 4.6, blue arrow) which was lacking in *spa* mutant strains. This reaction was likely due to the use of horseradish peroxidase-conjugated goat anti-rabbit polyclonal IgG as secondary antibody. Protein A binds to the Fc region of rabbit IgG in an antigen non-specific manner. The goat anti-rabbit IgG used as secondary antibody recognises the rabbit IgG bound by protein A.

Taken together these data show that the mutants constructed had the genotypes and phenotypes expected. These strains were used in analysis of resistance to phagocytosis described in Chapter 5.

4.2.2 Construction and validation of pTS*ermC* and pTS*tetK*

The chloramphenicol resistance determinant in plasmid pTS2 (Greene *et al.*, 1995) is poorly expressed when present in the cell as a single copy. This makes allelic replacement with this vector difficult. In order to facilitate efficient allelic replacement and in particular to allow the introduction of mutations lacking antibiotic resistance markers, two new temperature-sensitive plasmids were constructed. Plasmids pTS*ermC* and pTS*tetK* incorporated the replication functions, copy control mechanism and multiple cloning site of pTS2 but replaced the chloramphenicol resistance determinant with the erythromycin resistance

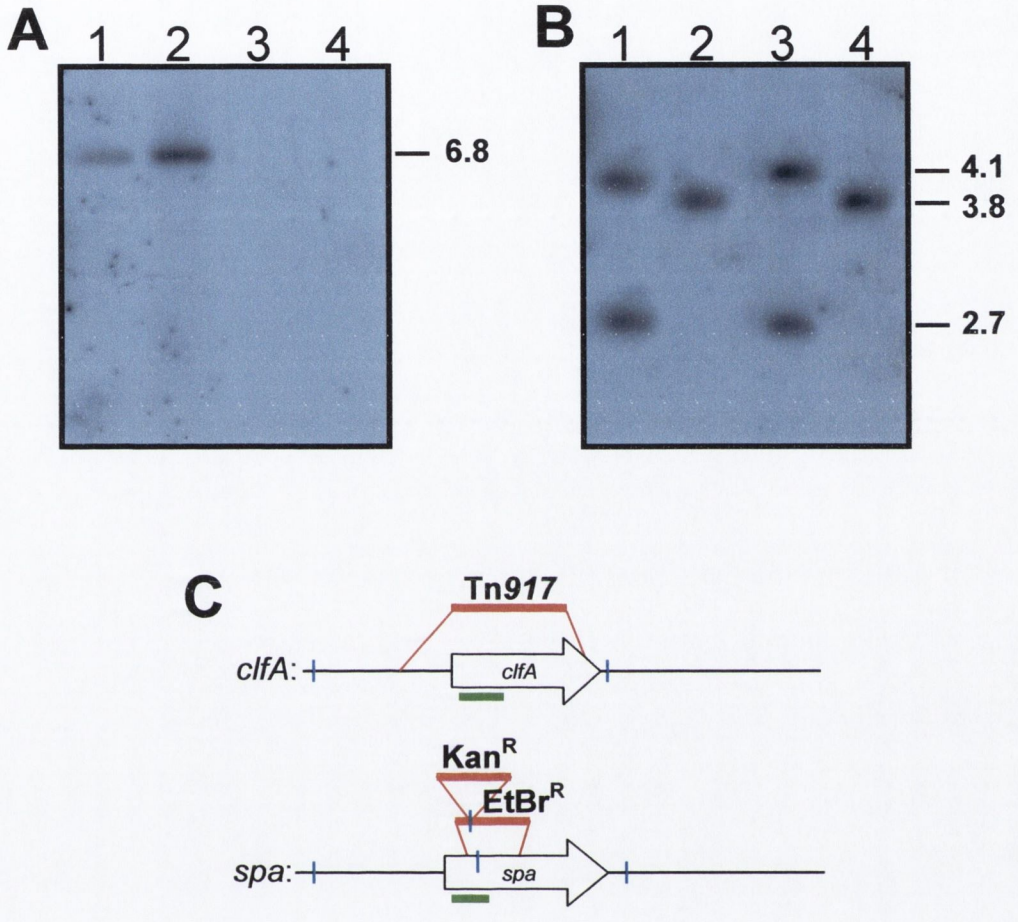


Fig 4.5 Southern hybridisation of Newman strains

A and B: Southern blotting of Newman strains. Genomic DNA was prepared and digested with *Hind*III before electrophoresis through 1% (w/v) agarose gels and transfer to positively charged membranes. DIG-labelled probes for *clfA* (A) and *spa* (B) were prepared by PCR and are detailed in panel C (green bars). Lane 1, Newman wild-type; lane 2, Newman *spa*; lane 3, Newman *clfA2*; lane 4, Newman *spa clfA2*. Sizes are indicated in kb.

C: *clfA* and *spa* loci. Insertions associated with null mutations are shown in red. Vertical blue marks denote *Hind*III restriction sites.

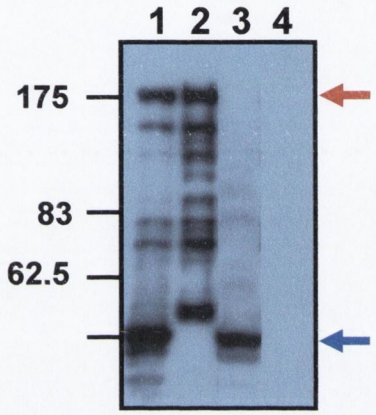


Fig 4.6 Western immunoblotting analysis of Newman strains

Cell wall-associated proteins of Newman strains were solublised by lysostaphin digestion and electrophoresed through a 7.5% (w/v) polyacrylamide gel. Proteins were transferred to a PVDF membrane and probed with rabbit anti-ClfA region A primary antibody and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. The red arrow indicates full length ClfA. The blue arrow indicates protein A. Lane 1, Newman wild-type; lane 2, Newman *spa*; lane 3, Newman *clfA2*; lane 4, Newman *spa clfA2*. Sizes are indicated in kDa.

determinant *ermC* from pE194 (in pTS*ermC*) or the tetracycline resistance determinant *tetK* from pT181 (in pTS*tetK*).

Primer pairs *ermC*F1*Sac*I/*ermC*R1*Nhe*I and *tetK*F1*Sac*I/*tetK*R1*Nhe*I were used to amplify fragments encoding the resistance markers from pE194 (*ermC*, 1.05 kb product) and pT181 (*tetK*, 1.60 kb product) (Figure 4.7, panel A). Primer pair pTSF2*Sac*I/pTSR2*Nhe*I was used to amplify the plasmid backbone of pTS2, excluding the chloramphenicol resistance marker (Figure 4.7, panel B). Several bands of smaller size than expected were amplified. However, the main product was of the expected size (2.9 kb) and was purified from the agarose gel. The purified plasmid and the *ermC* and *tetK* fragments were cut with *Sac*I and *Nhe*I at sites introduced in the PCR primers to facilitate ligation.

After ligation the DNA mixtures were transformed into *S. aureus* RN4220 by electroporation, as described in Section 2.17. Transformants were selected on tetracycline or erythromycin agar plates at 28 °C. Transformants of each plasmid ligation were single colony purified on their respective drugs overnight. Two colonies of each were retained and tested for temperature sensitivity.

Broth cultures were grown in the presence of drug at the permissive temperature and serial ten-fold dilutions (10^{-1} – 10^{-8}) were plated on duplicate plates containing the appropriate selective antibiotic. One set of plates was incubated at 28 ºC and the other at 42 ºC. The number of colonies was reduced from 1-2x10^{6} cfu/ml at 28 ºC to 0.8-1x10^{3} cfu/ml at 42 ºC, indicating that both plasmids pTS*ermC* and pTS*tetK* were temperature sensitive for replication. One representative of each plasmid was further used for restriction analysis.

Plasmid DNA was prepared from lysed *S. aureus* cells and digested overnight with the enzymes *Sac*I, *Bam*HI, *Eco*RI, *Hind*III, *Hpa*I, *Sph*I and *Sac*I/*Nhe*I (Figure 4.7, panel C). *Sac*I, *Bam*HI and *Hind*III linearised the plasmids (Figure 4.7, panel C, lanes 1,2 and 4). *Hpa*I cut both plasmids twice, yielding fragments of 1.3 kb and 2.6 kb from pTS*ermC* and of 1 kb and 3.5 kb from pTS*tetK* (Figure 4.7, panel C, lane 5). One *Hpa*I site in each plasmid was introduced with the resistance marker, demonstrating correct insertion of the fragments (see Figure 4.8). *Sac*I and *Nhe*I in combination dropped out the antibiotic resistance markers that had been inserted, leaving the backbone fragment of 2.9 kb and the inserts of 1.0 kb (*ermC*) and 1.6 kb (*tetK*) (Figure 4.7, panel C, lane 6). However *Eco*RI did not cut either plasmid (Figure 4.7, panel C, lane 3). To eliminate the possibility that the *Eco*RI site within the multiple cloning site was lost or destroyed during cloning,

Qiagen Midi plasmid preparations of both plasmids were digested with *Eco*RI. Both Wizard and Qiagen plasmid isolation kits are optimised for use with *E. coli* K12 hosts. It is clear that some enzymes do not optimally cleave the Wizard DNA, possibly due to the presence of proteins or other contaminants remaining attached to the DNA. As can be seen in panel D of Figure 4.7, the Qiagen plasmid DNA was digested to linear bands by *Eco*RI over 2 hours. The Wizard plasmid preparations were only partially linearised within that time.

These data show the construction of two new vectors to facilitate more straightforward allelic replacement in *S. aureus*. The use of pTS*ermC* in allelic replacement is described in Section 4.2.3, below.

4.2.3 Allelic replacement mutagenesis of *clfA*

In order to construct a *clfA* mutation that did not contain an inserted antibiotic resistance marker, *clfA* was mutated by the introduction of a frameshift marked by the presence of a cleavage site for the restriction endonuclease *Hind*III. The replacement of 5 bases at the end of the signal sequence with the *Hind*III site would result in a +1 frameshift in the downstream coding sequence. This frameshift would change the ADA signal sequence cleavage site and result in the termination of translation of the polypeptide at the third codon downstream (Figure 4.9). The phenotype of a *clfA* mutant was easily tested by a slide clumping test (Section 2.20).

The *clfA* mutation was constructed in a cloning vector in *E. coli* (pBlueΔ*clfA*) and subsequently introduced on a shuttle plasmid (pJH1) into *S. aureus*, where allelic replacement mutagenesis was carried out.

4.2.3.1 Construction of pBlueΔ*clfA*

Two segments of *clfA* were amplified by PCR from plasmid pCF77 (Hartford *et al.*, 1997) excluding bases 110 to 114 of the coding sequence. The central primers included *Hind*III sites and the flanking primers included *Eco*RI and *Bam*HI sites to facilitate cloning into the multiple cloning site (MCS) of pBluescript in *E. coli* (Figure 4.9). Primer pairs F1*Eco*RI/R6*Hind*III and F7*Hind*III/R7*Bam*HI (Table 2.3) were used to make upstream and downstream *clfA* products, respectively (Figure 4.10, panel A). PCR products were digested with *Eco*RI + *Hind*III and

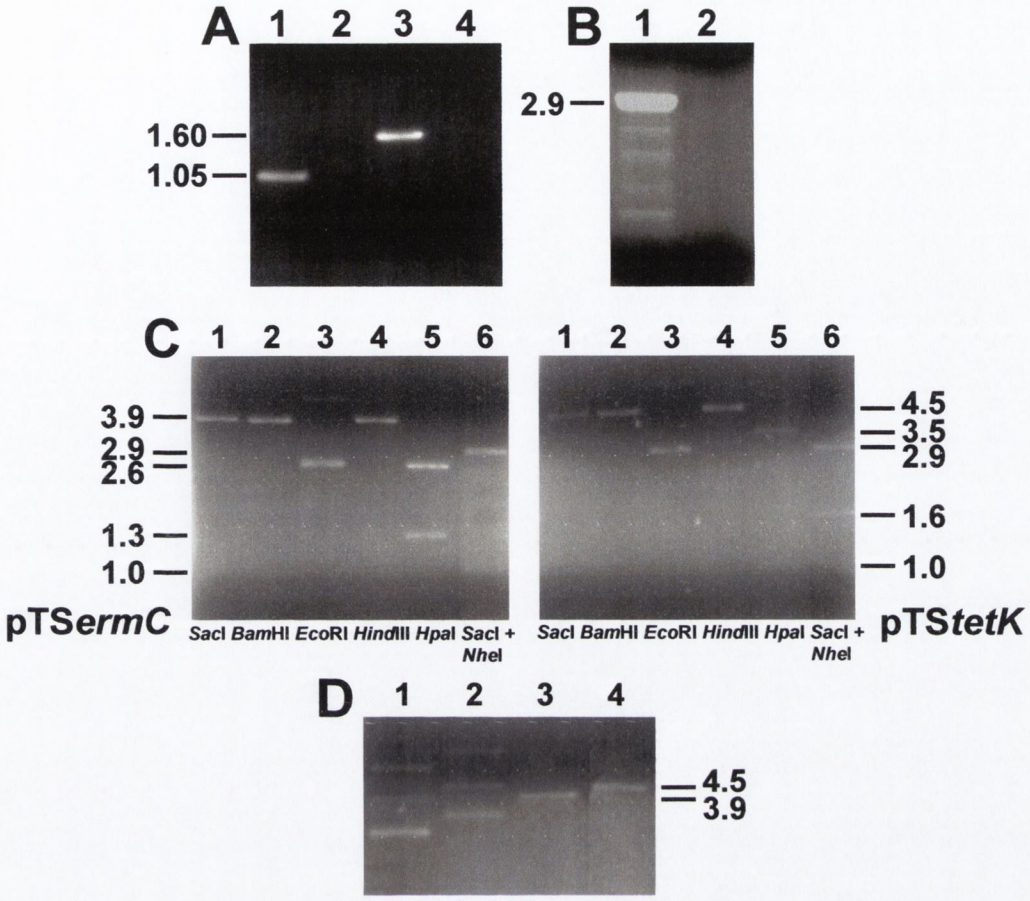


Fig 4.7 Construction of pTS*ermC* and pTS*tetK*

A. PCR amplification of resistance determinants. Lane 1, *ermC* product; lane 2, *ermC* template-free PCR control; lane 3, *tetK* product; lane 4, *tetK* template-free PCR control. Sizes are in kb.

B. PCR amplification of plasmid backbone. Lane 1, pTS product; lane 2, pTS template-free PCR control. Expected product was 2.9 kb.

C. Restriction analysis of pTS*ermC* (left panel) and pTS*tetK* (right panel). Lane 1, *Sac*I; lane 2, *Bam*HI; lane 3, *Eco*RI; lane 4, *Hind*III; lane 5, *Hpa*I; lane 6, *Sac*I + *Nhe*I. Digestions were for 16 hours at 37°C.

D. *Eco*RI restriction analysis of pTS*ermC* and pTS*tetK*. Lane 1, pTS*ermC* (Wizard preparation) incubated with *Eco*RI; lane 2, pTS*tetK* (Wizard preparation) incubated with *Eco*RI; lane 3, pTS*ermC* (Qiagen preparation) incubated with *Eco*RI; lane 4, pTS*tetK* (Qiagen preparation) incubated with *Eco*RI. Digestion was for 2 hours at 37°C; sizes of cut plasmids are indicated in kb.

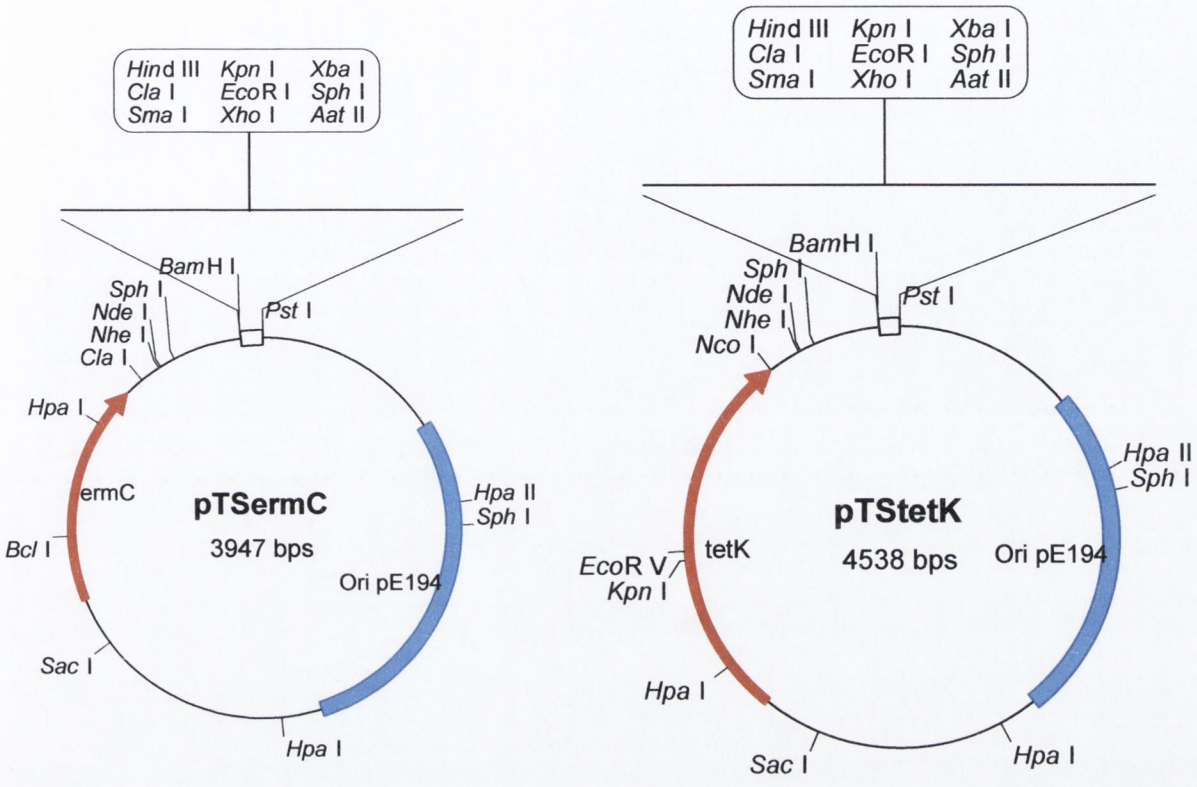


Fig 4.8 Maps of pTS*ermC* and pTS*tetK* plasmids

pTS*ermC* and pTS*tetK* were constructed by PCR using pTS2 as template for the replication functions and multiple cloning site (*Nhe*I-*Sac*I fragment) and pE194 and pT181 as template for the antibiotic resistance genes *ermC* and *tetK* (*Sac*I-*Nhe*I fragments). Temperature sensitive replicon is coloured blue. Resistance determinants are coloured red. Multiple cloning site is the white box. pTS*tetK* has two *Kpn*I sites, one within the tetracycline resistance cassette and the other in the multiple cloning site. pTS*ermC* has two *Cla*I sites, one in the multiple cloning site and one following the *ermC* resistance determinant. Both plasmids contain two *Hpa*I sites, one close to the replication functions and the other at different locations within the resistance marker.

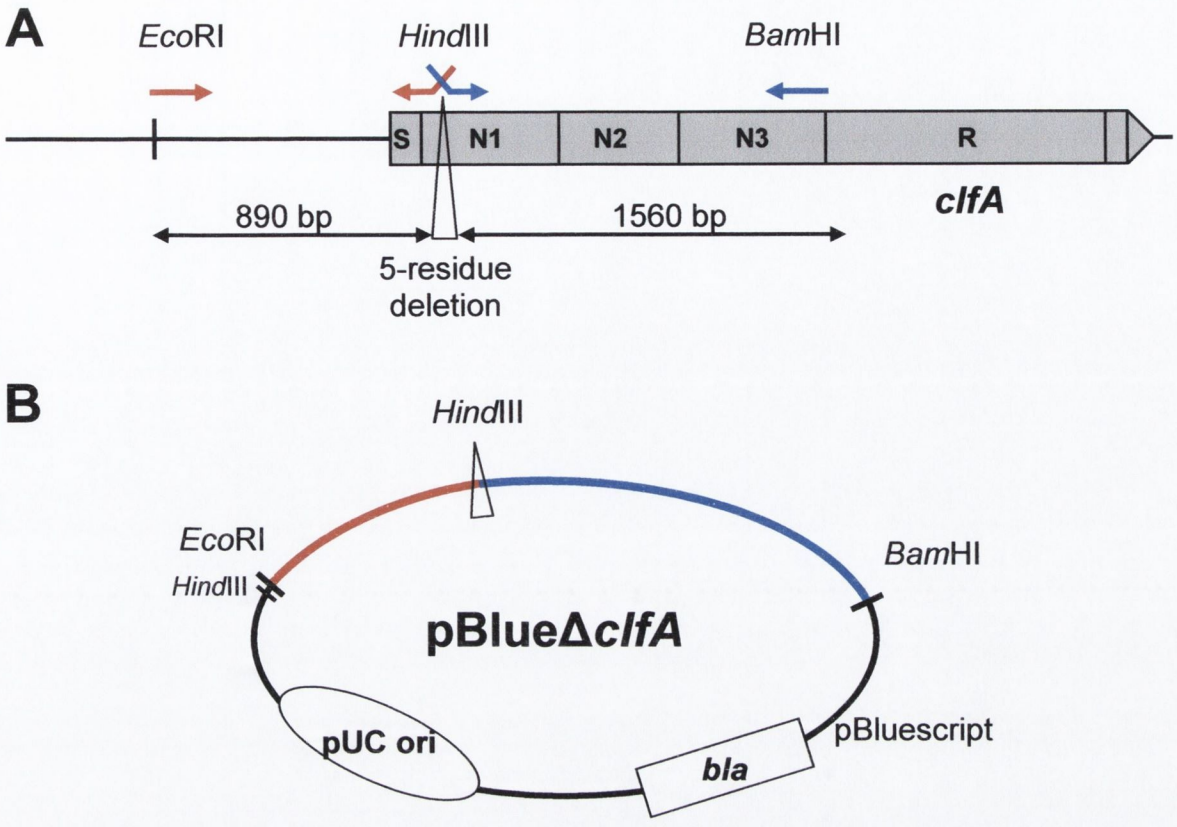


Fig 4.9 Construction of *clfA5* null mutation

A. PCR products of the regions upstream (red) and downstream (blue) of the mutation. Central primers were designed to remove five bases of the coding sequence and replace them with a *Hind*III restriction site.

B. pBlueΔ*clfA*. Following cleavage the PCR products were ligated together at the common *Hind*III site and cloned into the *Eco*RI and *Bam*HI sites in the MCS of pBluescript.

C. Introduction of the *Hind*III site resulted in a +1 frameshift in the downstream coding sequence. This frameshift changed the ADA signal sequence cleavage site (underlined), resulting in translation termination at the 3rd codon downstream.

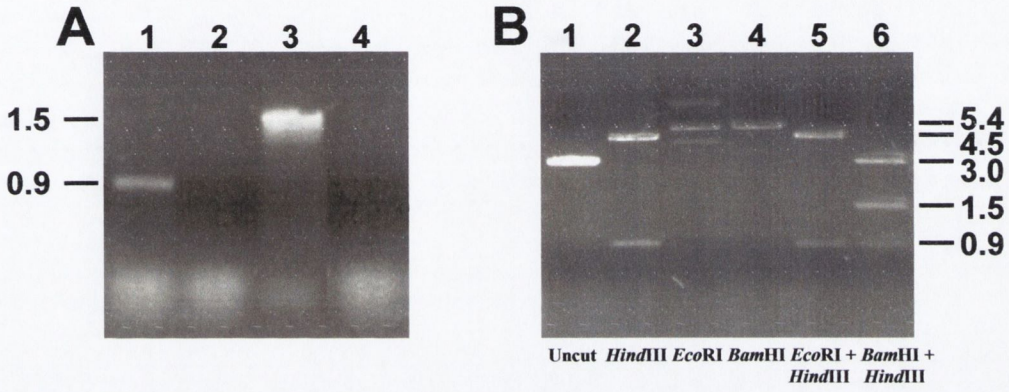


Fig 4.10 Construction of pBlueΔ*clfA*

A. Upstream and downstream PCR products. Lane 1, upstream product; lane 2, upstream template-free PCR control; lane 3, downstream product; lane 4, downstream template-free PCR control.

B. Restriction analysis of pBlueΔ*clfA* transformant. Lane 1, uncut plasmid; lane 2, *Hind*III; lane 3, *Eco*RI; lane 4, *Bam*HI; lane 5, *Eco*RI + *Hind*III; lane 6, *Bam*HI + *Hind*III.

*Bam*HI + *Hind*III, ligated with *Eco*RI/*Bam*HI-cut pBluescript and transformed into *E. coli*, as described in Sections 2.15 and 2.18.

Plasmid DNA was prepared from a transformant and digested with *Hind*III. The *Hind*III site of the MCS should have been present directly 5' to the *Eco*RI site used for insertion of the *clfA* fragments (Figure 4.9, panel B). Cleavage at this site and at the *Hind*III site introduced in the mutation between the two PCR fragments yielded a ca. 0.9 kb fragment (Figure 4.10, panel B, lane 2).

Additional restriction analysis demonstrated that the structure of the plasmid was as predicted. Digestion with *Hind*III or *Eco*RI/*Hind*III dropped out the ca. 0.9 kb *clfA* upstream fragment (Figure 4.10, panel B, lanes 2 and 5). Digestion with *Eco*RI or *Bam*HI alone caused linearization of the plasmid (Figure 4.10, panel B, lanes 3 and 4). Double digestion with *Bam*HI and *Hind*III yielded bands of ca. 3.0 kb, corresponding to pBluescript backbone, ca. 1.5 kb, corresponding to the *Hind*III-*Bam*HI downstream *clfA* fragment, and 0.9 kb, corresponding to the *Hind*III(*Eco*RI)-*Hind*III upstream *clfA* fragment (Figure 4.10, panel B, lane 6; Figure 4.9, panel B).

Removal of the 5 bases (110 to 114) and their replacement with the 6 bases of the *Hind*III site (AAGCTT) caused a +1 frameshift that introduced a premature stop codon in the *clfA* coding sequence (Figure 4.9, panel C). This plasmid was designated pBlueΔ*clfA*.

4.2.3.2 Construction of pJH1

In order to introduce the silent mutation carried by pBlueΔ*clfA* into *S. aureus* and facilitate allelic replacement with the chromosomal *clfA* gene, the plasmid was ligated to the temperature sensitive *S. aureus* plasmid pTS*ermC* (see Section 4.2.2). pBlueΔ*clfA* was linearised with *Bam*HI and treated with alkaline phosphatase to prevent re-ligation. pTS*ermC* was cut with *Bam*HI at a unique site within the MCS (Figure 4.8), ligated to pBlueΔ*clfA* and transformed into *E. coli*, with selection for ampicillin resistance and screening for resistance to erythromycin (100 µg/ml).

Plasmid DNA was purified from ampicillin- and erythromycin-resistant transformants and digested with *Hind*III (Figure 4.11, panel A). Following further restriction analysis, plasmid 18 was retained as pJH1. This plasmid was linearised by *Pst*I (Figure 4.11, panel B, lane 4), cut into fragments of 3.9 kb and 5.4 kb by

*Bam*HI (Figure 4.11, panel B, lane 2), and fragments of 0.9 kb and 1.5 kb were released by *Hind*III from a ca. 7 kb backbone (Figure 4.11, panel B, lane 3). This correlated exactly with the expected map of pJH1 (Figure 4.11, panel C). Plasmids were also isolated in which pTS*ermC* inserted in the opposite orientation relative to pBlueΔ*clfA* (data not shown). For the purpose of allelic replacement, however, the relative orientation of these fragments was not important and only pJH1 was used.

4.2.3.3 Allelic replacement in *S. aureus*

In order to perform allelic replacement mutagenesis of the *clfA* gene in *S. aureus* strains, the pJH1 shuttle plasmid was transferred into Newman *spa* and 8325-4 *spa*, ensuring the plasmid was selected at 28 °C with erythromycin. The plasmid was shown to be temperature sensitive by a 10^{-3}-fold lower colony count on selective agar incubated at the restrictive temperature. Single colonies from restrictive growth contain a copy of the plasmid integrated into the chromosomal *clfA* gene, as indicated in Figure 4.12, panel B. As the duplicated *clfA* DNA downstream of the mutation is twice the length of that upstream, it is more likely that the integration occurred in that region, though this was not tested.

In order to encourage plasmid excision the cultures grown at the restrictive temperature with antibiotic selection were diluted and grown without selection at the temperature permissive for replication. It is thought that rolling circle replication of a chromosomally integrated plasmid stimulates excision (Biswas *et al.*, 1993). Loss of excised plasmids was encouraged by dilution in drug-free broth and growth at the restrictive temperature. This process was repeated twice more to enrich for plasmid-free excisants. Finally colonies were grown on drug-free agar and screened for loss of resistance to erythromycin. For Newman *spa* 16 out of 90 colonies were erythromycin sensitive, while for 8325-4 *spa* 73 out of 90 colonies were erythromycin sensitive.

4.2.3.4 Identification of *clfA*-null (*clfA5*) mutants in *S. aureus*

The predicted result of the introduction of the +1 frameshift mutation in the chromosomal gene was complete absence of secreted or anchored ClfA on the staphylococcal cell surface. Erythromycin-sensitive Newman *spa* colonies were

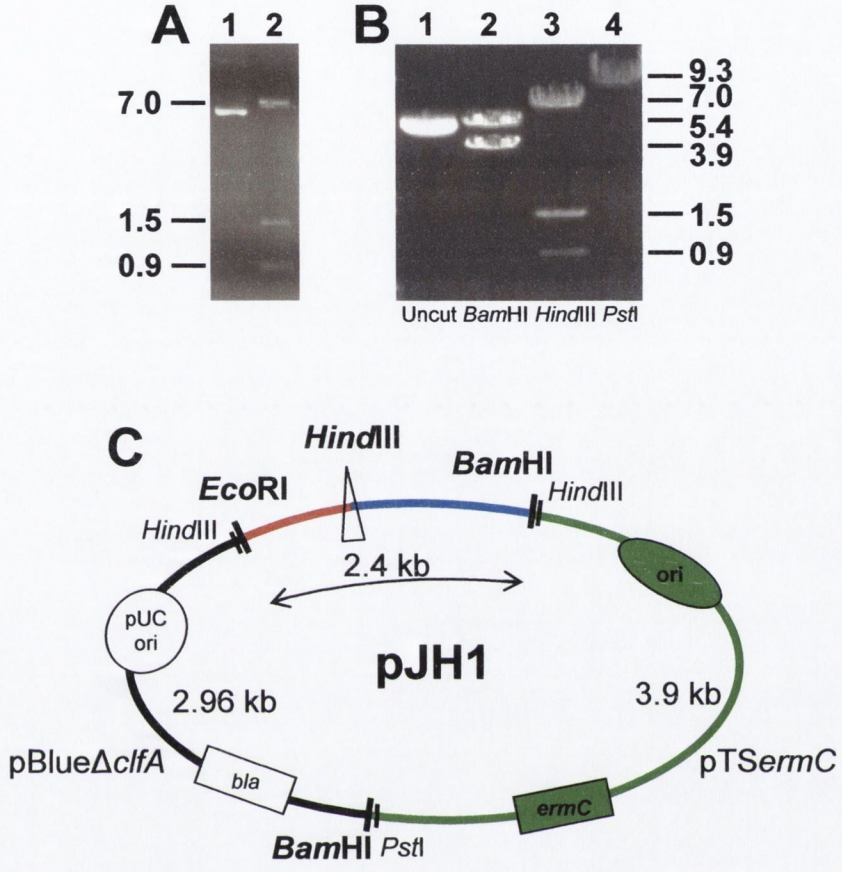


Fig 4.11 Construction of pJH1

A. Restriction analysis of transformed plasmid. Lane 1, plasmid 18; lane 2, plasmid 18 + *Hind*III.

B. Restriction analysis of plasmid 18 (pJH1). Lane 1, uncut plasmid; lane 2, *Bam*HI; lane 3, *Hind*III; lane 4, *Pst*I.

C. Map of pJH1. The red and blue lines indicate upstream and downstream inserts, respectively. The black line is pBluescript, including pUC origin of replication and *bla* (ampicillin resistance). The green line is pTS*ermC*, including pE194 origin.

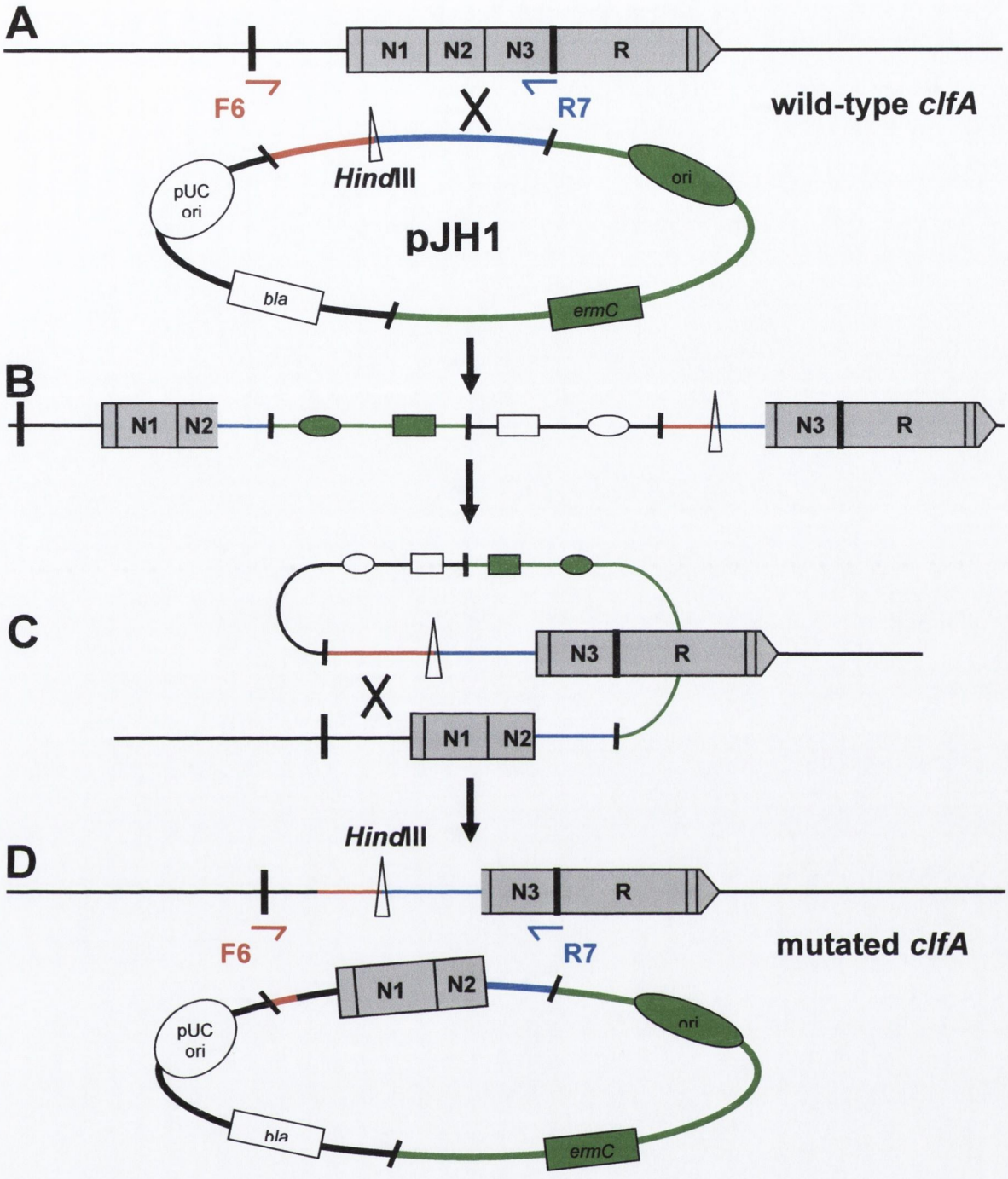


Fig 4.12 Allelic replacement mutagenesis with pJH1

The temperature-sensitive plasmid pJH1 (A) integrated into the chromosome within the region of homology (B). The plasmid was induced to excise, via an intermediate (C). The result of excision was that either the wild-type allele or the mutant allele was retained in the chromosome. This depended on which side of the mutation the second (excising) cross-over occurred, relative to the first cross-over that caused integration. After excision the plasmid was lost by growth in non-selective media at 42ºC. Loss of clumping by cells from the stationary phase and PCR using primers F6 and R7, followed by *Hind*III digestion of the product, indicated mutation of *clfA*.

screened for clumping activity in a solution of fibrinogen, as described in Section 2.20. All 16 of the Newman *spa* pJH1 derivatives failed to clump in soluble fibrinogen. However, 8325-4 does not form visible clumps in soluble fibrinogen (see Chapter 6), so clumping was not used as a screen for those strains.

Genomic DNA was prepared from parental strains and erythromycin-sensitive derivatives. Primers F1 and R7 were used to prepare PCR products that spanned the region of the mutation. *Hind*III digestion of these PCR products was a diagnostic test for the presence or absence of the mutation. If the mutated allele was retained in the chromosome, a *Hind*III site would have been introduced, causing the frameshift mutation. If the wild-type allele was retained the *Hind*III site would not be present (Figure 4.12).

The PCR product in all cases was 2.4 kb in length. The fragments amplified from the parental strains were not cut by *Hind*III (Figure 4.13, panels A and B, lane 1). Fragments containing the mutation were cut by *Hind*III into two fragments of 1.5 kb and 0.9 kb (Figure 4.13, panel A, lanes 2-4; panel B, lanes 2 and 4). Plasmid pBlueΔ*clfA* was used as a template for control PCR reaction and *Hind*III digestion (Figure 4.13, panels A and B, lane 5).

Of the 16 Newman *spa* pJH1 erythromycin sensitive, clumping negative derivatives screened, all 16 had the *Hind*III frameshift mutation. In strain 8325-4 *spa* pJH1, 6 of 11 erythromycin sensitive derivatives tested by PCR and *Hind*III digestion contained the mutation. The remainder retained the wild-type gene.

The mutation was designated *clfA5*. Western immunoblotting with anti-ClfA antibodies demonstrated the absence of ClfA in solublised cell wall extracts of Newman *spa clfA5* (Figure 4.13, panel C) compared to Newman *spa*. ClfA was not detected in cell wall extracts of 8325-4 *spa* (Figure 4.13, panel C), due to poor expression (see Chapter 6).

Despite failure to detect cell wall-associated ClfA under the conditions used for Western immunoblotting, sufficient protein is expressed by 8325-4 to promote adherence to immobilised fibrinogen, albeit less efficiently than Newman. The parental strains bound well to immobilised fibrinogen in stationary phase in a bacterial cell binding assay (see Section 2.14), but both *clfA5* mutants failed to bind under the same conditions (Figure 4.10, panel D). These data demonstrated that the ClfA-dependent fibrinogen-binding activity had been removed by introduction of the *clfA5* mutation. The cells used in this assay had been grown to stationary phase in order to avoid any possible contribution to fibrinogen binding

by ClfB or the fibronectin-binding proteins (FnBPs). In strain Newman the FnBPs are not functional due to truncation as a result of a point mutation in each gene (Grundmeier *et al.*, 2004). The FnBPs are present in 8325-4 in exponential phase only. ClfB is present in both strains only in early exponential phase. ClfB and the FnBPs lose fibrinogen-binding activity due to proteolysis as the cells enter stationary phase (McAleese *et al.*, 2001; McGavin *et al.*, 1997).

4.2.4 Isolation and validation of non-fibrinogen-binding mutant of ClfA

In order to study the role of fibrinogen binding in the possible antiphagocytic effect of ClfA it was necessary to isolate a mutant of ClfA that was expressed at normal levels on the cell surface but which could not bind fibrinogen. Studies with recombinant ClfA region A identified residues P_{336} and Y_{338} as being important for fibrinogen binding (Deivanayagam *et al.*, 2002). In order to increase the likelihood of isolating a completely non-functional mutant both $P_{336}S$ and $Y_{338}A$ substitutions were introduced into the *clfA* gene located on the shuttle plasmid pCF77 (Hartford *et al.*, 1997; see Table 2.2 for plasmids).

Overlap primer PCR (O'Connell *et al.*, 1998) was used to amplify two fragments that comprised 5' and 3' sequences of region A with an overlapping central segment generated by complementary reverse and forward primers (Figure 4.14, panel A). The primer pairs F10*Kpn*I/R$P_{336}SY_{338}A$ and F$P_{336}SY_{338}A$/R7*BamH*I (see Table 2.3 for primers) yielded upstream and downstream products of 1.4 kb and 0.7 kb, respectively. The complementary primers F$P_{336}SY_{338}A$ and R$P_{336}SY_{338}A$ contained the double mutation. A second round of PCR using the initial PCR products and flanking primers (Figure 4.14) allowed annealing of the complementary region and the generation of a fragment that could be cloned into the *clfA* gene of pCF77 between *Kpn*I and *Bam*HI sites, in the place of the wild-type fragment (Figure 4.15, panels A and B; see Section 2.21 for methods).

After preliminary restriction analysis (Figure 4.15, panel C), sequencing across the *Kpn*I-*Bam*HI fragment identified clones that carried the mutation and which were otherwise unchanged (data not shown).

The ability of the pCF77PY mutant to promote bacterial adherence to immobilised fibrinogen was investigated and compared with the wild-type pCF77 and pCF77EV (Hartford *et al.*, 2001a). pCF77EV expresses a protein with reduced binding to fibrinogen due to amino acid substitutions within the fibrinogen-

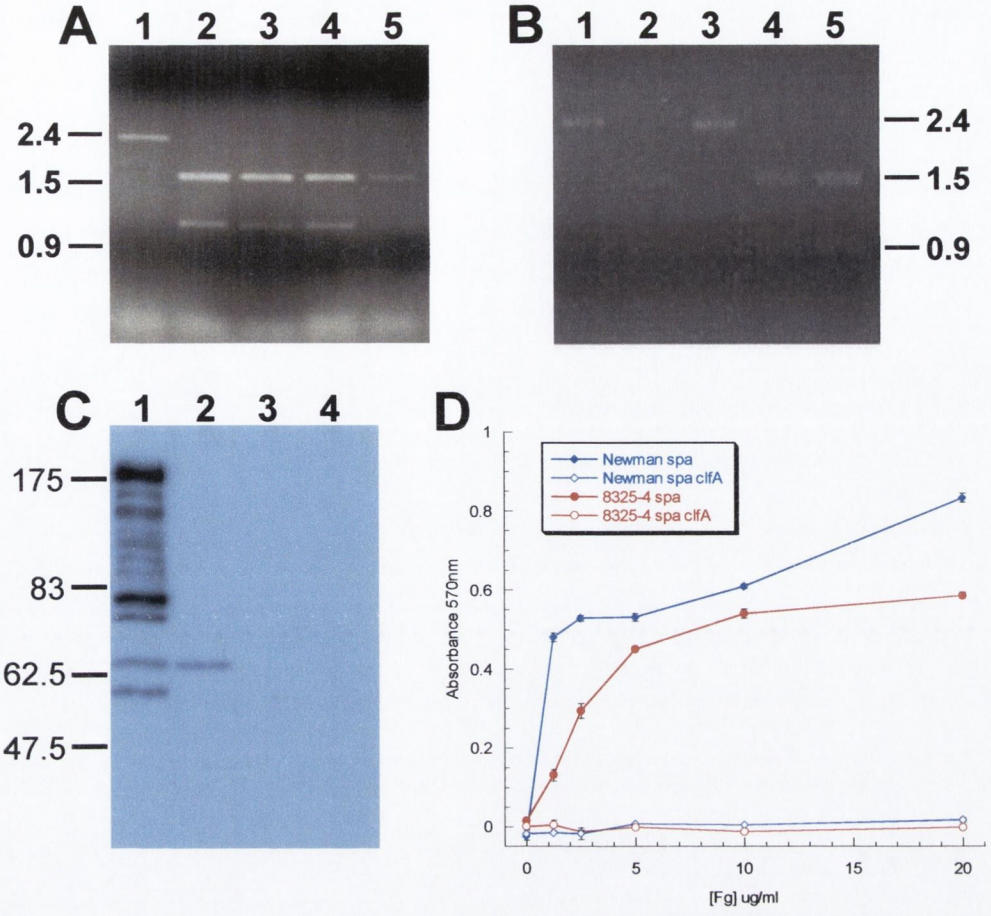


Fig 4.13 Validation of Newman *spa clfA* and 8325-4 *spa clfA* strains

A. Diagnostic PCR and digestion from Newman *spa* strains. Following PCR with primers F6 and R7 products were digested with *Hind*III. Lane 1, Newman *spa*; lane 2, colony 1; lane 3, colony 2; lane 4, colony 3; lane 5, pBlueΔ*clfA* control. Sizes are in kb.

B. Diagnostic PCR and digestion from 8325-4 *spa* strains. Following PCR with primers F6 and R7 products were digested with *Hind*III. Lane 1, 8325-4 *spa*; lane 2, colony 1; lane 3, colony 2; lane 4, colony 3; lane 5, pBlueΔ*clfA* control. Sizes are in kb.

C. Western immunoblot of solublised cell wall proteins of Newman *spa*, 8325-4 *spa* and their *clfA* derivatives, probed with rabbit anti-ClfA, and peroxidase-conjugated goat anti-rabbit. Lane 1, Newman *spa*; lane 2, Newman *spa clfA*; lane 3, 8325-4 *spa*; lane 4, 8325-4 *spa clfA*. Sizes are in kDa.

D. Adherence to immobilised fibrinogen by Newman *spa*, 8325-4 *spa* and their *clfA* derivatives. Fibrinogen was immobilised on the wells of a microtitre plate, incubated with washed whole cells grown to stationary phase, washed and fixed. Bound cells were stained with crystal violet and absorbance at 570 nm measured in an ELISA plate reader.

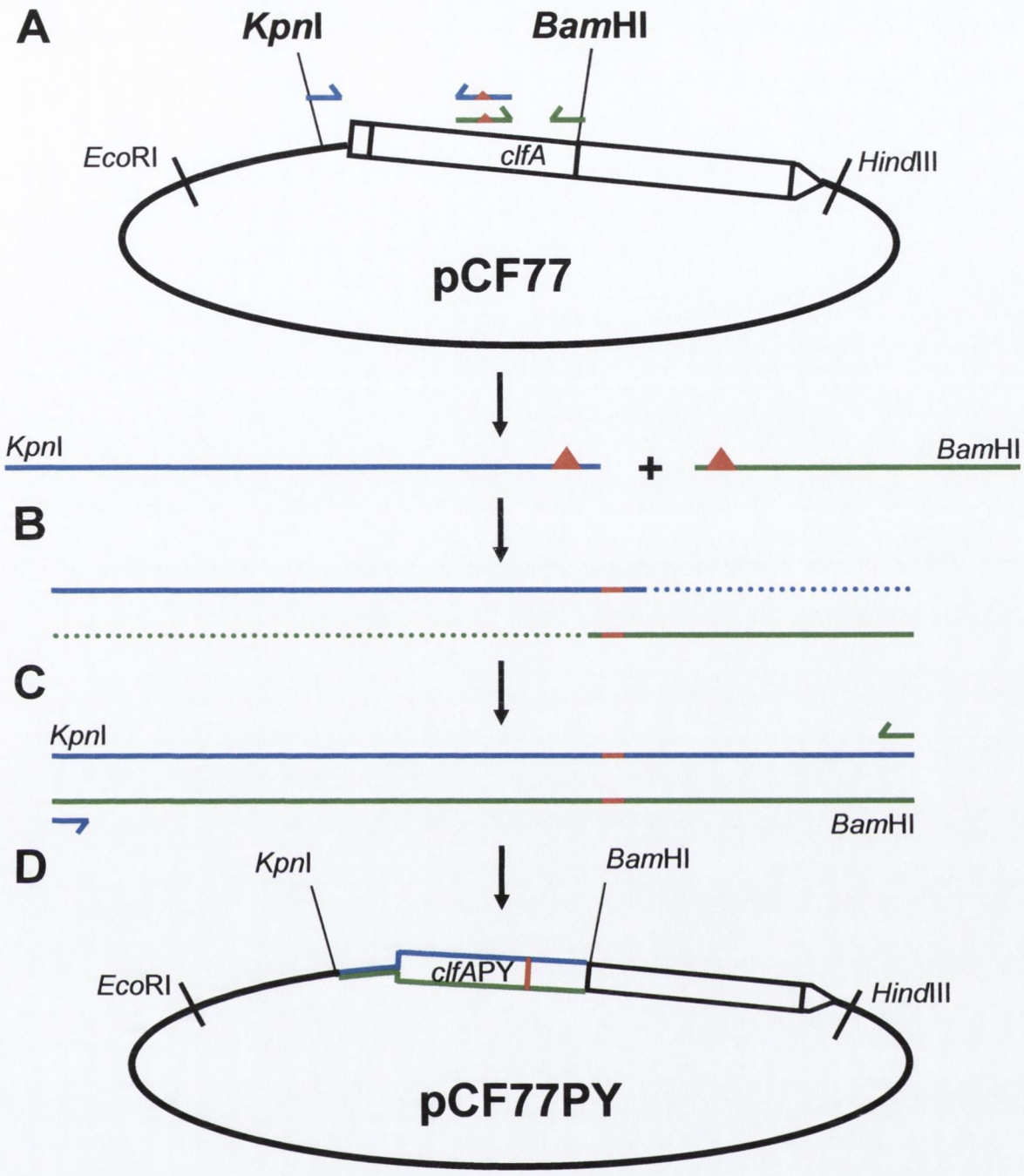


Fig 4.14 Overlap primer PCR mutagenesis of *clfA* in pCF77

A. Two initial PCR products containing the mutations were made from pCF77. Mutations are indicated in red and initial mismatches between primer and template are indicated as triangles.

B. Purified products were combined in a single PCR, where they annealed at the region of complimentarity and self-primed elongation.

C. Inclusion of flanking primers in the second PCR allowed amplification of the mutated products, which incorporated the restriction sites *Kpn*I and *Bam*HI.

D. The final double-stranded mutated PCR product was cloned into pCF77 in place of the wild-type *Kpn*I-*Bam*HI fragment to produce pCF77PY.

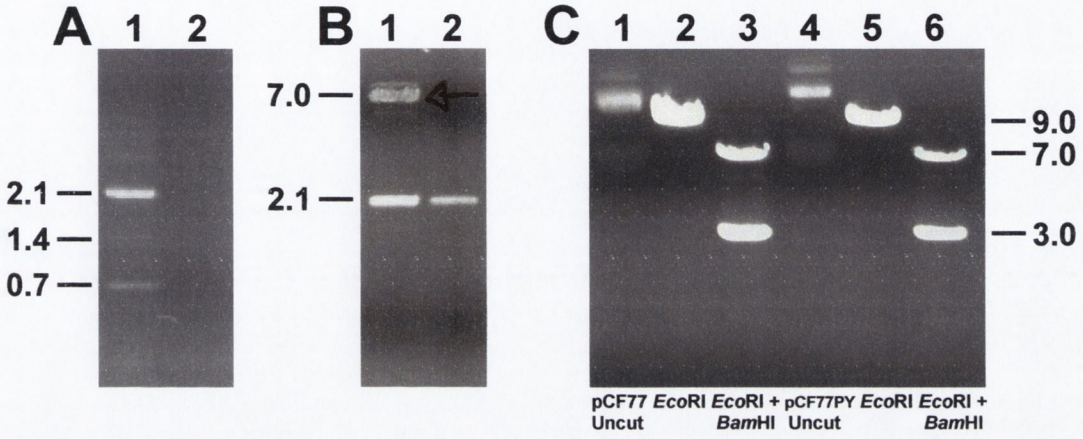


Fig 4.15 Construction of pCF77PY

A. Product of second PCR step. Lane 1, product of second PCR; lane 2, template-free PCR control. Sizes are in kb. Template molecules that were the products of the first PCR step are visible in lane 1 at 1.4 kb and 0.7 kb. Full-length product of second PCR step is 2.1 kb.

B. Digested plasmid backbone and insert. Lane 1, plasmid pCF77 digested with *Kpn*I and *Bam*HI; lane 2: mutated PCR product insert digested with *Kpn*I and *Bam*HI. The linearised plasmid backbone is approximately 7 kb.

C. Restriction digestion of plasmids. Lane 1, pCF77 uncut; lane 2, pCF77 digested with *Eco*RI; lane 3, pCF77 digested with *Eco*RI and *Bam*HI; lane 4, pCF77PY uncut; lane 5, pCF77PY digested with *Eco*RI; lane 6, pCF77PY digested with *Eco*RI and *Bam*HI.

binding site. Adherence by 8325-4 *spa clfA5* carrying pCF77 was dose-dependent and saturable. In contrast, pCF77EV adhered very weakly, whereas very little, if any adherence was detected for pCF77PY (Figure 4.16, panel A). Cell wall-associated proteins were solublised from these strains and Western immunoblotting demonstrated the presence of a 150-kDa protein that reacted with anti-ClfA antibodies (Figure 4.16, panel B). Full length ClfA (ca. 185 kDa) was not observed, but the 150-kDa band was likely the product of aureolysin degradation of ClfA. 8325-4 produces high levels of aureolysin (Shaw *et al.*, 2004) and low levels of ClfA (see Chapter 6), though it is over-expressed from multicopy plasmids. The PY mutation was subsequently transferred to *clfA* in plasmid pNZ8037 in *L. lactis* (Loughman *et al.*, 2005).

4.2.5 Construction of *clfA::lacZ* transcriptional fusion

In order to investigate the regulation of *clfA* transcription, *clfA::lacZ* transcriptional fusions were generated by directed plasmid integration into the *clfA* gene of suicide vector pAZ106 (Kemp *et al.*, 1991; see Section 4.1.3.2, above). This plasmid contains a replicon that functions in *E. coli* but it cannot replicate in *S. aureus* (Figure 4.17, panel A). Immediately 5' to the *lacZ* is the ribosome-binding site of the *Bacillus subtilis spoVG* gene. As a result, transcription of the *lacZ* element is fused to the target promoter but translation is independent. Cloning of an internal fragment generated by amplification of *clfA* DNA using primers F13*Bam*HI and R12*Eco*RI (Table 2.3) created an integrating plasmid pAZ106F13R12. Integration of this plasmid would generate a *clfA::lacZ* transcriptional fusion and would also allow expression of a truncated copy of ClfA, which would lack anchoring signals and be secreted into the growth medium (Figure 4.17, panel B). Primers F12*Bam*HI and R12*Eco*RI amplified a fragment including upstream sequences and the 5' end of *clfA*, to generate pAZ106F12R12. Upon integration of this plasmid a *clfA::lacZ* transcriptional fusion would be generated, and a truncated copy of ClfA would be expressed, as above. However, in addition, the *clfA* upstream sequences included on the plasmid would drive transcription of the copy of *clfA* located downstream of the plasmid (Figure 4.17, panel C). This construct was designated *clfA*$^{+}$ *clfA::lacZ*.

F13*Bam*HI/R12*Eco*RI and F12*Bam*HI/R12*Eco*RI PCR products were cloned into pAZ106 and transformed into *E. coli*. Restriction digestion of pAZ106F13R12

and pAZ106F12R12 dropped out the cloned PCR fragments (Figure 4.18, panel A). The plasmids were transformed to *S. aureus* RN4220 and genomic DNA was isolated from representative transformants and used as a template for PCR using the *clfA* forward primer F13 and a reverse *lacZ* primer R14 (Table 2.3). Primer positions are indicated in Figure 4.17, panels B and C. The presence of a 2.6 kb fragment that was absent from the wild-type strain was indicative of successful integration and fusion (Figure 4.18, panel B). Additional products in these reactions are likely due to mispriming.

The *clfA::lacZ* element (derived from pAZ106F13R12) was transduced selecting for the erythromycin resistance marker, to the following *S. aureus* strains: Newman, Newman *agr*, Newman *sarA*, Newman *sigB rsbU*, Newman *sigB rsbU agr,* Newman *sigB rsbU sarA,* 8325-4, 8325-4 SH1000 (see Table 2.1 for bacterial strains).

4.2.5.1 Validation of *clfA::lacZ* transcriptional fusion

Genomic DNA was prepared, cleaved with *Hind*III and Southern hybridisation was used to confirm linkage of the *clfA* and *lacZ* elements (Figure 4.19). The *lacZ* probe was made using primers F14*lacZ* and R14*lacZ* from pAZ106F13R12. The *clfA* probe was made using primers F13*Bam*HI and R12*Eco*RI from plasmid pAZ106F13R12. Strain Newman contains a single 6.8 kb *clfA*-specific *Hind*III fragment (McDevitt *et al.*, 1994). Plasmid pAZ106F13R12 is 10.2 kb in size (Figure 4.18). The *lacZ* probe hybridised to a single *Hind*III fragment of ca. 17 kb, in *clfA::lacZ* strains but not in the wild-type strains (Figure 4.19, panel A). The *clfA* probe hybridised to a 6.8 kb *Hind*III fragment in parental strains and to a ca. 17 kb fragment in *clfA::lacZ* strains (Figure 4.19, panel B). The *clfA*-reactive fragment and the *lacZ*-reactive fragment were the same size in both constructs. This indicated that fusion between *clfA* and *lacZ* had occurred as designed.

Activity of the construct was validated by comparison with Newman *clfB::lacZ*, Newman *spa*$^+$ *spa::lacZ* and Newman *hla*$^+$ *hla::lacZ* (McAleese *et al.*, 2003) in a β-galactosidase assay (Figure 4.20; see Section 2.24). *clfB::lacZ* was produced in mid-exponential phase only, stopping abruptly in late exponential phase. The activity of *clfB::lacZ* was approximately 10-fold lower than the peak activities of *spa* or *hla*. *clfB::lacZ* fusion activity was comparable to that observed previously (McAleese *et al.*, 2003). Activities of the *spa*+ *spa::lacZ* and *hla*+ *hla::lacZ* fusions

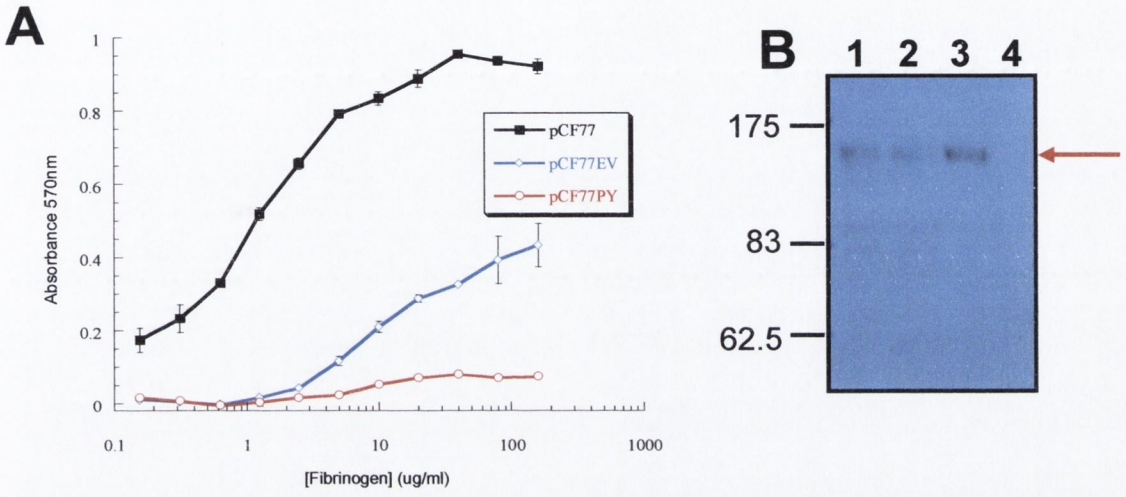


Fig 4.16 Validation of pCF77PY in 8325-4 *spa clfA5*

A. Adherence to immobilised fibrinogen of 8325-4 *spa clfA5* pCF77, pCF77EV and pCF77PY strains. Fibrinogen was immobilised on the wells of a microtitre plate, incubated with washed whole cells grown to stationary phase, washed and fixed. Bound cells were stained with crystal violet and absorbance at 570 nm measured in an ELISA plate reader, as a measure of adherence.

B. Western immunoblotting of solublised cell wall extracts of 8325-4 *spa clfA5* pCF77 and pCF77PY strains. Cell wall proteins were solublised by lysostaphin digestion, electrophoresed through 7.5% (w/v) polyacrylamide, transferred to PVDF membrane and probed with rabbit anti-ClfA region A antisera, followed by peroxidase-conjugated goat anti-rabbit antibody. Lane 1, 8325-4 *spa clfA5* pCF77; lane 2, 8325-4 *spa clfA5* pCF77PY; lane 3, 8325-4 *spa clfA5* pCF77EV; lane 4, 8325-4 *spa clfA5*. Sizes are in kDa.

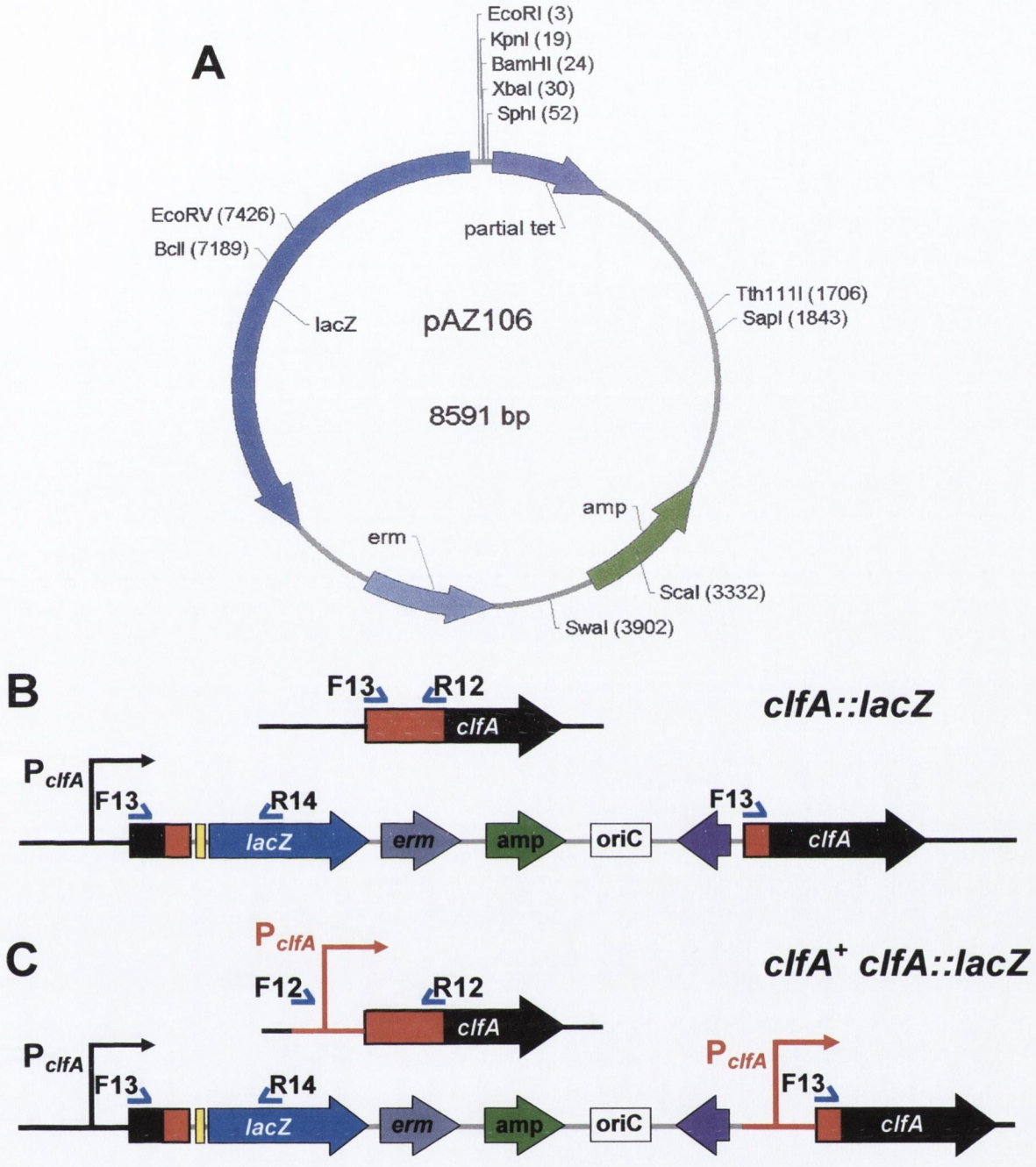


Fig 4.17 pAZ106 and integration strategy

A. Shuttle plasmid pAZ106. *lac*Z encodes a Gram-positive β-galactosidase.

B. Integration strategy for *clfA::lacZ*. DNA encoding signal sequence and region A of *clfA* were inserted into pAZ106 to make pAZ106F13R12. Integration into the chromosome occurred at this region of homology, resulting in the fusion shown. *clfA* fragments inserted in the pAZ106 vector are shown in red. Chromosomal DNA is pictured in black, with plasmid DNA as panel A. The *lacZ*-associated ribosome binding site is shown in yellow.

C. Integration strategy for *clfA*$^+$ *clfA::lacZ*. Upstream DNA, signal sequence and region A were inserted into pAZ106 to make pAZ106F12R12. Fusion in this case duplicated the promoter and *clfA* transcription continued.

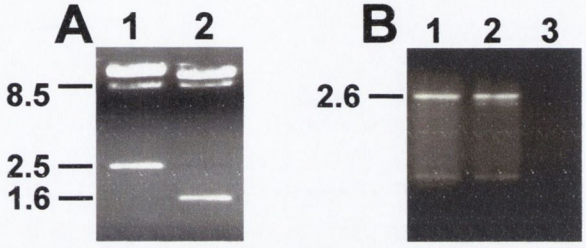


Fig 4.18 Construction of pAZ106F12R12 and pAZ106F13R12

A. Restriction digestion of pAZ106F12R12 and pAZ106F13R12. Lane 1, pAZ106F12R12 + *Eco*RI + *Bam*HI; lane 2, pAZ106F13R12 + *Eco*RI + *Bam*HI.

B. PCR across *clfA::lacZ* fusion. Primers F13 and R14*lacZ* were used to amplify across the fusion region from genomic DNA. Presence of a full-length 2.6 kb product indicated the presence of the fusion. Lane 1, pAZ106F12R12; lane 2: pAZ106F13R12; lane 3: pCF77 control.

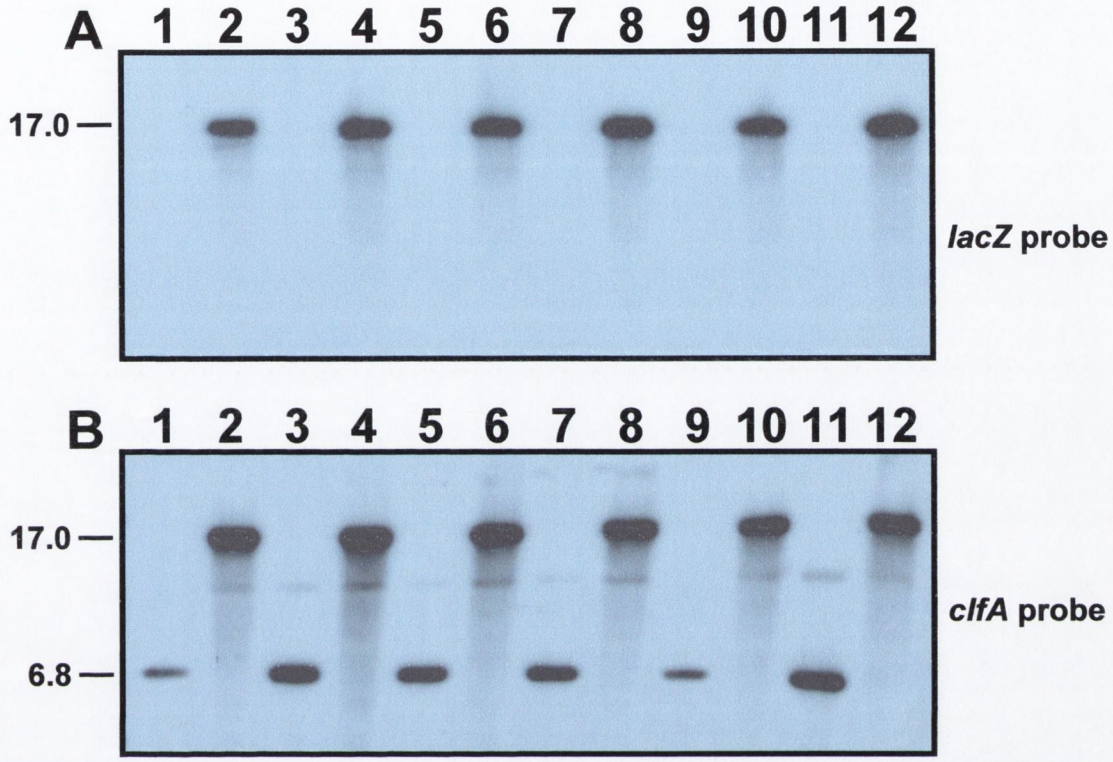


Fig 4.19 Southern hybridisation of *clfA::lacZ* strains

Southern hybridisation with DIG-labelled *lacZ* probe. DNA was digested with *Hin*dIII, seperated through a 1% (w/v) agarose gel and transferred to a positively charged nylon membrane. The *lacZ* probe (A) was generated by PCR from pAZ106. The *clfA* region A probe (B) was generated by PCR from pCF77. Lane 1, Newman wild-type; lane 2, Newman *clfA::lacZ*; lane 3, Newman *agr*; lane 4, Newman *agr clfA::lacZ*; lane 5, Newman *sarA*; lane 6, Newman *sarA clfA::lacZ*; lane 7, Newman *sigB rsbU*; lane 8, Newman *sigB rsbU clfA::lacZ*; lane 9, 8325-4 wild-type; lane 10, 8325-4 *clfA::lacZ*; lane 11, 8325-4 SH1000; lane 12, 8325-4 SH1000 *clfA::lacZ*.

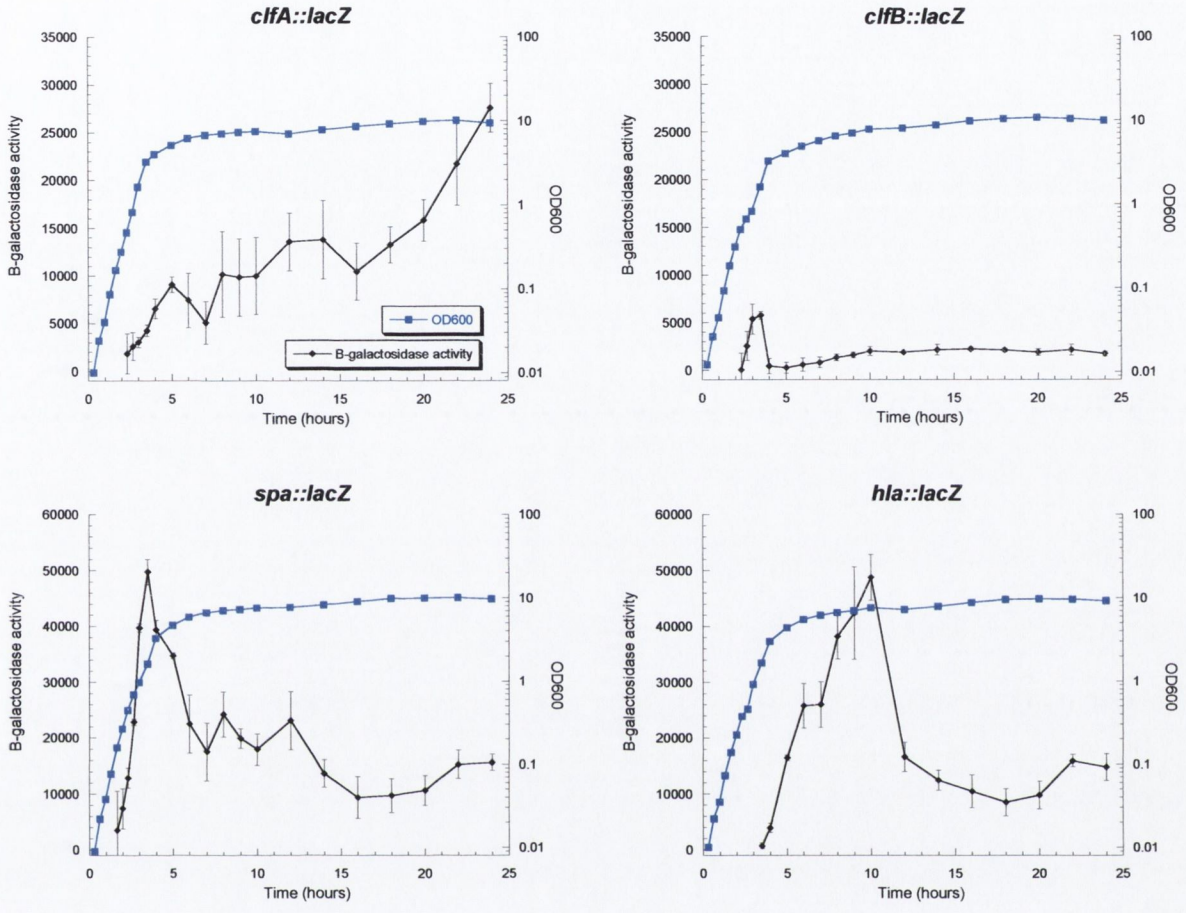


Fig 4.20 β-galactosidase activity of Newman strains bearing *lacZ* fusions

Strains Newman *clfA::lacZ*, Newman *clfB::lacZ*, Newman *spa*$^{+}$ *spa::lacZ* and Newman *hla*$^{+}$ *hla::lacZ* were grown for 24 hours in BHI broth. Mid-exponential phase cultures were used as inocula. Samples were taken at intervals and snap frozen. Thawed cells were assayed for β-galactosidase activity using MUG. Optical density at 600 nm (OD600) is marked with blue squares and β-galactosidase activity (in Miller Units) is marked with black diamonds. Activity was calculated according to the formula: Activity (Miller Units) = pmol 4-MU produced/(dilution factor x time x OD_{600}). β-galactosidase activity values below zero were omitted due to intrinsic inaccuracy. Data are representative of three independent experiments.

were in the same range as data observed in 8325-4 (Chan and Foster, 1998). Protein A was produced at high levels in mid-exponential phase, after which production ceased. α-haemolysin (Hla) was produced in late exponential phase and stationary phase (Figure 4.20). The expression profiles of all three fusions correlated well with published data, validating the methodology employed in the β-galactosidase assay. The *clfA::lacZ* fusion showed high level expression in stationary phase (Figure 4.20). These and further *clfA* regulation data are explored in Chapter 6.

4.3 Discussion:

The small number of resistance markers available in *S. aureus* has recently posed a problem in constructing strains with multiple mutations in loci with redundancy of function. In order to study a phenotypically-null strain, multiple loci must be inactivated. For example, ClfA, clumping factor B (ClfB), fibronectin-binding proteins A and B (FnBPA and FnBPB) have all been shown to bind fibrinogen in *S. aureus*. The *clfA2* mutation bears erythromycin resistance and the *clfB* mutation bears tetracycline resistance (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998). The *fnbA* and *fnbB* loci are linked, while the *clfA* and *clfB* loci are not. Mutations in both *fnb* loci are available, marked with the same erythromycin and tetracycline resistance markers as *clfA* and *clfB* (McGavin et al., 1997). Any two of these mutations can be combined, but not three or all four, which might give a fibrinogen-binding null mutant.

The availability of an unmarked *clfA* mutation allowed the construction of such a strain in the following way: *clfB* was transduced into a *clfA5* (unmarked) strain, with selection on tetracycline. Erythromycin resistance was then be used to select for transductants containing mutations in *fnbB*. The linked *fnbA* and *fnbB* genes are co-transduced at a high frequency. Though *fnbAB* mutations bear both erythromycin and tetracycline markers, selection for erythromycin is sufficient to obtain transductants. Co-transduction of the tetracycline resistance marker in *fnbA* was subsequently verified by PCR and Southern hybridisation. The existence of the *clfA5* null mutation allowed construction of *clfA clfB fnbA fnbB* strains in this manner in strains P1 and LS-1 that were used in studies of the role of fibrinogen-binding proteins in platelet aggregation caused by *S. aureus* (Fitzgerald *et al.*, 2006) and in a murine model of sepsis and septic arthritis (Palmqvist *et al.*, 2005). In the platelet study the ability to generate mutants defective in multiple factors (*clfA clfB fnbA fnbB* and *clfA clfB sdrC sdrD sdrE spa* mutants) allowed the identification of FnBPA and FnBPB as the dominant surface proteins promoting platelet aggregation on bacteria from the exponential phase of growth.

It was important to develop a new methodology to allow the establishment of an unmarked null mutation in *S. aureus*. Using the pTS*ermC* and pTS*tetK* plasmids described here, any non-essential gene in *S. aureus* can now be mutated. A phenotypic screen is not necessary, as demonstrated by the screening of *clfA*

mutants in 8325-4 solely on the basis of presence or absence of the *Hind*III site marking the mutation. This genetic screen was enough to allow easy identification of correct mutants, which arose at high frequency using the temperature-shifting method. Allelic replacement mutagenesis using these temperature-sensitive plasmids removes the requirement for a mutation-associated resistance marker. In addition to the generation of polar mutations (e.g. *clfA5*), this allows the construction of non-polar mutations by the generation of in-frame deletions, insertions, and single-base pair substitutions. The plasmid pTS*tetK* has been used to introduce unmarked point mutations by allelic replacement in the region upstream of *norA* (encoding the NorA multidrug efflux pump), facilitating studies of its regulation (Kaatz *et al.*, 2005).

Unmarked mutations are more difficult to work with than resistance-associated mutations only in that they must be transduced between strains on temperature-sensitive plasmids, before allelic replacement in each strain. However, as the antibiotic resistance associated with the plasmid is lost during allelic replacement, this method allows the construction of multiple mutations in different loci using a single plasmid, sequentially introduced into one strain. This has removed the previous limit on the number of mutations that can be combined in a single strain. An additional advantage of generating mutations directly in the strain of interest is that no linked mutations or polymorphisms are introduced. This is a risk of using directed plasmid integration with suicide vectors, which must be integrated in RN4220 and transduced to other strains.

The ability of ClfA to bind fibrinogen was first shown in 1994 (McDevitt *et al.*). A variety of properties of the ClfA protein have since been investigated. These include the ability of region R to act as a stalk for the functional projection of the ligand-binding domain away from the cell surface (Hartford *et al.*, 1997) and interactions between ClfA and human platelets (O'Brien *et al.*, 2002a; Siboo *et al.*, 2001; Loughman *et al.*, 2005). Part of this study focuses on the possible properties of ClfA involved in resistance to phagocytosis by human PMNL (see Chapter 5). Some properties may depend upon fibrinogen binding by ClfA, however, fibrinogen-independent functions of ClfA may also exist.

To investigate this, a mutant was made by overlap primer PCR in plasmid pCF77, lacking two residues identified in the crystal structure of $rClfA_{(221-559)}$ as necessary for fibrinogen binding (Deivanayagam *et al.*, 2002). Circular dichromism analysis of these mutations in the recombinant protein showed they

did not alter the general structure of N23. This PY mutation was introduced to *S. aureus* on a multicopy plasmid, pCF77PY. In an ELISA-type binding assay, 8325-4 *spa clfA5* pCF77PY cells failed to adhere to fibrinogen, although an immunoreactive ClfA protein of ca. 150 kDa was produced by the strain. The plasmid expressed non-fibrinogen-binding ClfA on the *S. aureus* cell surface. This mutant was used to study the fibrinogen-independent effects in resistance to phagocytosis by ClfA, and is discussed in Chapter 5. *clfA* and the PY mutation were also transferred to a nisin-regulated expression vector in *Lactococcus lactis*, where they were used to study interactions with platelets (Loughman *et al.*, 2005). ClfA-expressing strains caused rapid activation of platelets, which was dependent on a fibrinogen bridge between ClfA and the platelet integrin GpIIb/IIIa, as well as specific IgG to engage the platelet IgG receptor, FcγRIIa. The PY mutant had a greatly extended lag time to platelet activation compared to the wild-type protein, but platelet activation occurred by a second, fibrinogen-independent mechanism. This mechanism involved specific IgG and complement activation (Loughman *et al.*, 2005).

Two other mutations in domain N3 of region A, $E_{526}A$ and $V_{527}S$, had been shown to reduce binding to fibrinogen by ClfA on the surface *of S. aureus* and by $rClfA_{(220-559)}$ (Hartford *et al.*, 2001a). In the crystal structure of $rClfA_{(221-559)}$ these residues were identified as part of the hydrophobic binding pocket between domains N2 and N3 and it was postulated that the substitutions displaced the charge balance in the pocket, causing a reduction in binding (Deivanayagam *et al.*, 2002). The data presented here agree with the opinion of Deivanayagam *et al.*, in that ClfAEV (produced from pCF77EV) bound fibrinogen at a reduced level compared to wild type, but that residual fibrinogen-binding activity remained.

The expression and regulation of *clfA* have not so far been studied in detail. In order to analyse expression and regulation of *clfA*, transcriptional reporter fusions were generated using the plasmid pAZ106 (Kemp *et al.*, 1991). Two fusions were made, one that interrupted the production of ClfA (*clfA::lacZ*) and one that did not (*clfA*$^+$ *clfA::lacZ*). The ability to retain full functionality in the gene being studied is very important for essential genes. The *clfA::lacZ* fusion was used to study the pattern of expression of *clfA* in a variety of *S. aureus* strains and is discussed in Chapter 6.

Chapter 5

Antiphagocytic Activity of Clumping Factor A

5.1 Introduction:

Neutrophils, or polymorphonuclear leucocytes (PMNL), are the critical first line of defence against *S. aureus* once the organism has penetrated the skin and entered the body. Two lines of evidence suggest that this interaction is pivotal in host defence from staphylococcal disease. Sufferers of chronic granulomatous disease, a range of inherited disorders of PMNL function, frequently present with recurrent *S. aureus* pneumonia and abscesses (Liese *et al.*, 1996). In addition, in a mouse model of *S. aureus* sepsis and septic arthritis, neutrophil-depleted mice died of sepsis within 3 days at an inoculum level that allowed normal mice to survive (Verdrengh and Tarkowski, 1997).

S. aureus expresses several factors that promote avoidance of opsonophagocytosis by PMNL, as discussed in Section 1.3.4. Known surface-expressed antiphagocytic factors include protein A and capsular polysaccharide. The importance of protein A is indicated by the observation that it is a virulence factor in several experimental infection models. The use of specific protein A-deficient mutants allowed demonstration that protein A is required for full virulence in models of murine sepsis and septic arthritis (Palmqvist *et al.*, 2002), staphylococcal pneumonia (Gomez *et al.*, 2004) and subcutaneous infection (Patel *et al.*, 1987). Protein A binds both the Fc region of immunoglobulin G (IgG; Forsgren and Sjoquist, 1966) and the Fab regions of V_H3-class immunoglobulins (Hillson *et al.*, 1993), allowing it to recognise immunoglobulin in a non-antigen-specific manner. Protein A is believed to be antiphagocytic due to its IgG-binding activity (Gemmell *et al.*, 1991) although Fab binding activates complement via the classical pathway (Kozlowski *et al.*, 1996).

Through the use of capsule-deficient mutants it was shown that capsular polysaccharide is a virulence factor in a murine bacteraemia model (Thakker *et al.*, 1998), but not in infective endocarditis. Encapsulated strains were actually less virulent than non-encapsulated mutants in experimental endocarditis (Baddour *et al.*, 1992). This is believed to be due to an anti-adhesive property of capsular polysaccharide, which masks adhesins that are important for attachment to the sterile thrombus in experimental endocarditis and blocks their interactions with components of the extracellular matrix (Baddour *et al.*, 1992; O'Riordan and Lee, 2004). The antiphagocytic effect of capsule is due to its ability to impede binding by opsonising antibodies that recognise proteins, teichoic acids and peptidoglycan

on the cell surface, as well as to inhibit complement fixation (Thakker *et al.*, 1998; Cunnion *et al.*, 2003). Specific anti-capsule antibodies can overcome this and promote opsonophagocytosis (Thakker *et al.*, 1998). Capsule is not expressed in the exponential phase of growth. Comparison of wild-type and capsular mutant cells grown to exponential phase before inoculation in the murine bacteraemia model failed to show a role for capsular polysaccharide in virulence (Wiley and Maverakis, 1974), whereas a role for capsule was clearly seen with stationary-phase cells (Thakker *et al.*, 1998). Inhibition of opsonophagocytosis by capsule contributes to the virulence of *S. aureus* by preventing bacterial clearance from the bloodstream, liver and spleen (Luong and Lee, 2002).

Clumping factor A has been shown to be an important virulence factor in several infection models, including rat experimental endocarditis (Moreillon *et al.*, 1995), murine sepsis and septic arthritis (Josefsson *et al.*, 2001) and rabbit infective endocarditis (Vernachio *et al.*, 2003). The role of ClfA in experimental endocarditis was shown both by comparison of wild-type *S. aureus* with ClfA-deficient mutants and by transfer of *clfA* to *Streptococcus gordonii* (Stutzmann Meier *et al.*, 2001) and to *Lactococcus lactis*, where ClfA was identified as a critical adhesin independently capable of mediating endovascular infection (Que *et al.*, 2001). ClfA is an important virulence factor in murine sepsis and septic arthritis, as demonstrated by the reduced virulence of ClfA-deficient strains, and ClfA was identified as mediator of inflammation within knee joints (Josefsson *et al.*, 2001). In a similar study protein A was identified as a virulence factor, though less important than ClfA, but protein A did not promote inflammation in the knee (Palmqvist *et al.*, 2002).

Immunisation with recombinant region A of ClfA protected against arthritis, and passive immunisation with human IgG containing a high titre against ClfA protected mice from septic death (Josefsson *et al.*, 2001). The use of ClfA-deficient mutants showed that ClfA is a virulence factor in a rabbit model of infective endocarditis, where passive immunisation against ClfA was also shown to give protection (Vernachio *et al.*, 2003; Hall *et al.*, 2003). It has been suggested that fibrinogen binding is not necessary for ClfA-mediated virulence in the murine sepsis and septic arthritis model (Palmquist *et al.*, 2004a) and fibrinogen may in fact protect the animals from severe ClfA-dependent *S. aureus*-induced arthritis. This may be related to the pro-inflammatory activity of ClfA in arthritic joints or some other activity of ClfA.

Anti-ClfA antibodies have been detected in pooled human IgG (Vernachio *et al.*, 2003; Loughman *et al.*, 2005) and in normal human sera (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005). One study found that convalescent sera contained higher levels of anti-ClfA antibodies than normal sera, particularly in patients recovering from osteitis, septic arthritis or endocarditis, but no such increase was seen in patients recovering from *S. aureus* abscesses (Colque-Navarro *et al.*, 2000). Another recent study found no significant difference in anti-ClfA antibody levels between patients being treated for non-staphylococcal bacteraemia (*S. aureus* carriers or non-carriers) and convalescent *S. aureus* bacteraemia patients (Dryla *et al.*, 2005).

All sera tested in both studies contained detectable anti-ClfA antibodies (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005). It is likely, as indicated by Colque-Navarro *et al.* (2000) that at least some types of *S. aureus* infection lead to the formation of anti-ClfA antibodies. Perhaps the presence of antibodies in control sera prevented the observation of significant differences by Dryla *et al.* (2005). The observation of anti-ClfA antibodies in both normal and convalescent sera implies that normal exposure to *S. aureus* as a commensal of the skin stimulates their formation, and that these antibodies in normal sera are not protective against invasive *S. aureus* disease.

Immunity to staphylococcal disease involves the classical, alternative and mannose-binding lectin (MBL) complement pathways (Wilkinson *et al.*, 1978; Verbrugh *et al.*, 1979; Neth *et al.*, 2002). Binding of complement to the bacterial surface and to opsonising antibodies leads to activation (Wilkinson *et al.*, 1978) and highly efficient opsonophagocytosis by human PMNL (Cunnion *et al.*, 2001). In addition to the anti-opsonic properties of capsular polysaccharide, *S. aureus* avoids complement deposition by secreting Efb, which can bind and sequester complement protein C3, inhibiting deposition on the bacterial cell surface and hence complement activation (Section 1.3.4.3; Lee *et al.*, 2004).

S. aureus cells are capable of survival within the phagosome of PMNL in a process that is SarA-dependent (Section 1.3.6.1; Gresham *et al.*, 2000). In addition, resistance to killing in the PMNL phagolysosome is conferred both by modification of teichoic acids and lipoteichoic acids with D-alanine and an unusual L-lysine modification of membrane phosphatidylglycerol (see Section 1.3.5.1). These chemical modifications at the cell surface cause reduced overall negative

surface charge and repulsion of cationic antimicrobial peptides (Collins *et al.*, 2002; Peschel *et al.*, 2001).

The importance of ClfA in *S. aureus* virulence in a variety of animal models suggests that it is involved in subversion of the host immune response in a general way that is important for each of these pathogenic processes. These observations led to the current investigation of whether ClfA is itself an antiphagocytic factor, and whether its antiphagocytic properties were based on its ability to bind fibrinogen.

5.2 Results:

5.2.1 Antiphagocytic activity of ClfA and protein A in *S. aureus* Newman

A phagocytosis assay was used to measure the uptake of bacterial cells by freshly isolated human PMNL, as described in Sections 2.25 and 2.26. Fluorescently labelled bacteria were opsonised with antibody, complement and other factors in pooled normal human serum (NHS) and incubated with PMNL. The percentage of PMNL that had taken up the fluorescent bacteria was then measured by flow cytometry. Data were gated on the basis of fluorescence, so that the percentage of positive PMNL (fluorescent PMNL due to bacterial uptake) could be measured. Some examples of this are shown in Figure 5.1. Samples were prepared in triplicate and all experiments were repeated a minimum of three times using the blood of different donors. Mean '% gated' values for each triplicate dataset were then plotted against percentage NHS, to show the relationship between serum concentration and phagocytic uptake for each strain (as in Figure 5.2). Although experiments with cultured monocytes/macrophages or neutrophil-like cell lines would have been less difficult, they are far less representative of the *in vivo* situation than the use of fresh human neutrophils, as the development of PMNL, and granules in particular, is complex.

In order to measure the effect of ClfA and protein A in the inhibition of opsono-phagocytosis by *S. aureus*, Newman wild type, a ClfA-defective mutant (Newman *clfA2*), a protein A-defective mutant (Newman *spa*) and a double mutant (Newman *spa clfA2*) were opsonised with normal human serum with or without fibrinogen, incubated with fresh human PMNL and their uptake was measured (see Table 2.1 for strains). At a concentration of 1% (v/v) NHS, Newman, Newman *clfA2* and Newman *spa* were each taken up by about 45% of PMNL, whereas Newman cells deficient in both ClfA and protein A (Newman *spa clfA2*) were taken up by 72% of PMNL ($P < 0.001$) (Figure 5.2, upper panel). This demonstrated that both protein A and ClfA contribute to resistance to phagocytosis by *S. aureus* Newman, and that the absence of one factor was compensated for by the presence of the other. The absence of both factors led to a significant increase in phagocytic uptake.

The addition of a physiologically relevant concentration of fibrinogen to the opsonising material had no significant impact on the level of uptake of the ClfA-deficient strains Newman *clfA2* and Newman *spa clfA2* (Figure 5.2, lower panel).

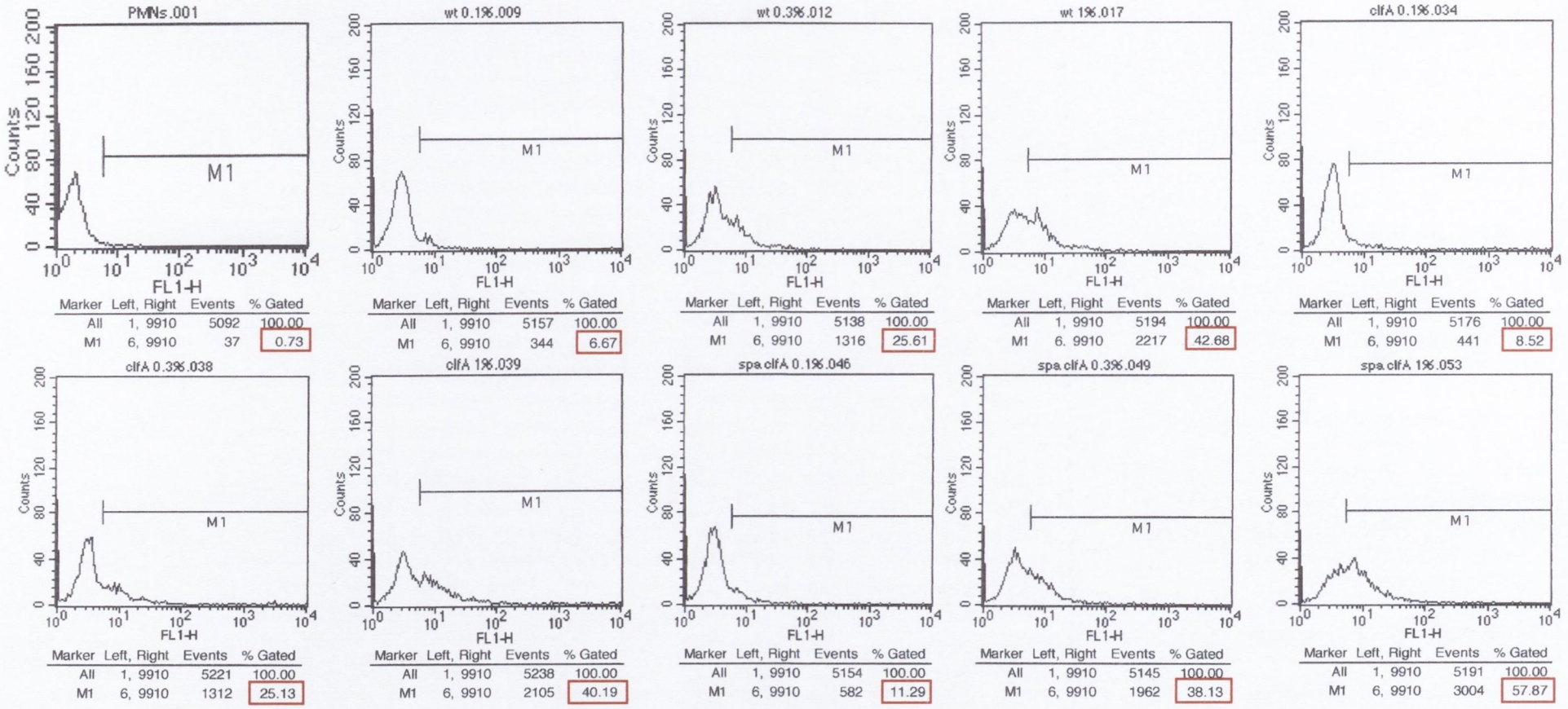


Fig 5.1 Phagocytosis of *S. aureus* strains by human PMNL

Histograms of phagocytosis of selected representative datapoints of Newman strains. Histograms plot number of PMNL against fluorescence. A shift towards fluorescence indicates phagocytic uptake of fluorescent bacteria. Gate (M1) was manually applied and identical for all datasets. Data below each plot indicate gate M1. Gated cells were considered positive by comparison to PMNL alone. The percentage of positive cells is indicated in a red box for each dataset. All samples were prepared and measured in triplicate.

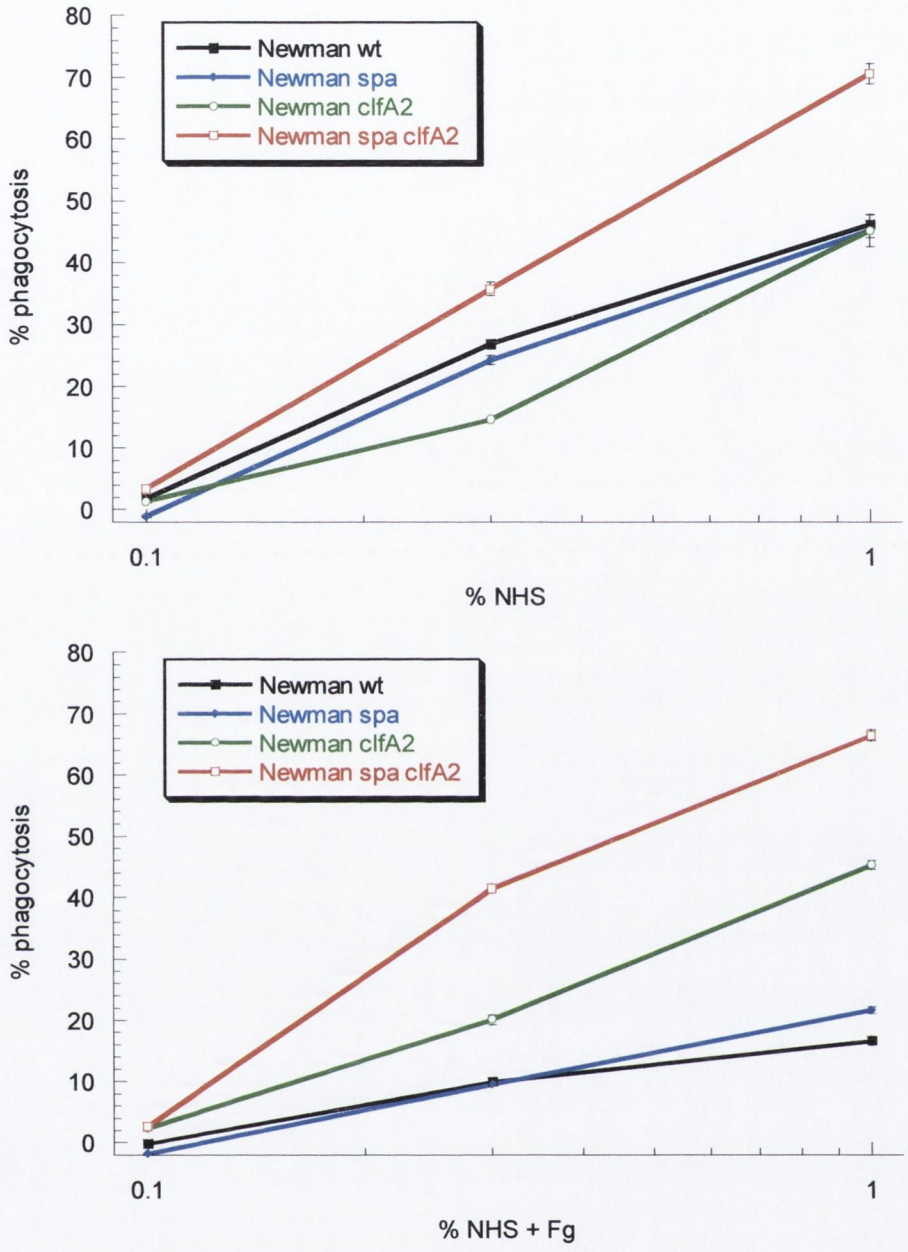


Fig 5.2 Phagocytosis of Newman strains by human PMNL

Freshly isolated human PMNL were incubated with *S. aureus* Newman strains grown to stationary phase and opsonised with normal human serum (NHS) in the absence (top panel) or presence (bottom panel) of physiologically relevant concentrations of fibrinogen (Fg). The percentage of PMNL containing bacteria ('% gated' in histograms) is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors. Error bars for all points are included.

The levels of uptake of Newman wild-type and Newman *spa* were significantly reduced in the presence of fibrinogen compared to NHS alone ($P < 0.005$ and $P < 0.01$, respectively). This indicates that strains bearing ClfA on their cell surface have enhanced antiphagocytic properties in the presence of soluble fibrinogen, whereas the presence of fibrinogen has no effect on the phagocytic uptake of cells lacking ClfA.

There was an approximately logarithmic correlation between serum concentration and uptake in all cases, indicating that the process is dependent on host serum opsonins. Bacterial cells used in this assay were grown to stationary phase to minimise any possible contribution of clumping factor B, which is absent in stationary phase due to proteolysis and cessation of *clfB* transcription (McAleese *et al.*, 2001). The fibronectin-binding proteins are also predominantly expressed during the exponential phase of growth and can also bind fibrinogen. However the *fnbA* and *fnbB* genes of Newman each have a frameshift mutation that results in secretion of truncated forms of the proteins due to loss of the wall-anchoring domains at the C-termini (Grundmeier *et al.*, 2004). This leaves ClfA as the only fibrinogen-binding surface protein on these cells. This was important as it is possible that the other fibrinogen-binding surface proteins might also contribute to antiphagocytic effects. Additionally, Newman can produce a serotype 5 micro-capsule in stationary phase. Although the growth conditions used did not promote high-level production of capsular polysaccharide, it is likely that some capsule was produced by these cells. This may have had an impact on the level of uptake of these strains, depending on the presence of capsular polysaccharide-specific antibodies in the NHS, which was not tested. Microscopic examination of samples following FACS analysis showed no significant extracellular clumping of bacteria, either in NHS alone, or in NHS containing fibrinogen (data not shown). The final concentration of fibrinogen in the samples was insufficient to cause large-scale bacterial clumping, which might interfere with phagocytic uptake.

These data show that ClfA and protein A are antiphagocytic, and that one factor can compensate for the absence of the other. The presence of fibrinogen contributed to the antiphagocytic effect of ClfA, but not to that of protein A.

5.2.2 Antiphagocytic activity of ClfA in *S. aureus* 8325-4

In order to investigate the contribution of fibrinogen binding by ClfA to its inhibition of opsonophagocytosis by human PMNL, ClfA derivatives defective in binding to fibrinogen were expressed from the multicopy plasmid pCU1 in strain 8325-4 defective in ClfA and protein A. The ClfA derivative 'EV' had amino acid substitutions $E_{526}A$ and $V_{527}S$, which reduced binding to fibrinogen (Hartford *et al.*, 2001a), while the ClfA derivative 'PY' had amino acid substitutions $P_{336}S$ and $Y_{338}A$, which completely abolished fibrinogen binding (see Section 4.2.4). The wild-type ClfA was expressed from plasmid pCF77, while the ClfA'EV' derivative was expressed from the plasmid pCF77EV and the ClfA'PY' derivative from plasmid pCF77PY, with pCU1 included as an empty vector control.

S. aureus 8325-4 *spa clfA5* (pCF77), 8325-4 *spa clfA5* (pCF77EV) and 8325-4 *spa clfA5* (pCF77PY) (see Table 2.2 for plasmids) were tested in the same phagocytosis assay as above. These strains expressed ClfA and its derivatives (ClfAEV and ClfAPY) on the bacterial surface in a *clfA*- and *spa*-defective background. Levels of phagocytic uptake of the ClfA, ClfAEV and ClfAPY-expressing strains were higher than the control strain *S. aureus* 8325-4 *spa clfA5* (pCU1) (Figure 5.3, upper panel; $P < 0.005$ in all cases). This may have been due to the presence of opsonising ClfA-specific antibodies in NHS that gained access to the cell surface due to the absence of capsular polysaccharide on 8325-4. 8325-4 is non-encapsulated due to a mutation in *cap5E*, a gene encoding a vital component of the capsular polysaccharide biosynthetic pathway (Kneidinger *et al.*, 2003).

Following opsonisation with fibrinogen in addition to NHS the level of uptake of each of these strains was somewhat reduced, although it did not reach significance (Figure 5.3, lower panel). This reduction of uptake due to fibrinogen binding in all strains was unexpected, as ClfAEV was shown to bind fibrinogen less avidly than ClfA, and ClfAPY was shown not to bind fibrinogen (Figure 4.16, panel A). This binding of fibrinogen must have been ClfA-independent and may have involved fibrinogen binding by ClfB, FnBPA, FnBPB or other factors on the 8325-4 surface. The contribution of ClfA in 8325-4 *spa clfA5* (pCF77) seems to have been insignificant in this background. 8325-4 is a laboratory strain and is atypical of natural isolates in many regards. In addition to being capsule deficient, it is defective in the stationary phase sigma factor SigB, which affects the

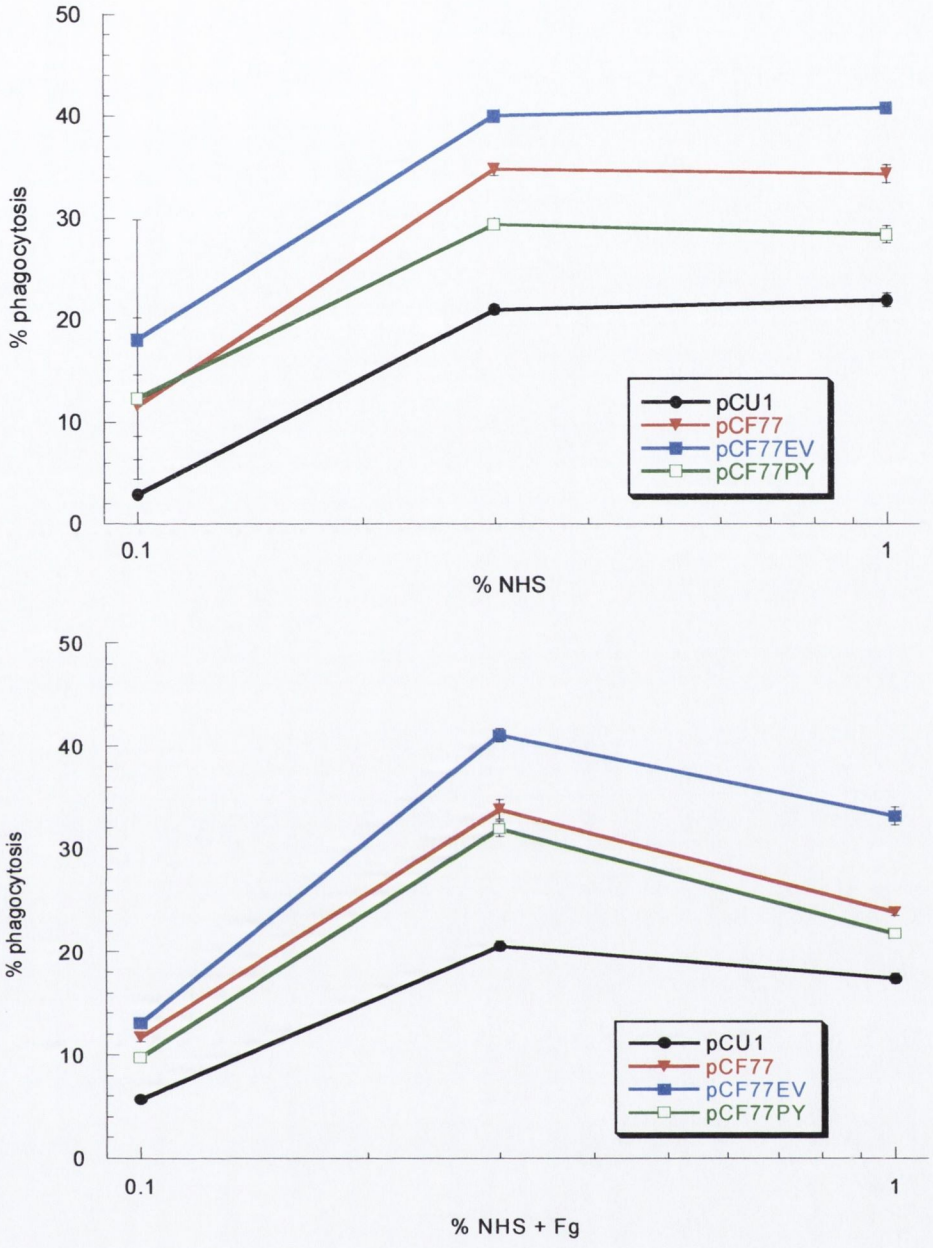


Fig 5.3 Phagocytosis of 8325-4 *spa clfA5* strains expressing ClfA derivatives

Freshly isolated human PMNL were incubated with *S. aureus* 8325-4 *spa clfA5* strains expressing no protein (pCU1) or ClfA (pCF77) or its derivatives ClfAEV (pCF77EV) and ClfAPY (pCF77PY) grown to stationary phase and opsonised with NHS in the absence (top panel) or presence (bottom panel) of fibrinogen. The percentage of PMNL containing bacteria is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors. pCU1 is included as protein negative control. Error bars are included for all points.

starvation-survival response and the regulation of *clfA* (see Chapter 6). It is possible that due to this defect, ClfA and its derivatives were weakly expressed in these strains (compared to Newman) despite being expressed from multicopy plasmids. In these 8325-4 strains ClfA did not inhibit phagocytic uptake of *S. aureus* by human PMNL.

5.2.3 Antiphagocytic activity of ClfA in *Lactococcus lactis*

It appeared that staphylococcal factors other than ClfA, protein A and capsular polysaccharide might be involved in inhibition of opsonophagocytosis. As the phagocytosis experiments were labour-intensive, using fresh donor human blood, it was important to minimise the number of inconclusive experiments. An alternative approach to studying the role of ClfA in inhibition of phagocytosis was employed. ClfA expressed on the surface of *Lactococcus lactis* (O'Brien *et al.*, 2002a) was used to verify that ClfA is an antiphagocytic factor, as had been demonstrated in *S. aureus* Newman.

L. lactis is a Gram-positive bacterium that has been used to study interactions between surface proteins and platelets (O'Brien *et al.*, 2002a; Loughman *et al.*, 2005) as well as the role of ClfA in infective endocarditis (Que *et al.*, 2001). The bacterium expresses few surface proteins but possesses the sorting system required for the anchoring of LPXTG proteins to peptidoglycan. Expression of individual *S. aureus* surface proteins in *L. lactis* avoids the complication of functional redundancy of surface proteins that recognise fibrinogen and/or are antiphagocytic.

ClfA was expressed constitutively from plasmid pKS80 in *L. lactis* strain MG1363. *L. lactis* MG1363 (pKS80), MG1363 (pKS80*clfA*) and MG1363 (pKS80*spa*) (see Table 2.2 for plasmids) were grown to stationary phase and tested in the phagocytosis assay. As protein A and ClfA were expressed from the same plasmid-based expression system, it was assumed that their levels were equivalent, although this was not tested. Both in the absence and presence of fibrinogen at 1% (v/v) NHS *L. lactis* bearing the empty vector was taken up by about 50% of PMNL (Figure 5.4). *L. lactis* expressing either protein A or ClfA showed a significant reduction in phagocytic uptake compared to wild-type in the absence of fibrinogen (P = 0.003 and P = 0.002, respectively, at 1% (v/v) NHS;

Figure 5.4, upper panel). ClfA showed a further reduction upon addition of fibrinogen (P = 0.001 at 1% (v/v) NHS; Figure 5.4, lower panel). Protein A was a very potent inhibitor of phagocytosis, and showed no fibrinogen-dependent reduction of uptake. As *L. lactis* is not encapsulated (Gilbert *et al.*, 2000), capsular polysaccharide was not a factor in these assays, and it is likely that antibodies in NHS to peptidoglycan and cell wall teichoic acids were sufficient to promote efficient opsonisation of the bacteria bearing the empty vector.

These data correlate with the data from *S. aureus* Newman (Section 5.2.1), showing that both protein A and ClfA are antiphagocytic factors and that the antiphagocytic activity of ClfA is in part fibrinogen dependent. It appeared that ClfA may also have fibrinogen-independent antiphagocytic properties.

5.2.4 Effect of fibrinogen on the antiphagocytic effect of ClfA

The open reading frames encoding wild-type ClfA and the non-fibrinogen-binding derivative ClfAPY were cloned into the nisin-inducible expression vector pNZ8037 in the *L. lactis* NZ9800 host (Loughman *et al.*, 2005). In order to test the contribution of fibrinogen binding by ClfA to resistance to phagocytosis, strains *L. lactis* NZ9800 (pNZ8037), which bears the empty vector, NZ9800 (pNZ8037*clfA*), which expresses ClfA, and NZ9800 (pNZ8037*clfA*PY), which expresses the non-fibrinogen-binding ClfAPY were tested in the phagocytosis assay (see Table 2.1 for strains and Table 2.2 for plasmids).

The pNZ8037 plasmids allow expression of proteins from nisin-inducible rather than constitutive promoters. Prior to labelling bacterial cells with fluorescein, the levels of expression of ClfA and ClfAPY were measured by whole cell dot-immunoblotting. Expression levels were optimised, by induction with increasing concentrations of nisin, for maximum expression without inhibition of cell growth (Loughman *et al.*, 2005) and equalised between the strains. ClfAPY has two amino acid substitutions in positions that are not surface-exposed (Deivanayagam *et al.*, 2002), and reacts strongly with polyclonal anti-ClfA antibodies. When fully induced the proteins were shown by dot immunoblotting to be expressed at a slightly higher level than was expressed from the constitutive expression vector pKS80 (Figure 5.5).

At 1% (v/v) NHS the control *L. lactis* (pNZ8037) was taken up by about 50% of PMNL both in the absence and the presence of fibrinogen (Figure 5.6). Both *L.*

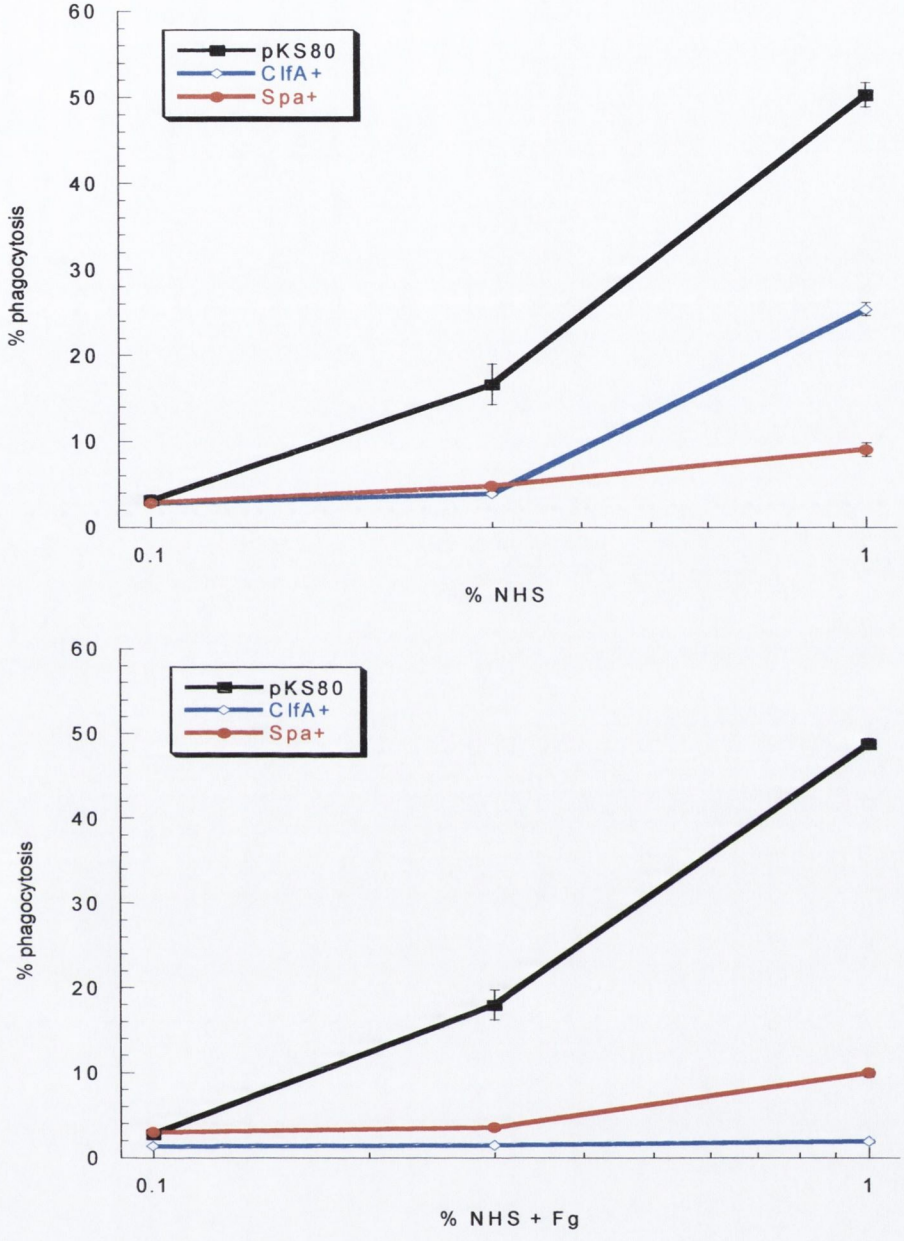


Fig 5.4 Phagocytosis of *L. lactis* strains by human PMNL

Freshly isolated human PMNL were incubated with *L. lactis* strains expressing no protein (pKS80), ClfA ($ClfA^+$) or protein A (Spa^+) opsonised with NHS in the absence (top panel) or presence (bottom panel) of physiologically relevant concentrations of fibrinogen. The percentage of PMNL containing bacteria is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors. Error bars are included for all points.

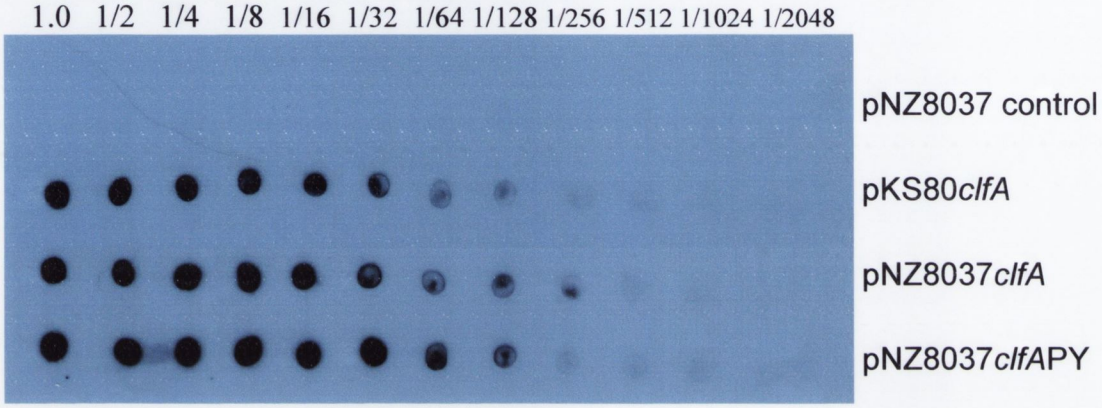


Fig 5.5 Dot immunoblotting of *L. lactis* strains

L. lactis harbouring pNZ8037 plasmids were grown to exponential phase before induction with 0.8 μg/ml nisin and growth to stationary phase. *L. lactis* harbouring pKS80*clfA* was grown to stationary phase. Cells were washed twice and resuspended at an optical density (OD_{600}) of 1.0 per ml. Doubling dilutions were prepared in PBS and dotted on a nitrocellulose membrane. After drying and blocking in 5% (w/v) skimmed milk powder, the membrane was incubated with rabbit anti-ClfA region A polyclonal primary antibody, followed by horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody. Cells are those used for Figure 5.6, and are representative of three experiments.

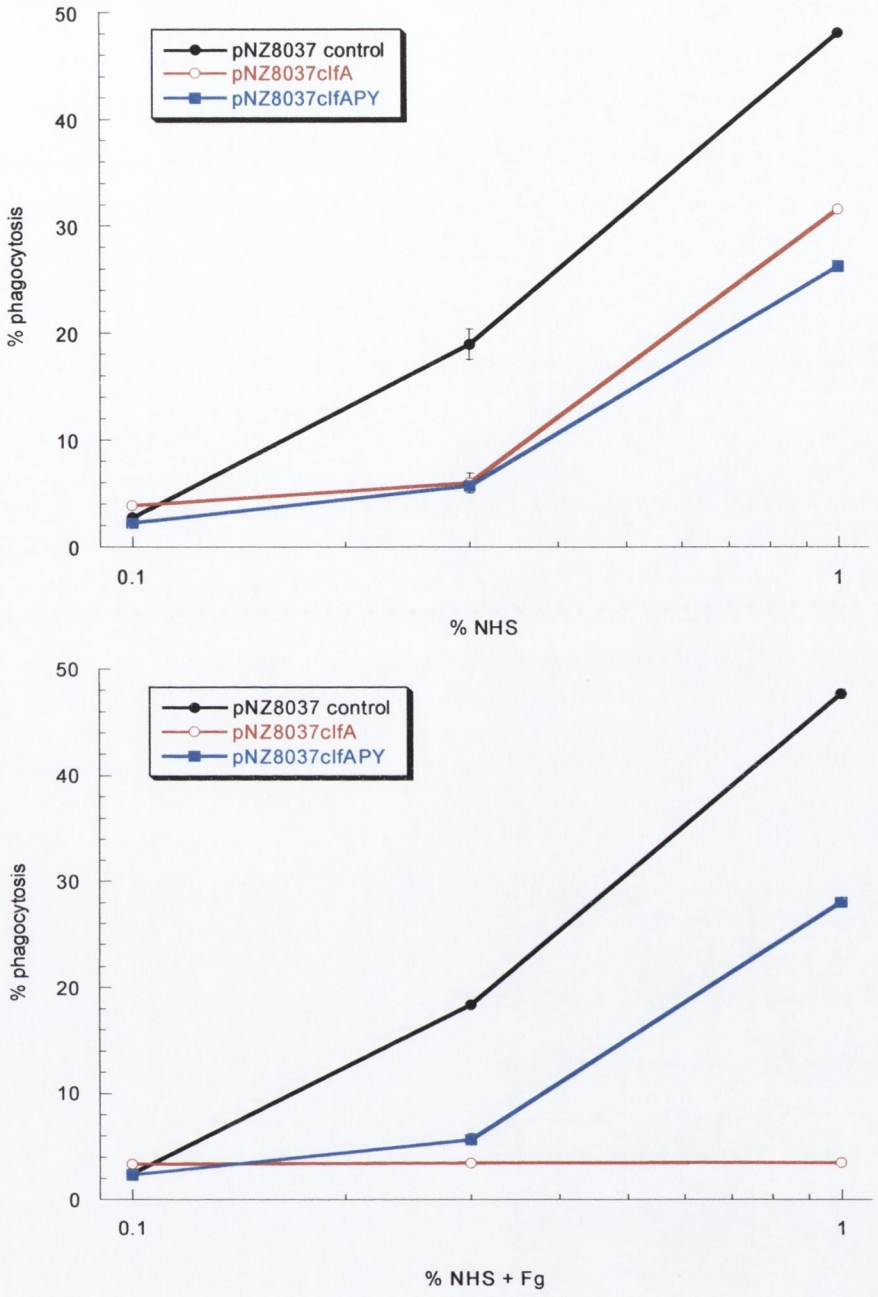


Fig 5.6 Phagocytosis of *L. lactis* ClfAPY strains by human PMNL

Freshly isolated human PMNL were incubated with stationary phase *L. lactis* strains expressing no ClfA protein (pNZ8037), wild-type ClfA (pNZ8037*clfA*) or ClfAPY (pNZ8037*clfA*PY) opsonised with NHS in the absence (top panel) or presence (bottom panel) of physiologically relevant concentrations of fibrinogen. The percentage of PMNL containing bacteria is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors. Error bars are included for all points.

lactis expressing ClfA and ClfAPY were taken up at a significantly lower level than wild-type at 1% (v/v) NHS ($P < 0.001$ for both proteins; Figure 5.6, upper panel). The difference in uptake between *L. lactis* expressing ClfA and ClfAPY was not significant. Addition of fibrinogen did not affect the level of uptake of either wild-type or *L. lactis* expressing ClfAPY (Figure 5.6). At 1% (v/v) NHS containing fibrinogen the level of uptake of *L. lactis* expressing ClfA was reduced to 4%, which was a significant decrease ($P < 0.001$).

These data demonstrate that in the absence of fibrinogen, ClfA is antiphagocytic. In the presence of fibrinogen ClfA-promoted resistance to phagocytosis was enhanced. The ClfA derivative that was incapable of binding fibrinogen inhibited phagocytosis to the same level as wild-type ClfA in the absence of fibrinogen. This showed that ClfA uses fibrinogen to inhibit phagocytosis, but that in the absence of soluble fibrinogen, it is nonetheless antiphagocytic by another mechanism. This may involve physical masking of antigens on the cell-surface such as peptidoglycan and teichoic acids or may be a novel function of ClfA.

5.2.5 Antiphagocytic properties of other surface proteins

As ClfA is one of many surface proteins capable of binding proteins in plasma, the ability of various fibrinogen-binding surface proteins to inhibit phagocytosis by human PMNL was investigated. Included in this analysis were SdrG and Fbl, fibrinogen binding proteins of *S. epidermidis* and *S. lugdunensis*, respectively. ClfB was also tested, and protein A and ClfA were included for comparative purposes. With the exception of protein A, which binds immunoglobulins, each protein shares significant homology with ClfA and binds fibrinogen. The proteins were expressed constitutively in *L. lactis* MG1363 from pKS80 (plasmids are listed in Table 2.2; see Figure 1.2 for schematic diagrams of protein structures).

The control strain *L. lactis* (pKS80) was taken up by approximately 50% of PMNL at 1% (v/v) NHS, both in the absence and the presence of fibrinogen (Figure 5.7). *L. lactis* expressing ClfA, ClfB, SdrG, Fbl or protein A were taken up at a lower level in NHS (Figure 5.7 upper panel; $P < 0.01$ in all cases). These strains showed a further significant reduction in uptake upon addition of fibrinogen (Figure 5.7, lower panel; $P < 0.01$) with the exception of protein A. Thus the

fibrinogen binding proteins ClfB, SdrG and Fbl displayed both a fibrinogen-dependent and a fibrinogen-independent inhibition of phagocytosis, similar to ClfA.

L. lactis strains expressing non-fibrinogen-binding surface proteins were also tested in the phagocytosis assay. SdrD is a surface protein of *S. aureus* that contains an SD repeat region, but has no known function. It has a region A that is related to that of ClfA, as well as 5 B repeats (Figure 1.2; Josefsson *et al.*, 1998). SdrH is a *S. epidermidis* protein that is an atypical member of the Sdr family. It has a short 60-residue N-terminal potentially surface-exposed A domain followed by a very short SD-repeat region, a unique 277-residue C region and a C-terminal hydrophobic segment. SdrH lacks a LPXTG motif (Figure 1.2; McCrea *et al.*, 2000; Mitchell, 2004).

SdrD appeared antiphagocytic, both in the absence and presence of fibrinogen (Figure 5.8). The uptake of *L. lactis* expressing SdrD was significantly different from wild type ($P < 0.001$ in both cases). The uptake of *L. lactis* expressing SdrH was very similar to *L. lactis* expressing no surface protein.

These data indicate that SdrD, but not SdrH, is an antiphagocytic factor. The inhibition of phagocytic uptake by SdrD was fibrinogen-independent.

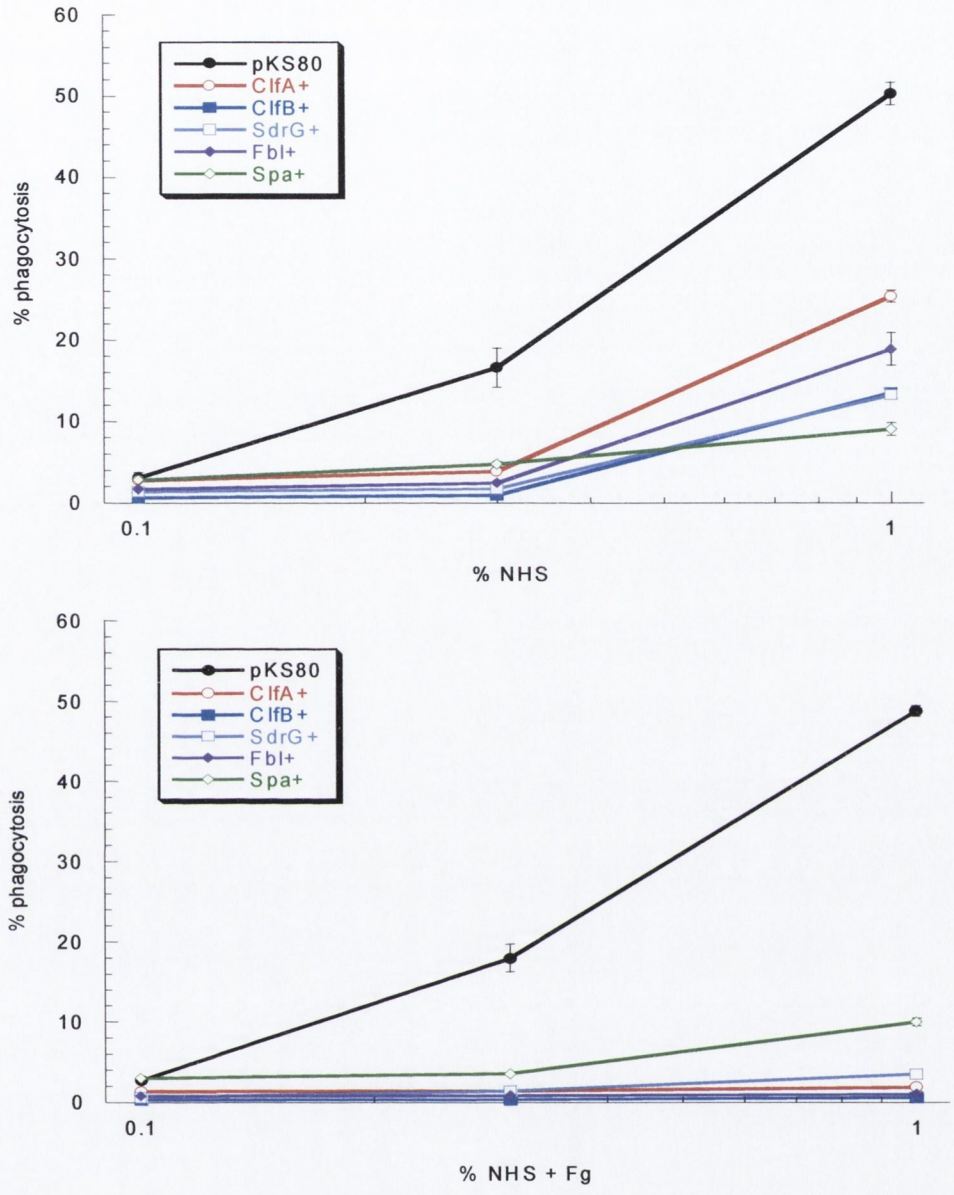


Fig 5.7 Phagocytosis of *L. lactis* expressing fibrinogen-binding proteins

Freshly isolated human PMNL were incubated with stationary phase *L. lactis* strains expressing no protein (pKS80), ClfA, ClfB, SdrG, Fbl or protein A (Spa^+) opsonised with NHS in the absence (top panel) or presence (bottom panel) of physiologically relevant concentrations of fibrinogen. The percentage of PMNL containing bacteria is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors. Error bars are included.

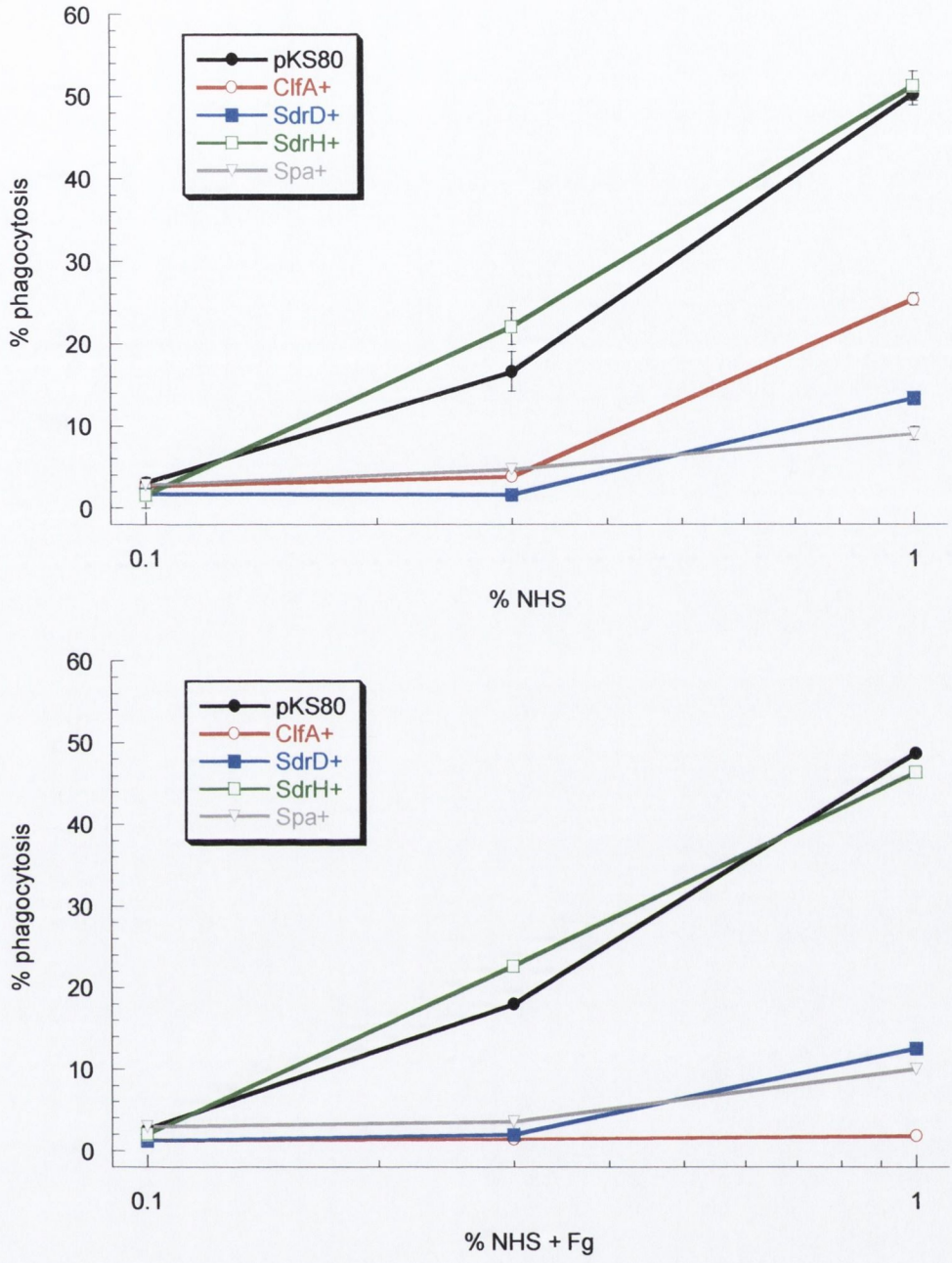


Fig 5.8 Phagocytosis of *L. lactis* strains expressing surface proteins

Freshly isolated human PMNL were incubated with stationary phase *L. lactis* strains expressing no protein (pKS80), ClfA, SdrD, SdrH or Spa opsonised with NHS in the absence (top panel) or presence (bottom panel) of physiologically relevant concentrations of fibrinogen. The percentage of PMNL containing bacteria is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors. Error bars are included.

5.3 Discussion:

This Chapter investigated a possible role for ClfA in resistance to phagocytosis by human PMNL. It was shown that ClfA is an antiphagocytic factor for *S. aureus*, with both fibrinogen-dependent and fibrinogen-independent mechanisms. In *S. aureus* Newman, ClfA was shown to be of equal importance to protein A, the antiphagocytic properties of which are well established. ClfA expressed in a surrogate Gram-positive host was also found to be antiphagocytic. A ClfA mutant that was unable to bind fibrinogen had a similar antiphagocytic effect as wild-type ClfA in the absence of fibrinogen, further establishing the existence of a fibrinogen-independent mechanism.

Using the surrogate host *L. lactis* it was shown that the staphylococcal fibrinogen-binding proteins ClfB, SdrG and Fbl were also antiphagocytic, and that this was also partially fibrinogen-independent. It was also shown that SdrD is antiphagocytic in a fibrinogen-independent manner. SdrH was found not to be antiphagocytic.

When *S. aureus* is grown in plasma it becomes coated with soluble plasma components that interfere with bacterial adherence to immobilised ligands (Massey *et al.*, 2002). It was suggested that binding of host proteins may impede clearance of bacteria by phagocytic cells. In this study, inactivation of both *clfA* and *spa* in a microencapsulated strain of *S. aureus* caused a significant increase in phagocytosis. Although Newman can express a type 5 capsular polysaccharide, the growth conditions used here would not have promoted its expression at high levels. In addition, the presence of either protein alone seemed to be sufficient to inhibit phagocytosis. It seems that being coated with either immunoglobulin or fibrinogen, or a mixture of both, is sufficient to impede phagocytosis. This indicates that protein A and ClfA are two major antiphagocytic factors for *S. aureus*.

Attempts to determine the contribution of fibrinogen binding in the anti-phagocytic effect of ClfA in *S. aureus* strain 8325-4 gave inconclusive results. This may have been caused in part by the lack of capsular polysaccharide, expression of other fibrinogen-binding proteins (ClfB or FnBPs), or indeed other non-fibrinogen-binding surface proteins. It was subsequently determined that ClfA is poorly expressed in this strain (see Chapter 6), although the *clfA* gene was present on a multicopy plasmid. Protease activity might also have had some

impact on the protein repertoire on the cell surface. In particular, high levels of aureolysin production by 8325-4 most likely caused removal of domain N1 of region A of ClfA from the cells in stationary phase (see Chapter 3). Although the proteolytic removal of domain N1 would not have reduced fibrinogen binding, it might have influenced the antiphagocytic properties of ClfA. At the time these experiments were performed little was known about the expression of *clfA* in *S. aureus*. It transpired that strain 8325-4 was an unsuitable choice for these experiments, and, in retrospect, strain Newman *spa clfA2* would have been a more suitable host.

Following preliminary results with *L. lactis* that showed significant differences in the uptake of ClfA$^+$ cells, it was decided to proceed with *L. lactis* as a host rather than *S. aureus*. The advantage of *L. lactis* is that a single surface factor can be studied in the absence of other surface components from the native host. *L. lactis* has been used previously to verify the role of ClfA in experimental endocarditis (Que *et al.*, 2001), and in studies of the interactions of *S. aureus* surface proteins with platelets (O'Brien *et al.*, 2002a) and with squamous epithelial cells (O'Brien *et al.*, 2002b). In *L. lactis*, ClfA was shown to be antiphagocytic, and the antiphagocytic effect was partially fibrinogen-dependent. Expression in *L. lactis* also showed that ClfB, SdrG, Fbl and SdrD had antiphagocytic properties.

Normal human sera contain antibodies to ClfA (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005). However, in pooled human sera the titre is low (Loughman *et al.*, 2005). High-titre antibodies are found only in approximately 1 in 100 community plasma donors (Vernachio *et al.*, 2003). In the pooled NHS used in the phagocytosis study it is likely that anti-ClfA antibodies were present, but any high-titre antibodies would be diluted. In immune animals (either vaccinated with recombinant ClfA or passively immunised with IgG containing high titre against ClfA or humanised monoclonal antibodies) the level of antibody is sufficient to be protective, and this is due at least in part to their opsonising activity (Josefsson *et al.*, 2001; Vernachio *et al.*, 2003; Patti, 2004). The protective antibodies might interact with ClfA in one of two ways. They may compete with fibrinogen to bind ClfA and promote phagocytosis, either alone via Fc receptors on the PMNL surface or by stimulating complement activation. Alternatively they may promote opsonophagocytosis by binding to ClfA along with bound fibrinogen.

In 'normal' individuals the level of anti-ClfA antibodies is insufficient to be protective (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005). Immunisation of mice

with recombinant ClfA ($ClfA_{(40-559)}$) induced anti-ClfA antibody production, whereas infection of naïve mice with *S. aureus* Newman did not (Josefsson *et al.*, 2001). Perhaps the ability of ClfA on the surface of *S. aureus* cells to bind fibrinogen in the bloodstream prevents the development of specific antibodies directed against it. This might be tested using an appropriate strain in which the chromosomal *clfA* gene was replaced with the *clfA*PY mutation.

Paradoxically, the low level of antibodies to ClfA in normal sera is exploited by *S. aureus* in its interaction with platelets. It has recently been shown that the binding of specific IgG to surface proteins is crucial for stimulating rapid activation and aggregation of platelets, which is correlated with the pathogenesis of endovascular infection (e.g. endocarditis) (see Section 1.3.8.1; Loughman *et al.*, 2005; Fitzgerald *et al.*, 2006). The observation that low levels of anti-ClfA antibodies stimulate platelet aggregation may go some way to explaining why these antibodies in normal serum are not protective against staphylococcal infection (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005; Loughman *et al.*, 2005).

Thus ClfA is antiphagocytic despite the presence of low levels of anti-ClfA antibodies in normal human sera. One would expect the binding of specific antibody to ClfA or other surface proteins to initiate complement activation and phagocytosis. Indeed, it has been shown that low levels of anti-ClfA antibodies are sufficient to promote complement fixation and lead to slow activation of platelet aggregation (Loughman *et al.*, 2005). Perhaps the bound opsonins are prevented from interacting productively with Fc receptors and complement receptors on the PMNL surface, possibly through steric hindrance by bound fibrinogen, immunoglobulins bound to protein A, or other serum or bacterial factors.

The presence of two mechanisms of resistance to phagocytosis, one fibrinogen-dependent and the other fibrinogen-independent may reflect the presence of different niches for the bacteria within the host during disease progression where fibrinogen is available or unavailable.

ClfA is an important virulence factor in the murine sepsis and septic arthritis model (Josefsson *et al.*, 2001). This model is complex, involving an initial bacteraemic phase, followed by extravasation and finally invasion of the joints, causing tissue damage and inflammation. During the bacteraemic phase PMNL are the primary line of defence against *S. aureus* (Verdrengh and Tarkowski, 1997). Extravasation may involve other surface proteins, particularly FnBPs, which promote invasion of endothelial cells (Section 1.3.6.2; Massey *et al.*, 2001).

Inside the joint inflammation is associated with an influx of PMNL. By treating mice with Ancrod, a serine protease with thrombin-like activity, free fibrinogen in the bloodstream was depleted. This aggravated septic arthritis caused by ClfA-producing *S. aureus* strains (Palmqvist *et al.*, 2004a). It appears likely that the ability of ClfA to inhibit opsonophagocytosis independently of fibrinogen may be important in this model.

In a study of murine macrophages, the presence of ClfA on the surface of *S. aureus* cells inhibited phagocytosis *in vitro*, an effect that was not influenced by the presence of fibrinogen (Palmqvist *et al.*, 2004b). However, it is unclear whether the macrophage has a significant role in the bacteraemic phase of infection. The major phagocytic cell is the PMNL (Verdrengh and Tarkowski, 1997; 2000). The data presented in this Chapter used fresh PMNL obtained from human donors and are more likely to represent the *in vivo* situation, at least with respect to bacteraemia.

At the physiological concentration of free Ca^{2+} in the bloodstream (2-3 mM) it is likely that only about 50% of ClfA molecules will have fibrinogen bound, as Ca^{2+} inhibits fibrinogen binding by ClfA with an IC_{50} of 2.5 mM (O'Connell *et al.*, 1998). Thus the ability of ClfA to inhibit phagocytosis independently of fibrinogen may be important *in vivo*, particularly in environments where the Ca^{2+} concentration is elevated. There is increasing evidence that ClfA has functions that are independent of fibrinogen-binding activity and contribute to virulence in *S. aureus* (Josefsson *et al.*, 2001; Palmqvist *et al.*, 2004a; Loughman *et al.*, 2005) and that fibrinogen-independent properties of ClfA may be important during the infectious process.

The present study has shown that a surface coat of immunoglobulin or fibrinogen, or a mixture of both, appears sufficient to impede phagocytosis. Indeed, in the absence of both protein A and fibrinogen, $ClfA^+$ *S. aureus* cells remained resistant to phagocytosis at the same level as wild-type. The antiphagocytic effect of ClfA may be partly due to the blocking of productive antibody-antigen interactions by bound fibrinogen. Fibrinogen (and IgG bound to protein A) may form a protective layer of host protein around the bacterium, preventing access by antibodies to antigens on the cell wall surface such as peptidoglycan, teichoic acids and other proteins, or preventing interactions between antibodies bound to these antigens and Fc receptors on the PMNL surface. Such a protective layer might also interfere with complement deposition.

It is clear that ClfA also impedes phagocytosis by macrophages by a mechanism that is independent of fibrinogen (Palmqvist *et al.*, 2004b). The study in which fibrinogen was depleted in mice also suggesed that fibrinogen binding by ClfA is not required for virulence in septic arthritis (Palmqvist *et al.*, 2004a). The use of a non-fibrinogen-binding mutant of *clfA* (such as *clfA*PY) in this study, rather than Ancrod, might have eliminated the pleiotropic effects of fibrinogen-depletion on arthritis in the mice. The fibrinogen-independent mechanism of inhibition of phagocytosis by ClfA may involve prevention of antibody binding and complement formation on the cell surface by the protein itself. This may be due to steric hindrance, or repulsion of one or more serum factors involved in opsonisation. The protein might also repel PMNL.

The common feature of the Clf-Sdr proteins is the SD repeat region R, which projects the ligand-binding domain away from the cell surface (Hartford *et al.*, 1999). It is possible that the negative charge contributed by the aspartate residues in the repeat regions or hydrophobicity provided by the N-terminal A regions might contribute to the repulsion of PMNL in the absence of efficient opsonisation. The three-dimensional structure of domains N2 and N3 of region A of ClfA, SdrG and ClfB are known and the majority of hydrophobic residues are buried in the globular domains and are not surface-exposed. The structure of domain N1, which migrates aberrantly in SDS-PAGE, is not known. Any contribution of domain N1 to inhibition of opsonophagocytosis could be tested using a mutant of ClfA lacking this domain.

The presence of large amounts of proteins on the surface might prevent access of antibodies in NHS that bind antigens on the cell wall surface, such as teichoic acids and peptidoglycan, and might prevent complement binding by the classical, alternative or mannose-binding lectin pathways. The binding of fibrinogen may exacerbate this steric blocking or masking of the cell surface. SdrH may have failed to prevent phagocytosis of *L. lactis* in this manner because of the small size of the surface-exposed N-terminal domain (Mitchell, 2004).

A model for the interactions between *S. aureus* surface factors and human PMNL is depicted in Figure 5.9. Phagocytosis by PMNL occurs following opsonisation of bacteria with specific antibody and complement components. *S. aureus* inhibits this process in a number of ways. Secreted Efb binds and sequesters complement protein C3. Capsular polysaccharide inhibits complement deposition on the bacterial cell surface and interferes with the deposition of

antibodies directed against peptidoglycan, teichoic acid and other surface antigens. Protein A inhibits phagocytosis by binding the Fc regions of IgG and preventing productive interactions between Fc and the Fcγ receptors on the PMNL surface. ClfA and other fibrinogen-binding proteins inhibit phagocytosis partially through binding fibrinogen that coats the bacteria with host protein, shielding the surface from host antibodies. In addition, large surface proteins inhibit phagocytosis independently of fibrinogen, possibly by interfering with opsonin deposition on the cell surface, or by inhibiting productive interactions with PMNL.

In considering the diverse staphylococcal factors that can affect opsonophagocytosis in NHS by human PMNL it is relevant to discuss their likely roles under different growth conditions. Variation in protein expression occurs between exponential-phase and stationary-phase cells grown *in vitro* in broth. However, the growth conditions encountered by *S. aureus in vivo* are unlikely to be reflected accurately by the exponential or stationary phases of *in vitro* growth. The study described here used *S. aureus* cells that had been grown to stationary phase in broth. The fibrinogen-binding proteins FnBPA, FnBPB and ClfB are expressed in exponential phase and are lost in stationary phase due to cessation of transcription before growth stops, proteolytic degradation and dilution of the remaining molecules as the cells continue to grow. Protein A is expressed at high levels in exponential phase and transcription ceases before cells enter stationary phase, but the protein still appears abundantly on the cell surface in stationary phase. Capsular polysaccharide is produced only in stationary phase and is more abundant in cells grown under sessile conditions, and is associated with *in vitro* biofilm formation. ClfA is expressed at high levels in stationary phase (see Chapter 6) although it is also present at lower levels on cells from exponential phase.

The relationship between *in vitro* growth conditions and *in vivo* growth in a variety of host niches has not been defined. *In vivo* growth conditions in the bloodstream or body tissues are likely to be anaerobic and iron-limited. Under these conditions the expression of antiphagocytic factors may differ from growth in broth. The Isd surface proteins, which are involved in iron acquisition (Skaar and Schneewind, 2004) are upregulated under iron-limiting conditions. At least one of these proteins is capable of binding multiple host ligands, including fibrinogen and fibronectin (Clarke *et al.*, 2004). Polysaccharide intercellular adhesin (PIA), which is associated with biofilm formation, is produced by *S. aureus* strains *in vivo* in late

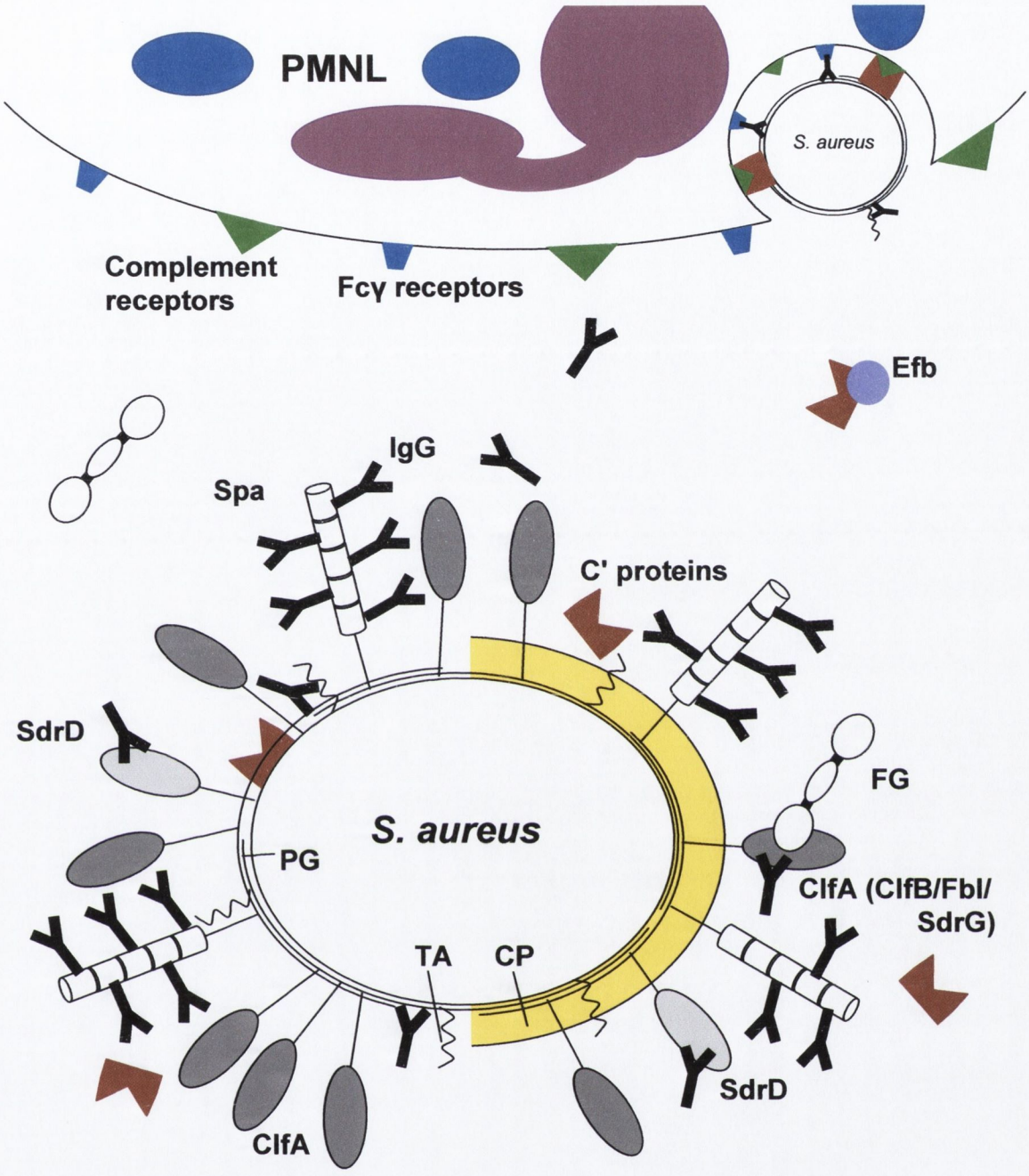


Fig 5.9 Schematic diagram of possible interactions between *S. aureus* and human polymorphonuclear leucocytes

Capsular polysaccharide (CP) inhibits complement (C') deposition on the cell surface. Secreted Efb binds complement protein C3. Protein A (Spa) inhibits phagocytosis by binding the Fc regions of IgG and preventing interactions between Fc and the Fcγ receptors on the PMNL surface. ClfA (and ClfB, Fbl and SdrG) inhibit phagocytosis, partially through fibrinogen (FG) binding that allows coating of the bacteria with host protein, shielding the surface from host antibodies. SdrD inhibits phagocytosis by an undefined mechanism. PG, peptidoglycan; TA, teichoic acid.

infection (Fluckiger *et al.*, 2005). In *S. epidermidis* PIA confers resistance to phagocytosis by human PMNL *in vitro* (Vuong *et al.*, 2004), although its role has not been tested in *S. aureus*, which expresses little or no PIA *in vitro*. These and other unknown factors may contribute to resistance to opsonophagocytosis in *S. aureus* and may be induced under *in vivo* conditions, allowing resistance in or escape from numerous situations that may be encountered by the bacterium. It would be important to evaluate of the roles of potentially antiphagocytic factors following growth in conditions more closely resembling those encountered *in vivo*.

Chapter 6

Regulation of Expression of Clumping Factor A

6.1 Introduction:

The generally accepted dogma of expression of *S. aureus* virulence factors is that surface proteins are expressed during the exponential phase of growth and that secreted proteins and toxins are expressed only when the cells reach stationary phase (Chan *et al.*, 1998; Novick and Jiang, 2003; Cheung *et al.*, 2004). This reflects the switch that occurs as cell density increases, mediated by the *agr* quorum sensing regulatory system (see Section 1.5; Novick 2003).

S. aureus expresses several surface-associated fibrinogen-binding proteins in addition to ClfA. ClfB is expressed exclusively during the early exponential phase of growth and is lost from stationary phase cells by proteolysis that removes domain N1 of the ligand binding region A, causing a loss of biological activity, and by dilution of the protein on the cell surface as the cells grow (McAleese *et al.*, 2003). The fibrinogen binding proteins FnBPA and FnBPB are also absent from or are greatly reduced in abundance on cells from stationary phase, as a result of proteolysis and a cessation of transcription before cells stop growing (Proctor *et al.*, 1982; McGavin *et al.*, 1997).

In contrast, expression of ClfA by strain Newman occurs in cells from stationary phase, which can form clumps in a solution of soluble fibrinogen and adhere avidly to fibrinogen-coated surfaces (McDevitt *et al.*, 1994; Wolz *et al.*, 1996). Results described in Chapter 3 show that ClfA is subject to proteolytic cleavage that removes domain N1 from the ligand-binding region A. However, in contrast to ClfB, this does not cause loss of fibrinogen-binding activity. A question that needs to be addressed is whether the *clfA* gene continues to be transcribed and translated as cells enter stationary phase or if there is such an abundance of the protein on the cell surface in exponential phase that sufficient molecules remain to support fibrinogen binding in stationary phase.

ClfA was first characterised due to its ability to promote clumping of stationary phase *S. aureus* Newman cells in soluble fibrinogen and their adherence to immobilised fibrinogen (McDevitt *et al.*, 1994). In contrast to strain Newman, the laboratory strain 8325-4 had very low clumping factor activity and adhered less avidly to immobilised fibrinogen (McDevitt *et al.*, 1994).

Nicholas *et al.* (1999) reported that a *sigB::*Tet^R mutant was deficient in clumping in soluble fibrinogen, and noted a consensus *Bacillus subtilis* sigma factor B (SigB) binding site upstream of the *clfA* gene. SigB is one of 16

alternative sigma (σ) factors of RNA polymerase in *B. subtilis*, and is involved in the stress response (Hecker *et al.*, 1996). *S. aureus* has three known σ factors, SigA (σ^A), SigB (σ^B) and SigH (σ^H) (Morikawa *et al.*, 2003; see Section 1.5.3). SigA can replace the functions of σ^{70} in *E. coli* and is the primary σ factor in *S. aureus*. SigB is an accessory σ-factor involved in the *S. aureus* stress response, allowing transcription of a specific subset of genes (Kullik *et al.*, 1998; Gertz *et al.*, 2000; Bischoff *et al.*, 2004). SigH is an accessory σ-factor orthologous to a *B. subtilis* sporulation-associated σ-factor and in *S. aureus* appears likely to be involved in natural DNA competence (Morikawa *et al.*, 2003).

The *sigB* locus in *S. aureus* encodes post-translational regulators of SigB (Figure 1.10). RsbW (for regulator of SigB) is an anti-σ-factor that binds and sequesters SigB. Induction of SigB activity in stationary phase involves the dephosphorylation of RsbV by RsbU, allowing RsbV to bind to RsbW, releasing SigB (Voelker *et al.*, 1995; Giachino *et al.*, 2001; Palma and Cheung, 2001).

An 11-base-pair (bp) deletion in *rsbU* that causes a frameshift mutation and premature termination of the RsbU polypeptide was discovered in 8325 (Kullik *et al.*, 1998), the strain from which 8325-4 is derived. This mutation means that SigB activity is not induced in stationary phase. A mutant of 8325-4 was constructed in which the defective *rsbU* was replaced with that of wild type strain COL (SH1000, Horsburgh *et al.*, 2002). Differences were observed between SH1000 and the parental 8325-4 in the expression of some exoproteins and the global regulator Agr, but not SarA. SH1000 expresses lower levels than 8325-4 of the proteases aureolysin, SspA and staphopain A (ScpA) (Shaw *et al.*, 2004). This appears to be SarA-independent and may be due to the severely decreased expression of *agr* in SH1000 compared to 8325-4 (Horsburgh *et al.*, 2002; Shaw *et al.*, 2004). In 8325-4 SigB is present but sequestered by RsbW due to the absence of RsbU, and this must be taken into account in the interpretation of genetic analyses in this strain.

Despite its importance as a virulence factor in animal models of septic arthritis and endocarditis (Josefsson *et al.*, 2001; Moreillon *et al.*, 1995), the expression and regulation of *clfA* has not been systematically investigated. Microarray analyses of the *agr*, *sarA* and *sigB* regulons has concluded that *clfA* is not regulated either by *agr* or *sarA* (Dunman *et al.*, 2001), but that it is regulated by *sigB* (Bischoff *et al.*, 2004). It has also been shown by Northern hybridisation

analysis and adherence to immobilised fibrinogen that ClfA is produced in stationary phase but is not regulated by *agr* in strain Newman (Wolz *et al.*, 1996).

The main objective of this study was to determine whether *clfA* is expressed constitutively, building up on the cell surface through the growth cycle, or is growth-phase dependent, and if such an abundance of the protein is present on the cell surface in exponential phase that sufficient molecules remain to support fibrinogen binding in stationary phase. It was also important to determine whether the differences observed between strains 8325-4 and Newman were due to SigB or some other regulator. In order to examine the observed differences, protein expression on the *S. aureus* cell surface was analysed, followed by studies on transcription of *clfA* using a *clfA::lacZ* fusion and by Northern hybridisation. Primer extension analysis was used to determine transcriptional start sites upstream of *clfA* and rifampicin treatment was used to determine the mRNA half-life in stationary phase.

6.2 Results:

6.2.1 Expression of clumping factor A on *S. aureus* cell surface

S. aureus Newman expresses large amounts of ClfA in stationary phase (Duthie, 1954; McDevitt *et al.*, 1994). Preliminary observations using the laboratory strain 8325-4 indicated that the level of expression in this strain might be lower, though sufficient to promote some limited binding to immobilised fibrinogen (see Figure 4.13, panel D). To investigate this further, cell wall-associated proteins were solublised by lysostaphin digestion and analysed by Western immunoblotting for the presence of ClfA, as described in Sections 2.7 and 2.8.

Following the observation that 8325-4 was an RsbU-defective mutant, protein expression was analysed in 8325-4 and the *rsbU*$^+$ derivative SH1000 (Horsburgh *et al.*, 2002). In addition, protein expression in strain Newman and a Newman *sigB rsbU* mutant, constructed by transduction of a *sigB::*Tet^R insertion mutation first isolated in 8325-4, were studied (see Table 2.1 for strains). The co-transduction of the 11-bp *rsbU* defect from 8325-4 was confirmed by PCR using the method of Kullik and Giachino (1997). Strains Newman and SH1000 each have a fully functional SigB/RsbU system. 8325-4 is RsbU-defective, while Newman *sigB rsbU* is defective in both SigB and RsbU.

In order to study expression of proteins on the surface of cells from the early exponential phase of growth it was necessary to prevent 'carry-over' of protein from stationary phase cells. Cultures were diluted 1/100 into fresh broth, grown for 2.5 hours (3-4 generations) and then diluted again into fresh broth. Samples were taken at intervals throughout the growth cycle, up to 24 hours after the second dilution.

ClfA was detected in the solublised cell wall protein fraction from the RsbU$^+$ strains Newman and SH1000 (Figure 6.1). In both strains the amount of ClfA present increased as the cells reached late stationary phase (14-24 hours). In early to mid-exponential phase (1-2.5 hours) the protein appeared to be produced mainly in the full-length form, with apparent molecular weight of 185 kDa (see Chapter 3). As the cells reached late exponential (SH1000) or late stationary phase (Newman) the 150-kDa form of ClfA became more abundant in cell wall extracts (Figure 6.1). This is likely to be the result of proteolytic removal of domain

N1 of region A (see Chapter 3). The 150-kDa form was particularly abundant in mid to late-exponential phase SH1000 cells. In Newman the 150-kDa form appeared later in growth (predominantly in late stationary phase) than in SH1000. This difference is likely to reflect a difference in protease expression between these strains. Considerable amounts of the full-length 185-kDa form of ClfA were observed in both strains in late stationary phase (Figure 6.1).

Significant degradation of ClfA was observed in late stationary phase Newman cell wall extracts. In Western immunoblotting proteins reacting with anti-ClfA region A antibodies must contain at least part of region A. Since recombinant region A alone migrates with an apparent molecular weight of 80 kDa (see Chapter 3) and full-length ClfA has an apparent molecular weight, by SDS-PAGE, of 185 kDa, the remainder of cell-wall associated ClfA (region R plus the wall spanning region) should migrate with an apparent molecular weight of ca. 105 kDa. ClfA-reactive products in these extracts migrating at less than 105 kDa are unlikely to exist on intact cells and remain attached to the surface of the cell. Proteolytic cleavage towards the C-terminus during growth would cause them to be released into the medium or washes. This suggests that some or all of the C-terminal proteolysis occurred during the lysostaphin digestion process. Proteases trapped in the cell wall peptidoglycan or capsular polysaccharide layer might be solublised and begin to degrade surface proteins, despite the presence of protease inhibitors. An alternative hypothesis is that the proteolysis has occurred during growth and the cleaved proteins were held in the cell wall or capsular polysaccharide, by some mechanism other than covalent anchoring at the LPXTG motif by sortase, and were released upon lysostaphin digestion without further proteolytic breakdown. These shorter products may be a product of the extraction process and might not be found in intact, growing cells. They are observed in smaller amounts in SH1000 than in Newman (Figure 6.1).

No immunoreactive ClfA was detected in solublised cell wall protein preparations from 8325-4 and very little was detected in Newman *sigB rsbU* (Figure 6.1). In Newman *sigB rsbU* only full-length ClfA (185 kDa) was observed in early exponential phase and only the N1-truncated 150-kDa form was observed in stationary phase. All samples in Figure 6.1 were prepared from the same number of cells, and samples from all strains were prepared simultaneously. Following SDS-PAGE all samples were transferred to a single membrane. The exposure time in each panel of Figure 6.1 is the same, so that comparisons of

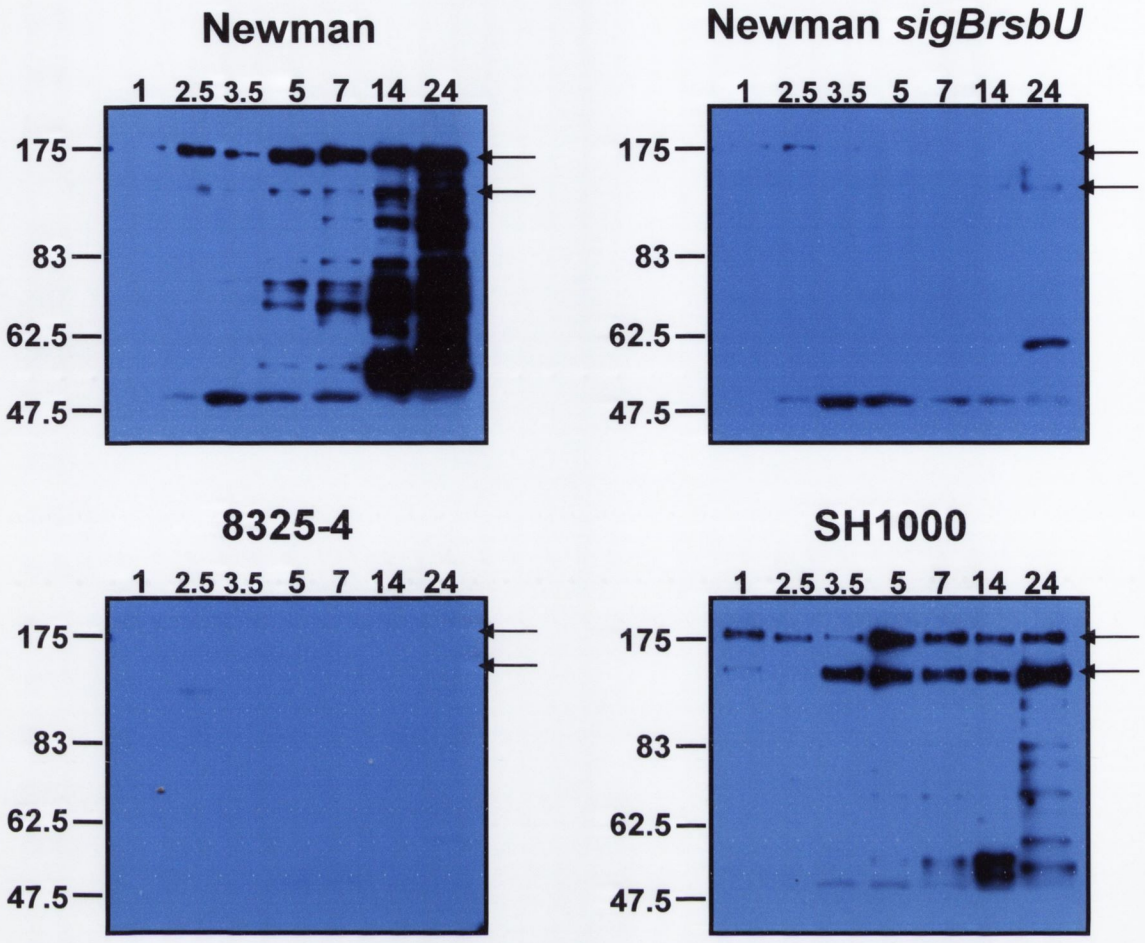


Fig 6.1 Western immunoblotting of solublised cell wall-associated proteins of *S. aureus* strains

Cells were grown in BHI broth for 2.5 hours, subcultured to fresh medium and samples taken at intervals. Equal numbers of cells from each timepoint were digested for 10 min with lysostaphin in osmotically supportive medium, pelleted and supernatants containing solublised cell wall proteins were subjected to SDS-PAGE on 7.5% (w/v) acrylamide gels, transferred to PVDF membrane and probed with anti-ClfA region A primary antibody, followed by peroxidase-conjugated protein A. Time of growth, in hours, is marked above each lane. Sizes are marked in kDa. Arrows indicate full-length and N1-truncated forms of ClfA. To standardise the experiment, samples for each time-point were prepared together and all samples were transferred to a single membrane, which was probed with antibodies in solution. As a result, signal intensities reflected strain-dependent differences.

band intensities represent differences in the amount of protein present in the solublised cell wall extracts. The amount of ClfA isolated from the SigB/RsbU-defective strains was almost undetectable compared to their isogenic *sigB/rsbU*$^+$ counterparts.

In order to quantify the differences in expression between SigB/RsbU$^+$ and SigB/RsbU$^-$ strains without subjecting the cells to lysostaphin treatment, whole cell dot immunoblotting was used. This is a less disruptive method of visualising proteins on the bacterial cell surface, although it does not distinguish between full-length and truncated forms of ClfA. Again, cells were subcultured in mid-exponential phase to prevent protein carry-over from stationary phase. After washing, samples were diluted to a standard optical density, and a series of doubling dilutions prepared from this were analysed. In order to minimise cross-reaction due to IgG binding by protein A on the cell surface, bound anti-ClfA antibody was detected with peroxidase-conjugated protein A. This binds the exposed Fc regions of IgG bound to ClfA preferentially over antibodies bound to protein A on the cell surface. In addition, a *clfA* deletion mutant was included as a control for the contribution of protein A.

In wild-type Newman cells ClfA was detected in early to mid-exponential phase (1-2.5 hours), in smaller amounts in late-exponential phase (3.5 hours) and in steadily increasing amounts in post-exponential phase (5 hours) and stationary phase (7-24 hours) (Figure 6.2). In Newman *sigB rsbU* ClfA was detected only in early to mid-exponential phase (1-2.5 hours). No expression was detected in stationary phase in this strain. Approximately 8-fold more ClfA was detected on stationary phase Newman cells than exponential phase (Figure 6.2). The amounts of ClfA detected in Newman and Newman *sigB rsbU* in early to mid-exponential phase were approximately equal (Figure 6.2).

By comparison with isogenic SigB/RsbU$^+$ strains, it appeared that high-level expression of ClfA in stationary phase on the *S. aureus* cell surface is dependent on SigB and/or RsbU.

6.2.2 Measurement of *clfA* promoter activity using a β-galactosidase reporter

In order to determine whether the presence of ClfA on stationary phase SigB/RsbU$^+$ cells was due to constitutive or growth-phase-dependent expression,

clfA transcription was measured using a *clfA::lacZ* fusion that expressed a β-galactosidase enzyme of Gram-positive origin in the place of ClfA (*clfA::lacZ* derived from pAZ106F13R12; see Section 4.2.5 for construction). β-galactosidase activity in cell samples was measured quantitatively by its conversion of the fluorogenic substrate 4-methylumbelliferyl-β-D-galactoside (4-MUG). This allowed comparison of the level of *clfA* transcription that occurred in different strains and under a variety of growth conditions. Cultures were grown under the same conditions as were used for measurement of protein levels in Section 6.2.1, above.

In strains Newman *clfA::lacZ* and SH1000 *clfA::lacZ* a short burst of β-galactosidase activity was detected in early to mid-exponential phase, followed by a decline in late exponential phase and a large increase in activity as cells entered stationary phase (Figure 6.3). In Newman *sigB rsbU clfA::lacZ* and 8325-4 *clfA::lacZ*, which is $SigB^+$ but $RsbU^-$, a peak of activity occurred in early to mid-exponential phase, followed by a decline in late exponential phase. Little activity was detected in stationary phase (Figure 6.3).

SigB is a regulator of gene expression that interacts directly with DNA to promote transcription at specific promoters under certain growth conditions, particularly under cellular stress. The activity of the alternative sigma factor SigB is regulated post-translationally by RsbW and RsbU. The anti-sigma factor, RsbW, binds and sequesters SigB, preventing its interaction with promoters. RsbU is a phosphatase that causes the release of SigB from RsbW (via RsbV) when the cell is under certain types of stress. The effect of the *rsbU* defect is mediated through SigB by a lack of derepression by RsbW. Promoters that depend upon RsbU are, in fact, dependent on SigB. The requirement for RsbU is to allow the release of SigB from RsbW by RsbV, which is activated by RsbU (see Section 1.5.3.2 and Figure 1.10).

These data showed that the stationary-phase expression of β-galactosidase as a reporter of *clfA* transcription was dependent on SigB (or derepression of sequestered SigB by RsbU, as indicated by 8325-4 *clfA::lacZ*). It also showed that in the absence of SigB, *clfA* transcription occurred in early to mid-exponential phase, but not in stationary phase. These results correlated with observations of protein expression on the *S. aureus* cell surface (Section 6.2.1), and with the low-level binding of 8325-4 to immobilised fibrinogen (Figure 4.13, panel D; McDevitt *et al.*, 1994). Expression of *lacZ,* as a reporter of *clfA* promoter activity, appeared to be SigB-dependent in stationary phase and SigB-independent in exponential

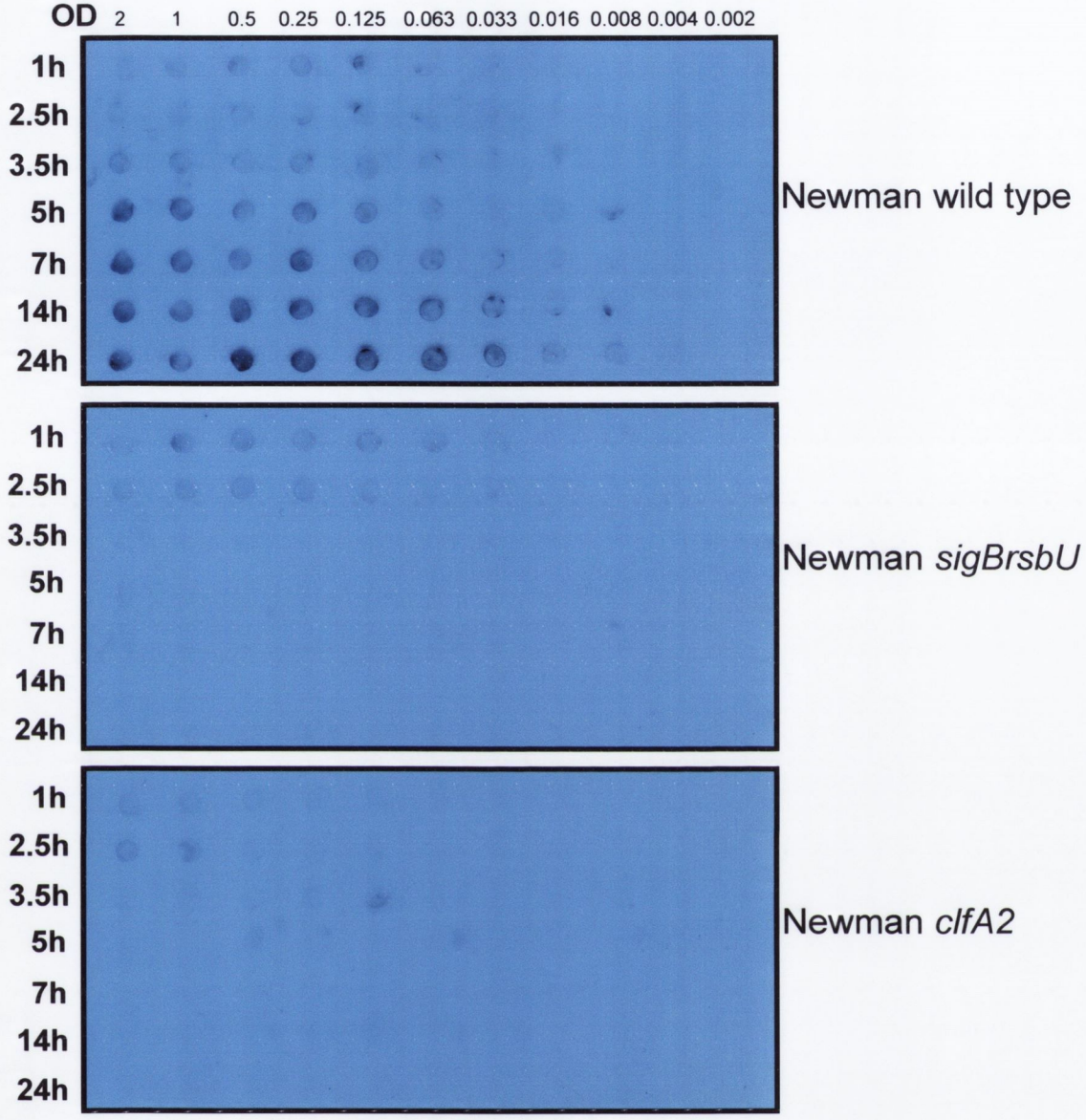


Fig 6.2 Whole cell dot immunoblotting of *S. aureus* strains

Cells were grown for 2.5 hours, subcultured to fresh medium and samples taken, pelleted and snap frozen at intervals. Thawed cells were washed in PBS before dilution to OD_{600} 2.0 per ml. Doubling dilutions were made for each sample and 5 µl of each dotted on nitrocellulose, which was probed with anti-ClfA region A primary antibody, followed by peroxidase-conjugated protein A. Newman *clfA2* was included as a control for protein A expression.

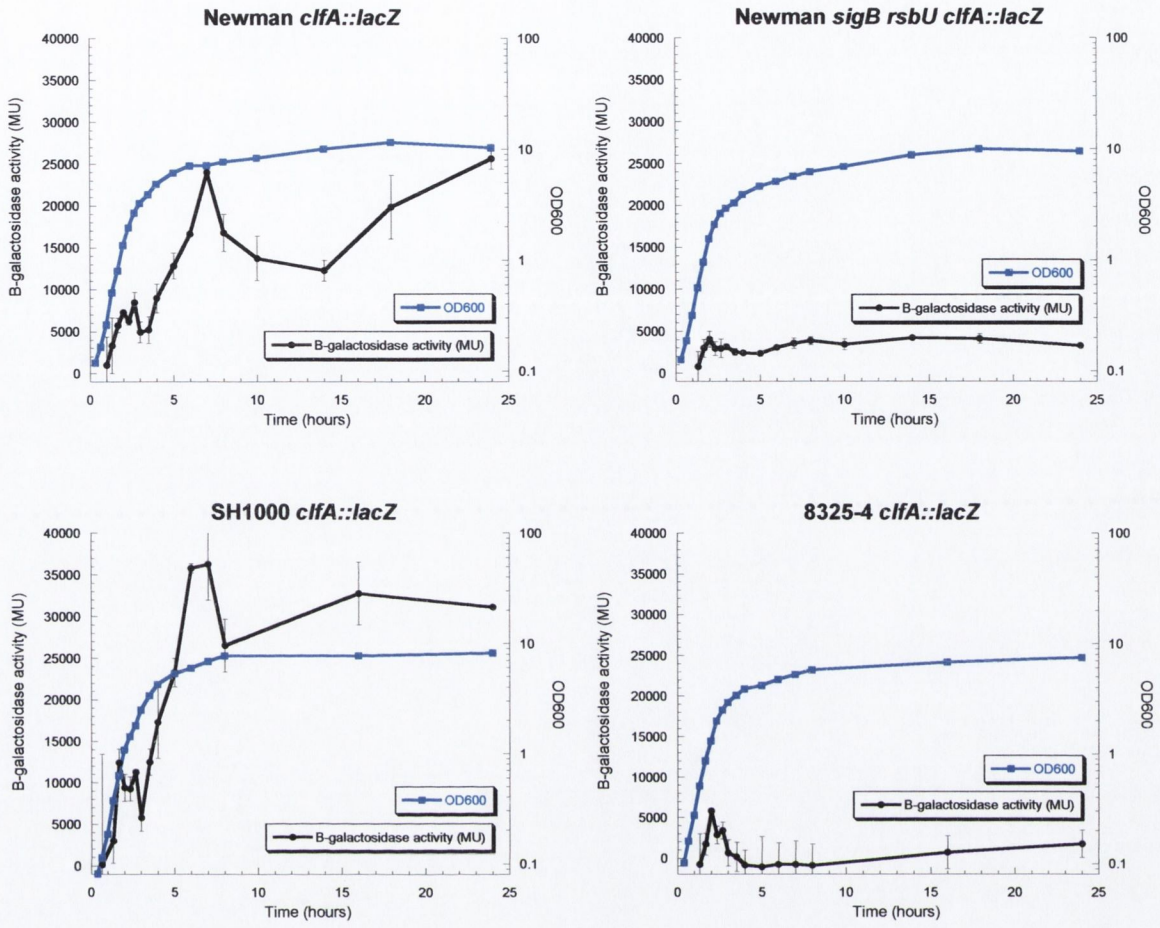


Fig 6.3 β-galactosidase activity of *S. aureus* strains bearing *clfA::lacZ*

Cells were grown for 2.5 hours in BHI broth, subcultured in fresh BHI broth, samples taken and snap frozen at intervals. Thawed samples were resuspended in assay buffer, the fluorogenic substrate 4-MUG was added and incubated for 60 minutes. Reactions were stopped by addition of Na_2CO_3 and fluorescence was measured. Strains are identified above each individual panel. β-galactosidase activity as Miller Units (MU) was calculated according to the formula: Miller Units = pmol 4-MU produced/ (1/dilution factor) x Time x OD_{600}. All panels are plotted on the same axes. β-galactosidase activity values below zero were omitted due to intrinsic inaccuracy. Results are representative of at least three independent experiments.

phase, allowing low-level expression in 8325-4 and high-level expression in Newman.

6.2.3 Effect of SarA and Agr on *clfA* promoter activity

Two major regulators of virulence gene expression in *S. aureus* are *agr*, the accessory gene regulator, and *sarA*, the staphylococcal accessory regulator (see Sections 1.5.1 and 1.5.2). In order to establish if either of these regulators influenced the expression of *clfA* during exponential or stationary phase growth, the *clfA::lacZ* fusion was transduced to Newman *sarA*, Newman *agr*, Newman *sigB rsbU sarA* and Newman *sigB rsbU agr* (see Table 2.1 for strains). Newman *sigB rsbU sarA* was constructed by transduction from 8325-4 *sarA* (Chan and Foster, 1998) and Newman *sigB rsbU agr* was constructed by transduction from Newman *agr* (McAleese *et al.*, 2001).

Measurement of β-galactosidase activity showed that levels of expression displayed less than 2-fold differences between parental strains and their isogenic *sarA* and *agr* mutants (Figures 6.3 and 6.4). These results were not perfectly reproducible, possibly due to differences in batches of BHI broth, but reproducibly showed the same trends, and less that 2-fold differences compared to the parental strains. In this experiment the early peak activities in Newman, Newman *sarA* and Newman *agr* (at 7 hours) were 24017 ± 122 Miller Units (MU), 18119 ± 3377 MU and 35803 ± 4821 MU, respectively. The peak activities of Newman *sigB rsbU*, Newman *sigB rsbU sarA* and Newman *sigB rsbU agr* (at 2 hours) were 4030 ± 958 MU, 5998 ± 1405 MU and 5670 ± 1059 MU, respectively.

In particular, the differences in *clfA* transcription between Newman wild type and the isogenic *sarA* and *agr* mutants in stationary phase were not significant ($P = 0.4709$ and $P = 0.2319$, respectively). This correlated with previous reports that *clfA* was not regulated by either *agr* or *sarA* (Dunman *et al.*, 2001). In Newman *sigB rsbU* a similar burst of transcription was seen in early exponential phase in *agr* or *sarA* mutants, relative to the parental strain (Figures 6.3 and 6.4). These data show that both SigB-independent and SigB-dependent transcription of *clfA* is independent of SarA and Agr.

6.2.4 Effect of salicylic acid on *clfA* promoter activity

It was recently reported that binding of *S. aureus* laboratory and clinical isolates to immobilised fibrinogen was reduced by exposure to salicylic acid (Kupferwasser *et al.*, 2003). Despite demonstrating that *sigB* was induced by growth in salicylic acid, the authors speculated that the decrease in fibrinogen binding was due to downregulation of *clfA*. To investigate this Newman *clfA::lacZ* was grown in the absence or presence of 50 µg/ml salicylic acid (Figure 6.5).

A salicylic acid-dependent increase in β-galactosidase activity of less than 50% was observed in early stationary phase, though no increase was observed in late stationary phase. The slight increase in *clfA::lacZ* transcription in early stationary phase is likely to have been mediated by induction of *sigB* by salicylic acid. These data show that *clfA* transcription was not downregulated either directly or indirectly by salicylic acid. The effect observed by Kupferwasser and co-workers may have been due to downregulation of other fibrinogen-binding proteins on exposure of the bacteria to salicylic acid.

6.2.5 Measurement of *clfA* transcription by Northern hybridisation

In order to identify and characterise *clfA* transcripts, to confirm that *clfA* expression is regulated by SigB at the transcriptional level and that the observations made in the β-galactosidase assays were not due to differences in protein stability, Northern hybridisation studies were carried out. RNA was isolated from strains Newman, Newman *sigB rsbU* and Newman *clfA2*, separated by electrophoresis and hybridised with DNA probes for region A of *clfA* and the 16S rRNA gene. The 16S rRNA gene was used as an internal control rather than *gyrA* because the latter is upregulated by *agr* in stationary phase in *S. aureus* (Dunman *et al.*, 2001; Vaudaux *et al.*, 2002). The same cell samples were used for RNA extraction as were used for dot immunoblotting in Figure 6.2.

Two *clfA*-specific transcripts of different sizes were observed in Newman, one of ca. 2900 nucleotides and the other of ca. 4000 nucleotides (Figure 6.6). The longer transcript was observed only in samples from early to mid-exponential phase. The shorter transcript was observed in late exponential phase and stationary phase samples. High levels were present in samples from late stationary phase (24 hours). In Newman *sigB rsbU* only the longer transcript was

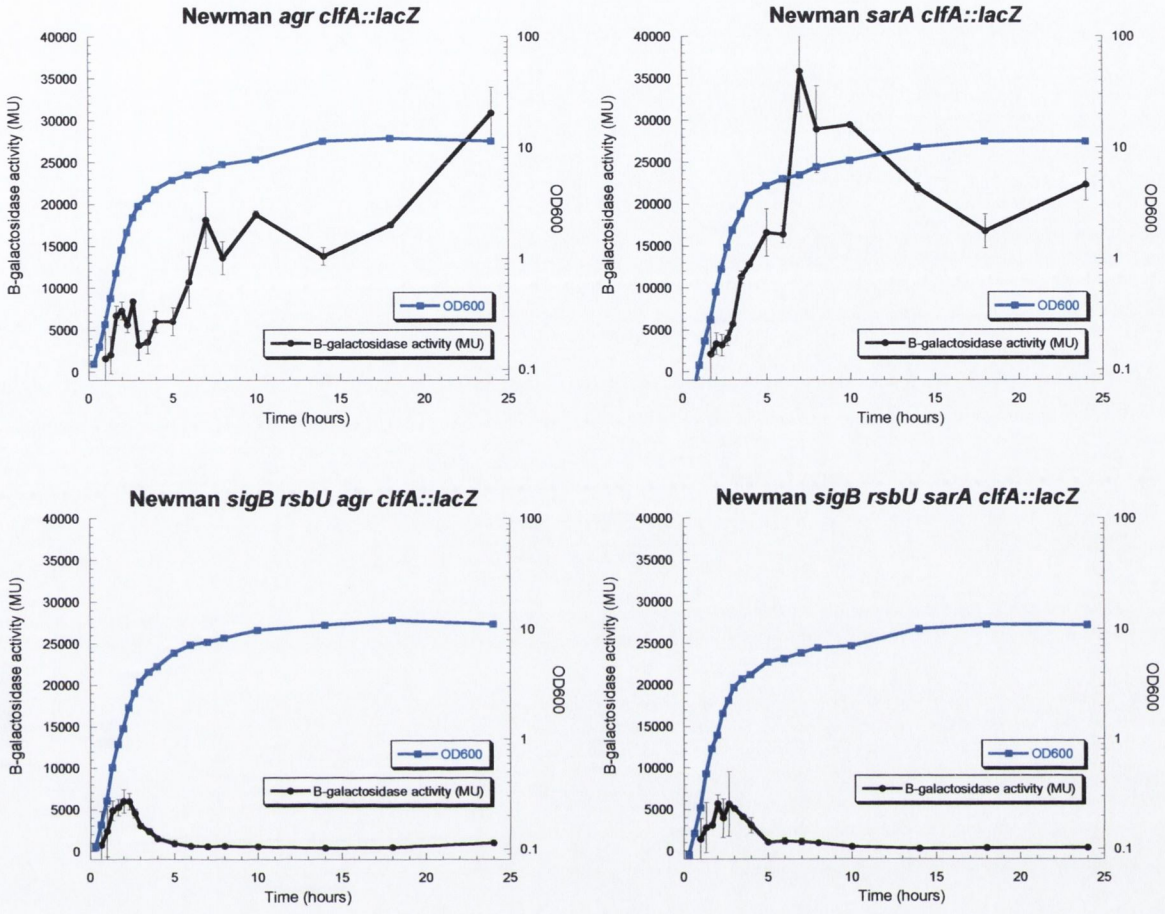


Fig 6.4 β-galactosidase activity of *agr* and *sarA* strains bearing *clfA::lacZ*

Cells were grown for 2.5 hours in BHI broth, subcultured to fresh BHI broth, samples taken and snap frozen at intervals. Thawed samples were resuspended in assay buffer, the fluorogenic substrate 4-MUG was added and incubated for 60 minutes. Reactions were stopped by addition of Na_2CO_3 and fluorescence was measured. Strains are identified above each individual panel. β-galactosidase activity as Miller Units (MU) was calculated according to the formula: Miller Units = pmol 4-MU produced/ (1/dilution factor) x Time x OD_{600}. All panels are plotted on the same axes. β-galactosidase activity values below zero were omitted due to intrinsic inaccuracy. Results are representative of at least three independent experiments.

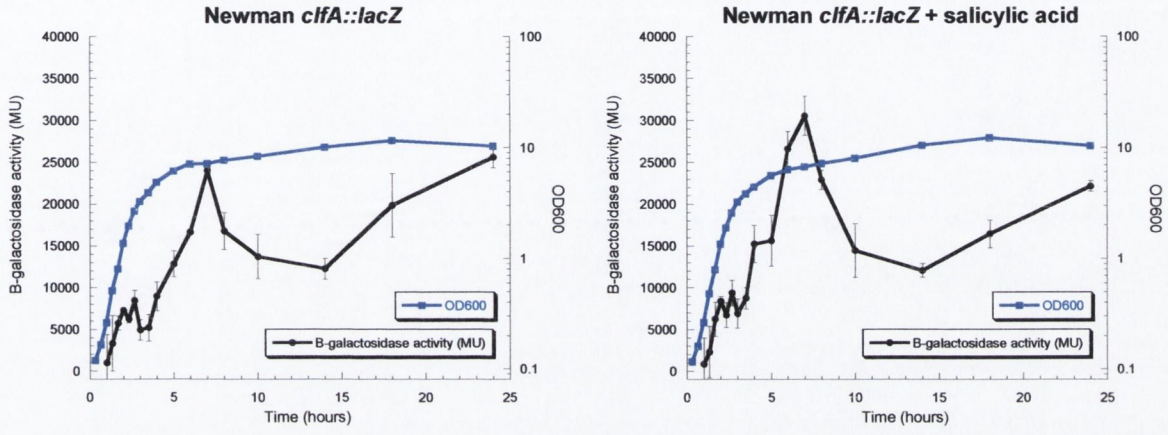


Fig 6.5 β-galactosidase activity of *S. aureus* Newman in salicylic acid

Cells were grown for 2.5 hours in BHI broth, subcultured to fresh BHI broth, with or without 50 μg/ml salicylic acid, samples were taken and snap frozen at intervals. Thawed samples were resuspended in assay buffer, the fluorogenic substrate 4-MUG was added and incubated for 60 minutes. Reactions were stopped by addition of Na_2CO_3 and fluorescence was measured. β-galactosidase activity as Miller Units (MU) was calculated according to the formula: Miller Units = pmol 4-MU produced/ (1/dilution factor) x Time x OD_{600}. Panels are plotted on the same axes. β-galactosidase activity values below zero were omitted due to intrinsic inaccuracy. Results are representative of three independent experiments.

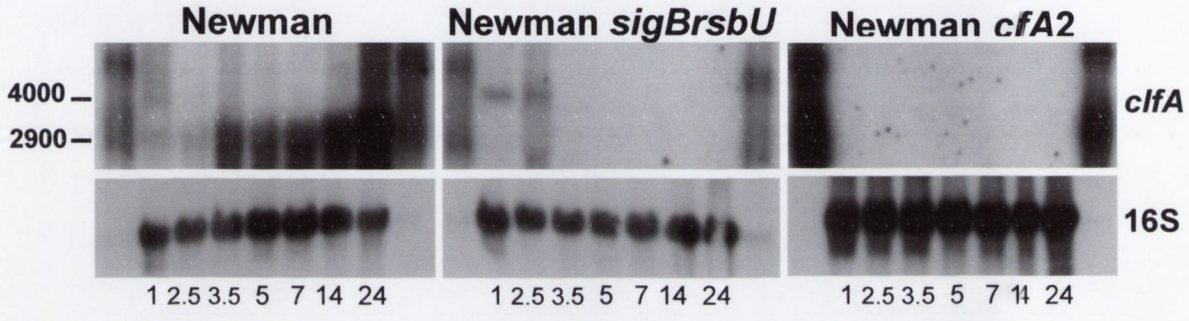


Fig 6.6 Northern blotting of *S. aureus* strains

Cells were grown for 2.5 hours, subcultured to fresh BHI broth, samples taken and snap-frozen at intervals. Total cellular RNA was extracted and electophoresed through 1% (w/v) agarose, 2% (v/v) formaldehyde gels in MOPS buffer before transfer to positively charged membranes. Membranes were probed with PCR-generated DIG-labelled DNA probes encompassing the entire length of region A of *clfA* (upper panel) or 16S rRNA gene (lower panel). Newman *clfA*2 is a *clfA*-null strain containing a transposon insertion and deletion of *clfA*, and is included as a control. 16S rRNA is included as a control. Lanes at either side of each panel contain RNA size standards. Time of growth (in hours) is indicated below lanes. Sizes of *clfA*-reactive transcripts are indicated in nucleotides.

observed, also in early to mid-exponential phase. This transcript must be produced independently of SigB. The shorter transcript is SigB-dependent as it was absent in the *sigB rsbU* mutant. No *clfA*-specific transcripts were detected in Newman *clfA2* samples. These data show that two different transcripts are responsible for SigB-dependent and SigB-independent expression of *clfA* in *S. aureus*. The presence of the SigB-independent (ca. 4000 nt) transcript correlated with the appearance of ClfA protein on the cell surface in Newman *sigB rsbU* (Figure 6.2) and the peak of β-galactosidase activity in exponential phase (Figure 6.3), whilst the appearance of the SigB-dependent transcript (ca. 2900 nt) correlated with the appearance of ClfA protein on the surface of stationary phase Newman cells (Figure 6.2) and the high levels of β-galactosidase activity in stationary phase in that strain (Figure 6.3).

6.2.6 Mapping of *clfA* transcripts by Northern hybridisation

In order to determine whether *clfA* is transcribed from two separate promoters to generate the 4000 nt and 2900 nt transcripts, or whether both are derived from the same promoter and are the result of processing or termination, RNA samples were hybridised with labelled probes representing sequences 5' and 3' to *clfA*.

The region 5' to *clfA* contains two predicted open reading frames (ORFs) of unknown function, ORF1 and ORF2 (Figure 6.7, panel B). Directly 3' to *clfA* lies *vwb*, the gene encoding a secreted von Willebrand factor-binding protein (Bjerketorp *et al.*, 2002). When *clfA* was cloned in the shuttle vector pCU1 with 250 bp of 5' sequence, no expression was detected in 8325-4 (Hartford *et al.*, 1997). The addition of 1 kb of additional 5' sequence, to generate pCF77, allowed expression to be detected. The pCF77 plasmid contains 4150 bp of *clfA* sequence between the upstream *Eco*RI site and the downstream *Hind*III site (Figure 6.7, panel B; Hartford *et al.*, 1997). This region includes the 3' end of ORF1, the complete sequences of ORF2 and *clfA*, and the 5' end of *vwb*. The plasmid that expressed *clfA* poorly in 8325-4 contained only the sequences between the upstream *Kpn*I and downstream *Hind*III sites (Hartford *et al.*, 1997; see Figure 6.7, panel B).

RNA was isolated from cells expressing only the SigB-independent transcript or the SigB-dependent transcript. As 8325-4 is RsbU-deficient, transcription in that strain is SigB-independent. pCF77 (the multi-copy plasmid bearing *clfA*) was used

to increase the level of transcription of *clfA* from the SigB-independent promoter in 8325-4. In stationary phase Newman cells transcription of *clfA* is mainly SigB-dependent (Figure 6.6). Exponential phase 8325-4 *clfA2* (pCF77) and stationary phase Newman cells were used as sources of RNA for these experiments. Newman *clfA2,* a mutant containing a transposon insertion in *clfA* combined with a deletion of 5' sequences (McDevitt *et al.*, 1994), was included as a control. In previous experiments no transcripts hybridising with a *clfA* region A probe could be detected in this strain (Figure 6.6).

RNA samples were hybridised with a probe representing region A of *clfA* as a control that revealed all *clfA*-specific transcripts (Figure 6.7, panel A). In 8325-4 (pCF77) transcripts corresponding in size to both SigB-independent and SigB-dependent transcripts were present (Figure 6.7, panel A, lane 2). This is likely due to expression of the transcripts from the multi-copy plasmid in a background where SigB is present but sequestered. The presence of multiple copies of the promoter sequences may have been sufficient to allow some transcription from the SigB-dependent promoter. In stationary phase Newman cells very large amounts of the SigB-dependent transcript were observed, relative to 8325-4, resulting in the gel appearing overloaded (Figure 6.7, panel A, lane 3). In this overloaded lane, a third *clfA*-specific transcript of approximately 4500 nt was also observed. This transcript was not present in RNA from exponential-phase cells. In Newman *clfA2* no hybridisation was observed (Figure 6.7, panel A, lanes 4 and 5).

Probe 3, which hybridised with sequences towards the end of the *clfA* coding sequence (Figure 6.9), revealed transcripts of the same size as the region A probe (Figure 6.7, panel A), although the 4500 nt transcript was extremely faint. The lower intensity of the reaction reflected the difference in the length of the probes, although a 5-fold higher concentration of the shorter probe was used.

Probe 4 corresponded to DNA located 3' to the *clfA* stop codon but 5' to the *Hind*III site used in cloning *clfA* in pCF77 (Figure 6.9). This sequence encodes part of the secretory signal sequence of the von Willebrand-binding protein Vwb (Bjerketorp *et al.*, 2002). This probe hybridised with transcripts of different sizes to the *clfA* transcripts. The transcripts were present only in exponential phase cells and were present in Newman *clfA2*, indicating that they are *clfA*-independent (Figure 6.7, panel A). Again, the weak reaction reflected the short length of the probe.

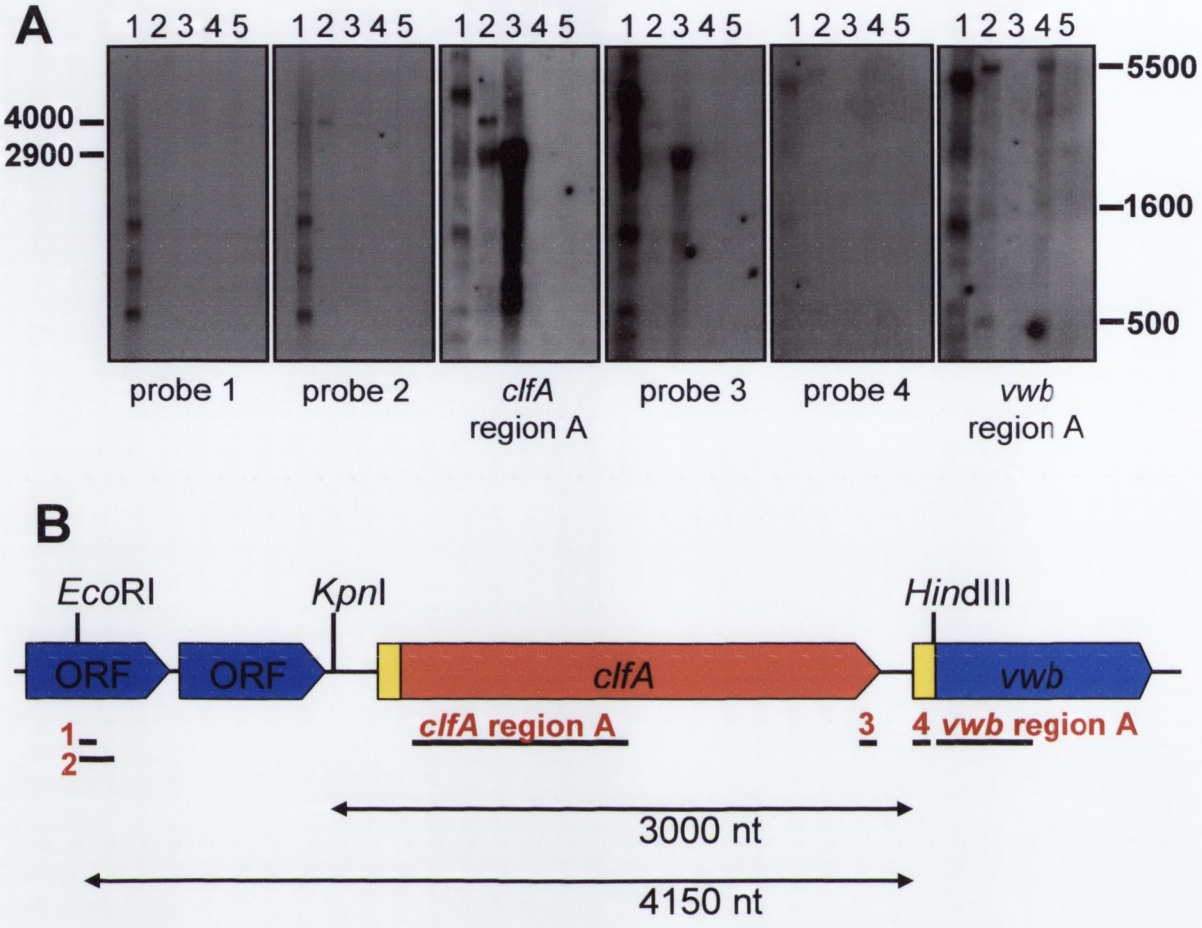


Fig 6.7 Localisation of 5' and 3' ends of *clfA* mRNAs by Northern blotting

A. Northern hybridisation. Probes are indicated below each panel and are depicted in panel B. In each panel: lane 1, RNA size standards; lane 2, RNA from early-exponential phase 8325-4 pCF77 (containing *clfA* on a multicopy plasmid); lane 3, RNA from stationary phase Newman; lane 4, RNA from early-exponential phase Newman *clfA2*; lane 5, RNA from stationary phase Newman *clfA2*. Sizes of transcripts are indicated in nucleotides.

B. DIG-labelled DNA probes generated by PCR. Total length of *Eco*RI-*Hin*dIII insert in pCF77 is 4150bp. ORF, putative gene of no known function; *vwb*, gene encoding von Willebrand-binding protein. Precise locations of probes are shown in Figures 6.8 and 6.9.

atttatttcg caaaaattta ttttaaaaac tctaaatgac ttatcatttt gagtaattaa 60
acaaagttga tattttgtga gattctaaga tgatattaaa taattcttgt aataatgatc 120
ctatgtattg ttgcaataaa ttaatgaaac tataattact aatattatat tacttttatt 180
gatagaaata tattactttt ttaaaaaaac ttgtaatata tcgaaagatt taaatgtaaa 240
attttgattt gttaagaaat tacgtttgta aaaataaaaa aatcaactta tttgtatgag 300
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tacttgatgg ttaaaaaatt acttaaggct ataaagcaaa acttttttata tgagcagtcg 480
aatataacgt ttaaaatgat tgtttttgga tataaacgat taagtaaaat gctttttcag 540
tttgaaatta atcatataaa tttcttatgg gagggttgat **atcttaatga ttaacattat** 600
ttcagctata ggatctattg gaacatttat tatggcttta ttttattttg tatcagtttc 660
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taaagattat attaaactat ataatttagg tggcggtgct gctaaaaaaa ttgcaataga 840
ggttttattg gggaaggata aagtcattca gaaaaaatac gtgcatattt tacctagtaa 900
agaagggtac atgttaccaa ttaataaaaa tgtgtacgaa gaattagaaa gaacgattga 960
gaacaatggt catgaagctg atttgaatgt acgtatgact tattatcata atgtaagtcg 1020
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acgagaagca atattaaaag gatataattt tatcagtgta ttaggatggc cgacgtatta 1620
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accagacgaa gcgtttttaa ttaaagaatt aaaagtgaac agtttagcgg gaaaaacagg 1740
taccataaat tacacatctg cttttgaaaa aatatgattt caagctagga ttacattagg 1800
tagagttcat attaataata aaaaatgttt gcaatcaaat cgtacgttgt cgtttgtaat 1860
tcttaaaata gcaataaata aaatgtttgt tagtaaagta ttattgtgga taataaaata 1920
tcgatacaaa ttaattgcta taatgcaatt ttagtgtata attccattga cagagattaa 1980
atatatcttt aaagggtata tagttaatat aaaatgactt tttaaaaaga gggaataaa**a** 2040
tgaatatgaa gaaaaaagaa aaacacgcaa ttcggaaaaa atcgattggc gtggcttcag 2100
tgcttgtagg tacgttaatc ggttttggac tactcagcag taaagaagca gatgcaagtg 2160
aaaatagtgt tacgcaatct gatagcgcaa gtaacgaaag caaaagtaat gattcaagta 2220
gcgttagtgc tgcacctaaa acagacgaca caaacgtgag tgatactaaa acatcgtcaa 2280
acactaataa tggcgaaacg agtgtggcgc aaaatccagc acaacaggaa acgacacaat 2340
catcatcaac aaatgcaact acggaagaaa cgccggtaac tggtgaagct actactacga 2400
caacgaatca agctaataca ccggcaacaa ctcaatcaag caatacaaat gcggaggaat 2460
tagtgaatca aacaagtaat gaaacgactt ctaatgatac taatacagta tcatctgtaa 2520
attcacctca aaattctaca aatgcggaaa atgtttcaac aacgcaagat acttcaactg 2580
aagcaacacc ttcaaacaat gaatcagctc cacagagtac agatgcaagt aataaagatg 2640
tagttaatca agcggttaat acaagtgcgc ctagaatgag agcatttagt ttagcggcag 2700

Fig 6.8 Nucleotide sequence 5' to *clfA*

Includes sequence of region A of *clfA* and upstream ORFs of unknown function. Numbering is arbitrary. Nucleotides in bold lie within the open reading frames ORF 1, ORF 2 and *clfA*. Probe 1 is depicted in red. Probe 2 encompasses all of probe 1 and the sequence depicted in green. *clfA* region A probe is blue-grey and continues in the 3' direction. *Eco*RI site is indicated in blue. Nucleotides upstream of this site are not present in pCF77.

tagctgcaga tgcaccggta gctggcacag atattacgaa tcagttgacg aatgtgacag 2760
ttggtattga ctctggtacg actgtgtatc cgcaccaagc aggttatgtc aaactgaatt 2820
atggtttttc agtgcctaat tctgctgtta aaggtgacac attcaaaata actgtaccta 2880
aagaattaaa cttaaatggt gtaacttcaa ctgctaaagt gccaccaatt atggctggag 2940
atcaagtatt ggcaaatggt gtaatcgata gtgatggtaa tgttatttat acatttacag 3000
actatgtaaa tactaaagat gatgtaaaag caactttgac catgcccgct tatattgacc 3060
ctgaaaatgt taaaaagaca ggtaatgtga cattggctac tggcataggt agtacaacag 3120
caaacaaaac agtattagta gattatgaaa aatatggtaa gttttataac ttatctatta 3180
aaggtacaat tgaccaaatc gataaaacaa ataatacgta tcgtcagaca atttatgtca 3240
atccaagtgg agataacgtt attgcgccgg ttttaacagg taatttaaaa ccaaatacgg 3300
atagtaatgc attaatagat cagcaaaata caagtattaa agtatataaa gtagataatg 3360
cagctgattt atctgaaagt tactttgtga atccagaaaa ctttgaggat gtcactaata 3420
gtgtgaatat tacattccca aatccaaatc aatataaagt agagtttaat acgcctgatg 3480
atcaaattac aacaccgtat atagtagttg ttaatggtca tattgatccg aatagcaaag 3540
gtgatttagc tttacgttca actttatatg ggtataactc gaatataatt tggcgctcta 3600
tgtcatggga caacgaagta gcatttaata acggatcagg ttctggtgac ggtatcgata 3660
aaccagttgt tcctgaacaa cctgatgagc ctggtgaaat tgaaccaatt ccagaggatt 3720
cagattctga cccaggttca gattctggca gcgattctaa ttcagatagc ggttcagatt 3780
cgggtagtga ttctacatca gatagtggtt cagattcagc gagtgattca gattcagcaa 3840
gtgattcaga ctcagcgagt gattcagatt cagcaagcga ttccgactca gcgagcgatt 3900
ccgactcaga caatgactcg gattcagata gcgattctga ctcagacagt gactcagatt 3960
ccgacagtga ctcagattca gatagcgatt ctgactcaga cagtgactcg gattcagata 4020
gcgattcaga ttcagatagc gattcagatt ccgacagtga ttccgactca gacagcgatt 4080
ctgactccga cagtgattcc gactcagaca gcgattcaga ttccgacagt gattccgact 4140
cagatagcga ttccgactca gatagcgact cagattcaga cagcgattca gattcagaca 4200
gcgattcaga ttcagatagc gattcagatt ccgacagtga ctcagattcc gacagtgact 4260
cggattcaga tagcgattca gattccgaca gtgactcaga ttccgacagt gactcagact 4320
cagacagtga ttcggattca gcgagtgatt cggattcaga tagtgattcc gactccgaca 4380
gtgactcgga ttcagatagc gactcagact cggatagcga ctcggattca gatagcgatt 4440
cggactcaga tagcgattca gaatcagaca gcgattcaga ttcagacagc gactcagaca 4500
gtgactcaga ttcagatagt gactcggatt cagcgagtga ttcagactca ggtagtgact 4560
ccgattcatc aagtgattcc gactcagaaa gtgattcaaa tagcgattcc gagtcagttt 4620
ctaacaataa tgtagttccg cctaattcac ctaaaaatgg tactaatgct tctaataaaa 4680
atgaggctaa agatagtaaa gaaccattac cagatacagg ttctgaagat gaagcaaata 4740
cgtcactaat ttggggatta ttagcatcaa **taggttcatt actacttttc agaagaaaaa** 4800
aagaaaataa agataagaaa taagtaataa tgatattaaa ttaatcatat gattcatgaa 4860
gaagccacct taaaaggtgg ctttttttact tggattttcc aaatatattg tttgaatata 4920
attaataatt aattcatcaa cagttaatta ttttaaaaag gtagatgtta tataatttgg 4980
cttggcgaaa aaatagggtg taaggtaggt tgttaattag ggaaaattaa ggagaaaata 5040
cag**ttgaaaa ataaattgct agttttatca** ttgggagcat tatgtgtatc acaaatttgg 5100
gaaagtaatc gtgcgagtgc agtggtttct ggggagaaga atccatatgt atctagtcgt 5160
tgaaactgac taataataaa aataaatcta gaacagtaga agagtataag aaaagcttgg 5220
atgatttaat atggtccttt ccaaacttag ataatgaaag atttgataat cctgaatata 5280
aagaagctat gaaaaaatat caacagagat ttatggctga agatgaggct ttgaagaaat 5340
tttttagtga agagaaaaaa ataaaaaatg gaaatactga taatttagat tatctaggat 5400
tatctcatga aagatatgaa agtgtattta atactttgaa aaaacaaagt gaggagttct 5460
taaaagaaat tgaagatata aaaaaagata accctgaatt gaaagacttt aatgaagagg 5520
agcaattaaa gtgcgactta gaattaaaca aattagaaaa tcagatatta atgttaggta 5580

Fig 6.9 Nucleotide sequence 3' to *clfA*

Including sequences within *clfA* and 5' sequences of the downstream gene *vwb*. Arbitrary numbering is continued from Figure 6.8. Nucleotides in bold lie within the open reading frames *clfA* and *vwb*. *clfA* region A probe is blue-grey and is continued from Figure 6.8. Probe 3 is depicted in red. Probe 4 is depicted in green. *vwb* region A probe overlaps part of probe 4 and is violet. *Hind*III site is indicated in blue. Nucleotides downstream of *Hind*III are not present in pCF77. Predicted rho-independent transcription terminator is underlined.

A probe for region A of *vwb* revealed the same sized transcripts as probe 4 (Figure 6.7, panel A). These transcripts, approximately 5500, 1600 and 500 nt in length, were present only in exponential phase cells, and were detected in Newman *clfA2* and 8325-4 *clfA2* (pCF77). These results showed that *clfA* and *vwb* are independently transcribed, and that the *clfA2* mutation, despite being a transposon insertion combined with a deletion of 5' sequences, has no polar effect on the downstream gene.

In order for *clfA* and *vwb* to be independently transcribed, a transcriptional terminator must be located between the 3' end of *clfA* and the promoter of *vwb*. A predicted stem-loop structure, followed by a run of 6 Ts, is present in the sequence downstream of the *clfA* stop codon and is underlined in Figure 6.9.

The length of the shorter SigB-dependent *clfA* transcript (2900 nt) is consistent with the location of a transcriptional start site close to the 5' end of *clfA* and a transcriptional terminator in the region between *clfA* and *vwb*. The presence of this putative transcriptional terminator indicates that the longer transcripts must be accounted for by additional upstream sequences. As ClfA was not expressed from a plasmid containing only 250 bp of upstream sequence in the SigB-defective 8325-4, whereas it is expressed from pCF77, it appeared that the transcriptional start site of the SigB-independent transcript was between 250 bp and 1280 bp upstream of the *clfA* coding sequence, between the *Kpn*I and *Eco*RI sites (see Figure 6.7, panel B). The promoter driving transcription of the 4500 nt transcript, observed only in overloaded samples containing RNA from stationary phase cells, may be a second SigB-dependent promoter located upstream of ORF1. Transcription from this promoter appeared to be relatively weak, in comparison to the more proximal promoters, and its location was not further investigated.

In order to investigate the location of the SigB-independent promoter, probes to sequences close to the *Eco*RI site 1280 bp upstream of *clfA* were designed (Figures 6.7, panel B and 6.8). Probes 1 and 2 were overlapping, using the same forward primer, F33, and two reverse primers, F33 and F34 respectively (see Table 2.3 for primers). The sequences to which these probes hybridise are within the coding sequence of the hypothetical ORF1. Probe 2 hybridised with the SigB-independent *clfA* transcript, but not the SigB-dependent transcript (Figure 6.7, panel A). Probe 1 failed to hybridise to any fragment, despite the use of 500 ng/ml of probe (10 times the recommended maximum amount). The failure of probe 1 to

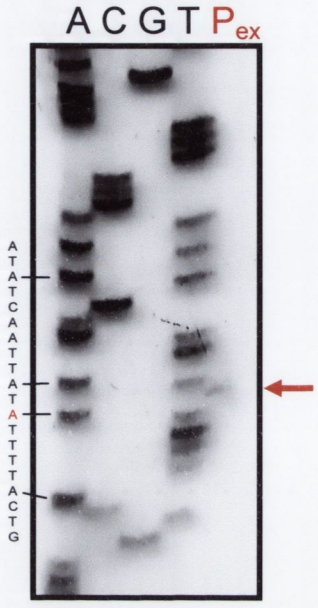
hybridise indicates that the sequence is not transcribed under any of the growth conditions used.

These data indicated that the SigB-dependent transcript began just upstream of the *clfA* coding sequence, that the SigB-independent transcript began 1200-1260 bp upstream of *clfA* and that both transcripts most likely ended at a transcriptional terminator located between 42 and 68 bp downstream of the *clfA* stop codon. An additional SigB-dependent promoter may be present upstream of ORF1 that is involved in the transcription of *clfA* in stationary phase.

6.2.7 Identification of transcriptional start sites by primer extension

In order to identify the transcriptional start sites upstream of *clfA* that yielded the major SigB-dependent (2900 nt) and SigB-independent (4000 nt) transcripts, primer extension analysis was carried out. RNA was isolated from exponential-phase 8325-4 (pCF77) and stationary-phase Newman cells and was used for cDNA synthesis incorporating specific ^{32}P radiolabelled primers. The same primers were used to incorporate ^{35}S into sequencing ladders that were run alongside the primer extension reactions. The lengths of the primer extension products were then correlated with the DNA sequence data, identifying the transcriptional start sites (Figures 6.10 and 6.12, upper panels).

The proximal SigB-dependent transcriptional start site was identified 31 bp upstream of the *clfA* start codon. Putative *B. subtilis* SigB-dependent -10 and -35 consensus promoter sequences were identified upstream of this site (Figure 6.10). Shortly after this experiment was carried out, Homerova and co-workers published a study of SigB-dependent promoters identified using a two-plasmid system in *E. coli*. They established a consensus *S. aureus* SigB-dependent promoter sequence (GttTaa - N_{12-15} - gGGTAt), which is very similar to the *B. subtilis* consensus, and identified *clfA* as a SigB-dependent gene (Homerova *et al.*, 2004). The authors introduced *S. aureus sigB* into *E. coli* on a plasmid and used the gene product to produce *clfA* transcript in *E. coli*. Using S1 nuclease mapping with this RNA they identified a transcriptional start site three bases upstream from that identified here (Figure 6.10, lower panel). S1 nuclease mapping uses enzymatic digestion of DNA to reveal a length of sequence protected by hybridised mRNA. The authors allowed for this and deduced the transcriptional start site by subtracting 1.5 bases from the length of the protected fragment. Primer extension



SigB-dependent

```
tcttaaaata gcaataaata aaatgtttgt tagtaaagta ttattgtgga taataaaata    1920
tcgatacaaa ttaattgcta taatgcaatt ttagtgtata attccattga cagagattaa    1980
atatatcttt aaagggtata tagttaAtaT aaaatgactt tttaaaaaga gggaataaaa    2040
tgaatatgaa gaaaaaagaa aaacacgcaa ttcggaaaaa atcgattggc gtggcttcag    2100
tgcttgtagg tacgttaatc ggttttggac tactcagcag taaagaagca gatgcaagtg    2160
```

Fig 6.10 Primer extension analysis of SigB-dependent *clfA* transcriptional start site by autoradiography

Total RNA was isolated from Newman cells grown for 7 hours and used as template for cDNA synthesis using a *clfA*-specific ^{32}P end-labelled primer, Pex1, underlined in the sequence panel. pCF77 was used as template for sequencing using the same *clfA*-specific primer and incorporating ^{35}S. Reactions were electrophoresed through 6% (w/v) acrylamide, dried and exposed to X-ray film. A,C,G,T: DNA sequencing ladders. P_{ex}: primer extension reaction. Position of transcriptional start site is arrowed in the upper panel, and highlighted in red in the sequence. Nucleotides of ORFs are depicted in bold, as in Figure 6.8. Putative -10 and -35 boxes of the SigB-dependent promoter are depicted in red. Transcriptional start site (**A**) identified by Homerova *et al.* using S1 nuclease mapping is also indicated.

is more precise as it measures the length of the mRNA from the position of the primer used. Small (less than 1 bp) differences in the lengths of primer extension products and sequencing products are caused only by the difference in electrophoretic migration of the labels used (^{32}P versus ^{35}S). The small discrepancy in the reported transcriptional start sites may lie in the fact that in this study the RNA was prepared from *S. aureus*, whereas in the study of Homerova *et al.* (2004) the RNA was prepared from *E. coli*.

In order to test which of these transcriptional start sites is more likely to be accurate, primer extension was carried out independently, using a different primer, in the laboratory of our collaborators (Glenn Kaatz, personal communication). Both primer extension and sequencing reactions were prepared using ^{32}P end-labelled primers to avoid size discrepancies. The sequencing ladder was prepared from the *S. aureus norA* gene, using a *norA*-specific primer, to allow measurement of the length (in nt) of the primer extension product. As a result, the sequencing ladder did not correspond to *clfA* sequence. The image was captured digitally using a phosphorimaging system. These experiments determined that the main transcriptional start site for *clfA* in *S. aureus* Newman is 139 bp upstream of the 3' end of the primer (Figure 6.11). This position corresponds exactly to the T described above, rather than the A described by Homerova *et al.* (2004). No product corresponding to the A was detected. Putative -10 and -35 SigB-dependent promoter elements, corresponding to those described above that agree with the *S. aureus* concensus, were identified upstream of the transcriptional start site (Figure 6.11, lower panel). This indicates that the T 31 bp upstream of the *clfA* start codon is the main SigB-dependent transcriptional start site in Newman cells.

The SigB-independent transcriptional start site was identified 1222 bp upstream of the *clfA* start codon (Figure 6.12). No strict consensus SigA-dependent promoter sequence has been defined in *S. aureus* (Wang *et al.*, 2004), because of the AT-rich genome, although SigA (σ^{70}) sigma factors are highly conserved through all bacterial species (Morikawa *et al.*, 2003). Putative -10 and -35 promoter sequences were identified upstream of the SigB-independent transcriptional start site (Figure 6.12, lower panel). The -35 region in particular is a weak match to the *B. subtilis* SigA consensus (TTGACA - N_{13-14}- TATAAT), which correlates with the low level of expression from this promoter. As mutations in

sigA have not been isolated, it can only be assumed by the lack of consensus with SigB- and SigH-dependent promoters that this promoter is SigA-dependent.

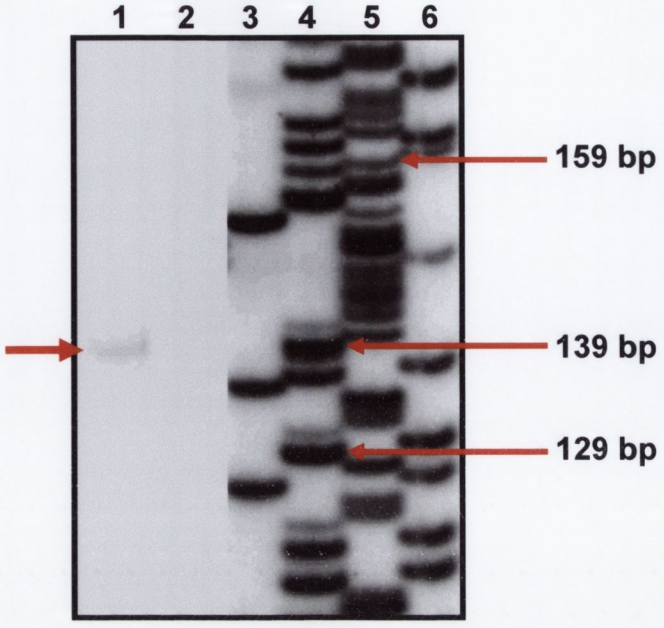
These data show that *clfA* is transcribed from at least two distinct promoters. A SigB-dependent *clfA* promoter with a transcriptional start site 31 bp upstream of the start codon was identified. An apparently SigA-dependent *clfA* promoter was identified with a transcriptional start site 1222 bp upstream of the start codon.

6.2.8 Measurement of *clfA* transcript half-life in stationary phase

The large amount of SigB-dependent *clfA* transcript present in stationary phase in SigB/RsbU$^+$ strains was unexpected, particularly for a gene encoding a surface protein. Its presence is due either to RNA stability or to continued SigB-dependent *clfA* transcription in stationary phase cells. In order to investigate this further, stationary phase Newman cells were treated with rifampicin, which stops transcription by binding to the RNA polymerase holoenzyme. Samples were taken at intervals and RNA levels were quantified by Northern hybridisation and densitometric analysis.

In Newman cells untreated with rifampicin the transcript was present 24 min after monitoring began, whereas cells treated with rifampicin rapidly lost the transcript (Figure 6.13). In Newman *clfA2* cells no transcript was observed. Percentage mRNA remaining at each time point was plotted against time on a semi-logarithmic scale (Figure 6.13, panel B). The amount of *clfA* mRNA appeared to rise slightly in the rifampicin-untreated cells, whereas it decreased significantly in rifampicin-treated cells (Figure 6.13, panel B). A best-fit line was plotted and used to calculate the half-life of *clfA* mRNA as ca. 3.5 minutes.

These data show that the SigB-dependent *clfA* transcript is short-lived in late stationary phase cells, with a half-life of ca. 3.5 minutes. The transcript is present at high levels in the absence of rifampicin treatment, and therefore must be continually produced by SigB/RsbU$^+$ *S. aureus* cells in late stationary phase.

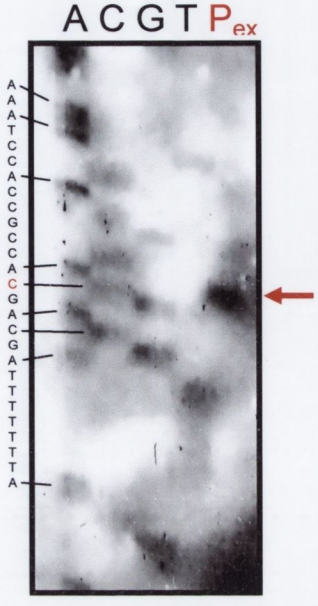


SigB-dependent

```
tcttaaaata gcaataaata aaatgtttgt tagtaaagta ttattgtgga taataaaata   1920
tcgatacaaa ttaattgcta taatgcaatt ttagtgtata attccattga cagagattaa   1980
atatatcttt aaagggtata tagttaAtaT aaaatgactt tttaaaaaga gggaataaaa   2040
tgaatatgaa gaaaaaagaa aaacacgcaa ttcggaaaaa atcgattggc gtggcttcag   2100
tgcttgtagg tacgttaatc ggttttggac tactcagcag taaagaagca gatgcaagtg   2160
```

Fig 6.11 Primer extension analysis of SigB-dependent *clfA* transcriptional start site by phosphorimaging

Total RNA was isolated from Newman or Newman *clfA2* cells grown to OD_{600} 1.0 per ml and used as template for cDNA synthesis using a *clfA*-specific ^{32}P end-labelled primer, Pex2, underlined in the sequence panel. This primer differed from that used in Figure 6.10. A size ladder was derived from sequencing of the *norA* gene with a *norA*-specific ^{32}P end-labelled primer, to allow determination of product length. Reactions were electrophoresed through 6% (w/v) acrylamide, dried and bands were digitally quantitated using a phosphorimaging system. Lane 1, Newman wt; lane 2, Newman *clfA2*; lanes 3-6, *norA* sequencing ladder. Position of transcriptional start site is arrowed in the upper panel, and highlighted in red in the *clfA* sequence in the lower panel. Nucleotides of ORFs are depicted in bold, as in Figure 6.8. Putative -10 and -35 boxes of SigB-dependent promoter are depicted in red (G. Kaatz, personal communication). Transcriptional start site (A) identified by Homerova *et al.* using S1 nuclease mapping is also indicated.



SigB-independent

```
agttcaactt tatcaaatga aaattagctt tctgccagct ttaggtttta accaaatttt    720
attagaaagg gaggaggatc aacttaatat aatgaattcg gcaacagaag agcatcatca    780
taaagattat attaaactat ataatttagg tggcggtgct gctaaaaaaa ttgcaataga    840
ggttttattg gggaaggata aagtcattca gaaaaaatac gtgcatattt tacctagtaa    900
agaagggtac atgttaccaa ttaataaaaa tgtgtacgaa gaattagaaa gaacgattga    960
```

Fig 6.12 Primer extension analysis of SigB-independent *clfA* transcriptional start site

Total RNA was isolated from 8325-4 pCF77 grown for 2 hours and used as template for cDNA synthesis using a specific ^{32}P end-labelled primer, R35, underlined in the sequence panel. pCF77 was used as template for sequencing using the same primer and incorporating ^{35}S. Reactions were electrophoresed through 6% (w/v) acrylamide, dried and exposed to X-ray film. A,C,G,T: DNA sequencing ladders. P_{ex}: primer extension reaction. Position of transcriptional start site is arrowed in the upper panel and highlighted in red in the sequence. Nucleotides of ORFs are depicted in bold, as in Figure 6.8. Putative -10 and -35 boxes of the SigA-dependent promoter are depicted in red.

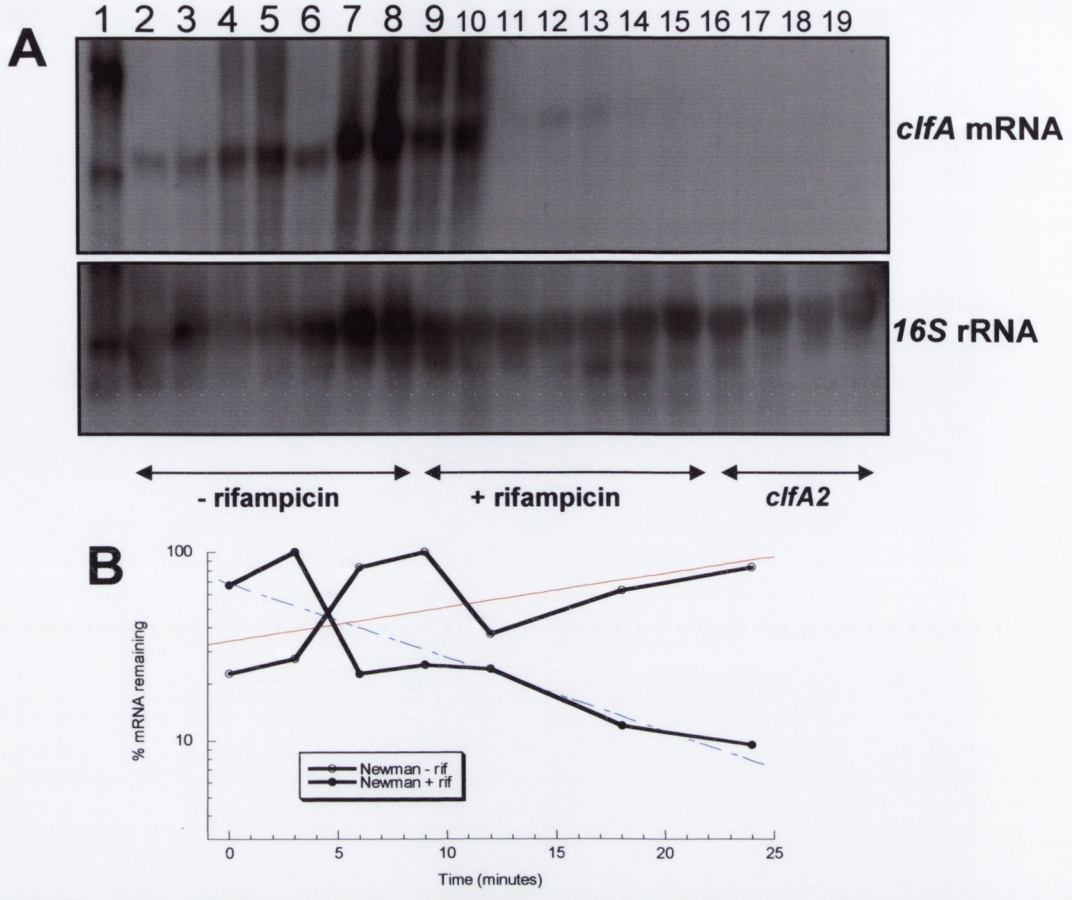


Fig 6.13 *clfA* mRNA levels in stationary phase following rifampicin treatment

A. Newman cells were grown for 20 hours before addition of rifampicin (rif). Samples were taken at intervals, RNA isolated and Northern blotting carried out using DNA probes to the entire length of region A of *clfA* (upper panel) or 16S rRNA gene (lower panel). Lane 1, RNA size standards; lanes 2, Newman (20 hours); lane 3, Newman (20 hours + 3 min); lane 4, Newman (20 hours + 6 min); lane 5, Newman (20 hours + 9 min); lane 6, Newman (20 hours + 12 min); lane 7, Newman (20 hours + 18 min); lane 8, Newman (20 hours + 24 min); lane 9, Newman + rif (20 hours + 0 min); lane 10, Newman + rif (20 hours + 3 min); lane 11, Newman + rif (20 hours + 6 min); lane 12, Newman + rif (20 hours + 9 min); lane 13, Newman+ rif (20 hours + 12 min); lane 14, Newman + rif (20 hours + 18 min); lane 15, Newman + rif (20 hours + 24 min); lane 16, Newman *clfA2* (20 hours); lane 17, Newman *clfA2* (20 hours + 24 min); lane 18, Newman *clfA2* + rif (20 hours + 0 min); lane 19, Newman *clfA2* + rif (20 hours + 24 min).

B. Following densitometric analysis, relative mRNA amounts for each *clfA*$^+$ sample were calculated by dividing *clfA* band volumes (intensity x area) by 16S band volumes for each sample. Percentage mRNA remaining was calculated, with the highest value as 100%, and plotted against time.

6.3 Discussion:

The data presented in this chapter demonstrate that the high level of ClfA expression on the surface of *S. aureus* cells from the stationary phase of growth is dependent on the accessory sigma factor, SigB. In early- to mid-exponential phase cells, however, transcription of *clfA* occurs independently of SigB.

Analysis of ClfA protein on the *S. aureus* cell surface showed that it is present throughout the growth cycle and at high levels in stationary phase in SigB/RsbU$^+$ strains. However, in *sigB* or *rsbU* mutant strains it was barely detected. These experiments also revealed that ClfA is present as a full-length (185-kDa) molecule and also as a truncated (150-kDa) form. The truncated form becomes more prevalent towards stationary phase, and is likely to be a product of the proteolytic breakdown of full length ClfA to the truncated form on the cell surface, as was described in Chapter 3. In late stationary phase additional, smaller breakdown products occur, particularly in strain Newman. These are shorter than the length of region R and are therefore likely to be C-terminally truncated. They might be the result of proteolysis during lysostaphin digestion of the cell wall or may be cleaved during growth and retained in the cell wall by a mechanism other than covalent attachment via sortase. They occur only in late stationary phase and are more abundant in Newman than SH1000. SH1000 has been shown to produce smaller amounts of both aureolysin and SspA than 8325-4 (Shaw *et al.*, 2004). These proteases both cleave region A of ClfA (see Chapter 3). The amounts of these and other proteases produced by Newman are not known. The additional smaller products observed in Newman compared to SH1000 may be due to a difference in protease expression in late stationary phase.

The relative amounts of ClfA on Newman and Newman *sigB rsbU* were measured by whole cell dot immunoblotting. This technique avoids the requirement for enzymatic digestion of cell wall proteins, but allows interference from other surface proteins that could be separated by SDS-PAGE. Peroxidase-conjugated protein A was used as secondary antibody to avoid non-immune binding of secondary antibody by protein A on the bacterial cell surface. Despite a small cross-reaction with protein A, the low level of ClfA produced by Newman *sigB rsbU* was better detected by dot immunoblotting than by Western immunoblotting (compare Figures 6.1 and 6.2).

The *clfA::lacZ* transcriptional fusion allowed quantification of transcriptional activity of the *clfA* promoters in different strains and under different growth conditions. Importantly, this method did not discriminate between promoters or exclude promoters more than a certain distance upstream. The use of a two-plasmid system in *E. coli* allowed Homerova and co-workers to describe the SigB-dependence of *clfA* but gave no clues as to the presence of an upstream SigA-dependent promoter. The two-plasmid system cannot identify *S. aureus* SigA-dependent promoters because of their similarity to *E. coli* promoter elements (Homerova *et al.*, 2004). The *clfA::lacZ* fusion allowed quantification of transcription from all promoters affecting *clfA*.

β-galactosidase assays showed a burst of *clfA* transcription in early exponential phase. The use of Newman *sigB rsbU* showed that this early transcription was SigB-independent. A second burst of transcription was detected as the cells entered stationary phase. This was absent in Newman *sigB rsbU* and 8325-4, indicating that it was SigB-dependent. High levels of transcription were detected in late stationary phase in SigB/RsbU$^+$ strains.

β-galactosidase measurements showed that *clfA* transcription was not influenced significantly by *agr* or *sarA* either in the exponential or stationary phases of growth. This implies that neither the SigB-dependent nor the SigA-dependent promoter driving transcription of *clfA* is directly regulated by SarA or Agr. This is unusual for genes encoding surface proteins, many of which are downregulated directly by *agr* in late exponential phase, or indirectly through its effects on the Sar family of transcription factors. The burst of β-galactosidase activity in early exponential phase is similar to that seen for *clfB* (McAleese *et al.*, 2001), which is upregulated in early exponential phase by Rot (Saïd-Salim *et al.*, 2003). However, *clfA* was not identified amongst 146 *S. aureus* genes regulated by Rot (Saïd-Salim *et al.*, 2003). In addition, *clfA* was shown not to be regulated either directly or indirectly by exposure of *S. aureus* to salicylic acid. The reduction in fibrinogen binding in *S. aureus* cells treated with salicylic acid (Kupferwasser *et al.*, 2003) must be accounted for by downregulation of other fibrinogen-binding proteins or other modifications to the cell wall or cell surface that reduce fibrinogen binding.

It was demonstrated by Northern hybridisation that at least two different transcripts are responsible for SigB-dependent and SigB-independent expression of *clfA* in *S. aureus*. A putative transcriptional terminator was identified shortly 3'

to the *clfA* stop codon. The 5' ends of two of the *clfA*-specfic transcripts were deduced from Northern hybridisation. The shorter (2900 nt) transcript began shortly upstream of the *clfA* coding sequence, whilst the longer (4000 nt) transcript began 1200-1260 bp upstream of *clfA*. A third (4500 nt) transcript was present in small amounts in stationary phase. The length of this transcript is consistent with possible expression of ORF1 and ORF2, two hypothetical proteins, one of which, ORF2, has significant homology to the GNAT family of acetyltransferases. A highly sensitive quantitative method, such as RT-PCR, might be used to verify the integrity and expression of this transcript, and to determine its start site.

Primer extension analysis in two regions upstream of *clfA* allowed the identification of a SigB-dependent *clfA* promoter, with a transcriptional start site 31 bp upstream of the start codon, and a SigA-dependent *clfA* promoter, with a transcriptional start site 1222 bp upstream of the start codon.

β-galactosidase assays showed a burst of *clfA* transcription in early exponential phase. The use of mutants and Northern hybridisation showed this transcription to be SigB-independent. This burst of transcription occurs from the SigA-dependent promoter identified 1222 bp upstream of *clfA* and is likely to be responsible for the ClfA found on the *S. aureus* cell surface in exponential phase. Although the amount of protein on the cell surface of 8325-4 cells was not detected under the conditions used to measure expression in strain Newman, there appears to be sufficient protein to support the low-level ClfA-dependent binding to immobilised fibrinogen observed in this strain (McDevitt *et al.*, 1994). After cessation of this early burst of transcription, cells continued to grow exponentially, leading to dilution of the protein on the cell surface in mid to late- exponential phase (2.5-3.5 hours, see Figure 6.3). These results indicated that the SigB-defective 8325-4 is a poor choice of strain for studying expression of ClfA on the cell surface in stationary phase. The location of the SigA-dependent promoter is consistent with the observation that *clfA* was not expressed from a plasmid containing only 250 bp of upstream sequence when present in 8325-4, whereas it was expressed from a plasmid (pCF77) containing 1300 bp of upstream sequence (Hartford *et al.*, 1997).

A second burst of transcription that was dependent on SigB was detected as the cells entered stationary phase. Transcription was confirmed by Northern hybridisation and occurred mainly from the SigB-dependent promoter located 31 bp upstream of *clfA*. It led to high levels of ClfA expression on the cell surface in SigB/RsbU$^+$ strains in late stationary phase. Analysis of the half-life of the SigB-

dependent transcript showed that it was continually produced in late stationary phase. ClfA protein produced as the cells entered stationary phase (3.5-5 hours), shortly after the onset of the second burst of transcription, was released by lysostaphin treatment (Figure 6.1). This indicates that protein production, secretion and cell wall anchoring of ClfA via sortase occurred rapidly after the commencement of transcription.

The exponential-phase SigA-dependent expression of *clfA* is similar to that observed for *clfB*, *fnbA* and *fnbB*. These genes, encoding other fibrinogen-binding MSCRAMMs of *S. aureus*, are expressed transiently in early exponential phase and are downregulated via SarA and its homologues in response to cell density detected by *agr*. However, *clfA* is not regulated through *sarA* or *agr*, and unlike the other MSCRAMMs, *clfA* is also expressed at a very high level in stationary phase. This expression of *clfA* both during exponential growth and also under stress may go some way to explaining its importance in the virulence of *S. aureus* in animal models of infection.

The differences between exponential and stationary phase conditions *in vitro* and those pertaining *in vivo* within the host during infection are poorly understood. A study relating the *clfA* transcript levels in broth cultures and in cells from device-related infection in guinea pigs revealed that levels increased during the initial stages of infection and remained high after 96 hours of infection, although they never reached those seen in stationary phase Newman cells (Wolz *et al.*, 2002). ClfA is not an essential virulence factor in this model of infection. The relationship between the expression of *clfA* in *in vitro*-grown bacteria and in those causing endocarditis or septic arthritis might be more difficult to establish due to the inaccessibility of the disease sites and smaller numbers of bacterial cells involved.

Virulence studies using *sigB* mutants have recently shown that this factor is not important in the murine model of skin abscess (Horsburgh *et al.*, 2002), rat osteomyelitis (Nicholas *et al.*, 1999) or rat endocarditis (Entenza *et al.*, 2005). ClfA is important in rat endocarditis (Moreillon *et al.*, 1995), although its importance in models of murine skin abscess and rat osteomyelitis has not been tested. Any effect of ClfA in these models must be mediated solely through SigA-dependent ClfA production. On the other hand, *sigB* is necessary for *S. aureus*-induced arthritis and sepsis in mice (Jonsson *et al.*, 2004). The role of ClfA has been well established in this model (Josefsson *et al.*, 2001). The dependence on SigB for virulence in the sepsis and septic arthritis model may reflect the more

extensive bacteraemic phase in this model and the need for bacteria to escape the blood vessels and to enter the joints. This model is more complex than experimental endocarditis or wound infection, with longer periods of bacterial growth *in vivo*. However, the influence of SigB on this model of infection might also be related to its control of ClfA production. Perhaps low-level expression of *clfA* is insufficient to promote virulence in this model, and high-level SigB-dependent expression of *clfA* is needed. A variant devoid of the SigB-dependent *clfA* promoter would be required to determine whether the importance of *sigB* in this model is mediated through its influence on *clfA*.

Further information on the expression of *clfA in vivo*, particularly in the case of endocarditis, may give important clues to its role in the pathogenicity of *S. aureus*.

Chapter 7

General Discussion

A role for clumping factor A in the virulence of *S. aureus* has been established in a variety of animal models of infection. Its importance might in part be explained by the observation that it has antiphagocytic properties. The antiphagocytic properties of ClfA were partially dependent on fibrinogen-binding activity. In the absence of fibrinogen-binding, however, significant antiphagocytic activity remained. This might be due to other properties of ClfA (e.g. the presence of SD dipeptide repeats, domain N1, the binding of additional ligands or the release of peptides with biological activity).

Domain N1 of region A of ClfA can be removed by proteolysis. The removal of domain N1 did not influence fibrinogen-binding activity. It may, however, influence the pathogenesis of *S. aureus* infection by releasing anti-inflammatory or pro-inflammatory peptides or by releasing *S. aureus* from an unknown ligand that might be bound by domain N1. Domain N1 might be the source of the fibrinogen-independent antiphagocytic activity of ClfA, possibly due to its structure, the binding of unknown ligands or the release of fragments by proteolysis. In addition, the ability of *S. aureus* to release domain N1 of ClfA may aid in evasion of host antibody responses through the removal of immunodominant epitopes. These hypotheses might be tested by the use of a mutant of *clfA* lacking the DNA encoding domain N1 in *S. aureus*. The generation of such a mutant was attempted during the course of this study, but was unsuccessful. Such a mutant would allow the evaluation of the properties and role of domain N1, which is highly conserved in *S. aureus* strains.

Knowledge of the regulation of *clfA in vitro* allows comparison to be drawn with its likely regulation *in vivo* during infection and with the regulation of ClfB and FnBPA *in vitro*. Unlike ClfB and FnBPA, ClfA is expressed in stationary phase and proteolysis does not destroy its fibrinogen-binding activity. The role of removal of domain N1 is likely to differ between ClfA and ClfB. In ClfB removal of domain N1 eliminates ligand-binding activity and may facilitate detachment from solid surfaces coated with fibrinogen or cytokeratin 10. Removal of domain N1 from ClfA did not reduce fibrinogen-binding activity, and must presumably serve some other function. The 'dock, lock and latch' model of fibrinogen binding suggests that when the fibrinogen γ-chain peptide ligand is bound, domains N2 and N3 lie folded back with respect to region R instead of being projected in a linear sequence (see Figure 3.11). Perhaps in this conformation, domain N1 has properties that are not observed with recombinant protein or in the absence of ligand. Domain N1 may

interact with region R or be protected from proteolysis when ligand is bound. These possibilities were not investigated in this study.

The expression of *clfA* in both the stationary and, to a lesser extent, the exponential phase of growth *in vitro* may help to explain the important role of ClfA in animal models of infection. ClfA plays a more pro-inflammatory role in the progression of disease in septic arthritis than FnBPA (Palmqvist *et al.*, 2005), implying that fibrinogen binding, which is also a property of FnBPA, might not be the only property of ClfA with biological function in this animal model. The antiphagocytic activity of ClfA is likely to play an important role in this model of infection. The possibility of antiphagocytic properties of FnBPA is not known and was not tested in this study. Further analysis of the molecular basis of the antiphagocytic properties of ClfA, including analysis of the role of domain N1, might reveal a mechanism common to ClfB, SdrG, Fbl and SdrD.

The reduced virulence of a *sigB* mutant of *S. aureus* in the murine model of sepsis and septic arthritis might be due to reduced expression of *clfA*. Demonstration that the half-life of *clfA* mRNA is relatively short in stationary phase implies that the high-levels of ClfA on stationary phase cells are due to continued expression of *clfA* from the SigB-dependent promoter. During stationary phase cells are expending energy in order to continue to make ClfA. This is likely to serve an important biological function *in vivo* during infection, and may be related to the antiphagocytic activity of ClfA. The presence of large amounts of ClfA on the surface of *S. aureus* cells may inhibit opsonophagocytosis *in vivo* in abscesses and bacterial vegetations, where cells are present at high density and may be in a state resembling stationary phase. The dependence of ClfA on SigB *in vivo* might be tested using a mutant in which the SigB-dependent *clfA* promoter has been inactivated. Such a mutant would express *clfA* only during the exponential phase of growth *in vitro*.

Any role of ClfA in virulence that is independent of fibrinogen binding might be tested by introduction of the *clfA*PY mutation into the chromosomal *clfA* gene by allelic replacement using the vectors described in this study.

During this study new vectors were developed to allow the establishment of a null mutation in *S. aureus* that is not marked by antibiotic resistance. Using the pTS*ermC* and pTS*tetK* plasmids non-essential genes in *S. aureus* can be mutated by allelic replacement. The availability of the *clfA5* null mutation facilitated the construction of *clfA clfB fnbA fnbB* strains that were used in studies of the role of

fibrinogen-binding proteins in platelet aggregation caused by *S. aureus* and in a murine model of sepsis and septic arthritis. These plasmids also allow the construction of non-polar mutations by the generation of in-frame deletions, insertions and single-base pair substitutions. Finally, the construction of multiple mutations in different loci using a single plasmid, sequentially introduced into one strain, has removed the previous limit on the number of mutations that can be combined in a single strain.

This work has gone some way to explaining the influence of ClfA in the virulence of *S. aureus*. The differences in regulation and the lack of influence of proteolytic removal of domain N1 distinguish ClfA from the other fibrinogen-binding proteins (ClfB and FnBPs). The antiphagocytic activity of ClfA, which appears to be shared by ClfB and some other fibrinogen-binding proteins (SdrG and Fbl), is also likely to influence its important role in the virulence of *S. aureus*.

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