



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

Copyright statement

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

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

Liability statement

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

Access Agreement

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

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

**Analysis of the Mechanism of Protein A Release and its
Contribution to Immune Evasion by *Staphylococcus*
*aureus***

A thesis submitted for the degree of Doctor in Philosophy

by

Dara O'Halloran

Moyne Institute of Preventive Medicine

Department of Microbiology

School of Genetics and Microbiology

Trinity College Dublin



PM
GENETICS
& MICROBIOLOGY

Declaration

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

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.



Thesis 11290

Acknowledgements

Firstly I would like to sincerely thank my supervisor Dr. Joan Geoghegan. There aren't enough words to measure my gratitude to the endless guidance and patience that you have given me. It has been a pleasure to work with you and to see your lab start from the beginning and grow into what it is today. Thank you for everything! Especially for somehow always having the time to help, and then always having the answer! I couldn't have asked for a better mentor.

My deepest thanks to all those, past and present, in the Geoghegan lab. Thank you Orla, Leanne, Joana, Keenan, Aisling, Emma and Marta; there's always been a friendly atmosphere in the lab that has made it so much easier to be here. A special thanks has to go to Dee. From finding those hidden stocks of whatever I need in those desperate moments to purifying proteins for me, I wish you and your family all the best. Thank you to Jayne, Caroline, Stephen, Ronan, and the rest of the Moyne staff, I don't think I will ever find a building full of friendlier people.

To all those who donated blood for me, especially Orla and Mick who were my personal pin-cushions at one stage, thank you for your kindness and willingness to help me whenever you could. This thesis literally could not have happened without you.

To all my friends who regularly help me forget about science, at least for an evening anyway. A special shout-out must go to Marc, Chandie and Mick. Thank you for always being there, and Mick I will be forever grateful to you for those final few hours on judgement day, I couldn't have done it without you. I'll be there for your turn brother!

To my parents, you made this all possible. You always believed in me and gave me the confidence to believe I could do this. Thank you so much for everything and for always being supportive, even when I seem to disappear for weeks on end into the lab. I wouldn't be who I am today if it weren't for you.

Laura, you have been through this with me every step of the way. You have made those dark days, when everything fails, seem brighter. You pulled me through when everything seemed impossible and kept me going. T'estimo carinyo!

Summary

The immunoglobulin binding protein A (SpA) of *Staphylococcus aureus* was one of the first cell wall-associated proteins of Gram-positive bacteria to be identified and characterised. It is synthesized as a precursor with a C-terminal sorting signal and becomes covalently anchored to the cell wall peptidoglycan by the sortase A enzyme. However, in addition to being displayed on the surface of the bacterium, SpA is also released into the extracellular medium. While cell wall anchored SpA and its role in *S. aureus* infection has been studied extensively, little is known about how SpA is released or the biological significance of extracellular SpA.

LytM is a glycylglycine endopeptidase of *S. aureus* that was previously shown to promote partial release of SpA from the *S. aureus* lab strain Newman. Here the influence of LytM on SpA surface-expression and release was analysed in strain Newman and the community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain LAC. While disruption of *lytM* did not alter the levels of surface-displayed SpA, a significant reduction in SpA release was measured. However, as the influence of LytM only accounted for a portion of SpA release from *S. aureus* it was evident that other mechanism(s) of SpA release must exist. A protease-deficient variant of LAC produced equal amounts of CWA and released SpA as wild-type LAC, indicating that the *S. aureus* extracellular proteases are not involved in this process.

To gain a better understanding of the nature of the extracellular form of the protein, released SpA was purified from the supernatant of a LAC culture and analysed by liquid chromatography tandem mass spectrometry. Analysis of the amino acid sequence of released SpA revealed that a portion of SpA is released into the supernatant of LAC with an intact sorting signal, indicating that it has not been processed by sortase A. Release of SpA was significantly reduced when the native sorting signal was replaced with the corresponding region of another sortase-anchored protein, SdrE. Altering the number of positively charged residues at the extreme C-terminus, or the substitution of certain amino acids in the sorting signal, also reduced SpA release.

SpA is best known for its ability to bind to the Fc region of immunoglobulin G (IgG). In this way cell wall-associated SpA sequesters IgG at the *S. aureus* cell surface in the incorrect orientation for immune recognition, thereby protecting *S. aureus* from phagocytic uptake. However a role for released SpA in *S. aureus* immune evasion had previously not

been investigated. Here released SpA was shown to promote the growth of LAC in whole human blood. Furthermore purified recombinant SpA was shown to protect *S. aureus* from phagocytosis by purified human neutrophils. In this work, a model is proposed whereby released SpA binds to the Fc region of *S. aureus*-specific IgG on the surface of the bacterium thereby blocking immunoglobulin receptor recognition and phagocytic uptake of *S. aureus*.

SasY is an uncharacterised protein that is encoded on a plasmid carried by the newly described multi-locus sequence type (ST) 2371 MRSA strain first identified in the UK in 2011. SasY has a domain organisation typical of cell wall-associated proteins. Accordingly it was found to be associated with the cell wall and displayed on the surface of *S. aureus*. SasY significantly increased the ability of ST2371 and LAC to grow in human blood, but did not alter bacterial growth in plasma, indicating that SasY protects *S. aureus* from phagocytic killing. It was shown that bacteria expressing SasY were better able to resist phagocytic uptake by human neutrophils, further indicating that SasY protects *S. aureus* from killing in blood by interfering with neutrophil phagocytosis. The identification and characterisation of this new immune evasion protein of *S. aureus* could prove to be invaluable to the treatment, and possibly prevention, of MRSA infections in the future.

Publications

O'Halloran DP, Wynne K, Geoghegan JA. 2015. Protein A is released into the *Staphylococcus aureus* culture supernatant with an unprocessed sorting signal. Infect Immun 83:1598–1609

Table of contents

Declaration	ii
Acknowledgements	iii
Summary	iv
Publications	vi
List of tables	xii
List of figures	xii
Key to abbreviations	xv

Chapter 1 Introduction

1.1 Biology of the staphylococci.	2
1.1.1 Classification and identification	2
1.1.2 Colonisation and disease.....	2
1.1.3 Methicillin-resistant <i>Staphylococcus aureus</i>	3
1.2 Virulence factors	5
1.2.1 Cell envelope components	5
1.2.2 Cell wall-anchored proteins	6
1.2.2.1 Export of cell wall-anchored proteins from the cytoplasm.....	7
1.2.2.2 Sortase A-mediated anchoring of cell wall-anchored proteins	8
1.2.2.3 Release of cell wall-anchored proteins	9
1.2.2.4 Structural classification of cell wall-anchored proteins of <i>S. aureus</i> ...	10
1.2.2.4.1 The MSCRAMM family.....	10
1.2.2.4.1.1 The Clf/Sdr subfamily of MSCRAMMs.....	10
1.2.2.4.1.2 The fibronectin-binding protein subfamily of MSCRAMMs.....	11
1.2.2.4.1.3 Collagen binding protein.....	11
1.2.2.4.2 NEAT family motif.....	11
1.2.2.4.3 Three helical bundle family	12
1.2.2.4.4 The G5-E repeat family	12
1.2.2.4.5 Legume-lectin domain	12
1.2.2.5 Contribution of cell wall-anchored proteins to virulence	13
1.2.2.5.1 Invasion of epithelial and endothelial cells.....	13
1.2.2.5.2 Evasion of immune responses.....	14
1.2.2.5.3 Biofilm formation	14
1.2.3 Immunoglobulin-binding proteins of Gram-positive bacteria	15

1.2.3.1 IgG-binding proteins of <i>S. aureus</i>	15
1.2.3.1.1 Protein A	15
1.2.3.1.2 Sbi	18
1.2.3.1.3 Staphylococcal superantigen like proteins.....	19
1.2.3.2 Streptococcal IgG-binding proteins	19
1.2.4 Extracellular proteases	20
1.3 Immune response to bacterial infection	22
1.4 Complement.....	23
1.5 Immune evasion by <i>S. aureus</i>	24
1.5.1 Inhibition of neutrophil migration	24
1.5.2 Inhibition of neutrophil phagocytosis	25
1.5.2.1 Capsule.....	25
1.5.2.2 Cell wall-anchored proteins	26
1.5.2.3 Secreted proteins	27
1.5.2.4 Cytolytic pore-forming toxins.....	28
1.5.2.4.1 Alpha toxin.....	28
1.5.2.4.2 Bi-component pore-forming toxins.....	28
1.5.2.4.2.1 PVL	29
1.5.2.4.2.2 Gamma-haemolysin	29
1.5.2.4.2.3 LukAB.....	30
1.5.2.4.2.4 LukED.....	30
1.5.2.4.3 Phenol-soluble modulins.....	30
1.5.3 Disruption of NETs.....	31
1.5.4 <i>S. aureus</i> survival following neutrophil uptake	32
1.5.4.1 Resistance to antimicrobial peptides and bactericidal proteins	32
1.5.4.2 Resistance to oxidative stress.....	32
1.6 Vaccine design	33
1.6.1 Vaccination strategies for the prevention of <i>S. aureus</i> infection	33
1.6.2 Protein A as a vaccine candidate	34
1.7 Genetic manipulation of <i>S. aureus</i>	35
1.7.1 Transposon mutagenesis	35
1.7.2 Allelic exchange.....	35
1.7.3.1 Temperature-sensitive plasmids.....	36
1.7.3.1.1 The pVW01ts replicon.....	36
1.7.3.1.2 Counter-selection in <i>S. aureus</i>	36

1.7.4 Complementation.....	37
1.7.5 Bypassing the <i>S. aureus</i> restriction modification barriers.....	37
1.8 Aims and Objectives.....	38

Chapter 2 Material and Methods

2.1 Bacterial culture conditions and reagents	40
2.2 Plasmids	40
2.3 DNA manipulation.....	40
2.3.1 Isolation of plasmid and genomic DNA	40
2.3.2 Polymerase chain reaction	40
2.3.3 Agarose electrophoresis	41
2.3.4 Sequence and ligase-independent cloning	41
2.4 Strain and plasmid construction.....	42
2.4.1 Strain construction	42
2.4.2 Plasmid construction.....	42
2.4.3 Allelic exchange.....	43
2.5 Transformation.....	44
2.5.1 Preparation and electroporation of <i>E. coli</i>	44
2.5.2 Preparation and electroporation of <i>S. aureus</i>	45
2.5.3 Colony PCR screening of <i>S. aureus</i>	45
2.6 Isolation of cell wall associated and extracellular proteins	45
2.7 Protein electrophoresis and Western immunoblotting.....	46
2.7.1 SDS-PAGE	46
2.7.2 Western immunoblotting	46
2.7.3 Ligand affinity blotting.....	46
2.8 Analysis of surface-displayed SpA by flow cytometry	47
2.9 Enzyme-linked immunosorbent assay (ELISA)	47
2.10 Protein purification	48
2.10.1 Purification of SpA from <i>S. aureus</i> culture supernatants by affinity chromatography	48
2.10.2 Purification of GST-tagged recombinant proteins	48
2.11 Mass spectrometry	49
2.11.1 Trypsin Digestion.....	49
2.11.2 Liquid chromatography and tandem mass spectrometry (LC-MS/MS)	49

2.11.3 Data Base Search.....	49
2.12 Whole blood survival assay.....	50
2.13 Bacterial growth in human plasma.....	50
2.14 Neutrophil isolation.....	50
2.15 Neutrophil assays.....	51
2.15.1 Phagocytosis assay.....	51
2.15.2 Bacterial survival in neutrophils.....	51
2.16 Binding of recombinant proteins to opsonised <i>S. aureus</i>	52

Chapter 3 The role of extracellular proteases and the endopeptidase LytM in the production of released protein A in *Staphylococcus aureus*

3.1 Introduction.....	55
3.2 Results.....	57
3.2.1 Release of SpA from the CA-MRSA strain LAC*.....	57
3.2.2 The role of staphylococcal extracellular proteases in the generation of released SpA in LAC*.....	58
3.2.3 Generation of LAC* <i>sbilym</i> by allelic exchange.....	59
3.2.4 The influence of the endopeptidase LytM on the generation of SpA in LAC*.....	60
3.2.5 The influence of the endopeptidase LytM on the generation of SpA in NM.	61
3.3 Discussion.....	63
3.4 Summary.....	67

Chapter 4 Protein A is released into the *S. aureus* culture supernatant with an unprocessed sorting signal

4.1 Introduction.....	69
4.2 Results.....	72
4.2.1 Identification of a protein A precursor with an unprocessed sorting signal in <i>S. aureus</i> culture supernatants.....	72
4.2.2 Released SpA does not become anchored to the cell wall of <i>S. aureus</i>	73
4.2.3 The vector pRMC2 allows inducible expression of SpA in <i>S. aureus</i>	73
4.2.4 Replacing the SpA sorting signal with the sorting signal of SdrE reduces release of SpA.....	72
4.2.5 The C-terminal sorting signal of SpA allows release of cell wall-anchored proteins.....	75
4.2.6 The release of SpA by LAC* is mediated by both LytM and the native SpA sorting signal.....	76

4.2.7 Exchanging the SpA sorting signal for the sorting signal of SdrE on the chromosome of NM reduces the release of SpA.	77
4.2.8 Amino acid substitutions in the SpA sorting signal reduce SpA release.....	78
4.2.9 Increasing the number of positively-charged amino acids at the C-terminus does not influence release of SpA.....	79
4.3 Discussion.....	79
4.4 Summary.....	86

Chapter 5 Released SpA promotes the survival and growth of *S. aureus* in human blood

5.1 Introduction.....	87
5.2 Results.....	91
5.2.1 Released SpA contributes to the survival of LAC* in whole human blood.	91
5.2.2 Recombinant GST-SpA reduces neutrophil phagocytosis of <i>S. aureus</i> in an IgG-dependent manner.	93
5.2.3 Recombinant GST-SpA binds to the surface of serum-opsonised <i>S. aureus</i>	94
5.3 Discussion.....	97
5.4 Summary.....	104

Chapter 6 SasY: a novel cell wall-anchored protein that enhances the survival and growth of *S. aureus* in human blood

6.1 Introduction.....	104
6.2 Results.....	107
6.2.1 SasY is associated with the cell wall of <i>S. aureus</i>	107
6.2.2 SasY promotes the growth of <i>S. aureus</i> in human blood.....	108
6.2.3 Cell wall-anchored SasY reduces neutrophil phagocytosis of LAC*.....	109
6.2.4 Recombinant SasY does not protect <i>S. aureus</i> from phagocytosis by human neutrophils.....	110
6.2.5 SasY promotes survival of ST2371 in human blood.	111
6.3 Discussion.....	112
6.4 Summary.....	117

Chapter 7 Discussion

7.1 Discussion.....	118
---------------------	-----

References

References.....	126
-----------------	-----

List of Tables

	Following Page
Table 1.1 Cell wall-anchored proteins of <i>S. aureus</i>	13
Table 1.2 <i>S. aureus</i> cytolytic pore-forming toxins.	30
Table 2.1 Bacterial strains.....	40
Table 2.2 Plasmids.....	40
Table 2.3 Oligonucleotides.....	41
Table 2.4 Antibodies used in this study.....	46
Table 4.1. Peptides identified by liquid chromatography tandem mass spectrometry.	72

List of Figures

	Following Page
Fig. 1.1 CWA protein anchoring in <i>S. aureus</i>	9
Fig. 1.2 Schematic representation of the common motif organisation of the <i>S. aureus</i> CWA proteins.....	10
Fig. 1.3 Immunoglobulin-binding protein A.....	15
Fig. 1.4 Neutrophil extravasation to the site of infection and killing mechanisms.	23
Fig. 1.5 Schematic representation of pIMAY.....	36
Fig. 1.6 Schematic of allelic exchange in <i>S. aureus</i> using pIMAY.....	36
Fig. 3.1 Staphylococcus aureus LAC* releases SpA.....	57
Fig. 3.2 Comparing the levels of cell wall-associated and released SpA from LAC*.	58
Fig. 3.3 Extracellular proteases of LAC* are not required for the release of protein A.....	58
Fig. 3.4 V8 is not involved of the generation of released SpA in LAC*.....	59
Fig. 3.5 Schematic representation of pIMAY:: Δ lytM plasmid..	59
Fig. 3.6 Transformation and integration of pIMAY:: Δ lytM into LAC* <i>sbi</i>	60
Fig. 3.7 Investigating the side of integration of pIMAY:: Δ lytM into the LAC* <i>sbi</i> chromosome.....	60
Fig. 3.8 Excision of pIMAY:: Δ lytM from the LAC* <i>sbi</i> chromosome.	60
Fig. 3.9 Comparing the levels of cell wall-associated and surface-displayed SpA between LAC* and a LytM-deficient mutant.	61

Fig. 3.10 Comparing the levels of released SpA generated by LAC* *sbi* and LAC* *sbi* *lytM*..... 61

Fig. 3.11 LytM-mediated release of SpA by *S. aureus* Newman (NM). 61

Fig. 4.1 Schematic representation of the anchoring of protein A to cell wall peptidoglycan by the sortase A enzyme. 70

Fig. 4.2 Released SpA purified from culture supernatant of LAC* *sbi*..... 72

Fig. 4.3 Alignment of peptides identified by LC-MS/MS to the amino acid sequence of SpA. 72

Fig. 4.4 Released SpA from LAC* is not anchored to the cell wall of *S. aureus*..... 73

Fig. 4.5 Genetic map of pRMC2..... 73

Fig. 4.6 Generation of pSpA by SLIC. 73

Fig. 4.7 Measuring the levels of surface-displayed SpA by LAC* *spa sbi* pSpA. 74

Fig. 4.8 Release of protein A into *S. aureus* culture supernatants can be reduced by altering the sorting signal. 75

Fig. 4.9 Release of D3D4 reporter protein into *S. aureus* culture supernatants can be reduced by altering the sorting signal..... 75

Fig. 4.10 Construction of pIMAY::SdrESS..... 76

Fig. 4.11 Replacing the SpA sorting signal with the sorting signal from SdrE on the chromosome of LAC* reduces release of SpA. 76

Fig. 4.12 Replacing the SpA sorting signal with the sorting signal from SdrE on the chromosome of Newman reduces release of SpA. 77

Fig. 4.13 Clustal alignment of SpA and SdrE sorting signals (SS) and modifications of the SpA SS to become more “SdrE-like” and their effect on SpA release. 78

Fig. 4.14 Increasing the number of positively charged residues at the extreme C-terminus of SpAΩSdrESS does not influence SpA release. 80

Fig. 5.1 A SpA-deficient mutant of LAC* has decreased growth in human blood compared to wild-type bacteria, and released SpA from the supernatant of a LAC* culture can increase growth. 91

Fig. 5.2 Deletion of the SpA sorting signal (SS) increases SpA release and abrogates cell surface-display..... 92

Fig. 5.3 Both cell wall-associated and released SpA protect LAC* from killing in human blood. 92

Fig. 5.4 Released SpA protects *S. aureus* from killing in human blood..... 92

Fig. 5.5 Recombinant GST-SpA and purified released SpA from LAC* *sbi* reduce neutrophil phagocytosis of *S. aureus*. 93

Fig. 5.6 GST-SpA can interact with the surface of serum-coated *S. aureus*. 94

Fig. 5.7 The interaction of GST-SpA with the surface of serum-coated <i>S. aureus</i> is dependent on IgG.	95
Fig. 6.1 Schematic representation of the domain organisation of SasY.	104
Fig. 6.2 SasY is a cell wall-associated protein that is displayed on the surface of <i>S. aureus</i>	107
Fig. 6.3 SasY promotes the growth of LAC* <i>spa sbi</i> in human blood.	108
Fig. 6.4 SasY does not promote the growth of <i>S. aureus</i> EMRSA-15 in human blood.	109
Fig. 6.5 Cell wall-associated SasY protects LAC* from phagocytosis by human neutrophils.	110
Fig. 6.6 Recombinant HIS-SasY does not reduce neutrophil phagocytosis of <i>S. aureus</i>	110
Fig. 6.7 SasY protects ST2371 from killing in human blood.	111
Fig. 7.1 The different forms of protein A and their roles during <i>S. aureus</i> infection.	119

Key to abbreviations

SpA	Staphylococcal protein A
CWA	Cell wall anchored
SN	Supernatant
SasY	<i>Staphylococcus aureus</i> surface protein Y
Ig	Immunoglobulin
SS	Sorting signal
CA-MRSA	Community-associated methicillin resistant <i>Staphylococcus aureus</i>
IC	Intracellular
HGT	Horizontal gene transfer
WGS	Whole genome sequencing
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
ELISA	Enzyme-linked immunosorbent assay
GST	Glutathione S-transferase
PVDF	Polyvinylidene fluoride
w/v	Weight per volume
v/v	Volume per volume
kDa	Kilodalton
MGE	Mobile genetic element
Agr	Accessory gene regulator
SLIC	Sequence and ligation-independent cloning

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 three planes to form grape-like structures (Tzagoloff & Novick, 1977). Historically, taxonomic studies positioned *Staphylococcus* in the *Bacillus-Lactobacillus-Streptococcus* cluster of the *Micrococcaceae* (Ludwig *et al.*, 1985; Stackebrandt & Teuber, 1988), however more recent studies have placed the staphylococci in a new family, the *Staphylococcaceae*, order *Bacillales*, class *Bacilli* (Becker *et al.*, 2011). Staphylococci are most closely related to *Enterococcus*, *Bacillus*, and *Listeria*, and their genomes contain DNA of low G+C content (30-39%) (Ludwig *et al.*, 1985; Stackebrandt & Teuber, 1988). The staphylococci are robust organisms that are resistant to desiccation and are extremely halotolerant, growing at up to 3.5 M NaCl.

The secretion of the coagulases, which bind to and activate prothrombin, is used to divide the staphylococci into two major groups: coagulase-positive and coagulase-negative staphylococci (CoNS). *Staphylococcus aureus* and the *Staphylococcus intermedius* group make up the coagulase-positive staphylococci. *S. aureus* is a catalase-positive, non-spore forming and non-motile coccus that forms smooth, entire, raised colonies. *S. aureus* can be distinguished from other staphylococcal species by the presence of the carotenoid pigment staphyloxanthin which gives *S. aureus* its characteristic golden colour, its ability to ferment mannitol, and production of thermostable DNase. *S. aureus* is considered to be more virulent than CoNS, however a number of CoNS, particularly *S. epidermidis* (Sievert *et al.*, 2013) and *S. lugdunensis* (Anguera *et al.*, 2005), can cause serious infections in humans.

1.1.2 Colonisation and disease

Staphylococcus aureus is a commensal and pathogen of humans that persistently colonises the nasal cavity of 20% of the population, and the remaining 80% are transiently colonised throughout their lives (van Belkum *et al.*, 2009). Nasal carriage is an established risk factor for *S. aureus* infection and individuals are usually infected with the strain that they carry (Munoz *et al.*, 2008; Wertheim *et al.*, 2004). However, colonisation is also associated with increased rates of survival as *S. aureus*-mediated bacteraemia-related deaths are significantly higher in infected non-carriers, suggesting that nasal carriage may contribute to the development of immunological memory (Wertheim *et al.*, 2004).

A number of studies have suggested that the primary nasal colonisation site of *S. aureus* is the anterior nares (Cole *et al.*, 2001; Peacock *et al.*, 2001), which is made up of

layers of dead keratinised cells called desquamated epithelial cells, or squames. However there is also evidence that indicates that *S. aureus* can bind to ciliated nasal epithelial cells that are positioned deeper within the nasal cavity (Clement *et al.*, 2005; Plouin-Gaudon *et al.*, 2006). *S. aureus* can also be found at secondary sites throughout the body such as the skin, pharynx, and perineum (Wertheim *et al.*, 2005). *S. aureus* expresses a number of cell wall-anchored (CWA) proteins that have been shown to promote adherence to squames. Clumping factor B (ClfB) mediates adhesion of *S. aureus* to squames through its affinity for the host ligands loricrin (Mulcahy *et al.*, 2012), which accounts for 80% of the protein mass of squames (Steven & Steinert, 1994), and cytokeratin 10 (O'Brien *et al.*, 2002). The iron regulated surface determinant A (IsdA) protein has also been shown to promote adherence to squames (Clarke & Foster, 2008). *S. aureus* mutants deficient in either ClfB or IsdA had reduced ability to adhere to squames *in vitro* (Clarke *et al.*, 2006; O'Brien *et al.*, 2002) and poorly colonised the nares of rodents (Clarke *et al.*, 2006; Schaffer *et al.*, 2006), and in the case of ClfB, humans (Wertheim *et al.*, 2008). The CWA proteins SasX (Li *et al.*, 2012), SasG (Corrigan *et al.*, 2007) and the serine-aspartate repeat proteins SdrC and SdrD (Corrigan *et al.*, 2009) promote adherence of *S. aureus* to squames *in vitro*. However further study is necessary to identify their host ligands and to determine if these factors mediate nasal colonisation *in vivo*.

Wall teichoic acids (WTA) are a major component of the *S. aureus* cell wall that are covalently anchored to peptidoglycan and have been implicated in nasal colonisation. A WTA-deficient mutant of *S. aureus* displayed decreased adherence to human nasal epithelial cells and poorly colonised the nares of cotton rats (Weidenmaier *et al.*, 2004). WTA has been shown to promote adherence to SREC-I, a type F scavenger receptor present on the surface of nasal epithelial cells (Baur *et al.*, 2014). Pre-incubating the nares of cotton rats with SREC-I function-blocking antibodies significantly impaired the early stages of *S. aureus* colonisation (Baur *et al.*, 2014).

1.1.3 Methicillin-resistant *Staphylococcus aureus*

From a clinical perspective, a major difficulty in the treatment of *S. aureus* infection is the increasing prevalence of antibiotic resistance. Penicillin was the first antibiotic discovered and was initially an effective treatment against *S. aureus* infections. Penicillin is a β -lactam antibiotic which inhibits the transpeptidation step during peptidoglycan biosynthesis. Two years following its introduction penicillin-resistant strains of *S. aureus* emerged which possessed an enzyme, β -lactamase, that cleaves the β -lactam ring present in

penicillin and its derivatives. Methicillin is a semi-synthetic derivative of penicillin that is not susceptible to β -lactamase inactivation and was introduced into the clinical setting to replace penicillin in the treatment of *S. aureus* infection. However, only one year after its introduction strains of methicillin-resistant *S. aureus* (MRSA) were detected in the UK (Jevons *et al.*, 1963), and unlike resistance to penicillin, the mechanism underlying methicillin resistance (the *mecA*-encoded alternative transpeptidase PBP2a) rendered *S. aureus* resistant to the entire class of β -lactam antibiotics.

MRSA is traditionally associated with hospitals, where it has developed a niche among the immunocompromised and patients possessing indwelling medical devices such as catheters. These strains are referred to as healthcare-associated (HA) MRSA. However, in the past decade there has been a significant increase in the number of healthy individuals developing MRSA infections in the community setting (Diep *et al.*, 2006a; Seybold *et al.*, 2006). These highly transmissible and virulent strains are referred to as community-associated (CA-) MRSA. CA-MRSA of the USA300 lineage are the major cause of skin and soft tissue infections (Johnson *et al.*, 2007) and community-associated bacterial infections in the US (Talan *et al.*, 2011).

The success of the USA300 lineage has, in-part, been attributed to an increased repertoire of virulence factors present on mobile genetic elements (MGEs) (Diep & Otto, 2008), and the differential expression of core genomic virulence factors (Li *et al.*, 2010). The SCC*mec* elements present in CA-MRSA strains, mainly types IV and V, are smaller and more mobile than those more commonly found in HA-MRSA strains, typically types I-III (Cameron *et al.*, 2011; David & Daum, 2010). The larger SCC*mec* elements in HA-MRSA strains are associated with a reduction in bacterial fitness coupled with decreased toxin production (Collins *et al.*, 2010). It has been suggested that the increased virulence of USA300 in comparison to HA-MRSA strains can partly be attributed to differential expression of the *mecA* gene encoded on their respective SCC*mec* elements. High-level expression of the *mecA*-encoded alternative penicillin-binding protein, PBP2a, in HA-MRSA isolates affects the responsiveness of the accessory gene regulator (Agr) system, which results in low-level toxin production (Rudkin *et al.*, 2012). PBP2a is expressed at relatively lower levels in CA-MRSA strains (Rudkin *et al.*, 2012), and it has been shown that induction of increased PBP2a expression in strains of the USA300 and USA400 lineages repressed Agr activity and reduced phenol-soluble modulins (PSM) activity in culture supernatants (Rudkin *et al.*, 2014). PSMs are a family of amphipathic peptides that are able to recruit, activate and

lyse human neutrophils (Peschel & Otto, 2013; Surewaard *et al.*, 2013; Wang *et al.*, 2007). CA-MRSA strains produce higher levels of PSMs compared to HA-MRSA strains (Li *et al.*, 2009).

USA300 strains possess a MGE known as the arginine catabolic mobile genetic element (ACME) (Diep *et al.*, 2008) that harbours the *speG* gene that encodes a spermidine acetyltransferase which confers resistance to spermidine and other polyamines (Joshi *et al.*, 2011). ACME also possesses a cluster of genes encoding enzymes of the arginine deiminase pathway which converts arginine into carbon dioxide, ATP, and ammonia, which may aid in the survival of USA300 on the skin (Otto, 2010). Panton–Valentine leucocidin (PVL) is a bi-component toxin that lyses human neutrophils (Loffler *et al.*, 2010), that is encoded by the *lukF-PV* and *lukS-PV* genes which are carried on a prophage that is highly prevalent among CA-MRSA strains worldwide (Monecke *et al.*, 2011).

1.2 Virulence factors

The wide range of infections and disease caused by *S. aureus* can be attributed to the equally diverse range of virulence factors produced by this organism. Many virulence factors have important roles in a number of key activities that enhance colonisation and the progression of *S. aureus* disease.

S. aureus produces a number of cell wall-associated (CWA) proteins which recognise and promote binding to various host proteins, such as those present in the extracellular matrix (ECM) and components of blood. Adhesion is recognised as a key step in the initiation of infection, and expression of these proteins can aid in the adhesion of *S. aureus* to implanted medical devices, host cells, and damaged tissue (Foster *et al.*, 2014). A number of CWA and secreted proteins are involved in the evasion and suppression of both the innate and adaptive immune response to *S. aureus* infection (Foster *et al.*, 2014; Thammavongsa *et al.*, 2015).

1.2.1 Cell envelope components

The Gram-positive cell wall is made up of a thick peptidoglycan layer, which accounts for approximately 60% of the envelope mass, and the remainder is comprised of wall teichoic acid (WTA), and CWA proteins (Navarre & Schneewind, 1999). The peptidoglycan layer protects Gram-positive bacteria from osmotic lysis and serves as a barrier against environmental hydrolases or membrane-damaging agents (Shockman & Barrett, 1983). Peptidoglycan also serves as a scaffold structure for the anchoring of other envelope components, such as WTA (Coley *et al.*, 1976) and the CWA proteins (Schneewind *et al.*,

1995). Peptidoglycan is composed of a repeating disaccharide, N-acetylglucosaminyl-N-acetylmuramic acid, polymerised in β -1, 4 linkage (Ghuysen & Strominger, 1963). The glycan chains are crosslinked by tetrapeptides (L-Ala-D-Glu-L-Lys-D-Ala) to the N-acetylmuramic acid (MurNAc) moieties (Ghuysen *et al.*, 1965). The tetrapeptides on neighbouring glycan strands are cross-linked by a reaction known as transpeptidation that is catalysed by penicillin-binding protein 2 (PBP2). *S. aureus* possesses a pentaglycine crossbridge that renders it susceptible to cleavage by the glycyl-glycine endopeptidase lysostaphin (Schleifer & Kandler, 1972). However, *S. aureus* is resistant to lysozyme due to the O-acetylation of MurNAc (Bera *et al.*, 2005).

WTA is covalently linked to peptidoglycan via a disaccharide linkage unit and is composed of a main chain polymer of phosphodiester-linked polyol repeat units substituted with N-acetylglucosamine and D-alanine residues (Collins *et al.*, 2002; Neuhaus & Baddiley, 2003; Ward, 1981). Lipoteichoic acid (LTA) typically consists of a polyglycerolphosphate chain that is linked via a glycolipid anchor to the bacterial membrane and protrudes into the cell wall (Fischer *et al.*, 1990; Xia *et al.*, 2010). Both WTA and LTA appear to be essential in maintaining the uniform structure of the cell envelope as WTA- and LTA-deficient mutants of *S. aureus* display cell-division and morphological defects (Corrigan *et al.*, 2011; Park *et al.*, 1974; Schlag *et al.*, 2010). Glycosylation of WTA is required for β -lactam resistance in MRSA (Brown *et al.*, 2012) and the degree of D-alanine substitution of teichoic acids regulates the net ionic charge of the *S. aureus* bacterial surface which is believed to play a role in resistance to defensins, kinocidins and cationic antibiotics like vancomycin (Collins *et al.*, 2002; Peschel *et al.*, 2001; Weidenmaier *et al.*, 2005).

1.2.2 Cell wall-anchored proteins

S. aureus can express more than 20 distinct CWA proteins that promote important functions such as adhesion, invasion, nutrient acquisition, biofilm formation, and evasion of immune responses (Foster *et al.*, 2014). All CWA protein precursors contain a Sec-dependent signal sequence at the amino terminus which targets pre-proteins for secretion from the cytoplasm. The C-terminus of a CWA protein precursor comprises a sorting signal which targets proteins for attachment to the cell wall via covalent linkage to uncrosslinked pentaglycine crossbridges of peptidoglycan by the membrane-associated sortase enzymes (Mazmanian *et al.*, 2001).

1.2.2.1 Export of cell wall-anchored proteins from the cytoplasm

The Sec-dependent N-terminal signal sequence terminates with an “AXA” motif which is recognised and cleaved by the membrane-anchored signal peptidase SpsB during or shortly after translocation (Cregg *et al.*, 1996). Most components of the Sec pathway were identified from genetic screens conducted in *Escherichia coli* and *Saccharomyces cerevisiae* (Deshaies & Schekman, 1987; Emr *et al.*, 1981; Oliver & Beckwith, 1981). The core Sec membrane translocon in bacteria consists of three protein subunits: SecY, SecE and SecG (Zimmermann *et al.*, 2011). SecD, SecF and YajC are additional components of the secretion machinery that are required for *in vivo* secretion but are dispensable for *in vitro* translocation of precursors (Duong & Wickner, 1997; Gardel *et al.*, 1987). The SecA ATPase powers the Sec machinery by inserting into the base of the translocase and facilitates forward motion of the pre-protein through repeated rounds of ATP hydrolysis (Pugsley, 1993). As most genetic analyses of the prokaryotic Sec machinery have been performed using *E. coli* relatively little in comparison is known about the mechanisms of protein secretion in Gram-positive bacteria. The Sec system in *S. aureus* harbours homologs to the canonical SecYEG translocase and the SecA ATPase (Sibbald *et al.*, 2006). Some Gram-positive bacteria also express an accessory Sec system composed of homologs to SecY and SecA, termed SecY2 and SecA2 (Siboo *et al.*, 2008). *S. aureus* encodes the *secY2* and *secA2* genes along with genes for the accessory proteins Asp1, Asp2 and Asp3. The serine-rich adhesin of platelets (SraP), which is the sole member of the legume-lectin domain family of CWA proteins in *Staphylococcus aureus* (Table 1.1), is encoded within the *secA2Y2* locus. SraP is the only protein of *S. aureus* that has been shown to be translocated by this system (Siboo *et al.*, 2008).

CWA proteins of *S. aureus* present on the cell surface in ring-like distributions, such as staphylococcal protein A (SpA), clumping factor A (ClfA) and fibronectin-binding protein B (FnBPB), possess a signal sequence that harbours a YSIRK/GS motif (Bae & Schneewind, 2003). Deletion or modification of the YSIRK/GS motif of SpA was shown to significantly reduce signal peptide processing and secretion from the cytoplasm. However these modifications did not impact cell wall-anchoring or the functional display of SpA (Bae & Schneewind, 2003). The presence of a signal sequence possessing the YSIRK/GS motif in ClfA is essential for direction of the protein to the septum of growing cells (DeDent *et al.*, 2008). Replacement of the ClfA signal sequence with the signal sequence from the surface protein SasF, which does not possess the YSIRK/GS motif, resulted in the deposition of ClfA at alternative sites in the cell (DeDent *et al.*, 2008).

However mutations altering only the YSIRK/GS motif of ClfA were not sufficient to abolish localisation of the protein to the septum. Thus the exact role of the YSIRK/GS motif in protein secretion is unclear.

1.2.2.2 Sortase A-mediated anchoring of cell wall-anchored proteins

All proteins of Gram-positive bacteria destined to be covalently anchored to cell wall peptidoglycan possess a C-terminal sorting signal (SS) (Schneewind *et al.*, 1992; Schneewind *et al.*, 1993). The SS comprises an LPXTG motif, which is recognised by the membrane-bound enzyme sortase A, followed by a hydrophobic membrane-spanning domain and, at the extreme C-terminus, a stretch of positively charged residues (Schneewind *et al.*, 1993). The last two elements delay secretion across the membrane and facilitate recognition and cleavage by sortase A (Schneewind *et al.*, 1993). All known sortase enzymes of Gram-positive organisms are cysteine transpeptidases that recognise and process proteins possessing a C-terminal SS and attach them to the peptidoglycan precursor lipid II (Clancy *et al.*, 2010; Marraffini & Schneewind, 2006). The gene encoding the *S. aureus* sortase A (*srtA*) enzyme, which is responsible for the covalent anchoring of the majority of the *S. aureus* CWA proteins (Ton-That *et al.*, 1999), was first identified in 1999 (Mazmanian *et al.*, 1999). *S. aureus* possesses a second sortase enzyme, sortase B (*srtB*), which is encoded within the iron-regulated surface determinant (*isd*) gene cluster of bacilli, clostridia, listeria and staphylococci (Mazmanian *et al.*, 2003). In *S. aureus* the only known protein that is anchored by sortase B is the iron-regulated surface determinant C (IsdC) (Mazmanian *et al.*, 2002).

The crystal structure of the N-terminal catalytic domain of sortase A has been solved in complex with an LPXTG peptide (Zong *et al.*, 2004a). Sortase A cleaves between the threonine and glycine of the LPXTG motif forming an acyl-enzyme intermediate capturing the C-terminal carboxyl group of the protein with its active site cysteine thiol (Mazmanian *et al.*, 1999). Acyl intermediates are relieved by the nucleophilic attack of the amino group of the pentaglycine crossbridge of lipid II, a peptidoglycan precursor (Perry *et al.*, 2002). Following transglycosylation and transpeptidation, the protein becomes covalently anchored to peptidoglycan and is displayed on the surface of the bacterium (Fig. 1.1) (Mazmanian *et al.*, 2001). Sortase B recognises the NPQTN motif present in the SS of IsdC (Mazmanian *et al.*, 2002). Sortase B assumes a similar fold and catalytic mechanism as sortase A (Zong *et al.*, 2004b). However the enzyme appears to accept the crossbridge residues of assembled

cell wall as a nucleophile, instead of the lipid II precursor, to anchor IsdC in the immediate vicinity of the membrane (Marraffini & Schneewind, 2005)

Sortase A is essential in the anchoring and surface-display of the CWA proteins. Deletion of the *srtA* gene in *S. aureus* strain Newman resulted in the release of the majority of CWA proteins from the staphylococcal cell and the unprocessed pre-proteins were detected in the bacterial cytoplasm, cytoplasmic membrane, and non-covalently associated with the cell wall (DeDent *et al.*, 2007; Mazmanian *et al.*, 2000). Sortase A-deficient mutants of *S. aureus* are defective in the colonisation of the nares of mice and cotton rats (Schaffer *et al.*, 2006; Weidenmaier *et al.*, 2008), and are attenuated in animal infection models of septic arthritis and endocarditis (Jonsson *et al.*, 2002; Weiss *et al.*, 2004).

1.2.2.3 Release of cell wall-anchored proteins

Recently a mechanism has been described for the release of a portion of CWA SpA from the surface of *S. aureus* strain Newman by the action of the autolysin LytM, a glycyl-glycine endopeptidase that cleaves within the pentaglycine crossbridge of peptidoglycan thereby releasing CW-anchored SpA (Becker *et al.*, 2014). This mechanism is predicted to release a portion of all CWA proteins covalently anchored to the pentaglycine crossbridge of *S. aureus*.

There is evidence that certain CWA proteins of Gram-positive bacteria can be modified or removed from the cell surface by extracellular proteases. The *S. aureus* extracellular serine protease V8 cleaves and removes the fibronectin-binding proteins from the staphylococcal cell surface (McGavin *et al.*, 1997). The *S. aureus* metalloprotease aureolysin (Aur) modifies CWA ClfB by cleaving at the junction of the N1 and N2 subdomains (see section 1.2.2.4.1.1) which removes N1 and abolishes the ligand-binding activity of ClfB (McAleese *et al.*, 2001). A cysteine protease, SpeB, present in the culture supernatants of M1 serotype group A streptococci modifies the CWA M1 protein, which binds all four subclasses of human immunoglobulin (Ig) G. SpeB removes 24 amino acids from the N-terminus of the mature CWA M1 protein which alters the IgG-binding profile of this protein so that modified M1 preferentially binds the IgG₃ subclass of human IgG (Raeder *et al.*, 1998). Furthermore, full-length released M protein forms complexes with human fibrinogen. These complexes bind to β_2 integrins on the surface of neutrophils, which induces the secretion of heparin-binding protein (Herwald *et al.*, 2004). Complexes of M protein and fibrinogen were isolated from samples of human tissue taken from a patient suffering from streptococcal toxic shock syndrome (STSS) and necrotising fasciitis (Herwald *et al.*, 2004).

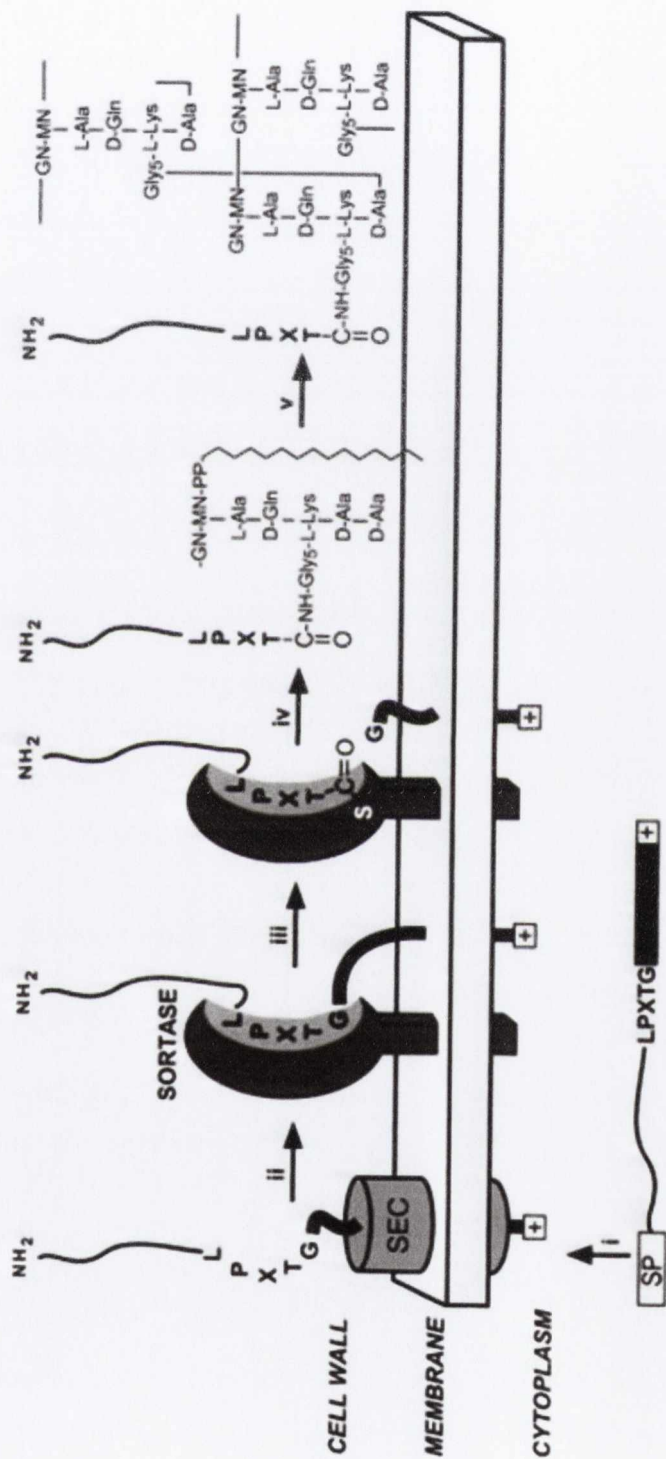


Fig. 1.1 CWA protein anchoring in *S. aureus*. (i) Precursor proteins with an N-terminal signal peptide (SP) are initiated into the secretory (Sec) pathway, and the signal peptide is removed. (ii) The C-terminal sorting signal retains polypeptides within the secretory pathway. (iii) Sortase cleaves between the threonine and the glycine of the LPXTG motif, resulting in the formation of a thioester enzyme intermediate. (iv) Nucleophilic attack of the free amino group of lipid II at the thioester bond resolves the acyl-enzyme intermediate, synthesizing the amide bond between surface proteins and the pentaglycine cross-bridge. (v) Lipid-linked surface protein is first incorporated into the cell wall via the transglycosylation reaction. The murein pentapeptide subunit with attached surface protein is then cross-linked to other cell wall peptides, generating the mature murein tetrapeptide. Taken from (Mazmanian *et al.*, 2001).

1.2.2.4 Structural classification of cell wall-anchored proteins of *S. aureus*

S. aureus can express more than 20 distinct CWA proteins, the number of which varies between strains (McCarthy & Lindsay, 2010). Historically all CWA proteins which adhere to components of the blood and ECM, such as fibrinogen, fibronectin and collagen were defined as members of the microbial surface component recognising adhesive matrix molecule (MSCRAMM) family (Patti *et al.*, 1994). No classification system existed for the remaining CWA proteins. However, recently the CWA proteins have been redefined and classified into five groups based on specific structural or functional domains (Fig. 1.2, (Foster *et al.*, 2014)).

1.2.2.4.1 The MSCRAMM family

Based on this new method of classification the term MSCRAMM is now used to describe the CWA proteins that possess at least two tandemly-linked IgG-like folded domains in the N-terminal A region (Fig. 1.2A) (Foster *et al.*, 2014).

1.2.2.4.1.1 The Clf/Sdr subfamily of MSCRAMMs

The largest subfamily of the MSCRAMMs is the Clf/Sdr subfamily which comprises the clumping factors A and B (ClfA, ClfB) and the serine aspartate repeat (Sdr) proteins (SdrC, SdrD and SdrE) (Foster *et al.*, 2014). Following the signal sequence, the Clf/Sdr proteins possess a ligand-binding A region which is made up of three independently folded subdomains: N1, N2 and N3 (Fig. 1.2A), where N2 and N3 comprise the IgG-like folded domains (Deivanayagam *et al.*, 2002; Foster & Hook, 1998; Ganesh *et al.*, 2008; Ganesh *et al.*, 2011). The N2 and N3 domains are essential for ligand binding (Ganesh *et al.*, 2011), however the function of the N1 subdomain is unclear. Recent work has identified a short stretch of residues at the border of the N1 and N2 subdomains of ClfA that is essential for the export of the pre-protein so that ClfA is displayed on the bacterial cell surface (McCormack *et al.*, 2014). The Clf/Sdr proteins also possess a flexible unstructured region (R) which connects the N-terminal ligand-binding A domain to the C-terminal SS (Fig. 1.2A). The R domain is composed of serine-aspartate dipeptide repeats and is predicted to function as a stalk to project the A domain away from the surface of the cell (Hartford *et al.*, 1997).

The Sdr proteins possess an additional domain composed of two or more independently folded repeat domains called B_{SDR} domains which are situated between the A region and the serine-aspartate repeats (Fig. 1.2A). The B_{SDR} domains are predicted to function as an additional stalk structure for projecting the A domain away from the cell surface (Josefsson *et al.*, 1998). Glycosylation of the SD repeats of ClfA contributes to the

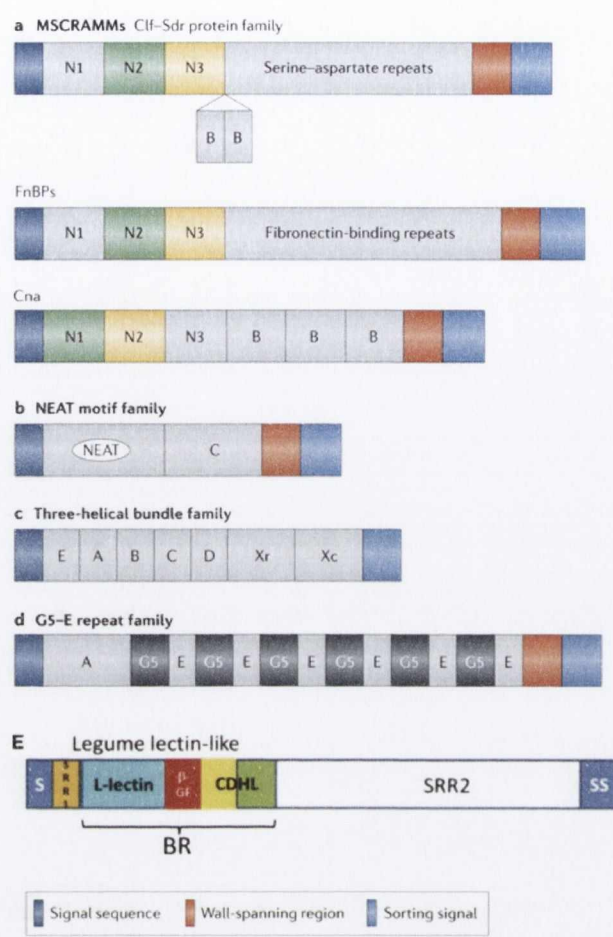


Fig. 1.2 Schematic representation of the common motif organisation of the *S. aureus* CWA proteins. An N-terminal signal sequence directs the pre-protein for secretion via the Sec machinery. The C-terminus is composed of the cell wall-spanning region, or Xc region in the case of the three-helical bundle family, and the sorting signal, which facilitates covalent anchoring of CWA proteins to peptidoglycan. CWA proteins are characterised based on shared structural and functional motifs. The MSCRAMMs (A) are the largest family of CWA proteins and possesses an N-terminal A domain which contains two consecutive IgG-fold like domains (green and yellow boxes). The domain structures of the neat motif family (B), three-helical bundle family (C) G5-E repeat family and (E) legume-lectin family are also shown. All major functions and ligands of the *S. aureus* CWA proteins are outlined in Table 1.1. Adapted from (Foster *et al.*, 2014) and (Speziale *et al.*, 2014).

agglutination of *S. aureus* in human plasma (Thomer *et al.*, 2014). Furthermore, a mutant of *S. aureus* unable to glycosylate the SD repeat proteins had reduced virulence in a mouse model for *S. aureus* bloodstream infection (Thomer *et al.*, 2014).

1.2.2.4.1.2 The fibronectin-binding protein subfamily of MSCRAMMs

The second subfamily of MSCRAMMs is the fibronectin-binding protein (FnBP) family. FnBPA and FnBPB are multifunctional proteins which promote bacterial attachment to fibronectin, fibrinogen and elastin (Keane *et al.*, 2007; Schwarz-Linek *et al.*, 2003), and mediate biofilm formation (McCourt *et al.*, 2014; O'Neill *et al.*, 2008a). Similar to the Clf/Sdr subfamily of MSCRAMMs the FnBPs possess a N-terminal A region composed of three subdomains (Fig. 1.2A). The fibrinogen and elastin binding sites and the residues involved in biofilm formation have been localised to the IgG-like folded N2 and N3 subdomains (Geoghegan *et al.*, 2013; Keane *et al.*, 2007; O'Neill *et al.*, 2008a). The A region of the FnBPs is linked to the SS by a long flexible unstructured repeat region comprised of 11 (FnBPA) or 10 (FnBPB) tandemly repeated motifs which mediate bacterial adherence to type I repeats of fibronectin (Schwarz-Linek *et al.*, 2003).

1.2.2.4.1.3 Collagen binding protein

The final subfamily of the MSCRAMMs is made up by the collagen-binding protein (Cna). Similar to the other subclasses of MSCRAMMs Cna possesses a N-terminal A region composed of three subdomains (Fig. 1.2A). However unlike other MSCRAMMs ligand-binding by Cna is mediated by the N1 and N2 domains rather than the N2 and N3 domains (Zong *et al.*, 2005). The A region of Cna is separated from the SS by a variable number of B_{CNA} repeated domains that differ in sequence from the B_{SDR} domains (Deivanayagam *et al.*, 2000). Cna also does not possess a flexible stalk region linking the B domains and the SS (Fig. 1.2A).

1.2.2.4.2 NEAT family motif

Near iron transporter (NEAT) motif proteins are involved in haem capture from haemoglobin and help bacteria to survive in the host where iron is limited (Mazmanian *et al.*, 2003; Sharp *et al.*, 2007). This family of proteins is comprised of the iron-regulated surface determinant A (IsdA), IsdB, IsdC, and IsdH. These proteins are defined by the presence of one or more central NEAT domains (Fig. 1.2B) which bind to haem (Grigg *et al.*, 2007; Grigg *et al.*, 2010). NEAT domains adopt IgG-like beta-sandwich folds of 7 or more beta-strands in two beta-sheets (Grigg *et al.*, 2010).

1.2.2.4.3 Three helical bundle family

The three helical bundle family (Fig. 1.2C) is comprised of the CWA protein staphylococcal protein A (SpA). Following the signal sequence SpA possesses 4-5 independently folded triple-helical bundles (known as domains EDABC, Fig. 1.2C) (Cedergren *et al.*, 1993; Deisenhofer, 1981) which can bind to several different ligands. The triple helical bundles are linked to the SS by a variable number of octapeptide repeats (Xr) and a predicted cell wall-spanning region (Xc, Fig. 1.2C). Although SpA is the only CWA protein that possesses this structural motif *S. aureus* secretes a number of small immune evasion proteins which also possess domains that fold into triple helical bundles. These are the *S. aureus* binder of IgG (Sbi) protein (Upadhyay *et al.*, 2008), the staphylococcal complement inhibitor (SCIN) and the extracellular fibrinogen-binding protein (Efb) (Lambris *et al.*, 2008). The structure and functions of SpA and Sbi are discussed in greater detail in section 1.2.3.

1.2.2.4.4 The G5-E repeat family

S. aureus surface protein G (SasG) is the prototype of the G5-E domain subfamily of CWA proteins in staphylococci. The G5-E repeat proteins possess a typical domain structure associated with the CWA proteins of *S. aureus* consisting of a signal sequence followed by an A domain. The A domain is linked to the SS by identical G5 (5 conserved glycines) domains in a tandem array separated by 50 residue sequences known as E domains (Conrady *et al.*, 2013; Gruszka *et al.*, 2012) (Fig. 1.2D). The alternation of the G5 and E domains prevents protein misfolding as the E domains form stable interfaces that facilitate the coupling of non-adjacent G5 domains (Gruszka *et al.*, 2015).

SasG is closely related to the accumulation-associated protein (Aap), and both proteins have been shown to promote cell-cell accumulation during biofilm formation via homophilic interactions between proteins on adjacent cells (Geoghegan *et al.*, 2010; Rohde *et al.*, 2005). The removal of the A domain is necessary to facilitate the biological functions of both proteins. This occurs by proteolytic removal in the case of Aap, and limited autocleavage within the G5-E domains of SasG (Geoghegan *et al.*, 2010; Rohde *et al.*, 2005).

1.2.2.4.5 Legume-lectin domain

The serine-rich adhesin of platelets (SraP) is the sole member of the legume-lectin domain family of CWA proteins in *S. aureus*. Following the signal sequence which directs export by the accessory Sec pathway, SraP possesses a short serine-rich repeat region (SSR1), which is followed by the ligand-binding domain (BR, Fig. 1.2E). The BR domain is

comprised of four subdomains: a legume-like lectin domain, a β -grasp fold domain, and two cadherin-like domains (CDHL, Fig. 1.2E). SraP is involved in *S. aureus*-mediated infective endocarditis via its affinity for human platelets (Siboo *et al.*, 2005), and can mediate bacterial adherence and invasion into host epithelial cells by binding to N-acetylneuraminic acid (Yang *et al.*, 2014), which is the predominant sialic acid found on mammalian cells. SraP promotes biofilm formation, through homophilic interactions of CHDL domains on neighbouring cells (Yang *et al.*, 2014).

1.2.2.5 Contribution of cell wall-anchored proteins to virulence

The success of *S. aureus* as a pathogen can largely be attributed to the array of multifunctional CWA proteins that are displayed on the bacterial surface. Many CWA proteins have important roles during infection and there is also a remarkable degree of functional redundancy among these proteins, for example at least five different CWA proteins bind the plasma glycoprotein fibrinogen (Table 1.1). The importance of these proteins in infection has been demonstrated experimentally, for example SpA-deficient mutants of *S. aureus* have been shown to have reduced virulence in mice (Falugi *et al.*, 2013; Gomez *et al.*, 2004; Kim *et al.*, 2012b; Palmqvist *et al.*, 2002; Patel *et al.*, 1987), and *S. aureus* mutants deficient in SpA, FnBPs, ClfA or SasF showed impaired virulence in a murine skin abscess model (Kwiecinski *et al.*, 2014). All known ligands and functions of the CWA proteins of *S. aureus* are outlined in Table 1.1.

1.2.2.5.1 Invasion of epithelial and endothelial cells

S. aureus can survive and proliferate within professional phagocytic cells such as neutrophils (Thwaites & Gant, 2011; Voyich *et al.*, 2005), macrophages (Koziel *et al.*, 2009; O'Keeffe *et al.*, 2015), and dendritic cells (O'Keeffe *et al.*, 2015), which can result in bacterial escape and dissemination throughout the host. *S. aureus* can also invade non-professional phagocytes, e.g. epithelial or endothelial cells, which protects *S. aureus* from detection and attack by the immune system (Peacock *et al.*, 1999; Zapotoczna *et al.*, 2013). FnBPs have been shown to promote bacterial adherence to and internalisation into mammalian cells (Dziewanowska *et al.*, 1999; Peacock *et al.*, 1999; Sinha *et al.*, 1999). FnBP-mediated internalisation of *S. aureus* has been shown *in vitro* and *in vivo* in a mouse model of mastitis (Brouillette *et al.*, 2003) and a rat model of endocarditis (Que *et al.*, 2005).

FnBP-mediated invasion is the best studied mechanism of *S. aureus* internalisation. However, recently other CWA proteins have been identified which can promote bacterial uptake into mammalian cells (Table 1.1). IsdB promotes bacterial adhesion and subsequent

Table 1.1 Cell wall-anchored proteins of *S. aureus*

Protein group	Known ligand	Function	Reference
<i>MSCRAMMs</i>			
Clumping factor A (ClfA)	Fibrinogen γ -chain carboxyl terminus	Adhesion to immobilized fibrinogen; immune evasion by binding soluble fibrinogen	(Deivanayagam <i>et al.</i> , 2002; Ganesh <i>et al.</i> , 2008)
	Complement factor I	Immune evasion; degradation of C3b	(Hair <i>et al.</i> , 2008; Hair <i>et al.</i> , 2010)
Clumping factor B (ClfB)	Fibrinogen α -chain repeat 5, keratin 10 and loricrin	Adhesion to desquamated epithelial cells; nasal colonization	(Ganesh <i>et al.</i> , 2011; Mulcahy <i>et al.</i> , 2012; Walsh <i>et al.</i> , 2004; Wertheim <i>et al.</i> , 2008; Xiang <i>et al.</i> , 2012)
Serine-aspartate repeat protein C (SdrC)	β -neurexin	Unknown	(Barbu <i>et al.</i> , 2010)
	Unknown receptor on desquamated epithelial cells	Adhesion to desquamated epithelial cells. Nasal or skin colonization?	(Corrigan <i>et al.</i> , 2009)
	SdrC	Homophilic SdrC-SdrC interaction. Biofilm accumulation	(Barbu <i>et al.</i> , 2014)
SdrD	Unknown receptor on desquamated epithelial cells	Adhesion to desquamated epithelial cells. Nasal or skin colonization?	(Corrigan <i>et al.</i> , 2009)
SdrE	Complement factor H	Immune evasion; degradation of C3b	(Sharp <i>et al.</i> , 2012)
Bone sialoprotein-binding protein (Bbp, isoform of SdrE)	Fibrinogen α -chain	Adhesion to fibrinogen	(Vazquez <i>et al.</i> , 2011)

Fibronectin-binding proteins A (FnBPA) and B (FnBPB)	Fibrinogen γ -chain carboxyl terminus and elastin. FnBPB A domain also binds fibronectin.	Adhesion to extracellular matrix	(Burke <i>et al.</i> , 2011; Keane <i>et al.</i> , 2007)
	Fibronectin (FnBPA and FnBPB C-terminal repeats)	Adhesion to ECM; invasion	(Peacock <i>et al.</i> , 1999; Sinha <i>et al.</i> , 1999)
	FnBPA	FnBPA-FnBPA domain homophillic interaction. Biofilm formation	(Geoghegan <i>et al.</i> , 2013; Herman-Bausier <i>et al.</i> , 2015)
Collagen adhesin (Cna)	Collagen triple helix	Adhesion to collagen-rich tissue	(Zong <i>et al.</i> , 2005)
	Complement protein C1q	Inhibition of classical pathway of complement activation	(Kang <i>et al.</i> , 2013)
<i>NEAT motif family</i>			
Iron-regulated surface protein A (IsdA)	Haem , fibrinogen , fibronectin, cytokeratin 10, loricrin (N-terminal NEAT-motif region)	Haem uptake and iron acquisition; adhesion to desquamated epithelial cells; nasal colonisation, resistance to lactoferrin	(Clarke <i>et al.</i> , 2004; Clarke <i>et al.</i> , 2006; Clarke & Foster, 2008; Clarke <i>et al.</i> , 2009; Grigg <i>et al.</i> , 2010)
	Unknown ligand (carboxyl terminal domain NEAT motif region)	Resistance to bactericidal lipids and antimicrobial peptides; survival in neutrophils	(Clarke <i>et al.</i> , 2007)
	Haemoglobin, haem (N-terminal NEAT motif region)	Haem uptake and iron acquisition	(Grigg <i>et al.</i> , 2010; Pilpa <i>et al.</i> , 2009)
IsdB	β 3 integrins (NEAT motif regions)	Invasion of non-professional phagocytic cells	(Zapotoczna <i>et al.</i> , 2013)

IsdH	Haem, haemoglobin (N-terminal and/or C-terminal NEAT motif region)	Haem uptake and iron acquisition	(Grigg <i>et al.</i> , 2010)
	Unknown ligand (N-terminal NEAT motif region)	Degradation of C3b	(Visai <i>et al.</i> , 2009)
Three-helical bundle			
Protein A	IgG Fc, IgM Fab VH3 subclass, TNFR1 (three-helical bundle domain)	Inhibition of opsonophagocytosis; B cell superantigen; inflammation	(Deis <i>et al.</i> , 2015; Deisenhofer, 1981; Gomez <i>et al.</i> , 2006; Kim <i>et al.</i> , 2015; Smith <i>et al.</i> , 2011)
	von Willebrand factor (three-helical bundle domain)	Endovascular infection; endocarditis	(Gomez <i>et al.</i> , 2006)
	Unknown ligand (region Xr)	Inflammation	(Martin <i>et al.</i> , 2009)
G5-E domain family			
<i>S. aureus</i> surface protein G (SasG) and plasmin-sensitive surface protein (Pls) (a SasG homologue in MRSA)	Unknown ligand (A domain)	Adhesion to desquamated epithelial cells. Nasal colonisation?	(Corrigan <i>et al.</i> , 2009; Roche <i>et al.</i> , 2003b)
	G5-E repeats (SasG)	Biofilm formation. Homophilic G5-E-G5-E interaction.	(Geoghegan <i>et al.</i> , 2010; Gruszka <i>et al.</i> , 2012)

<i>Legume-lectin domain family</i>			
Serine-rich adhesion for platelets (SraP)	Salivary agglutinin gp340 and N-acetylneuraminic acid	Endocarditis; and endovascular infection	(Kukita <i>et al.</i> , 2013; Yang <i>et al.</i> , 2014)
	SraP cadherin-like domain (CHDL)	Homophilic CHDL-CHDL interaction. Biofilm formation. Projection of L-lectin domain	(Yang <i>et al.</i> , 2014)
<i>Structurally uncharacterized proteins</i>			
Adenosine synthase A (AdsA)	Converts ATP to adenosine	Promotes survival in neutrophils by inhibiting the oxidative burst	(Thammavongsa <i>et al.</i> , 2009)
	2'-deoxyadenosine 3' monophosphate. Degradation product of DNA converted to 2'-deoxyadenosine	Macrophage and monocyte apoptosis	(Thammavongsa <i>et al.</i> , 2013)
	Unknown ligand	Biofilm formation; neutrophil evasion; and inhibition of phagocytosis. Nasal colonisation. Skin infections.	(Li <i>et al.</i> , 2012; Liu <i>et al.</i> , 2015)
<i>S. aureus</i> surface protein X (SasX)			
SasC	Unknown ligand	Promotes primary attachment and accumulation phases of biofilm formation	(Schroeder <i>et al.</i> , 2009)
SasB, SasD, SasF, SasJ, SasK and SasL.	Unknown ligand	Putative CWA proteins identified from genome sequences. No known structure or function	(Roche <i>et al.</i> , 2003a)

Adapted from (Foster *et al.*, 2014)

internalisation into mammalian cells expressing $\beta 3$ -containing integrins (Zapotoczna *et al.*, 2013). SraP is a CWA serine-rich glycoprotein involved in *S. aureus*-mediated infective endocarditis via its affinity for human platelets (Siboo *et al.*, 2005). SraP can mediate bacterial adherence and invasion into host epithelial cells by binding to N-acetylneuraminic acid (Yang *et al.*, 2014), which is the predominant sialic acid found on glycolipids and glycoproteins on the surface of mammalian cells.

1.2.2.5.2 Evasion of immune responses

S. aureus CWA proteins possess a number of functions that mediate the subversion and evasion of the human innate immune response, particularly the actions of the neutrophil which is the key immune effector cell in fighting *S. aureus* infection (Amulic *et al.*, 2012)(Table 1.1). The extensive role of the CWA proteins in assisting with evasion of killing of *S. aureus* by the human neutrophil is outlined in detail in section 1.3.

1.2.2.5.3 Biofilm formation

S. aureus is a leading cause of infections associated with indwelling medical devices such as central venous catheters, cardiovascular devices and artificial joints (O'Gara & Humphreys, 2001; Zimmerli *et al.*, 2004). The ability to form multicellular communities known as biofilm is crucial to success in device-related infection. Attachment to the surface of a biomedical device is the first stage in biofilm formation, which can be mediated by CWA proteins if the device has been coated in host plasma proteins (Vaudaux *et al.*, 1995). Adhesion is followed by bacterial accumulation, which was originally believed to be solely mediated by the *icaADBC*-encoded polysaccharide intercellular adhesin (PIA) (Heilmann *et al.*, 1996), but it is now clear that certain CWA proteins can promote bacterial accumulation in an *ica*-independent manner. This is especially true for HA- and CA-MRSA strains which depend on proteins, rather than polysaccharide, for biofilm formation (Boles *et al.*, 2010; Geoghegan *et al.*, 2013; McCourt *et al.*, 2014; O'Neill *et al.*, 2007; O'Neill *et al.*, 2008b). The CWA proteins that have been shown to mediate biofilm formation are: biofilm-associated protein (Bap) (Cucarella *et al.*, 2001), ClfB (Abraham & Jefferson, 2012), the FnBPs (Geoghegan *et al.*, 2013; McCourt *et al.*, 2014; O'Neill *et al.*, 2008a), SasC (Schroeder *et al.*, 2009), SasG (Corrigan *et al.*, 2007; Geoghegan *et al.*, 2010), SdrC (Barbu *et al.*, 2014), and SraP (Sanchez *et al.*, 2010) (Table 1.1).

1.2.3 Immunoglobulin-binding proteins of Gram-positive bacteria

1.2.3.1 IgG-binding proteins of *S. aureus*

1.2.3.1.1 Protein A

Almost all clinical isolates of *S. aureus* express the major virulence factor, SpA (Votintseva *et al.*, 2014). SpA is an approximately 55 kDa CWA protein which is well known for its ability to bind with high affinity to IgG. SpA contains 4-5, depending on the strain, N-terminal ligand-binding domains (named EDABC, Fig. 1.3A), each of which comprises a single separately folded three helical bundle composed of anti-parallel α -helices (Gouda *et al.*, 1992). Distal to the ligand-binding region is a short polymorphic region (Xr, Fig. 1.3A) which consists of between 1 and 22 octapeptide repeats that vary in number greatly between strains (Moks *et al.*, 1986; Uhlen *et al.*, 1984). Following this is the hydrophobic domain which is predicted to act as a cell wall-spanning region (Xc), which harbours a predicted lysin motif (LysM) motif which has no known function (Fig. 1.3A). The C-terminal SS mediates anchoring of SpA to cell wall peptidoglycan by sortase A (see section 1.2.2.2), and is comprised of an LPETG motif, a hydrophobic membrane-spanning domain (Xc) and, at the extreme C-terminus, a stretch of positively charged residues (+, Fig. 1.3A). The three helical bundles of SpA display a high degree of conformational heterogeneity (Deis *et al.*, 2015) which can facilitate binding to structurally different partners.

SpA is best known for its ability to bind to the Fc region of mammalian IgG (Fc γ) in a non-immune reaction (Deisenhofer, 1981). Other ligands of SpA include the Fab heavy chain of human V_H3 class IgM (Hillson *et al.*, 1993), von Willebrand factor (vWF) (Hartleib *et al.*, 2000; O'Seaghdha *et al.*, 2006) and tumour necrosis factor receptor 1 (TNFR1) (Gomez *et al.*, 2004). The crystal structure of domains B and C of SpA in complex with an Fc fragment has been solved and 11 residues which are present in helices I and II have been implicated in SpA recognition of IgG-Fc (Fig. 1.3B) (Deis *et al.*, 2015; Deisenhofer, 1981). The binding sites of vWF and TNFR1 have been mapped to a region clustered between helices I and II that overlap the Fc-binding domain (Gomez *et al.*, 2006; O'Seaghdha *et al.*, 2006). The crystal structure of SpA domain D in complex with V_H3-Fab showed that the residues involved in this interaction are confined to helices II and III and are distinct from the IgG-Fc binding site (Fig. 1.3B, 1.3C) (Graille *et al.*, 2000). Analysis of the crystal structure of domain C in complex with IgG-Fc highlighted a repression of conformational heterogeneity within the interfacial residues of helices I and II in comparison to the C domain apo structure (Deis *et al.*, 2015). This indicates that SpA cannot bind to different ligands

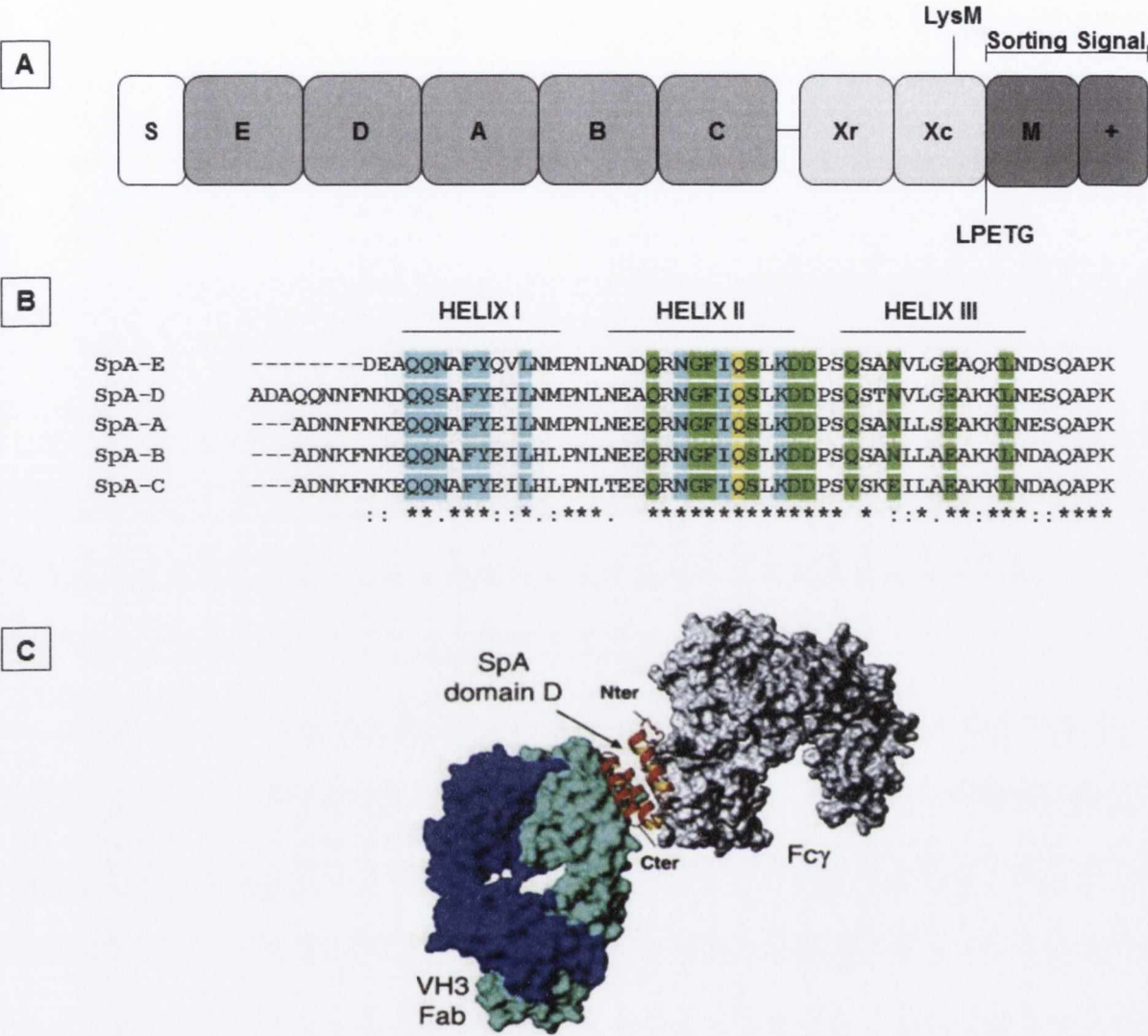


Fig. 1.3 Immunoglobulin-binding protein A. (A) Protein A consists of an N-terminal signal sequence (S) followed by up to 5 IgG-binding domains (E – C), an antigenic variable region (Xr) and a cell wall-spanning region (Xc), which harbours a predicted LysM domain. The sorting signal comprises an LPETG motif, a hydrophobic membrane spanning region (M) and a positively-charged tail region (+). (B) Alignment of amino acid sequence of the 5 SpA IgG-binding domains using ClustalΩ, with positions of α-helices indicated. Residues involved in Fc (blue), Fab (green) or both (yellow) indicated. * indicates positions which have a single, fully conserved residue, : indicates conservation between residues of strongly similar properties, . indicates conservation between residues of weakly similar properties. (C) Model of the SpA D-domain in complex with a V_H3 Fab (cyan surface) and a Fcγ (grey surface) molecule. Model taken from (Graille *et al.*, 2000).

simultaneously via helices I and II. SpA contains an important molecular recognition pocket for IgG-Fc between Q9 of helix 1 and the C-terminus of helix 2. Substitution of Q9 with an aromatic tryptophan residue is predicted to prefill the binding pocket and reduce the affinity of the SpA C domain for IgG-Fc (Deis *et al.*, 2015). In agreement with this substitution of residues Q9 and Q10 with lysines in all five IgG-binding domains significantly reduced the ability of strain Newman to bind IgG (Falugi *et al.*, 2013).

SpA is a potent immune evasion factor of *S. aureus*. SpA-deficient mutants of *S. aureus* have been shown to have reduced virulence in mice (Falugi *et al.*, 2013; Gomez *et al.*, 2004; Kim *et al.*, 2012b; Palmqvist *et al.*, 2002; Patel *et al.*, 1987), and survived poorly in an *ex vivo* human blood survival assay (Smith *et al.*, 2011). SpA is a potent inhibitor of phagocytosis *in vitro* (Atkins *et al.*, 2008; Smith *et al.*, 2011). The interaction of SpA with the Fc region of IgG is predicted to aid in evasion of the host immune system as *S. aureus* expressing a variant of SpA lacking the ability to recognise IgG Fc survives poorly in mouse blood, akin to a SpA-deficient mutant (Falugi *et al.*, 2013). In agreement with this, SpA-specific monoclonal antibodies which neutralise the Fc-binding ability promote opsonophagocytic killing of *S. aureus* in murine and human blood (Kim *et al.*, 2012b). The interaction between SpA and IgG Fc is hypothesised to sequester host IgG on the surface of the bacterium in the opposite orientation to that required for neutrophil recognition of pathogen-bound IgG via its Fc-receptors, and will also interrupt activation of the classical complement pathway by interfering with the interaction of IgG and C1q (Atkins *et al.*, 2008; Gemmell, 1991).

The development of a successful *S. aureus* vaccine has been hindered by the fact that *S. aureus* infections do not generate protective immunity in humans or animals (Kim *et al.*, 2012a) which has been attributed to the ability of SpA to modulate the host adaptive immune responsive. SpA binds specifically to the Fab region of the V_H3 subclass of human IgM, which is the predominant subclass in the mediation of antibody responses to infection and development of immunological memory (Graille *et al.*, 2000). The interaction of SpA with IgM Fab triggers the proliferation and depletion of B cells (Goodyear & Silverman, 2003) suppressing the development of adaptive immune responses. Thus infection with SpA-expressing bacteria does not provide protection against subsequent *S. aureus* infection (Falugi *et al.*, 2013). A study by Kim *et al.* (2010) showed that immunisation of mice with a variant of SpA unable to bind human IgM facilitated the production of protective antibodies against other staphylococcal antigens and the development of immunological protection against

subsequent challenge with highly virulent CA-MRSA. Further work has shown that purified recombinant SpA can promote crosslinking of IgM from humans and guinea pigs via high V_H3 binding activity (Kim *et al.*, 2015). This leads to significant depletion of potential antibody-secreting cells from the spleen and bone marrow (Goodyear & Silverman, 2003) and consequently dampens the adaptive immune response to *S. aureus* infection (Falugi *et al.*, 2013).

Activation and regulation of the TNF α signalling cascade is critically important in the host response to infection (Aggarwal, 2003; Chen & Goeddel, 2002). SpA is a potent activator of TNFR1 (Gomez *et al.*, 2004) which is essential at sites of infection where TNF α signalling is critical, such as the lung. SpA mimics TNF α by activating the TNFR1 proinflammatory signalling cascade and stimulating shedding of TNFR1 from the surface of epithelial cells and macrophages by TNF-converting enzyme which serves to limit TNF- α signalling (Gomez *et al.*, 2004; Gomez *et al.*, 2006; Gomez *et al.*, 2007). In agreement with this the interaction between SpA and TNFR1 has been shown to be essential for pathogenesis in a mouse model of *S. aureus*-mediated pneumonia (Gomez *et al.*, 2004). The interaction between SpA and TNFR1 has been shown using purified recombinant SpA variants incubated with human airway epithelial cells (Gomez *et al.*, 2006), keratinocytes (Classen *et al.*, 2011) and osteoblasts (Claro *et al.*, 2013). Incubation of recombinant SpA with human keratinocytes resulted in the activation of nuclear factor kappa B (NF κ B) followed by increased expression of the pro-inflammatory proteins IL-8 and COX-2 (Classen *et al.*, 2011). The interaction of SpA with TNFR1 has been implicated in the pathogenesis of *S. aureus*-mediated osteomyelitis. Adherence of *S. aureus* to osteoblasts via SpA leads to inhibition of proliferation and subsequent apoptosis of these cells while simultaneously increasing the production of the bone remodelling molecule RANKL (Claro *et al.*, 2011). Similar to its interaction with human keratinocytes, binding of recombinant SpA to TNFR1 on the surface of human osteoblasts stimulated the activation of NF κ B which resulted in the release of IL-6 (Claro *et al.*, 2013).

The hypervariable C-terminal region Xr of SpA triggers inflammation via induction of type I interferon (IFN) signalling in airway epithelial cells, in particular increasing IFN- β transcription (Martin *et al.*, 2009). Recombinant SpA was endocytosed by airway epithelial cells and subsequently induced IFN- β expression, JAK-STAT signaling, and IL-6 production (Martin *et al.*, 2009). Mice that lack the IFN- β receptor, and consequently cannot respond to type I IFN signalling, exhibited increased survival in a lethal pneumonia model of *S. aureus*

(Martin *et al.*, 2009) further indicating that SpA is an important virulence factor in the pathogenesis of *S. aureus*-mediated pneumonia.

Von Willebrand factor (vWF) is a blood glycoprotein involved in haemostasis. The main function of vWF is to capture platelets and direct them to a site of damage in a blood vessel to stimulate the formation of a blood clot. Heritable defects or deficiencies in vWF result in von Willebrand's disease, a common bleeding disorder. Recombinant SpA has been shown to bind to purified vWF in the presence of physiological concentrations of IgG (Hartleib *et al.*, 2000). Both purified full-length SpA and each of the five IgG-binding domains bound to purified vWF (O'Seaghdha *et al.*, 2006), and analysis of this interaction using a number of SpA variants harbouring amino acid substitutions within the IgG-binding domains showed that vWF binds to a region of SpA in helices I and II that overlaps with the IgG-Fc binding site (O'Seaghdha *et al.*, 2006).

SpA is located both on the surface of the bacterium and in the extracellular medium (Becker *et al.*, 2014; Forsgren & Nordstrom, 1974; Lindmark *et al.*, 1977; Movitz, 1976). The existence of released protein A was first shown in the *S. aureus* strain Cowan (Movitz, 1976), where it was measured that released SpA accounts for approximately 30% of total produced SpA in this strain (Movitz, 1976). Released SpA can be detected in the skin lesions of mice infected with a USA300 strain of community-associated methicillin-resistant *S. aureus* (CA-MRSA) and in fluids recovered from patients with *S. aureus* infection (Yung *et al.*, 2011). SpA is shed from the cell envelope of strain Newman into the culture medium following cleavage of the pentaglycine crossbridge of peptidoglycan by the *S. aureus* glycylglycine endopeptidase LytM. The murein hydrolase LytN cleaves the amine bonds between N-acetylmuramic acid and the tetrapeptide side chains of peptidoglycan so that peptidoglycan fragments linked to released SpA lack amino sugars (Becker *et al.*, 2014). This is hypothesized to allow released SpA to avoid activating nucleotide-binding and oligomerization domain-containing protein 2 (NOD2) *in vivo*. The release of SpA is not completely inhibited in a *lytM*-deficient mutant of strain Newman, indicating that LytM activity is not the only factor involved in the release of SpA. A definitive role for released SpA in the virulence of *S. aureus* during infection has not yet been shown.

1.2.3.1.2 Sbi

Sbi is a 50 kDa protein that can be found in the extracellular medium or linked to the cell membrane by binding lipoteichoic acid (LTA) (Smith *et al.*, 2011) but is not a CWA

protein as it does not possess a C-terminal SS (Zhang *et al.*, 1998). Sbi is comprised of a signal sequence followed by four N-terminal domains linked to a C-terminal proline-rich cell wall spanning region and a tyrosine-rich region which is necessary for the association of Sbi with the cell membrane (Smith *et al.*, 2012). Positioned at the N-terminus of Sbi are two IgG-binding domains folded as independent three helical bundles with sequence similarity to the IgG-binding domains of SpA. However, unlike SpA these domains have no affinity for V_H3-Fab (Atkins *et al.*, 2008). The D1 and D2 domains are predicted to interfere with C1q binding to IgG thus hindering activation of the classical complement pathway (Atkins *et al.*, 2008; Zhang *et al.*, 1998). In agreement with this a Sbi-deficient mutant of *S. aureus* survived poorly in human blood compared to wild-type bacteria (Smith *et al.*, 2011). The D3 and D4 domains associate with the human complement components C3 and Factor H to form tripartite complexes that inhibit activation of the alternative complement pathway (Haupt *et al.*, 2008; Upadhyay *et al.*, 2008).

1.2.3.1.3 Staphylococcal superantigen like proteins

The staphylococcal superantigen-like (SSL) proteins are a group of released proteins that share structural similarity with the classical superantigens (Arcus *et al.*, 2002; Langley *et al.*, 2010). SSL7 has been shown to interfere with IgA binding to Fc α RI, the production of C5a, and the oxidative burst of phagocytes *in vitro* (Bestebroer *et al.*, 2010). SSL10 binds to human IgG₁, but not to immunoglobulins of lower vertebrates. This interaction inhibits IgG₁ binding to Fc γ receptors on the surface of neutrophils thereby interfering with the phagocytic uptake of IgG₁-opsonized bacteria *in vitro* (Itoh *et al.*, 2013; Patel *et al.*, 2010).

1.2.3.2 Streptococcal IgG-binding proteins

Streptococcus pyogenes is a Gram-positive, catalase-negative organism. It is the sole species of Lancefield group A and is often referred to as group A *Streptococcus* (GAS) as it displays streptococcal group A antigen on its cell surface. An estimated 700 million GAS infections occur worldwide annually and while the overall mortality rate for these infections is 0.1% over 650,000 represent severe and invasive disease and have a mortality rate of 25% (Aziz *et al.*, 2010). Members of the M family of proteins, of which there are more than 80 different subtypes, dominate the cell surface of GAS isolates. M proteins are elongated dimeric alpha-helical coil-coiled CWA proteins that can bind a variety of host components, including immunoglobulins (Akerstrom *et al.*, 1991; Boyle *et al.*, 1994; Frick *et al.*, 1994). M proteins, despite a lack of sequence homology to the IgG-binding domains of SpA, bind to the same region of IgG-Fc (Frick *et al.*, 1992). Some M proteins have been

shown to bind human IgG subclasses IgG₁, IgG₂ and IgG₄, whereas others only display affinity for IgG₃. Only a small number of M proteins possess the ability to bind to all four subclasses of human IgG (Boyle *et al.*, 1994). The ability of the M proteins to bind to IgG-Fc has been suggested to inversely correlate with the concentration of environmental IgG. Protein H, a member of the M protein family, preferentially bound IgG-Fc following incubation of bacteria in human saliva, which has a low concentration of soluble IgG, whereas IgG-Fc binding was abolished following incubation in human serum (Nordenfelt *et al.*, 2012).

Protein G is a CWA IgG-binding protein expressed by group C and group G streptococci. Similar to the M proteins of *S. pyogenes* protein G interacts with the same domains of human IgG-Fc as SpA despite a lack of structural homology (Frick *et al.*, 1992; Guss *et al.*, 1986). Protein G possesses 2-3 repeating IgG-binding domains which recognise all four subclasses of human IgG (Akerstrom *et al.*, 1985; Bjorck & Kronvall, 1984) and also mediate binding to α 2-macroglobulin, a major protease inhibitor of human plasma (Godehardt *et al.*, 2004). Similar to SpA protein G can also interact with the Fab region of immunoglobulins. However this interaction is weak and only equates to approximately 10% of its affinity for IgG-Fc (Bjorck & Kronvall, 1984). The non-immune accumulation of IgG on the surface of group G streptococci via protein G-mediated interactions has been shown to reduce the accumulation of C1q on the bacterial surface which consequently prevents activation of the classical complement pathway (Nitsche-Schmitz *et al.*, 2007).

Peptostreptococcus magnus, a Gram-positive anaerobe, is a commensal of humans that can cause a variety of diseases including vaginosis and urethritis. Approximately 10% of *P. magnus* clinical isolates express a unique IgG-binding protein, protein L (Kastern *et al.*, 1992). Protein L, unlike many other Gram-positive IgG-binding proteins which bind the Fc fragment of IgG, has the unusual ability to bind the variable portions of IgG kappa light chains (Bjorck, 1988). The role of protein L in the pathogenesis of *P. magnus* infections is unclear. However one study has implicated protein L in the activation of human basophils and mast cells resulting in the release of histamines (Patella *et al.*, 1990).

1.2.4 Extracellular proteases

S. aureus can secrete up to 10 different proteolytic enzymes into the culture supernatant (Kolar *et al.*, 2013). These are the metalloprotease aureolysin (Aur), the serine protease V8 (or SspA), two cysteine proteases (staphopain A (ScpA) and staphopain B

(SspB)), and six serine-like proteases that are SspA homologues (SplABCDEF) (Reed *et al.*, 2001; Shaw *et al.*, 2004). While the six Spl enzymes are active upon secretion, the Aur, SspA, SspB, and ScpA proteases are produced as zymogens. The Aur and ScpA zymogens auto-activate outside the cell (Nickerson *et al.*, 2010; Nickerson *et al.*, 2008), V8 is enzymatically processed into its mature form by the mature form of Aur (Drapeau, 1978; Lindsay & Foster, 1999), and the cysteine protease SspB, which is encoded within the same operon as the *spsA* gene, is enzymatically processed by mature V8 (Chan & Foster, 1998; Rice *et al.*, 2001). Encoded directly downstream of each staphopain gene is a staphopain inhibitory protein, termed staphostatin. Each staphostatin interacts specifically with its cognate staphopain and together these proteins form a stable non-covalent complex (Rzychon *et al.*, 2003). These interactions are predicted to be required to protect staphylococcal cytoplasmic proteins from being degraded by prematurely activated or folded prostaphopains (Rzychon *et al.*, 2003). However, it has been shown that the proSspB precursor can be secreted and activated without detriment to *S. aureus* in the absence of its cognate staphostatin (Nickerson *et al.*, 2010).

Secreted proteases have been shown to be required for growth of USA300 strains of *S. aureus* in peptide-rich environments, serum, and in the presence of anti-microbial peptides (AMPs) (Kolar *et al.*, 2013) and a protease-deficient mutant of USA300 was more readily phagocytosed by human leukocytes (Kolar *et al.*, 2013). The staphylococcal accessory regulator (Sar) negatively regulates the expression of the extracellular proteases in the USA300 strain LAC (Zielinska *et al.*, 2012). A SarA-deficient mutant of LAC displayed increased expression of all 10 extracellular proteases and was attenuated in murine models of implant-associated biofilm infection and *S. aureus* bacteraemia (Zielinska *et al.*, 2012). Attenuation was reversed following the elimination of all 10 extracellular proteases (Zielinska *et al.*, 2012), indicating that extracellular proteases limit the accumulation of *S. aureus* virulence factors. Aur reduces the abundance of the secreted phenol-soluble modulins (PSMs) in strain LAC (Cassat *et al.*, 2013). Analysis of the proteome of a protease-deficient mutant of USA300 showed an increased abundance of both secreted (e.g. α -toxin, PSMs, Sbi) and CWA proteins (e.g. ClfA, FnbB, IsdA), which was reflected by the hypervirulence of the protease-deficient mutant in the context of mortality in a murine model of infection (Kolar *et al.*, 2013).

The extracellular proteases have also been shown to inhibit the formation of *S. aureus* protein-dependent biofilm (Lauderdale *et al.*, 2009; Loughran *et al.*, 2014; Marti *et*

al., 2010), presumably via their function in modulating the stability of a number of CWA proteins (Zielinska *et al.*, 2012). Furthermore it has been shown that recombinant Aur, ScpA and SspA can mediate the dispersal of established biofilms formed by the USA300 strain LAC (Loughran *et al.*, 2014).

1.3 Immune response to bacterial infection

Neutrophils, as circulating cells, are first alerted to the presence of an invading pathogen by chemical signals produced by activated host cells, such as leukocytes, or derived from bacterial activation of the human complement system (Kolaczowska & Kubes, 2013). These signals produce a chemical gradient which induces chemotaxis and diapedesis of neutrophils from the bloodstream to the site of infection (Fig. 1.4). Upon arrival at the site of infection the first step in the process of phagocytosis, i.e. the ingestion of bacterial cells by neutrophils, is bacterial recognition. Neutrophils can directly recognise a number of secreted and surface-associated bacterial molecules known as microbial-associated molecular patterns (MAMPs) and these are directly recognised by structures displayed on the surface of neutrophils known as pattern-recognition receptors (PRRs) (Bloes *et al.*, 2015). For more specific, and effective initiation of phagocytosis, neutrophils require the decoration of the bacterial surface with opsonins that are recognised by specific receptors on the neutrophil cell surface. The two major opsonin components of human serum are immunoglobulins (Ig) and complement (Fig. 1.4). As most humans from early life have been exposed to *S. aureus*, commonly without the development of infection or disease, we have naturally developed specific IgG which recognise *S. aureus* antigens, most of which are directed against components of the cell surface, e.g. peptidoglycan, wall teichoic acid (WTA), and CWA proteins. A study in 2005 demonstrated the presence of specific IgG to a number of CWA proteins, as well as other surface-associated and secreted antigens, circulating in the blood of healthy volunteers (Dryla *et al.*, 2005). Neutrophils possess a number of receptors which recognise pathogen-bound IgG, specifically the Fc region which is extended out from the antigen-IgG interface, called Fc-gamma receptors (FcγRs), which induces the activation of phagocytosis (Fig. 1.4). The activation of the classical complement pathway is also dependent on the presence of specific antibodies (IgG or IgM) on the bacterial surface, thus immunoglobulins are important for the clearance of invading pathogens by neutrophils. The role of the complement pathways in response to bacterial infection is discussed further in section 1.4.

Upon phagocytic uptake bacteria are transported to an internal compartment called the phagosome (Fig. 1.4). Neutrophils are end-stage cells which store most of their antimicrobial compounds and peptides in granules (Borregaard & Cowland, 1997). The next step within the neutrophil involves the fusion of the phagosome with different granule subtypes, which can be peroxidase positive or negative, resulting in the release of granule content which has strong bactericidal properties (Nordenfelt & Tapper, 2011). Simultaneously, the phagosome undergoes a strong oxidative burst which is initiated by NADPH-dependent oxidases, leading to the generation of highly toxic reactive oxygen species (ROS), which acts together with reactive nitrogen species to damage cells, causing nitrosative stress. Neutrophils have also evolved a strategy to kill extracellular bacteria by the release of neutrophil extracellular traps (NETs) in a process termed NETosis (Fig. 1.4). Here, nuclear contents along with antimicrobial peptides (AMPs) and granular peptides are expelled and can together trap and kill extracellular bacteria, as well as inhibiting further bacterial dissemination (Brinkmann *et al.*, 2004; McDonald *et al.*, 2012). The concentration of AMPs in the bloodstream alone is too low for effective microbial killing. However, in association with NETs bactericidal concentrations can be reached (Brinkmann *et al.*, 2004).

1.4 Complement

The complement system is a chief component of the innate immune system and is also involved in the initiation of the adaptive immune response (Carroll, 2004; Song *et al.*, 2000). It can rapidly recognise and opsonise bacteria to facilitate phagocytosis by professional phagocytes, or can mediate bacterial killing directly by membrane perturbations (Tegla *et al.*, 2011). The complement system is composed of a family of more than 30 proteins and their cleavage products present in host plasma and on cells. The complement system acts via three separate pathways that differ in their mode of recognition, but all converge at the same step: the cleavage of the central complement component C3 (Walport, 2001a; Walport, 2001b). The alternative and lectin pathways are components of innate immunity whereas the classical pathway is activated following the specific interactions with antibodies that have bound to antigens on the bacterial surface.

The activation of the classical complement pathway is dependent on the presence of specific antibodies (IgG or IgM) on the bacterial surface. Classical pathway activation occurs via the binding of the complement molecule C1q to surface-bound IgG/IgM (Duncan & Winter, 1988), which activates two serine proteases, C1r and C1s, in complex with C1q. This process triggers a complement cascade which ultimately generates the classical pathway C3

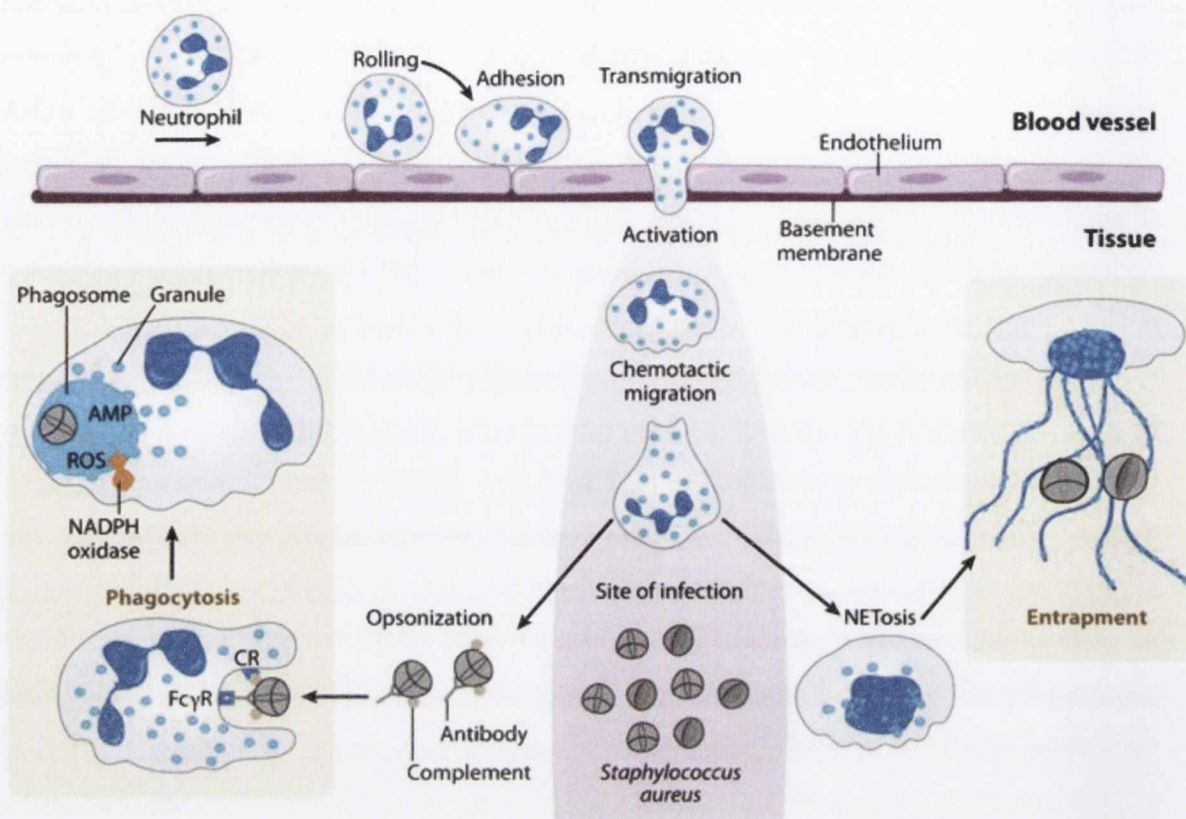


Fig. 1.4 Neutrophil extravasation to the site of infection and killing mechanisms.

Recruitment of neutrophils begins with the activation of the endothelium, followed by rolling on the activated endothelium. Upon stimulation, neutrophils stop rolling and firmly adhere to the endothelium. This step is followed by neutrophil transmigration through the endothelium into the tissue. A chemotactic gradient directs neutrophils toward the invading staphylococci, followed by recognition and subsequent phagocytosis. Phagocytosis of *S. aureus* is dependent on the deposition of complement and immunoglobulins, which are recognized by complement receptors (CRs) and Fcγ receptors (FcγRs). Inside the neutrophil phagosome, bacteria are killed by antimicrobial proteins (AMPs) released upon granule fusion and by NADPH oxidase-derived reactive oxygen species (ROS). Alternatively, neutrophils undergo a process called NETosis, in which neutrophils expel their DNA. The DNA is decorated with histones and AMPs that extracellularly capture and kill bacteria. Taken from (Spaan *et al.*, 2013).

convertase C4b2a, which cleaves C3 (Walport, 2001b). The deposition of C3 degradation products, which are recognised by complement receptors (CR, Fig. 1.4) on the neutrophil cell surface, is a pivotal step in the efficient initiation of neutrophil phagocytosis.

The lectin pathway is highly analogous to the classical pathway, and its activation also results in formation of the C3 convertase C4b2a (Matsushita & Fujita, 2001). However, the method of microbial recognition differentiates the two pathways. The recognition molecules for the lectin pathway, mannose-binding lectin (MBL) and ficolin (Fujita *et al.*, 2004), recognise microbial sugars instead of immune complexes (Thiel, 2007). Soluble MBL and ficolins are associated with several proteases called MBL-associated serine proteases (MASPs) which are structurally similar to C1q-associated serine proteases C1r and C1s (Matsushita *et al.*, 2000). MASP-2 cleaves C4 and C2 to generate the C4b2a.

The alternative pathway (AP) mainly functions as an amplification loop of the classical and lectin pathways after surface bound C3b is generated. However, the AP can also undergo spontaneous activation by hydrolysis of the internal thioester bond in C3 (Sahu & Lambris, 2001) generating C3H₂O which possesses a molecular conformation similar to C3b. Similar to particle-bound C3b, C3H₂O can participate in the generation of the AP C3 convertase C3bBb (Xu *et al.*, 2001).

1.5 Immune evasion by *S. aureus*

Neutrophils make up 60% of the leukocyte population in human blood and are the most important phagocytic cells for killing *S. aureus*. Patients that suffer from neutropenia or other congenital neutrophil deficiencies are at severe risk of bacterial infections that can often be fatal (Amulic *et al.*, 2012), and patients suffering from these disorders commonly develop *S. aureus* infections (Bogomolski-Yahalom & Matzner, 1995).

1.5.1 Inhibition of neutrophil migration

The first stage of neutrophil reaction to *S. aureus* invasion is extravasation from blood vessels into tissues and the site of infection. This action is triggered by a number of chemical signals such as chemokines produced by host cells, peptides secreted by bacterial cells or complement components. For example, C5a and N-formylated proteins are recognised by the surface-localised neutrophil receptors C5aR and human formyl-peptide receptor 1 (FPR1), respectively. The chemotaxis inhibitory protein of *S. aureus* (CHIPS) can bind to both of these receptors, blocking the cognate agonist from binding and inhibiting neutrophil migration (Postma *et al.*, 2004). A related protein, FLIPr, is also secreted by *S. aureus*.

FLIPr binds to FPR1 on the surface of neutrophils inhibiting recognition of FPR-like agonists (Prat *et al.*, 2006).

In order to exit the bloodstream neutrophils initiate a process known as neutrophil rolling. This process relies on the interaction between the endothelial cell receptor P-selectin and its corresponding ligand, P-selectin glycoprotein ligand 1 (PSGL1), which is present on the neutrophil surface (Moore *et al.*, 1995). *S. aureus* secretes a number of staphylococcal superantigen-like (SSL) proteins that interfere with neutrophil migration. Both SSL5 and SSL11 bind to PSGL1 on the surface of leukocytes, and have been shown to interfere with the binding of neutrophils to P-selectin and therefore disrupt adhesion and rolling (Bestebroer *et al.*, 2007; Chung *et al.*, 2007). Recently it has been shown that SEIX, a staphylococcal superantigen-like protein, also binds to PSGL1, and that the SSL6 protein binds to CD47, a receptor present on most host tissues that promotes migration, inhibits phagocytosis and proliferation of neutrophils (Fevre *et al.*, 2014).

The second step in neutrophil extravasation is the firm adhesion of neutrophils to the endothelium. The β 2-integrin Mac-1 and the leukocyte function associated antigen-1 (LFA-1) present on the surface of leukocytes mediate adhesion to the counter-receptor intercellular adhesion molecule-1 (ICAM-1) shortly before transmigration. The *S. aureus* extracellular adherence protein Eap recognises and adheres to ICAM-1, inhibiting leukocyte adhesion and transmigration (Chavakis *et al.*, 2002). The extracellular protease ScpA interferes with neutrophil migration by the enzymatic cleavage of CXCR2 so that it cannot bind to chemokines (Laarman *et al.*, 2012).

1.5.2 Inhibition of neutrophil phagocytosis

1.5.2.1 Capsule

S. aureus has also evolved a number of strategies to evade opsonophagocytosis by human neutrophils, and many of the effector molecules are components of the cell envelope. Up to 50% of *S. aureus* clinical isolates possess genes to produce one of the two types, type 5 or type 8, of capsular polysaccharide (O'Riordan & Lee, 2004). However the USA300 lineage possess four conserved mutations in the *cap5* locus which abrogates capsule production (Boyle-Vavra *et al.*, 2015). Production of a capsule is associated with increased virulence in animal infection models (Thakker *et al.*, 1998) and has been shown to protect *S. aureus* from phagocytosis *in vitro* (O'Riordan & Lee, 2004). Production of a capsule hinders

opsonisation by inhibiting antibody recognition of *S. aureus* surface components (Thakker *et al.*, 1998).

1.5.2.2 Cell wall-anchored proteins

ClfA can protect *S. aureus* from phagocytosis via its affinity for human fibrinogen and fibrin. Fibrinogen-coated bacteria are less likely to be killed by neutrophils and ClfA-expressing bacteria show an increased survival in a septicaemia model, which is dependent on fibrinogen binding (Flick *et al.*, 2013). ClfA is also involved in the agglutination of *S. aureus*, which reduces phagocytosis. The two secreted staphylococcal coagulases: coagulase (Coa) and von Willebrand factor-binding protein (vWbp) cleave host fibrinogen into fibrin fibrils (Friedrich *et al.*, 2003) which are recognised by CWA ClfA (McAdow *et al.*, 2011). In this way ClfA, Coa and vWbp act synergistically to protect *S. aureus* from phagocytosis (Ganesh *et al.*, 2008; McAdow *et al.*, 2011). Four other CWA proteins display functional redundancy in the agglutination pathway through their affinity for fibrinogen or fibrin: ClfB, the FnBPs, and bone sialoprotein binding protein (Bbp), all of which can influence the pathogenesis of *S. aureus* during bloodstream infection (Edwards *et al.*, 2010). ClfA also binds the complement regulator factor I and acts as a cofactor to trigger cleavage of C3b to its inactive form iC3b (Hair *et al.*, 2008; Hair *et al.*, 2010).

SdrE has been shown to bind and recruit complement regulatory factor H to the *S. aureus* cell surface (Sharp *et al.*, 2012), and SdrE-bound factor H exhibits cofactor functionality for factor I-mediated cleavage of C3b to the inactive iC3b. SdrE has also been implicated in the recruitment of C4b-binding protein (C4BP), a host molecule involved in the inhibition of classical pathway activation, to the bacterial cell surface (Hair *et al.*, 2013).

The collagen-binding protein, Cna, of *S. aureus* has been shown to inhibit activation of the classical complement pathway by binding to the complement molecule C1q (Kang *et al.*, 2013). C1q is comprised of six identical heterotrimeric proteins, each dominated by a collagen-like domain forming a characteristic triple helix structure. The C1r and C1s serine proteases associate to form the heterotetramer C1r2-C1s2 which binds to the individual collagenous stems of C1q (Bally *et al.*, 2009). Binding of C1q by Cna displaces C1r2-C1s2 deactivating C1 and inhibiting the activation of the C3 convertase by the classical complement pathway (Kang *et al.*, 2013).

CWA SpA also interferes with phagocytic uptake of *S. aureus* (see section 1.2.3.1.1).

1.5.2.3 Secreted proteins

S. aureus secretes several proteins that interfere with the deposition of complement on the bacterial surface. Aureolysin can block activation of all three complement pathways by cleaving soluble C3 to generate C3a and C3b. Soluble C3b is degraded or sequestered by complement regulatory factor I and factor H preventing its deposition on the staphylococcal surface (Laarman *et al.*, 2011), thus inhibiting complement-mediated phagocytosis.

The staphylococcal complement inhibitor (SCIN) is a small secreted protein of *S. aureus* (Rooijakkers *et al.*, 2005; Rooijakkers *et al.*, 2006). The genes encoding SCIN (*scn*) are located on a bacteriophage together with the genes encoding CHIPs (*chp*) and staphylokinase (*sak*) (van Wamel *et al.*, 2006). SCIN binds to and inhibits C3 convertases of the classical and alternative pathways, inhibiting the production and deposition of C3a, C3b and C5a, and thereby interfering with complement activation (Rooijakkers *et al.*, 2005). SCIN both stabilises C3bBb at the surface of the bacterium and impairs the enzymatic activity of the convertases (Rooijakkers *et al.*, 2005). Two homologs of SCIN, designated SCIN-B and SCIN-C, are expressed by many *S. aureus* clinical isolates and, similar to SCIN, specifically associate with human C3 convertases, interfere with complement-mediated phagocytosis and the activation of all three complement pathways (Jongerijs *et al.*, 2007).

Staphylokinase (SAK) is a secreted protein of *S. aureus* with potent anti-opsonic activity. SAK forms enzymatically active complexes with the host serum protein plasminogen on the bacterial surface, which results in the generation of plasmin (Collen, 1998), a potent serine protease. Plasmin cleaves human IgG as well as human C3b and iC3b from the bacterial surface leading to reduced neutrophil phagocytosis (Rooijakkers *et al.*, 2005). Plasmin removes the entire Fc fragment of surface-bound IgG thereby inhibiting bacterial recognition by neutrophil Fc-receptors and activation of the classical complement pathway (Rooijakkers *et al.*, 2005).

The extracellular fibrinogen-binding (Efb) protein and the extracellular complement-binding (Ecb) protein are small secreted proteins of *S. aureus* that can modulate the alternative pathway convertase by directly binding to the C3 molecule (Jongerijs *et al.*, 2010a). The crystal structure of both proteins in complex with C3 has been solved and revealed that both Efb and Ecb bind to the C3d thioester-containing domain of C3 (Hammel *et al.*, 2007). In a murine pneumonia infection model Efb and Ecb together promoted bacterial survival and blocked neutrophil influx into the lung (Jongerijs *et al.*, 2012). Efb attracts fibrinogen to the surface of C3b-coated *S. aureus*, and the resulting fibrinogen layer

prevents neutrophil recognition of surface-bound antibodies and C3b, thereby interfering with phagocytosis (Ko *et al.*, 2013).

The neutrophil serine proteases (NSPs) have a number of important roles in host defence against *S. aureus* infection, including generation of NETs (Papayannopoulos *et al.*, 2010), modulation of bacterial virulence factors (Weinrauch *et al.*, 2002), and regulating host responses by cleaving chemokines and receptors (Korkmaz *et al.*, 2010). Eap, and the Eap-homologue proteins (EapH) 1 and 2, specifically inhibit the functions of NSPs (Stapels *et al.*, 2014). A significant decrease in bacterial load was measured for *S. aureus* mutants deficient in all three Eap proteins, in comparison to wild-type bacteria, in a murine liver-abscess model (Stapels *et al.*, 2014).

1.5.2.4 Cytolytic pore-forming toxins

By secreting cytolytic toxins, *S. aureus* protects itself against killing by the host immune system both before and after engulfment by neutrophils. Beta (β)-barrel pore-forming toxins target the cell membrane and induce leakage and ultimately lysis of eukaryotic cells. The major properties of the cytolytic pore-forming toxins of *S. aureus* are outlined in Table 1.2.

1.5.2.4.1 Alpha-haemolysin

The single-component toxin alpha-haemolysin (Hla; or α -toxin) encoded by the *hla* gene is the archetypal cytolytic toxin produced by *S. aureus* (Bhakdi & Tranum-Jensen, 1991). Hla is secreted as a monomer but oligomerises into a heptameric pre-pore structure at the plasma membrane of target cells followed by formation of the β -barrel pore (Bhakdi & Tranum-Jensen, 1991). Hla is targeted to cell membranes by recognition of a disintegrin and metalloprotease 10 (ADAM10) (Wilke & Bubeck Wardenburg, 2010) which is expressed by certain eukaryotic cells. The interaction between Hla and ADAM10 is necessary to initiate the sequence of events that facilitate Hla pore formation (Wilke & Bubeck Wardenburg, 2010), and is essential for Hla cytolytic activity (Inoshima *et al.*, 2011). Although Hla is not cytotoxic to neutrophils, it mediates lysis of other immune cells such as monocytes and lymphocytes (Nygaard *et al.*, 2012; Valeva *et al.*, 1997).

1.5.2.4.2 Bi-component pore-forming toxins

The second class of *S. aureus* cytolytic toxins are the bi-component pore-forming toxins which are comprised of up to five leukocidins: the Pantón–Valentine leukocidin (LukSF-PV), leukotoxin ED (LukED), gamma hemolysin (which exists as two toxins: HlgAB

and HlgCB) and leukotoxin AB (LukAB, also known as LukHG) (Alonzo & Torres, 2014). One additional toxin, LukMF, is present in lineages of *S. aureus* isolated from ruminants and other mammals, but not humans. The leucocidins are comprised of two secreted monomeric water-soluble proteins denoted as the S and F subunits. Binding of the S component to its cellular receptor triggers the formation of an octameric heterodimer pore composed of an alternating arrangement of four S and four F subunits (Jayasinghe & Bayley, 2005). Additional structural evidence in support of an octameric pore structure was provided with the description of the crystal structure of a HlgAB pore (Yamashita *et al.*, 2011).

1.5.2.4.2.1 PVL

PVL is encoded by the genes *lukF-PV* and *lukS-PV*, which are carried on a prophage (ϕ Sa2, Table 1.2), and is highly prevalent among CA-MRSA strains worldwide (Monecke *et al.*, 2011). PVL mediates lysis of human and rabbit neutrophils via binding of the complement receptors C5aR and C5L2 (Spaan *et al.*, 2013a). The activity of PVL, however, demonstrates strict species specificity as PVL does not lyse either murine or simian neutrophils (Loffler *et al.*, 2010), which historically led to much debate over the function of PVL. This specificity has been attributed to species-specific variations in C5aR (Spaan *et al.*, 2013a). In rabbit models of necrotising pneumonia PVL has been shown to dramatically increase proinflammatory responses in the lung by mediating the release of neutrophil cytotoxic granular contents (Diep *et al.*, 2010).

1.5.2.4.2 2 Gamma-haemolysin

The expression of the gamma-haemolysin toxins Hlg AB and CB is significantly upregulated when the CA-MRSA strain USA300 is incubated in human blood (Malachowa *et al.*, 2011). In *ex vivo* whole-blood survival assays a HglABC-deficient mutant of *S. aureus* is more susceptible to bacterial killing in comparison to wild-type bacteria (Malachowa & DeLeo, 2011). Both purified HlgAB and HlgCB promote lysis of human neutrophils, monocytes, and macrophages, but display weaker activity towards murine and rabbit immune cells (Malachowa & DeLeo, 2011). HlgAB binds to the chemokine receptors CXCR1, CXCR2 and CC-chemokine receptor 2 (CCR2), whereas HlgCB recognises the complement receptors C5aR and C5L2 to initiate pore-formation on target cells (Spaan *et al.*, 2014). HlgAB, but not HlgCB, can also lyse human red blood cells (Malachowa & DeLeo, 2011), and the host receptor that mediates this function has recently been identified as the Duffy antigen receptor for chemokines (DARC) (Spaan *et al.*, 2015).

1.5.2.4.2.3 LukAB

LukAB (also known as LukGH) mediates lysis of neutrophils by binding the complement receptor CD11b (DuMont *et al.*, 2013a), and similar to PVL displays strict species specificity towards human and rabbit neutrophils (Malachowa *et al.*, 2013). Specifically, the I domain of CD11b has been shown to mediate the species-specific activity of LukAB (DuMont *et al.*, 2013a). Unlike the other bi-component toxins which exist as monomers in solution, soluble LukAB exists as a heterodimer (DuMont *et al.*, 2014). Recombinant heterodimeric LukAB displayed increased toxicity towards human neutrophils in comparison to when both subunits were added as monomers (DuMont *et al.*, 2014).

LukAB is required for survival of USA300 from bacterial killing in human blood and contributes to *S. aureus*-mediated killing of human neutrophils in *ex vivo* infection models (Dumont *et al.*, 2011). Lysis of neutrophils by LukAB has been shown to facilitate the escape of intracellular bacteria, enabling extracellular replication of *S. aureus* (DuMont *et al.*, 2013b).

1.5.2.4.2.4 LukED

LukED is one of the only leucocidins to exhibit broad activity on a wide variety of cell types from various species, including mice, which has greatly facilitated the studies of LukED function *in vivo* (Alonzo *et al.*, 2012; Morinaga *et al.*, 2003). LukED targets the chemokine receptors CCR5, CXCR1 and CXCR2 (Alonzo *et al.*, 2013; Reyes-Robles *et al.*, 2013), and a LukED-deficient mutant of *S. aureus* displayed reduced virulence in a murine model of systemic infection (Alonzo *et al.*, 2012). Similar to HlgAB, LukED also binds and lyses human erythrocytes via recognition of DARC (Spaan *et al.*, 2015).

Recently it has been shown that LukED and PVL antagonise each other's lytic activity via the formation of inactive complexes (Yoong & Torres, 2015). Recombinant LukED reduced PVL-mediated lysis of human neutrophils in a dose-dependent manner, and purified PVL inhibited LukED-mediated lysis of murine leukocytes and erythrocytes (Yoong & Torres, 2015). In agreement with this, expression of PVL in the MSSA strain Newman reduced the virulence and mortality of this strain in mice (Yoong & Torres, 2015).

1.5.2.4.3 Phenol-soluble modulins

Phenol-soluble modulins (PSMs) are a family of short formyl-peptides that are secreted by most staphylococci, and interfere with the physiological functions of immune cells (Peschel & Otto, 2013). All PSMs share an amphipathic, α -helical structure and the two

Table 1.2 *S. aureus* cytolytic pore-forming toxins

Name	Genome location	Cell type targeted	Receptor	Function	Species Specificity	Reference
Hla	Core	Erythrocytes, monocytes, lymphocytes	ADAM10	Cell lysis	Yes; toxic to human and rabbit erythrocytes,	(Nyggaard <i>et al.</i> , 2012; Wilke & Bubeck Wardenburg, 2010)
PVL	Prophage; ϕ Sa2	Monocytes, neutrophils, macrophages	C5aR, C5L2	Neutrophil lysis	Yes; human and rabbit immune cells only.	(Löffler <i>et al.</i> , 2010; Spaan <i>et al.</i> , 2013)
HlgAB	Core	Erythrocytes, monocytes, macrophages, T cells, neutrophils	DARC, CXCR1, CXCR2	Neutrophil lysis, phagosome escape	Unclear; toxic to immune cells of rabbits, humans, murine erythrocytes	(Spaan <i>et al.</i> , 2014; Spaan <i>et al.</i> , 2015)
HlgCB	Core	Monocytes, macrophages, neutrophils	C5aR, C5L2	Neutrophil lysis	Unclear; toxic to immune cells of rabbits, humans, and some cells of murine origin	(Spaan <i>et al.</i> , 2014)
LukAB	Core	Neutrophils, macrophages, monocytes, dendritic cells	CD11b	Neutrophil lysis	Yes; human and rabbit immune cells only	(DuMont <i>et al.</i> , 2013a; DuMont <i>et al.</i> , 2013b)
LukED	Pathogenicity island; vSa β	Monocytes, neutrophils, macrophages, T cells, dendritic cells, NK cells	DARC, CCR5, CXCR1, CXCR2	Erythrocyte lysis, neutrophil lysis	No	(Alonzo <i>et al.</i> , 2013; Reyes-Robles <i>et al.</i> , 2013; Spaan <i>et al.</i> , 2015)
PSMs	Core	Erythrocytes, monocytes, neutrophils	FPR2	Chemotaxis inhibition, neutrophil lysis, phagosome escape	No	(Forsman <i>et al.</i> , 2012; Kretschmer <i>et al.</i> , 2010; Surewaard <i>et al.</i> , 2013)

Adapted from (Alonzo & Torres, 2014).

major classes of PSMs can be distinguished according to peptide mass. PSMs of α -type are approximately 20-25 amino acids in length and the β -type PSMs are generally twice the size at approximately 44 amino acids in length (Mehlin *et al.*, 1999; Wang *et al.*, 2007).

Unlike Hla or the leucocidins, PSM cytolytic activity is mediated in a receptor-independent fashion (Kretschmer *et al.*, 2010) indicating that PSMs can target virtually any eukaryotic cell type, such as monocytes (Wang *et al.*, 2007), erythrocytes (Cheung *et al.*, 2012) and most importantly in the case of *S. aureus* infection, neutrophils (Forsman *et al.*, 2012; Wang *et al.*, 2007). On average, higher levels of PSMs are detected in the supernatants of CA-MRSA strains in comparison to HA-MRSA strains, indicating a correlation with increased virulence observed in CA-MRSA strains (Li *et al.*, 2010; Wang *et al.*, 2007). A PSM α -deficient mutant of USA300 showed reduced virulence in a murine model of bacteraemia, whereas deletion of β -type PSMs seemingly had no effect (Wang *et al.*, 2007). PSMs can be detected by formyl-peptide receptor 2 (FPR2) on human and mouse neutrophils which stimulates neutrophil chemotaxis and cytokine release (Kretschmer *et al.*, 2010).

PSMs are positively regulated by the Agr regulatory system (Wang *et al.*, 2007). The intraphagosomal environment is ideal to allow the concentration of *S. aureus* auto-inducing peptide (AIP) to reach critical activation levels that would be impossible to achieve in the diffuse extracellular environment (Carnes *et al.*, 2010). Increased levels of AIP activate the *agr* regulatory locus which in turn upregulates a number of secreted virulence factors, including PSMs. PSMs are also inactivated by host serum lipoproteins, indicating that it is likely that they only function within the neutrophil intracellular environment (Surewaard *et al.*, 2012; Surewaard *et al.*, 2013), and thereby allow *S. aureus* to escape from the phagosome.

1.5.3 Disruption of NETs

S. aureus secretes extracellular DNase which degrades the nuclear backbone of NETs (Berends *et al.*, 2010), releasing trapped bacteria and facilitating further proliferation and dissemination. *S. aureus* DNase production has been associated with delayed bacterial clearance in the lung and increased mortality after intranasal infection (Berends *et al.*, 2010). Adenosine synthase A (AdsA) is a CWA protein of *S. aureus* that degrades NETs, further facilitating bacterial escape and dissemination (Thammavongsa *et al.*, 2013). Specifically, AdsA converts the nuclear contents of NETs to deoxyadenosine which triggers caspase-3

mediated apoptosis of other immune cells, such as macrophages (Thammavongsa *et al.*, 2013).

1.5.4 *S. aureus* survival following neutrophil uptake

1.5.4.1 Resistance to antimicrobial peptides and bactericidal proteins

In response to intraphagosomal oxygen-independent bactericidal mechanisms, like the production of antimicrobial peptides (AMPs), *S. aureus* can modify its cell wall architecture, e.g. the incorporation of lysyl-phosphatidyl glycerol into the bacterial membrane and the D-alanylation of teichoic acids reduce the net negative charge of the bacterial surface (Peschel *et al.*, 2001), reducing the affinity of cationic AMPs for the cell surface. Aureolysin cleaves host LL-37, which is an AMP with potent anti-staphylococcal activity (Sieprawska-Lupa *et al.*, 2004 & Liu *et al.*, 2005). Carotenoid pigments also play a role in resistance against cationic AMPs. An increase in carotenoid rigidifies the cell membrane, making it less susceptible to AMPs (Mishra *et al.*, 2011).

Another important antibacterial enzyme produced by neutrophils is lysozyme, which degrades bacterial cell wall peptidoglycan. *S. aureus* however is intrinsically resistant to lysozyme via the action of the O-acetyltransferase A (OatA) enzyme which catalyses the O-acetylation of peptidoglycan altering the lysozyme target site (Bera *et al.*, 2005).

1.5.4.2 Resistance to oxidative stress

Following internalisation of bacteria the phagosome undergoes a strong oxidative burst which is initiated by NADPH-dependent oxidases, leading to the generation of highly toxic reactive oxygen species (ROS). Protection of *S. aureus* from oxygen-mediated antimicrobial mechanisms within the phagosome is in part reliant on the production of catalase, which catalyses the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen, and two superoxide dismutases, SodA and SodM, which together reduce ROS production (Liu *et al.*, 2005). *S. aureus* also produces staphyloxanthin, a carotenoid pigment which gives the bacterium its characteristic golden colour, which also aids in protection from attack from ROS (Liu *et al.*, 2005).

Adenosine is a potent immune-regulatory chemical that is synthesised by the host in response a number of conditions, such as hypoxia and exposure to ROS. The production of adenosine and its interaction with its receptors triggers an anti-inflammatory cascade which can inhibit neutrophil degranulation and superoxide burst. AdsA catalyses the dephosphorylation of adenosine mono-, di- and triphosphates to generate extracellular

adenosine, which consequently down-regulates neutrophil bactericidal activity (Thammavongsa *et al.*, 2009).

1.6 Vaccine design

1.6.1 Vaccination strategies for the prevention of *S. aureus* infection

The rapid development and spread of high level antibiotic resistance among *S. aureus* isolates worldwide (Chambers & Deleo, 2009) means that there is an urgent clinical need in the development of an effective vaccine against *S. aureus* infection. The ideal vaccine target would be present on the cell surface of all isolates *in vivo*, highly immunogenic, and would trigger the production of opsonising and neutralising antibodies as well generating immunological memory against recurrent infections. Numerous attempts to develop a vaccine against *S. aureus* infection have been undertaken, however no candidate has demonstrated the desired protection in human trials (Jansen *et al.*, 2013). The failure of these vaccines to develop an effective immune response can in part be attributed to the immunomodulatory functions of SpA (see section 1.2.3.1.1). However, a closer inspection at some of the strategies followed in *S. aureus* vaccine design also provides some insights.

The initial approaches to an *S. aureus* vaccine focused on targeting the capsule. The leading effort in this regard was StaphVAX, a bivalent vaccine comprising *S. aureus* capsular polysaccharide types 5 and 8. However, despite successful phase II trials which reported increased antibody titres in patients undergoing chronic hemodialysis (Fattom *et al.*, 2004; Robbins *et al.*, 2004; Shinefield *et al.*, 2002), StaphVAX did not reduce the incidence of invasive *S. aureus* infections in hemodialysis patients in a large phase III trial (Bagnoli *et al.*, 2012). One factor in the failure of a capsular polysaccharide approach is that not all clinical isolates of *S. aureus* produce a capsule, for example the CA-MRSA strains of the USA300 lineage possess four conserved mutations in the *cap5* locus that abrogates capsule production (Boyle-Vavra *et al.*, 2015). The failure of this vaccine, despite the presence of high titres of opsonising antibodies in immunized patients, indicates that the induction of specific immune serum to *S. aureus* does not necessarily result in protection.

Similarly, the single antigen approach that has provided successful vaccines in the case of other pathogens has not translated into *S. aureus* vaccine design. A number of individual envelope components and secreted proteins of *S. aureus*, e.g. ClfA, IsdB, α -haemolysin and the leukocidins, have been investigated as vaccine antigens. However vaccination with single antigens has not proven successful (Kuklin *et al.*, 2006; McKenney *et*

al., 1999; Menzies & Kernodle, 1996). The failures of this approach are perhaps not surprising as the expression of virulence factors by *S. aureus* is a dynamic process, often varied in response to certain stresses and conditions, and the functional redundancy demonstrated by many *S. aureus* proteins make single antigens weak targets. In agreement with this, a vaccine comprised of a number of immunogenic antigens, a combination of four CWA proteins (SdrD, SdrE, IsdA and IsdB), generated a much stronger protective effect than vaccination with any of the antigens individually in a murine sepsis model (Stranger-Jones *et al.*, 2006).

The choice of host organism used in early vaccine trials has come under scrutiny. Mice have been the animal models of choice for most pathogenesis studies, owing to their low cost, however, there is concern that protective immunity against *S. aureus* disease in mice is not predictive of vaccine success during clinical trials (Proctor, 2012). In contrast to humans, the mouse is not a natural host for *S. aureus* infection and in many ways does not mimic human infection. For example, the lethality of *S. aureus* disease is reliant on the production of superantigens, such as toxic shock syndrome toxin 1 (TSST1), and the cytolytic toxins (Spaulding *et al.*, 2013), however it has been shown that humans and mice react differently to these proteins (Seok *et al.*, 2013). Recently the guinea pig has been proposed as a more appropriate animal model to mimic natural *S. aureus* infection. Guinea pigs have been successfully used as models in the development of vaccines to prevent diphtheria and tetanus (Padilla-Carlin *et al.*, 2008). Furthermore, unlike mice, guinea pigs generate V_H3-type IgM, the predominant subclass in the development of immunological memory, in response to *S. aureus* infection (Kim *et al.*, 2015) and have recently been used in a model of *S. aureus* bloodstream infection (Kim *et al.*, 2015).

1.6.2 Protein A as a vaccine candidate

The development of a successful *S. aureus* vaccine has been hindered by the fact that *S. aureus* infections do not generate protective immunity in humans or animals (Kim *et al.*, 2012a) which has been attributed to the ability of SpA to modulate the host adaptive immune response (section 1.2.3.1.1). SpA binds specifically to the Fab region of the V_H3 subclass of human IgM (Graille *et al.*, 2000) which triggers the proliferation and depletion of B cells (Goodyear & Silverman, 2003) suppressing the development of adaptive immune responses.

For these reasons an approach has been undertaken that aims to neutralise the immunoglobulin-binding abilities of SpA to facilitate the development of immunological memory in response to vaccination. Immunisation of mice with a variant of SpA, termed

SpA_{KKAA}, unable to bind to either IgG or IgM facilitated the production of antibodies that promoted opsonophagocytic clearance, and protected mice (Kim *et al.*, 2010) and guinea pigs (Kim *et al.*, 2015) against challenge with highly virulent MRSA strains. The inclusion of SpA_{KKAA} in a multivalent vaccine has shown promising results. The vaccine comprising SpA_{KKAA} and the CWA proteins ClfA, FnBPB and SdrD, significantly increased the survival and delayed the time to death of mice in a *S. aureus* sepsis model, in comparison to a trivalent (ClfA/FnBPB/SdrD) vaccine alone (Kim *et al.*, 2011). These results suggest that any future active immunisation approaches would benefit from the inclusion of the SpA_{KKAA} variant, and this may be a vital step towards the generation of a successful *S. aureus* vaccine.

1.7 Genetic manipulation of *S. aureus*

The study of *S. aureus* virulence and pathogenesis has been greatly aided by the development of genetic techniques to manipulate or eliminate gene expression. A number of different methods have been developed for the generation of isogenic mutant strains of *S. aureus* which include transposon mutagenesis and allelic exchange.

1.7.1 Transposon mutagenesis

Transposon mutagenesis is commonly used to isolate insertion mutants in chromosomal genes encoding virulence factors. Transposable elements reside on an assortment of delivery vectors and encode a variety of antibiotic resistance markers. An advantage of using transposon elements as genetic tools for mutagenesis is the generation of ordered, or random, insertion mutant libraries without prior knowledge of the target gene. The *bursa aurealis* element has been used to create an extensive *S. aureus* mutant library consisting of 10,325 mutants of strain Newman, each with individually-defined insertion sites (Bae *et al.*, 2004). Screening this library for loss-of-function mutants in a nematode killing assay identified 71 genes important for the virulence of *S. aureus*, some of which were shown to be required for abscess formation in a murine infection model (Bae *et al.*, 2004). Recently, the *bursa aurealis* transposon element has been used by the Nebraska centre for staphylococcal research to generate a mutant library of the epidemic CA-MRSA isolate USA300 (Fey *et al.*, 2013). The library currently consists of 1,954 mutants, each containing a single mutation within a non-essential gene.

1.7.2 Allelic exchange

The process of allelic exchange is the method of choice to generate chromosomal site-specific mutations in *S. aureus*. This approach allows for the insertion or deletion of entire genes, the introduction of point mutations or the generation of hybrid genes. Initially, the

immediate 5' and 3' flanking regions of DNA of the target DNA are cloned into a multi-copy plasmid in *E. coli*. Following this, the vector is introduced into *S. aureus* and integrates into the bacterial chromosome by homologous recombination. This approach uses temperature-sensitive plasmids in *S. aureus*.

1.7.3.1 Temperature-sensitive plasmids

1.7.3.1.1 The pVW01ts replicon

Temperature-sensitive plasmids are vectors that contain a temperature-controlled origin of replication. The pWV01 plasmid was originally isolated from *Lactococcus lactis* subsp. *cremoris* (Otto *et al.*, 1982), and a temperature-sensitive derivative, containing the pWV01ts replicon, was first isolated in 1992 (Maguin *et al.*, 1992). Since then the pWV01ts replicon has been used to facilitate mutagenesis in a number of different Gram-positive organisms (Bruckner, 1997; Monk *et al.*, 2012; Nallapareddy *et al.*, 2006). Plasmid pKORI is a commonly used temperature-sensitive vector for mutagenesis in *S. aureus* (Bae & Schneewind, 2006). However, the temperature-sensitive pE194ts replicon of pKORI is restricted for replication at the high temperature of 43°C and it has been reported that the growth of *S. aureus* at non-physiological temperatures under antibiotic selective-pressure can induce alterations in the staphylococcal regulatory *sae* genes (Sun *et al.*, 2010). In contrast, the pWV01ts replicon is highly stable in *S. aureus* and allows for selection of integrants at the lower, physiological temperature of 37°C which eliminates the likelihood of secondary mutations. For this reason Monk *et al.*, (2012) harnessed the pWV01ts replicon to create the *S. aureus* mutagenesis vector pIMAY (Fig. 1.5).

1.7.3.1.2 Counter-selection in *S. aureus*

Replication of pIMAY is impaired following a temperature upshift to 37°C in the presence of antibiotic selective pressure, which selects for colonies where integration of the plasmid into the bacterial chromosome has occurred via a single cross-over event. A subsequent thermal shift to a temperature permissive for plasmid replication, in the absence of selective pressure, is performed to encourage a double cross-over event by homologous recombination (Biswas *et al.*, 1993; Monk *et al.*, 2012). Depending on where the double cross-over event occurred, either a wild-type or mutant gene product is obtained.

Counter-selectable markers are often used to enrich for cells that have lost the plasmid. Many counter-selection systems exploit antisense RNA fragments directed against an essential gene or the activity of an endonuclease. Plasmid pIMAY possesses a *secY*

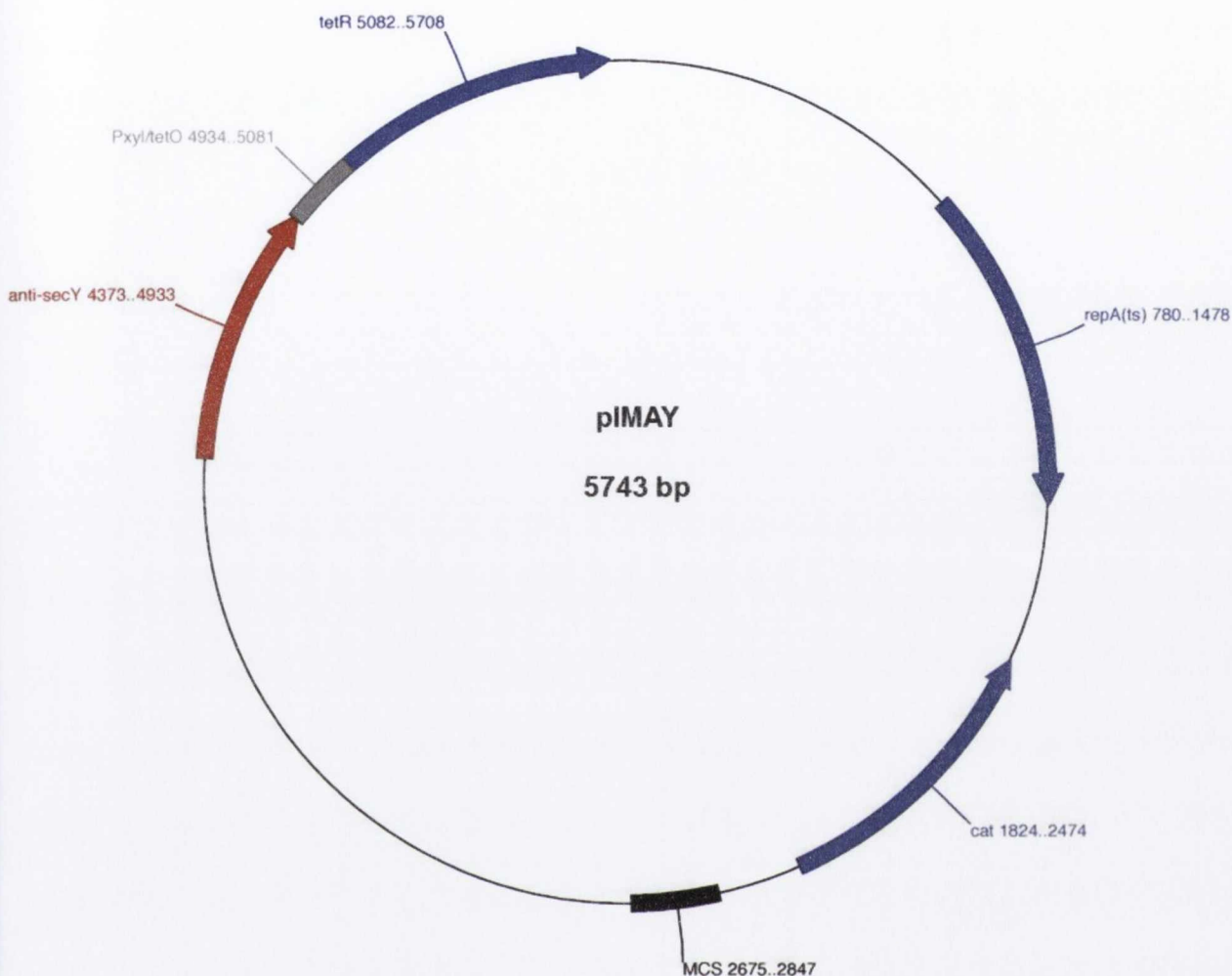


Fig. 1.5 Schematic representation of pIMAY. pIMAY possesses the temperature-sensitive *repA_{ts}* replicon from the plasmid pVE6007, which is restrictive for plasmid replication at 37°C. It also encodes a *secY* antisense RNA fragment (anti-*secY*) under the control of a tetracycline-regulatory (*tetR*) promoter. Expression of anti-*secY* allows enrichment for cells that have lost replicating and integrated pIMAY during plasmid excision. This vector also possess a chloramphenicol resistance gene (*cat*) which allows for selection of bacteria possessing replicating or integrated plasmid. Multiple cloning site (MCS) and plasmid coordinates are indicated. Plasmid map drawn using ApE software.

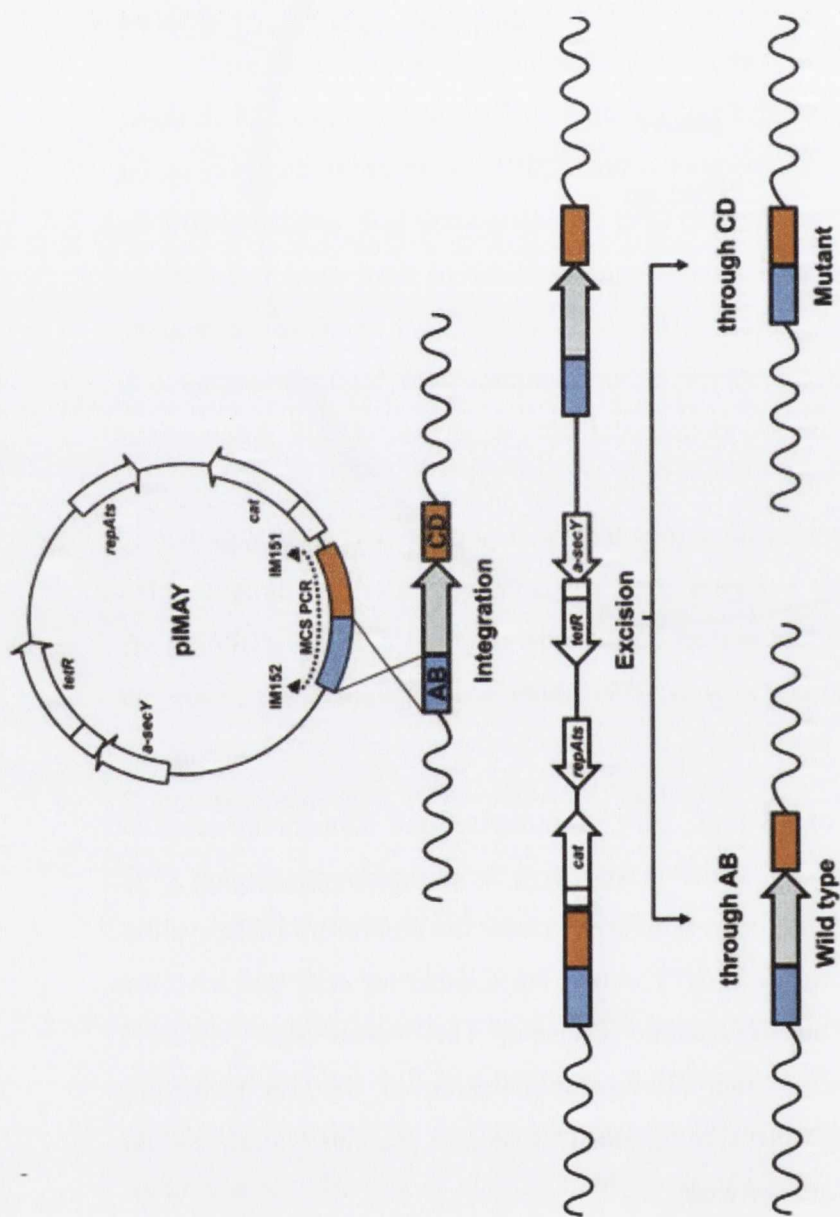


Fig. 1.6 Schematic of allelic exchange in *S. aureus* using pIMAY. Following transformation of pIMAY into *S. aureus* and growth at 28°C, which is permissive for plasmid replication, integration of pIMAY into the bacterial chromosome is stimulated by growth at 37°C with antibiotic selection. This diagram represents an integration event occurring through the AB side. Excision of integrated pIMAY is promoted following growth at 28°C without antibiotic selection. Bacteria are grown in the presence of anhydrotetracycline to induce the expression of *secY* anti-sense RNA and enrich for cells that have lost replicating and integrated pIMAY during plasmid excision. Plasmid excision through the AB side recreates the wild type locus, while CD excision yields a mutated gene. Adapted from (Monk *et al.*, 2012)

antisense RNA fragment under the tight control of the TetR repressor, flanked by an anhydrotetracycline (ATc)-inducible promoter ($P_{xyl/tetO}$, Fig. 1.5) (Monk *et al.*, 2012). SecY is a component of the essential protein secretory (Sec) machinery of prokaryotes (Manting & Driessen, 2000) and the inhibition of *secY* expression by antisense RNA is inhibitory to bacterial growth (Ji *et al.*, 2001). A schematic representation of the allelic exchange process in *S. aureus* using pIMAY is demonstrated in Fig. 1.6.

1.7.4 Complementation

Molecular Koch's postulates state that specific inactivation of the gene associated with a suspected virulence trait should result in a measurable loss in pathogenicity or virulence. Furthermore, the reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity or virulence. The latter activity is better known as gene complementation. Similar to generating site-specific mutations in *S. aureus*, allelic exchange vectors, such as pIMAY (see section 1.7.2), can also be used to restore, or reverse the mutation in the wild-type gene on the bacterial chromosome. However, the method of choice for gene complementation in molecular biology is plasmid-based gene expression.

The majority of *S. aureus* expression vectors are derived from the naturally-occurring plasmids pE194, pC194, pUB110 and pT181, which all replicate by a rolling circle mechanisms, or pI258 and pSK1, which rely on the theta-mode of replication (Prax *et al.*, 2013). A commonly used expression vector known as pALC2073 is a pC194-derived shuttle plasmid that was modified to possess the Tet regulatory system, i.e. TetR repressor and the anhydrotetracycline-inducible $P_{xyl/tetO}$ promoter (Bateman *et al.*, 2001), previously described in section 1.7.3.1.2. However, subsequent studies showed that gene expression in pALC2073 was not completely repressed in the absence of inducer (Corrigan *et al.*, 2009). Analysis and repair of the -10 box of the *tetR* promoter of pALC2073 repaired the repression defect to yield plasmid pRMC2 (Corrigan *et al.*, 2009). These plasmids have been, and continue to be, very important tools in fulfilling the molecular requirements for investigating virulence mechanisms and phenotypes in *S. aureus*.

1.7.5 Bypassing the *S. aureus* restriction modification barriers

Transformation of foreign DNA directly into many staphylococcal strains has previously been unachievable due to the strong restriction barrier present in this species (Corvaglia *et al.*, 2010; Veiga & Pinho, 2009; Waldron & Lindsay, 2006). *S. aureus* possesses two restriction modification (RM) systems, types I (Roberts *et al.*, 2013; Waldron

& Lindsay, 2006) and IV (Xu *et al.*, 2011), which detect and degrade foreign DNA that does not bear the same host-specific methylation profiles as the recipient strain. In particular, the distinct adenine methylation recognition sites for type I restriction enzymes have been identified for eight of the current dominant MRSA lineages, and these were shown to be sufficient to inhibit transfer of plasmids from foreign lineages (Monk *et al.*, 2015; Roberts *et al.*, 2013).

The *S. aureus* type IV RM system is widespread in *S. aureus* isolates, and relies on the recognition of cytosine-methylated DNA by the restriction endonuclease SauUS1 (Corvaglia *et al.*, 2010; Xu *et al.*, 2011). A Dcm (methylase)-deficient mutant of *E. coli*, called DC10B, has been engineered and can be used to transfer *E. coli*-derived un-methylated plasmid DNA directly into *S. aureus* with high efficiency (Monk *et al.*, 2012). However maximal transformation efficiency was shown to require a lineage-specific adenine methylation profile (Monk *et al.*, 2012). To address this, derivatives of DC10B have been created that possess lineage-specific type I modification genes that can tailor plasmid methylation profiles to be recognised by specific *S. aureus* lineages, which results in maximal transformation efficiency (Monk *et al.*, 2015).

1.8 Aims and objectives

The mechanism by which staphylococcal protein A (SpA) is anchored to the cell wall peptidoglycan of *S. aureus* and its numerous roles in infection are very well studied and understood. In comparison, relatively little is known about the release of SpA into the extracellular milieu and its role in advancing *S. aureus* infection. The major aims and objectives of this project have been to:

- Investigate the role of the *S. aureus* extracellular proteases in the generation of released SpA.
- Determine the influence of the glycylglycine endopeptidase LytM in the generation of released SpA in the community-associated MRSA strain LAC, of the USA300 lineage.
- Determine the influence of the SpA C-terminal sorting signal in the release of SpA from *S. aureus*.
- Investigate the ability of released SpA to protect *S. aureus* from phagocytic killing in whole human blood.

Chapter 2

Material and Methods

2.1 Bacterial culture conditions and reagents

The bacterial strains used in this study are listed in Table 2.1. *S. aureus* was grown on Tryptic Soy Agar (Oxoid), or in BHI (Difco) broth with shaking (200 rpm) at 37°C. *Escherichia coli* was grown on Luria agar or broth (Difco) at 37°C. Cultures were supplemented with ampicillin (100 µg/ml, Melford Laboratories) or chloramphenicol (10 µg/ml), as required. Bacteria from overnight cultures were diluted 1:200, washed in BHI and allowed to grow to the OD₆₀₀ required. Strains harbouring the pRMC2 expression vector were grown to OD₆₀₀ = 0.3 and induced with anhydrotetracycline (ATc; 9.77 - 625 ng/ml) until OD₆₀₀ = 1.2 was reached. Where indicated, broth was supplemented with V8 (1 U/ml) and 3, 4-dichloroisocoumarin (DCI; 200 µM). Unless otherwise stated all reagents were obtained from Sigma.

2.2 Plasmids

All plasmids and derivatives used in this study are listed in Table 2.2.

2.3 DNA manipulation

2.3.1 Isolation of plasmid and genomic DNA

Plasmid DNA was isolated using the Wizard® Plus SV Miniprep kit (Promega) or Qiagen® Plasmid Plus Midi Kit according to the manufacturer's instructions. DNA purification was carried out using the Wizard® SV Gel and PCR Clean-up System (Promega), or the High Pure PCR Product Purification Kit (Roche). Genomic DNA was prepared using Bacterial Genomic DNA purification kit (Edge BioSystems). Plasmid and genomic DNA extraction from *S. aureus* required the addition of lysostaphin (200 µg/ml; AMBI, New York) to digest cell wall peptidoglycan.

2.3.2 Polymerase chain reaction

Polymerase Chain Reaction (PCR) amplification was carried out in a DNA thermal cycler (Techne or PIKO). Reactions were typically carried out in 50 µl volumes using Phusion™ High-Fidelity polymerase (Thermo Fisher), Phire Hot Start DNA polymerase (Finnzymes), or Velocity DNA polymerase (Bioline) according to the manufacturer's instructions. Plasmid DNA (1 – 10 ng), genomic DNA (150 ng), or synthetic DNA fragments (10 ng) were used as template for PCR. Primers and dNTPs (Bioline) were used at final concentrations of 0.2 mM and 200 µM, respectively. Primers (Table 2.3) and synthetic DNA fragments (gBlocks®) were synthesised by Integrated DNA Technologies. Initial denaturation was carried out at 98°C (30 s) followed by 30-35 cycles of denaturation (10 s) at 98°C, 20 s annealing (temperature dependent on primer used) and extension at 72°C. When

Table 2.1 Bacterial strains

Strain	Description	Reference
<i>S. aureus</i>		
LAC*	Erythromycin-sensitive derivative of MRSA strain LAC. Clonal complex 8.	(Boles <i>et al.</i> , 2010)
LAC* <i>spa</i>	Derivative of LAC* deficient in protein A. <i>spa</i> ::Kan ^r	This study
LAC* <i>sbi</i>	Derivative of LAC* deficient in Sbi. <i>sbi</i> ::Em ^r .	(Smith <i>et al.</i> , 2011)
LAC* <i>spa sbi</i>	Derivative of LAC* deficient in protein A and Sbi. <i>spa</i> ::Kan ^r , <i>sbi</i> ::Em ^r .	This study
LAC* PD	Protease-deficient derivative of LAC*. Δaur , $\Delta sspAB$, Δscp , <i>spl</i> ::Em ^r .	(Wormann <i>et al.</i> , 2011)
LAC* <i>sbi lytM</i>	Derivative of LAC* <i>sbi</i> deficient in LytM. $\Delta lytM$.	This study
LAC* <i>sbi</i> [SpA Ω SdrESS]	Derivative of LAC* <i>sbi</i> where DNA encoding the SpA sorting signal has been replaced with DNA encoding the sorting signal of SdrE by allelic exchange.	This study
LAC* <i>sbi lytM</i> [SpA Ω SdrESS]	Derivative of LAC* <i>sbi</i> [SpA Ω SdrESS] deficient in LytM. $\Delta lytM$.	This study
Newman <i>sbi</i>	Derivative of Newman deficient in Sbi. <i>sbi</i> ::Em ^r . NCTC 8178. Clonal complex 8.	(Smith <i>et al.</i> , 2011)
Newman <i>sbi lytM</i>	Derivative of Newman <i>sbi</i> deficient in LytM. $\Delta lytM$.	This study
Newman <i>sbi</i> [SpA Ω SdrESS]	Derivative of Newman <i>sbi</i> where DNA encoding the SpA sorting signal has been replaced with DNA encoding the sorting signal of SdrE by allelic exchange.	This study

Table 2.1 Bacterial strains continued

Wood46	Produces undetectable levels of protein A. CC97.	(Amend <i>et al.</i> , 1984; Jongerius <i>et al.</i> , 2010)
EMRSA-15	Human clinical isolate. Sanger strain number HO50960412. Multilocus sequence type 22. SCC <i>mecIV</i> .	(Holden <i>et al.</i> , 2013)
ST2371	Strain SASCBU26. Human clinical isolate. Multilocus sequence type 2371. SCC <i>mecIV</i> . Contains pSCBU plasmid.	(Harris <i>et al.</i> , 2013)
ST2371 <i>sasY</i>	Derivative of SASCBU26 carrying plasmid pSCBU with a frameshift mutation within the <i>sasY</i> gene;	Emma J. Smith and Joan A. Geoghegan; unpublished
<i>E. coli</i>		
DC10B	<i>dam</i> ⁺ Δ <i>dcm</i> Δ <i>hsdRMS</i> <i>endA1</i> <i>recA1</i>	(Monk <i>et al.</i> , 2012)
TOPP3	Protease deficient strain used for recombinant protein expression. Rif ^r , [F' <i>proAB</i> ⁺ <i>lacI</i> ^r Z <i>ΔM15</i> Tn10 (Tet ^r) (Kan ^r)]	Stratagene

Table 2.2 Plasmids

Plasmids	Relevant Features	Reference
pRMC2	Anhydrotetracycline-inducible expression vector. Amp ^r , Cm ^r .	(Corrigan & Foster, 2009)
pSpA	Plasmid pRMC2 containing the full-length <i>spa</i> gene. Amp ^r , Cm ^r .	This study
pSpAΔSS	Plasmid pSpA lacking DNA encoding the SpA sorting signal (residues 474-508). Amp ^r , Cm ^r .	This study
pSpAΩSdrESS	Plasmid pSpA with DNA encoding the SpA sorting signal (residues 474-508) has been replaced with DNA encoding the SdrE sorting signal (residues 1117-1154). Amp ^r , Cm ^r .	This study
pSpA-KKQNK	Plasmid pSpA where DNA encoding residues 506 - 508 (REL) of SpA has been replaced with DNA encoding residues 1150-1154 (KKQNK) of SdrE. Amp ^r , Cm ^r .	This study
pSpA-KKQNK+A502F	Plasmid pSpA-KKQNK with alanine substitution of residue F502. Amp ^r , Cm ^r .	This study
pSpA-KKQNK+A502F/A499L	Plasmid pSpA-KKQNK+A502F with alanine substitution of residue L499. Amp ^r , Cm ^r .	This study
pSpAΩSdrESS-ENKDKK	Plasmid pSpAΩSdrESS where DNA encoding residues 1150-1154 (KKQNK) of SdrE has been replaced with residues 928-933 (ENKDKK) of ClfA. Amp ^r , Cm ^r .	This study

pRMC2- <i>sbi</i> ΔD1D2	Plasmid pRMC2 containing DNA encoding the Sbi signal sequence (residues 1-40) and the D3D4 domains (residues 153-253). Amp ^r , Cm ^r .	(Smith <i>et al.</i> , 2011)
pD3D4-SpA	Plasmid pRMC2 containing an in-frame fusion between DNA encoding Sbi D3D4 domains (residues 153-253) and DNA encoding the SpA sorting signal (residues 474-508). Amp ^r , Cm ^r .	This study
pD3D4-SdrESS	Plasmid pRMC2 containing an in-frame fusion between DNA encoding Sbi D3D4 domains (residues 153-253) and DNA encoding the SdrE sorting signal (residues 1117-1154). Amp ^r , Cm ^r .	This study
pSpAΩSdrESS-DS	Plasmid pSpAΩSdrESS containing 538 bp of sequence downstream of the <i>spa</i> gene. Amp ^r , Cm ^r .	This study
pRMC2:: <i>sasY</i>	Plasmid pRMC2 containing the full-length <i>sasY</i> gene from ST2371 strain SASCBU26. Amp ^r , Cm ^r .	Joan A. Geoghegan; unpublished
pALC::2073	Vector allowing high level gene expression in the absence of inducer. Amp ^R . Cm ^R	(Bateman <i>et al.</i> , 2001)
pALC:: <i>sasY</i>	Plasmid pALC::2073 containing the full-length <i>sasY</i> gene from ST2371 strain SASCBU26. Amp ^r , Cm ^r .	Emma J. Smith and Joan A. Geoghegan; unpublished
pIMAY	Temperature-sensitive vector for allelic exchange in <i>S. aureus</i> . Cm ^r .	(Monk <i>et al.</i> , 2012)
pIMAY::Δ <i>lytM</i>	Plasmid for creating a <i>lytM</i> deletion mutant. Carries 509 bp of DNA from upstream and 512 bp of DNA from downstream of the <i>lytM</i> gene amplified from LAC. Cm ^r .	Joan A. Geoghegan; This study

pIMAY::SpA Ω SdrESS	Plasmid for replacing DNA encoding the sorting signal of SpA with DNA encoding the sorting signal of SdrE on the chromosome of LAC*. Cm ^r .	This study
pCM29	Constitutive GFP-expressing plasmid. Cm ^R	(Pang <i>et al.</i> , 2010)
pGEX-4T2:: <i>spa</i>	<i>E. coli</i> vector for the expression of glutathione S-transferase tagged recombinant proteins. Derivative encoding the full-length <i>spa</i> gene. Amp ^r .	(Gomez <i>et al.</i> , 2006)
pGEX-4T2:: <i>spa</i> [L17A]	Derivative of pGEX-4T2:: <i>spa</i> possessing an alanine substitution for L17 in each of the five IgG-binding domains. Amp ^r .	(Gomez <i>et al.</i> , 2006)

amplifying plasmid DNA, an extension time of 15 s/kb DNA was used. For genomic DNA a longer extension time was used (30 s/kb). A final extension step was carried out at 72°C for 5 min. PCR products amplified using plasmid DNA as template were incubated with 1U DpnI (NEB) at 37°C for 60 min to digest parental template DNA. PCR products were purified using Wizard® SV gel and PCR clean-up system (Promega) or the High Pure PCR Product Purification Kit (Roche).

Site directed mutagenesis was performed using the Quikchange method (Stratagene) and Velocity DNA polymerase (Bioline). Primer extension was carried out using a Techne thermal cycler. An initial denaturation step at 98°C for 30 s was followed by 18 cycles of denaturation for 10 s, annealing for 20 s at a temperature between 50 and 60°C dependent on the primers, and elongation at 72°C for 15 s/1kb being amplified. This was followed by incubation at 68°C for 10 min. PCR products were incubated with 1U DpnI (NEB) at 37°C for 60 min to digest parental template DNA.

2.3.3 Agarose electrophoresis

Gels containing 1-2% agarose were dissolved by boiling in Tris-acetate-EDTA (TAE) buffer, cooled to 65°C and were cast in mini trays (Life Technologies). DNA samples in loading buffer were added to wells along with DNA size markers (Bioline). Electrophoresis of samples was routinely performed at 90 V. Gels were bathed in ethidium bromide for 10 min, washed and viewed under UV light. Gel images were imaged using an Alpha Imager.

2.3.4 Sequence and ligase-independent cloning

The majority of cloning was carried out (Table 1.2) using the sequence and ligase-independent cloning (SLIC) procedure as described by Li and Elledge (2012). Here, both vector and insert were amplified by PCR. Primers for amplification of insert were designed with 5' extensions, typically 24 bp in length that possess sequence homology to the end regions on the amplified vector. Both vector and insert DNA (1 µg) were incubated with T4 polymerase (3 U; NEB) in a final reaction volume of 40 µl containing NEB Buffer 2, molecular grade BSA (NEB), dithiothreitol (DTT; 5 mM) and urea (200 mM). The reaction mix was incubated at 23°C for 20 min and stopped by the addition of EDTA (25 mM), followed by further incubation at 75°C for 20 min. 5 µl each of vector and insert with single-stranded ends were mixed and were allowed to anneal. This reaction was incubated at 65°C for 10 min followed by a slow decrease in temperature from 65 - 25°C with 1 min hold for each degree. The entire reaction was used for transformation into *E. coli* DC10B.

Table 2.3 Oligonucleotides

Oligonucleotide name	5' - 3' sequence
pSLF	<u>GAATTC</u> ACTGGCCGTCGTTTTACAACGTCG
pSLR	<u>GAGCTC</u> AGATCTGTTAACGGTACCATCATA
SpAF	<u>TATGATGGTACCGTTAACAGATCTGAGCTCTACAGGGGGTATT</u> AATTTG
SpAR	<u>CGACGTTGTAAAACGACGGCCAGTGAATTCTTATAGTTCGCGA</u> CGACG
dSSF	*TAAGAATTCACTGGCCGTCGTTT
dSSR	*TGCTTGAGCTTTGTTAGCATCTG
RESF	<u>CAGATGCTAACAAAGCTCAAGCATTACCAGAAACAGGTAGTG</u>
RESR	<u>GTAAAACGACGGCCAGTGAATTCTTATTTGTTTTGTTTTTGCG</u> AC
DWrR	TGGCGCCACTTTCTTTTCAGCATC
DSpF	<u>GATGCTGAAAAGAAAGTGGCGCCATTACCAGAAACTGGTGAA</u> G
DSdF	<u>GATGCTGAAAAGAAAGTGGCGCCATTACCAGAAACAGGTAGT</u> G
SEQF	ATTCAGGCTGCGCAAC
SEQR	TTGTTGACATTATATCATTG
lytm-A	GGAGGTACCCAATCACTATACGATGTATCAGAC
lytM-B	CATGTATAAAACATCCTCCATTAAAG
lytM-C	<u>TTAATGGAGGATGTTTTATACATGTAATACAGAAAATCCCAAG</u> TTG
lytM-D	GGAGAGCTCAAAGACAAATACAAAATCACATC
pSRER	TTATTTGTTTTGTTTTTTGCGAC
SpADSF	<u>CGTCGCAAAAAACAAAACAAATAAAAAACAAACAATACACAAC</u> GATAG
SpADSR	<u>CGACGTTGTAAAACGACGGCCAGTGAATTCATTTCACAAGCG</u> GTTCAATTAC
pIREF	<u>CCTCACTAAAGGGAACAAAAGCTGGGTACCAAAGCCTTAAAG</u> ACGATC
pIRER	<u>CGACTCACTATAGGGCGAATTGGAGCTCTCAACAAGCGGTTCA</u> TTAC
pIMAYF	GGTACCCAGCTTTTGTTCCTTTAGTGAGG

pIMAYR	GAGCTCCAATTGCGCCCTATAGTGAGTCG
OUTF	TACCGATTGGAATTGTACGTC
OUTR	ATGATGGAACATCAACTATTTCG
SPA _{KK} F	* <u>CAAAACAAATA</u> AGAATTCACTGGCCGTC
SPA _{KK} R	* <u>TTTTTT</u> ACGACGTCCAGCTAATAAC
SS+F	* <u>AAAGAAAATAAAGATAAGAAATAA</u> AGAATTCACTGGCCGTCGT TTTACAAC
SS+R	*TTTGCGACGACCGAATAAC
SS502F	GGTGCAGCGTTATTAT TTC GGACGTCGTAAAAAA
SS502R	TTTTTTACGACGTCC GA ATAATAACGCTGCACC
SS499F	CATTAGCCTTAGGT GCATT ATTATTATTCGGACG
SS499R	CGTCCGAATAATAA TATGC ACCTAAGGCTAATG

Underlined sequences represent 5' homologous overhangs designed for sequence and ligase-independent cloning.

* represents the presence of a 5' phosphorylation modification

* & underlined sequences indicate 5' overhangs for primer extension

Nucleotide changes in primers for site-directed mutagenesis are in bold

2.4 Strain and plasmid construction

2.4.1 Strain construction

Bacterial strains used in this study are listed in Table 2.1. Mutations marked with antibiotic resistance were moved between *S. aureus* strains by generalised bacteriophage 85-mediated transduction. Bacteriophages were propagated on the donor strain and used to infect the recipient strain with antibiotic selection for the required marker. Putative transductant colonies were single colony purified three times on TSA plates containing the appropriate antibiotic and 0.05% sodium citrate (w/v) to eliminate contaminating phage particles.

Strain LAC* *spa* was constructed by transduction of *spa*::Kan^r into strain LAC*. LAC* *spa sbi* was constructed by transduction of *sbi*::Em^r into strain LAC* *spa*::Kan^r. Transductants were screened by Western immunoblotting, for antibiotic resistance, tested for growth in BHI broth, and tested for alpha (α) and delta (δ)-toxin production on sheep blood agar plates.

2.4.2 Plasmid construction

Primers used in this study are listed in Table 1.3. Cloning was carried out using the SLIC procedure (see section 2.3.4). Primers for amplifying insert sequences contained 5' extensions with homology to the target vector. The complete *spa* gene from strain Newman (100% amino-acid sequence identity to the *spa* gene from LAC*) was amplified from genomic DNA by PCR using primers SpAF and SpAR. Plasmid pRMC2 was used as template for inverse PCR with primers pSLF and pSLR. Both amplimers were joined using SLIC to generate the plasmid pSpA, where *spa* is cloned between SacI and EcoRI sites of pRMC2.

DNA encoding the sorting signal of SdrE was amplified by PCR using primers RESF and RESR using synthetic DNA encoding the SdrE sorting signal from strain Newman as template. Plasmid pSpA was used as template for inverse PCR with primers dSSF and dSSR. The amplimers were joined using SLIC to generate pSpA Ω SdrESS. To generate plasmid pSpA Δ SS a PCR product generated using primers dSSF and dSSR was treated with DpnI and blunt-end ligation was carried out.

Plasmids pSpA-KKQNK and pSpA Ω SdrESS-ENKDKK were generated by primer extension during inverse PCR. Plasmid pSpA was used as template for inverse PCR using primers SPAkkF and SPAkkR to generate plasmid pSpA-KKQNK. Similarly, plasmid

pSpA Ω SdrESS was used as template for inverse PCR using primers SS+F and SS+R to generate plasmid pSpA Ω SdrESS-ENKDKK. Both PCR products were treated with DpnI and blunt-end ligation was carried out.

Plasmids pSpA-KKQNK+A502F and pSpA-KKQNK+A502F/A499L were generated using site-directed mutagenesis (see section 2.3.2). Overlapping complementary primers containing the appropriate base changes were used to amplify plasmid templates. Plasmid pSpA-KKQNK was used as template to generate pSpA-KKQNK+A502F using primers SS502F and SS502R, and plasmid pSpA-KKQNK+A502F was subsequently used to generate plasmid pSpA-KKQNK+A502F/A499L using primers SS499F and SS499R. Both PCR products were treated with DpnI prior to transformation into *E. coli*.

To generate plasmids pD3D4-SpASS and pD3D4-SdrESS PCR was performed using primers dSSF and DWrR and plasmid pRMC2-*sbi* Δ D1D2 as template. DNA encoding the SpA sorting signal was amplified using primers DSpF and SpAR and plasmid pSpA as template. DNA encoding the SdrE sorting signal was amplified using primers DSdF and RESR and plasmid pSpA Ω SdrESS as template. PCR products were joined by SLIC to generate pD3D4-SpASS and pD3D4-SdrESS.

All plasmids were transformed into *E. coli* strain DC10B (see section 2.5.1). Plasmids were isolated from DC10B, verified by DNA sequencing (Source Bioscience) using primers SEQF and SEQR and transformed (5 μ g) into *S. aureus* made electrocompetent as described in section 2.5.2.

2.4.3 Allelic exchange

S. aureus LAC* *sbi* *lytM*, Newman *sbi* *lytM*, LAC* *sbi* [SpA Ω SdrESS], and Newman *sbi* [SpA Ω SdrESS] were generated using plasmid pIMAY (Monk *et al.*, 2012). Deletion of the *lytM* gene was achieved following the creation of plasmid pIMAY:: Δ *lytM* (Joan A. Geoghegan). Primers *lytM*-A and *lytM*-B were designed to amplify 509 bp of DNA located upstream and primers *lytM*-C and *lytM*-D amplified 512 bp of DNA located downstream of the *lytM* gene. The upstream and downstream PCR products were denatured and allowed to re-anneal via the complementary sequences in primers *lytM*-B and *lytM*-C and this was used as template in a second PCR using primers *lytM*-A and *lytM*-D. The amplicon was cloned into pIMAY between KpnI and SacI restriction sites and the resulting plasmid (pIMAY:: Δ *lytM*) was transformed into *E. coli* DC10B and verified by DNA sequencing. The plasmid was transformed into *S. aureus* LAC* *sbi* and Newman *sbi* made electrocompetent

and deletion of the *lytM* gene was achieved by allelic exchange as previously described (see section 1.5.2). The resulting LytM-deficient mutants were confirmed by DNA sequencing of a PCR amplicon. The mutants were phenotypically identical to the parent strains in terms of growth rate and haemolysis on sheep blood agar.

DNA encoding the C-terminal sorting signal of SpA was exchanged for DNA encoding the corresponding region of SdrE on the chromosome of *S. aureus* LAC* *sbi*, LAC* *sbi lytM* and Newman *sbi* using plasmid pIMAY::SdrESS. Plasmid pSpA Ω SdrESS-DS was generated by amplifying 538 bp of DNA located downstream of the *spa* gene using primers SpADSF and SpADSR and genomic DNA from *S. aureus* LAC* as template and using primers pSLF and pSRER to amplify plasmid pSpA Ω SdrESS. The PCR products were joined by SLIC so that the 538 bp fragment was incorporated into plasmid pSpA Ω SdrESS directly downstream of the stop codon. Following confirmation of successful generation of plasmid pSpA Ω SdrESS-DS by DNA sequencing, primers pIREF and pIRER were used to amplify DNA encoding the SdrE sorting signal and 521 bp of DNA upstream and 538 bp of DNA downstream using plasmid pSpA Ω SdrESS-DS as template. The PCR amplicons were joined by SLIC and transformed into *E. coli* DC10B. The resulting plasmid (pIMAY::SpA Ω SdrESS) was transformed into *S. aureus* LAC* *sbi*, LAC* *sbi lytM* and Newman *sbi* made electrocompetent and replacement of DNA encoding the SpA sorting signal with DNA encoding the sorting signal of SdrE on the chromosome was achieved by allelic exchange as previously described. The resulting mutants were phenotypically identical to their respective parent strains in terms of growth rate and haemolysis on sheep blood agar. Mutations were confirmed by DNA sequencing (Source Bioscience) of a PCR amplicon generated using primers OUTF and OUTR.

2.5 Transformation

2.5.1 Preparation and electroporation of *E. coli*

Electrocompetent *E. coli* were prepared by inoculating L-broth (400 mL in a 2 L baffled flask) with 4 mL overnight culture of *E. coli*. The culture was incubated at 37°C with shaking (200 rpm) until an OD₆₀₀ of 0.6 was reached. The culture was then chilled on ice for 15 min. Cells were harvested at 8,000 x g for 10 min at 4°C and resuspended in 400 mL sterile ice-cold distilled water. Cells were harvested again at 8,000 x g for 10 min at 4°C and the pellet was resuspended in 5 mL 10% (v/v) glycerol. Aliquots (100 μ L) were snap-frozen in liquid nitrogen and were stored at -80°C. Cells were thawed for 5 min on ice before adding up to 10 ng plasmid in a volume of 5 μ L. Cells were then left on ice for 1 min before being

added to prechilled 0.1 cm electroporation cuvettes (Biorad) and were then pulsed at 1.5 kV/cm, 200 Ω and 25 μ F. Cells were immediately resuspended in LB and were incubated for 1 h at 37°C with shaking (200 rpm). Recovered cells were plated onto L-agar containing the appropriate antibiotic. Transformants were screened by PCR.

2.5.2 Preparation and electroporation of *S. aureus*

Plasmids isolated from *E. coli* DC10B were introduced into *S. aureus* by electroporation. Electrocompetent cells were prepared by diluting an overnight culture of *S. aureus* to an OD₅₇₈ of 0.5 in 50 ml pre-warmed BHI, and the culture was incubated at 37°C for 30 min. Cells were cooled in an ice-water slurry for 10 min. Cells were harvested at 3,900 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in 50 ml sterile filtered ice-cold water. Cells were harvested again and the pellet was then washed in decreasing volumes of sterile ice-cold 10% (v/v) glycerol (5 ml, 1 ml, 250 μ l). Aliquots (50 μ l) were snap-frozen in liquid nitrogen and stored at -80°C.

Aliquots were thawed on ice for 5 min and then at room temperature for 5 min. Cells were harvested at 5,000 x g for 1 min and were re-suspended in sterile 10% (v/v) glycerol / 500 mM sucrose (50 μ l). Up to 5 μ g of plasmid was added to the cells in a volume of 5 μ l. The cells were mixed before being added to a 0.1 cm electroporation cuvette (Biorad). The cells were pulsed at 21 kV/cm, 100 Ω and 25 μ F. Following electroporation cells were immediately resuspended in sterile BHI / 500 mM sucrose (1 ml) and were incubated for 1.5 h at 37°C with shaking (200 rpm). Cells were then plated out on TSA containing the appropriate antibiotic. Transformants were screened by PCR and Western immunoblotting.

2.5.3 Colony PCR screening of *S. aureus*

Colonies intended for screening were toothpicked onto the appropriate agar. Half a toothpicked colony was resuspended in 40 μ l TE buffer (1 mM EDTA / 10 mM Tris-HCl, pH 7.8, autoclaved) until a milky suspension was achieved. The suspension was then boiled at 100°C for 10 min and centrifuged at 6,000 rpm for 10 min. Supernatant (2 μ l) was used as a template for PCR using Phire polymerase.

2.6 Isolation of cell wall associated and extracellular proteins

To extract cell wall-associated proteins, cultures of *S. aureus* were harvested, washed in phosphate-buffered saline (PBS) and resuspended to an OD₆₀₀ of 5 or 10 in lysis buffer (50 mM Tris/HCl, 20 mM MgCl₂, pH 7.5) supplemented with raffinose (30% w/v) and complete protease inhibitors (40 μ l/ml, Roche). Cell wall proteins were solubilised by

incubation with lysostaphin (100 µg /ml; AMBI, New York) for 8 min at 37°C. Protoplasts were removed by centrifugation at 16,000 × g for 5 min and the supernatant containing solubilised cell wall proteins was aspirated and boiled for 10 min in Laemmli sample buffer. To isolate extracellular proteins, bacteria were removed from cultures by centrifugation at 4,000 × g for 5 min and the supernatant was passed through a 0.2 µm filter. Following this, supernatant samples, where indicated, were concentrated using a 30,000 molecular weight cut off spin column (Millipore). An equal volume of concentrated supernatant was boiled in Laemmli sample buffer for 10 min.

2.7 Protein electrophoresis and Western immunoblotting

2.7.1 SDS-PAGE

Typically 20 µl volumes of the diluted protein samples were loaded onto acrylamide gels (4.5% stacking and 7.5-12.5% separating gel) and separated by SDS-PAGE. Electrophoresis was carried out at 120 V after which proteins were electroblotted onto PVDF membranes (Roche) for Western immunoblotting.

2.7.2 Western immunoblotting

Non-specific binding to membranes was inhibited by incubation for 1 – 16 h in 10% (w/v) skimmed milk powder (Marvel) in TS buffer (10 mM Tris-HCl, 0.9% (w/v) NaCl, pH 7.4). Blots were probed with the appropriate primary antibody for 1 h and washed three times with gentle agitation for 10 min in TS buffer. Bound antibody was detected using a horseradish peroxidase (HRP)-conjugated antibody where appropriate. Primary and secondary antibodies used are listed in Table 2.4. Reactive bands were visualised using the LumiGLO reagent and peroxidase detection system (Cell Signalling Technology). Blots were exposed to X-Omat autoradiographic film (Kodak) and visualised using manual development with developer and fixer solutions (Kodak). Alternatively, blots were developed and visualised using ImageQuant TL software (GE).

2.7.3 Ligand affinity blotting

Fibronectin-binding proteins in *S. aureus* cell wall extracts were detected by probing membranes with biotinylated fibronectin. Human fibronectin (0.5 mg/ml, Calbiochem) was incubated with biotin (2 mg/ml) for 20 min at room temperature. The reaction was stopped by addition of NH₄Cl (10 mM). Excess biotin was removed by dialysis against PBS overnight at 4°C. PVDF membranes containing electroblotted protein samples were blocked by incubation for 1 – 16 h in 10% (w/v) skimmed milk powder (Marvel) in TS buffer. Blots

Table 2.4 Antibodies used in this study

Antibody	Working dilution / concentration	Reference
HRP-conjugated rabbit anti-mouse IgG	1:2000 / 1:500	DAKO
Polyclonal rabbit anti-SdrE IgG	1:2000	(Josefsson <i>et al.</i> , 1998)
Rabbit anti-V8 serum	1:250	M. McGavin
Rabbit anti-D3D4 IgG	1:500	(Smith <i>et al.</i> , 2011)
HRP-conjugated goat anti-rabbit IgG	1:1000	DAKO
FITC-labelled rabbit anti-mouse IgG	1:3000	DAKO
FITC-labelled goat anti-mouse (Fab) ₂ fragments	1:100	DAKO
Monoclonal mouse anti-GST 10G7	1:50	P. Speziale
Polyclonal mouse anti-SasY IgG	1:1000	P. Speziale
Polyclonal chicken anti-protein A IgY	1 µg/ml	Genscript
Biotin-conjugated monoclonal mouse anti- protein A IgG	1 µg/ml	Genscript

were probed with biotin-labelled human fibronectin (15 µg/ml, Calbiochem) for 45 min and washed three times with gentle agitation for 10 min in TS buffer. Bound fibronectin was detected with HRP-conjugated streptavidin (0.5 µg/ml, Genscript).

2.8 Analysis of surface-displayed SpA by flow cytometry

Cultures of *S. aureus* were grown to an OD₆₀₀ = 1.2, washed once with PBS and once in a solution of bovine serum albumin (BSA, 0.1% w/v) and then adjusted to an OD₆₀₀ of 0.3 in PBS. Bacteria were incubated with an equal volume of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG to detect surface-displayed protein A, or polyclonal mouse anti-SasY IgG followed by FITC-labelled rabbit anti-mouse IgG to detect surface-displayed SasY, for 30 min. Unbound antibody was removed by washing once in PBS. Bacteria were resuspended in formaldehyde (2% v/v) and bound IgG was detected using flow cytometry. Bacteria were gated on the basis of forward and side scatter. The fluorescence intensity of 20,000 bacteria was analysed and the median fluorescence was calculated.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Microtitre plates (Nunc Maxisorp) were coated with chicken anti-protein A polyclonal IgY (1 µg /ml, Genscript) diluted in coating buffer (100 mM NaHCO₃, 34mM Na₂CO₃, pH 9.6) at 4°C overnight. Wells were washed five times with PBS and blocked with 100 µl of BSA (5% w/v, Fisher Scientific) at 37°C for 2 h. Supernatant samples were diluted (1:8 or 1:50) and 100 µl of each added into the appropriate well and incubated with shaking at room temperature for 1 h, and at 37°C with no shaking for 1 h. Wells were washed five times with PBS, 100 µl of mouse monoclonal biotin-conjugated anti-protein A IgG (1 µg /ml, Genscript) was added and incubated at 37°C for 1 h. Wells were washed five times with PBS, 100 µl of HRP-conjugated streptavidin (0.5 mg /ml, Genscript) was added and incubated at 37°C for 40 min. Wells were washed five times with PBS, 100 µl of 3,3',5,5'-tetramethylbenzidine liquid substrate solution was applied and incubated at room temperature with shaking for 10 min. The reaction was stopped by the addition of 50 µl of H₂SO₄ (2 M) and absorbance was read at 450 nm in an ELISA plate reader. Wells incubated with supernatants from LAC* *spa sbi*, LAC* *spa sbi* (pRMC2) or Newman *spa sbi* were included to account for background and absorbance readings for these wells was subtracted from the values obtained from the sample wells.

2.10 Protein purification

2.10.1 Purification of SpA from *S. aureus* culture supernatants by affinity chromatography

S. aureus strain LAC* *sbi* was grown in BHI broth for 16 h to stationary phase. Bacteria were removed by centrifugation at 4,000 x *g* for 10 min and the supernatant was passed through a 0.2 µm filter. 150 ml of filtered supernatant was allowed to pass through a gravity-feed column packed with 4 ml bed volume IgG Sepharose (GE Healthcare). Briefly, the column was equilibrated with 16 ml of elution buffer (0.5 M HAc), followed by at least 20 ml of Tris-Saline Tween 20 (TST; 50 mM Tris buffer, pH 7.6, 150 mM NaCl and 0.05% Tween 20) until the column eluate was at neutral pH. The column was washed with 40 ml of TST, 10 ml of NH₄Ac (5 mM; pH 5.5), and SpA was eluted with 12 ml of elution buffer. The purified SpA was dialyzed against PBS at 4°C overnight. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting, and the protein concentration was determined with the BCA protein assay kit (Pierce). For N-terminal sequencing, samples were transferred to PVDF and sequencing was carried out by Abingdon Health Laboratory Services, UK.

2.10.2 Purification of GST-tagged recombinant proteins

Recombinant proteins were expressed from pGEX-4T2 with an N-terminal glutathione-S-transferase (GST) IPTG-inducible affinity tag in *E. coli* TOPP3. Cultures were grown to OD₆₀₀ of 0.5-0.6 and then induced with 1 mM IPTG for 3 h at 37°C with shaking (200 rpm). Cells were harvested by centrifugation and resuspended in 20 - 40 ml PBS containing protease inhibitors (EDTA-free; Roche) prior to lysis in a French pressure cell. The lysate was centrifuged at 15,000 x *g* for 20 min at 4°C. DNase, 20 – 80 µl (1 mg/ml) was added to the supernatant followed by filtration through a 0.45 µm filter (Whatman). A GSTrapTM FF column (5 ml; GE Healthcare) was used according to the manufacturer's instructions with the flow rate controlled using a peristaltic pump. The column was equilibrated using PBS. The filtered, cleared cell lysate was applied to the column. The column was washed with PBS and bound protein was eluted from the column using reduced glutathione elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). Eluted protein was collected in 2 ml fractions. Elution was monitored by measuring the A₂₈₀ of the eluate using a NanodropTM 1000 spectrophotometer. Positive fractions were analysed by SDS-PAGE after which proteins were visualised by Coomassie blue staining. Fractions containing the purified recombinant protein were dialysed against PBS for 16 h at 4°C. Protein concentrations were determined using the BCA assay kit (Pierce) and by measuring

absorbance at 280 nm using a Nanodrop™ 1000 spectrophotometer.

2.11 Mass spectrometry

Trypsin digests and mass spectrometry was carried out by Kieran Wynne in the proteomics core facility in UCD.

2.11.1 Trypsin Digestion.

Purified SpA (2 µg) from LAC* *sbi* supernatants was resuspended in a solution containing urea (6 M) and dithiothreitol (4 mM). The solution was heated at 60°C for 60 min. The sample was allowed to cool before iodoacetamide (15 mM) was added and incubated for 30 min at 37°C. A solution (120 µl) containing NH₄HCO₃ (50 mM; pH 7.8) and CaCl₂ (1 mM) was added to dilute the urea concentration to below 1 M. Sequencing Grade Modified Trypsin was added (1 µg, Promega) and the samples were incubated at 37°C overnight. Prior to MS analysis the samples were cleaned using ZipTips (Millipore).

2.11.2 Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

The samples were run on a Thermo Scientific Q Exactive mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were resuspended in formic acid (0.1%). Each sample was loaded onto Biobasic Picotip Emitter (120 mm length, 75 µm ID) packed with ReproCil Pur C18 (1.9 µm) reverse phase media and was separated by an increasing acetonitrile gradient over 37 min at a flow rate of 250 nL min⁻¹. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220°C, and with a potential of 2000 V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution (70,000) MS scan (300-2000 m/z) was performed using the Q Exactive to select the 12 most intense ions prior to MS/MS analysis using higher-energy collisional dissociation with stepped normalised collision energy.

2.11.3 Data Base Search.

The raw data was *de novo* sequenced and searched against the Homosapien subset of the Uniprot Swissprot database (to which the full-length amino acid sequence of SpA from strain LAC* was added) using the search engine PEAKS Studio 7 (Bioinformatics Solutions) for peptides cleaved with trypsin. Each peptide used for protein identification met specific Peaks parameters, i.e. only peptide scores that corresponded to a false discovery rate (FDR) of ≤1% were accepted from the Peaks PTM database search. The Peaks De Novo results were

filtered using an average local confidence (ALC) of $\geq 65\%$ and peptide score of $(-10\lg P)$ of ≥ 15 .

2.12 Whole blood survival assay

The ability of *S. aureus* to survive in whole human blood was studied as previously described (Visai *et al.*, 2009). Bacteria were grown to the desired OD₆₀₀ in BHI broth, and washed twice in Roswell Park Memorial Institute (RPMI) media before being diluted to give 5×10^3 CFU/ml. Blood was obtained from healthy volunteers and treated with the anticoagulant Hirudin (50 µg/ml, Refludan, Pharmion). 25 µl of bacteria was added to 475 µl of blood. Where indicated washed *S. aureus* was resuspended in cell-free supernatant from overnight cultures of *S. aureus* prior to inoculation in blood. Where necessary, ATc (312.5 ng/ml) was added to maintain expression of ATc inducible genes. Immediately 100 µl of each sample was added to 900 µl of ice cold endotoxin-free water, and 100 µl was plated out on TSA in triplicate to calculate input CFU. Tubes were incubated at 37°C with shaking (70 rpm) for 3 h. Following this, 100 µl of each sample was added to 900 µl of ice cold endotoxin-free water, and 100 µl of each was plated out on TSA in triplicate to calculate the number of surviving CFU. Percentage survival was determined by dividing the mean CFU after 3 h by the corresponding mean input CFU. Ethical approval for the use of human blood was obtained from the TCD Faculty of Health Sciences ethics committee.

2.13 Bacterial growth in human plasma

Bacteria were grown to the desired OD₆₀₀ in BHI broth, and washed twice in RPMI media before being diluted to give 5×10^3 CFU/ml. Plasma was isolated from whole blood treated with anticoagulant Hirudin (50 µg/ml, Refludan, Pharmion) by centrifugation at 1,500 rpm for 15 min at 4°C. Following sedimentation of blood cells plasma was aspirated and stored at -20°C or used immediately. 25 µl of bacteria was added to 475 µl of plasma. Immediately 100 µl of each sample was added to 900 µl of ice cold endotoxin-free water, and 100 µl was plated out on TSA in triplicate to calculate input CFU. Tubes were incubated at 37°C with shaking (70 rpm) for 3 h. Following this, 100 µl of each sample was added to 900 µl of ice cold endotoxin-free water, and 100 µl of each was plated out on TSA in triplicate to calculate the number of final CFU. Percentage growth was determined by dividing the mean CFU after 3 h by the corresponding mean input CFU.

2.14 Neutrophil isolation

Fresh whole blood was obtained from healthy volunteers, heparinised and mixed in a 2:1 ratio with 3% Dextran (in saline) to stimulate erythrocyte aggregation and allowed to

settle for 30 min at room temperature. Following erythrocyte separation (bottom layer) the top layer was aspirated from the solution and gently layered on top of 10 ml Ficol-Paque PLUS (GE Healthcare) and centrifuged for 20 min at 300 x g. Following this the supernatant was removed and the pellet was resuspended in 5 ml of 0.87% (w/v) ammonium chloride for 11 min to lyse remaining erythrocytes. Following this cells were centrifuged at 300 x g for 5 min and the pellet was resuspended in 5 ml Dulbecco's modified eagle's medium (DMEM). Neutrophils were counted on a haemocytometer (bright line, Neubauer) and adjusted to 5×10^6 cells/ml in DMEM. Purified neutrophils were assessed using flow cytometry. Cells were gated on the basis of forward and side scatter. The fluorescence intensity of 5,000 cells was analysed and only samples obtaining >95% purity were retained.

2.15 Neutrophil assays

2.15.1 Phagocytosis assay

S. aureus Wood46 harbouring plasmid pCM29 (constitutive GFP expression vector) was grown in 50 ml BHI until an OD₆₀₀ of 0.5 was achieved. Bacteria were centrifuged at 4,000 rpm for 10 min and washed in an equal volume of PBS. Bacteria were harvested once more and resuspended in an equal volume of RPMI. Following this the OD₆₀₀ was measured and bacteria were adjusted to a concentration of 5×10^7 CFU/ml in RPMI/BSA (0.1%, w/v). 50 µl of bacteria was incubated with 50 µl of recombinant protein (5 µM), or purified SpA from LAC* *sbi* and either: 10% pooled human serum (CompTech), purified IgG (0.8 mg/ml; Baxter), 10% IgG-depleted serum, or 10% IgG-depleted serum supplemented with purified IgG (0.8 mg/ml) in a final volume of 250 µl for 1 min at 37°C with shaking (200 rpm). Serum was depleted of IgG using a Protein A/G HP SpinTrap™ column. Following this 50 µl of purified neutrophils (5×10^6 cells/ml) was added to give a multiplicity of infection (MOI) of 10 and samples were incubated for a further 15 min at 37°C with shaking at 600 rpm to encourage phagocytosis. Reactions were stopped by addition of 100 µl ice-cold 1% (v/v) formaldehyde in PBS. The percentage of neutrophils bearing fluorescent bacteria was determined by flow cytometric analysis of 5,000 cells by gating on forward scatter and side scatter. Flow cytometric data were acquired with a BD Accuri C6 instrument (BD Biosciences). Quenching of extracellular fluorescence was carried out by addition of 20 µg/ml (final concentration) of trypan blue.

2.15.2 Bacterial survival in neutrophils

S. aureus LAC* *spa sbi* harbouring plasmid pRMC2 or pALC::*sasY* (Table 2.2) was grown, as described in section 2.14.1, and adjusted to a final concentration of (5×10^7)

CFU/ml in RPMI/BSA (0.1%, w/v). 25 μ l of bacteria was added to 25 μ l of purified human neutrophils (5×10^6) cells/ml in 10% pooled human serum (final concentration) in a final volume of 100 μ l. Immediately 40 μ l of each sample was added to 360 μ l of ice-cold endotoxin-free water and 100 μ l was plated out in triplicate on TSA to calculate input CFU. Samples were incubated at 37°C for 15 min with shaking (600 rpm) to encourage phagocytosis. The time-point directly following phagocytosis is termed time 0 (T0). Samples were incubated at 37°C with reduced shaking (200 rpm) from this point onwards. At 0, 15, 30 and 60 min post-phagocytosis 40 μ l of each sample was added to 360 μ l of ice-cold endotoxin-free water and 100 μ l was plated out in triplicate on TSA to calculate total viable CFU. At the same time-points a separate aliquot of each sample was removed and incubated with a mixture of lysostaphin (20 μ g/ml; final concentration) and gentamicin (100 μ g/ml; final concentration) at 37°C for 15 min to kill extracellular bacteria. Following this samples were centrifuged at 300 x g for 5 min and washed twice with 100 μ l of RPMI/BSA (0.05%; w/v). Samples were diluted 1:10 in ice-cold endotoxin-free water and 100 μ l was plated out in triplicate on TSA to calculate total viable intracellular CFU. Percentage total and intracellular CFU recovered was determined by dividing the respective mean CFU by the mean input CFU.

2.16 Binding of recombinant proteins to opsonised *S. aureus*

S. aureus Wood46 was swabbed from a blood agar plate and emulsified in 2 ml of HEPES++ buffer (10 mM HEPES, 150 μ M NaCl, 5 mM CaCl_2 , 2.5 mM MgCl_2 , pH 7.4) with 0.1% BSA (w/v in PBS). Following this the OD₆₀₀ was measured and bacteria were adjusted to an OD₆₀₀ = 1.0 in a final volume of 1 ml in HEPES++ / 0.1% BSA. Bacteria were centrifuged at 14,000 rpm for 1 min, resuspended in 1 ml of HEPES++ buffer and diluted 1:3 in HEPES++ buffer. 50 μ l of bacteria was mixed with 40 μ l of GST-linked recombinant protein (3.125 μ M) in either: 5% pooled human serum, 5% heat-inactivated (HI) serum, 1% serum, 1% HI-serum, purified IgG (0.08 mg/ml; Baxter), 1% IgG-depleted serum, or 1% IgG-depleted serum supplemented with purified IgG (0.08 mg/ml) in a final volume of 100 μ l. Samples were incubated for 30 min at 37°C with shaking (200 rpm). Serum was heat-inactivated by incubation at 56°C for 20 min. Serum was depleted of IgG using a Protein A/G HP SpinTrapTM column. Samples were washed once in 0.1% BSA (in PBS; w/v) and incubated with mouse monoclonal anti-GST IgG (1:50) in a final volume of 100 μ l PBS/0.1% BSA. Samples were incubated on ice for 30 min. Unbound antibody was removed by washing once in PBS. Bacteria were resuspended in 100 μ l of FITC-conjugated goat anti-

mouse F(ab)₂ fragments (1:100, DAKO) in PBS and incubated on ice for 30 min. Unbound antibody was removed by washing once in PBS. Bacteria were resuspended in formaldehyde (2% v/v) and bound FITC-conjugated (Fab)₂ fragments were detected using flow cytometry. Bacteria were gated on the basis of forward and side scatter. The fluorescence intensity of 20,000 bacteria was analysed and the median fluorescence was calculated.

2.17 Statistical analysis

Statistical analysis was performed using Prism Graphpad 5 software. P values were calculated using Student's t-test.

Chapter 3

The role of extracellular proteases and the endopeptidase LytM in the production of released protein A in *Staphylococcus aureus*

3.1 Introduction.

Almost all clinical isolates of *S. aureus* express the major virulence factor, staphylococcal protein A (SpA) (Votintseva *et al.*, 2014). SpA is located both on the surface of the bacterium and in the extracellular medium (Becker *et al.*, 2014; Forsgren & Nordstrom, 1974; Lindmark *et al.*, 1977; Movitz, 1976). The existence of released SpA was first reported in the *S. aureus* strain Cowan (Movitz, 1976), where it was shown that released SpA accounts for approximately 30% of total produced SpA in this strain (Movitz, 1976). Released SpA can be detected in the skin lesions of mice infected with a USA300 strain of community-associated methicillin-resistant *S. aureus* (CA-MRSA) and in fluids recovered from patients with *S. aureus* infection (Yung *et al.*, 2011). SpA is a potent activator of tumour necrosis factor α (TNF α) receptor 1 (TNFR1) signalling (Gomez *et al.*, 2004) which is essential at sites of infection where TNF α signalling is critical, such as the lung. SpA is an important factor during bloodstream infection due to its ability to bind to the Fab region of human B cells and thus subvert an adaptive immune response (Falugi *et al.*, 2013). Purified recombinant SpA promotes crosslinking of IgM from humans and guinea pigs via V_H3 binding activity (Kim *et al.*, 2015). These observations imply that released SpA alone may be sufficient for the suppression of the adaptive immune response during *S. aureus* bloodstream infections (Becker *et al.*, 2014).

MRSA is traditionally associated with hospitals, where it has developed a niche among the immunocompromised, and these strains are referred to as hospital-associated (HA) MRSA. The late 1990s/early 2000s marked the first appearance of the USA300 lineage of MRSA which rapidly spread across the US displacing the previously dominant MRSA strains (Tenover & Goering, 2009). These highly transmissible and virulent strains are referred to as community-associated (CA-) MRSA. CA-MRSA of the USA300 lineage are members of clonal complex (CC) 8, which possess the staphylococcal cassette chromosome *mec* (SCC*mec*) type IV element, and are the major cause of skin and soft tissue infections in the US (Johnson *et al.*, 2007). The success of CA-MRSA isolates has, in-part, been attributed to an increased repertoire of potent virulence factors, such as PVL and the arginine catabolic mobile element (ACME) which are encoded by mobile genetic elements (Diep *et al.*, 2006b; Diep *et al.*, 2008), or high expression of core genomic virulence determinants such as α -toxin, PSMs, or extracellular proteases (Li *et al.*, 2009). As well as causing severe skin and soft tissue infections (Moran *et al.*, 2006) USA300 is also a major cause of invasive disease such as community-acquired pneumonia and bloodstream infections (Klevens *et al.*, 2007),

S. aureus secretes up to 10 different proteolytic enzymes into the culture supernatant (Kolar *et al.*, 2013). Extracellular proteases are proposed to have a number of different roles in the virulence of *S. aureus*. It has been suggested that extracellular proteases can facilitate the generation of released SpA via enzymatic cleavage of CWA SpA from the surface of *S. aureus* (Kolar *et al.*, 2013). Additionally, Karlsson *et al.*, (2001) demonstrated an increase in the amount of surface-located fibronectin-binding proteins (FnBPs) and SpA when the *sspA* gene, encoding for the V8 serine protease, was mutated. However, further study has shown that while V8 does remove FnBPs from the cell surface of *S. aureus*, V8 did not remove SpA under the same conditions (McGavin *et al.*, 1997). These conflicting studies have led us to address the role of extracellular proteases in the generation of released SpA in LAC* during logarithmic growth.

Peptidoglycan must be continually synthesised to maintain cell viability and growth, for this a careful balance of peptidoglycan synthesis and degradation must be maintained, which is a vital function of the peptidoglycan hydrolases in *S. aureus*. A recent study has identified a mechanism for release of SpA from the surface of *S. aureus* strain NM by the action of autolysins (Becker *et al.*, 2014), specifically LytN, a cross-wall murein hydrolase, and LytM, a glycyl-glycine endopeptidase. Cell wall-located surface proteins of *S. aureus* are covalently anchored to the pentaglycine crossbridge of peptidoglycan via the actions of the membrane-bound enzyme sortase A. Becker *et al.*, (2014) described a mechanism whereby SpA is shed from the cell envelope of strain NM into the culture medium following cleavage of the pentaglycine crossbridge of peptidoglycan by the *S. aureus* glycyl-glycine endopeptidase LytM. The murein hydrolase LytN cleaves the amine bonds between N-acetylmuramic acid and the tetrapeptide side chains of peptidoglycan so that peptidoglycan fragments linked to SpA lack amino sugars (Becker *et al.*, 2014). This is hypothesized to allow released SpA to avoid activating nucleotide-binding and oligomerization domain-containing protein 2 (NOD2) *in vivo*. The release of SpA is not completely inhibited in a LytM-deficient mutant of strain NM, indicating that LytM activity is not the only factor involved in the release of SpA fragments.

This chapter investigates the role of extracellular proteases and LytM in the generation of released SpA in the CA-MRSA strain LAC*.

3.2 Results

3.2.1 Release of SpA from the CA-MRSA strain LAC*.

Surface-displayed SpA becomes linked covalently to the *S. aureus* cell wall by sortase A (Mazmanian *et al.*, 1999; Ton-That *et al.*, 1999). SpA is also released into culture supernatants (Becker *et al.*, 2014; Forsgren & Nordstrom, 1974; Movitz, 1976). This project set out to investigate mechanisms of SpA release using an erythromycin-sensitive derivative of the USA300 strain LAC (LAC*) (Boles *et al.*, 2010). Strain LAC expresses SpA at high levels (Herbert *et al.*, 2010; Ventura *et al.*, 2010) and produces an additional Ig-binding protein (staphylococcal binder of immunoglobulin, Sbi) which associates with lipoteichoic acid in the cell envelope (Smith *et al.*, 2012). Sbi is also found extracellularly (see section 1.2.3.1.2) (Smith *et al.*, 2011).

In order to determine if LAC* releases SpA, proteins solubilised from the cell wall by lysostaphin treatment during protoplast formation (cell wall extract, CW) and culture supernatants (SN) were analyzed by Western immunoblotting, probing with HRP-conjugated rabbit IgG (Fig. 3.1A). To distinguish between Sbi and SpA, isogenic *spa* and *sbi* mutants and a double mutant (LAC* *spa sbi*) were generated. A single band corresponding to SpA was detected in cell wall extracts from LAC* and LAC* *sbi* and was absent in extracts from LAC* *spa* and the LAC* *spa sbi* (Fig. 3.1A). Consistent with previous findings, Sbi was not detected in cell wall extracts (Fig. 3.1A) since it is not solubilised by lysostaphin during protoplast formation (Smith *et al.*, 2011). A band corresponding to Sbi was detected in culture supernatants of strains LAC* and LAC* *spa* (Fig. 3.1A) since Sbi binds to rabbit IgG. Therefore, to study the release of SpA, it was important to use the LAC* *sbi* mutant to avoid interference from extracellular Sbi in culture supernatants. As a control, the same cell wall extracts and supernatants were probed for the CWA protein SdrE (Fig. 3.1B) and V8, a secreted protease (Fig. 3.1C). The SdrE protein was detected only in the cell wall fraction (Fig. 3.1B) and V8 was detected only in supernatant samples indicating purity of the samples. Equal loading of protein is demonstrated by similar band intensities between strains (Fig. 3.1B, 3.1C).

Two bands of similar apparent molecular weight were detected in culture supernatants probed with anti-V8 serum (Fig. 3.1C). To determine if these bands corresponded to V8, culture supernatant from an isogenic mutant (LAC* PD) lacking all extracellular proteases of *S. aureus* (V8, SplABCDEF, ScpA, SspB and aureolysin) was analysed by Western immunoblotting probing with anti-V8 serum. Two bands were detected in culture

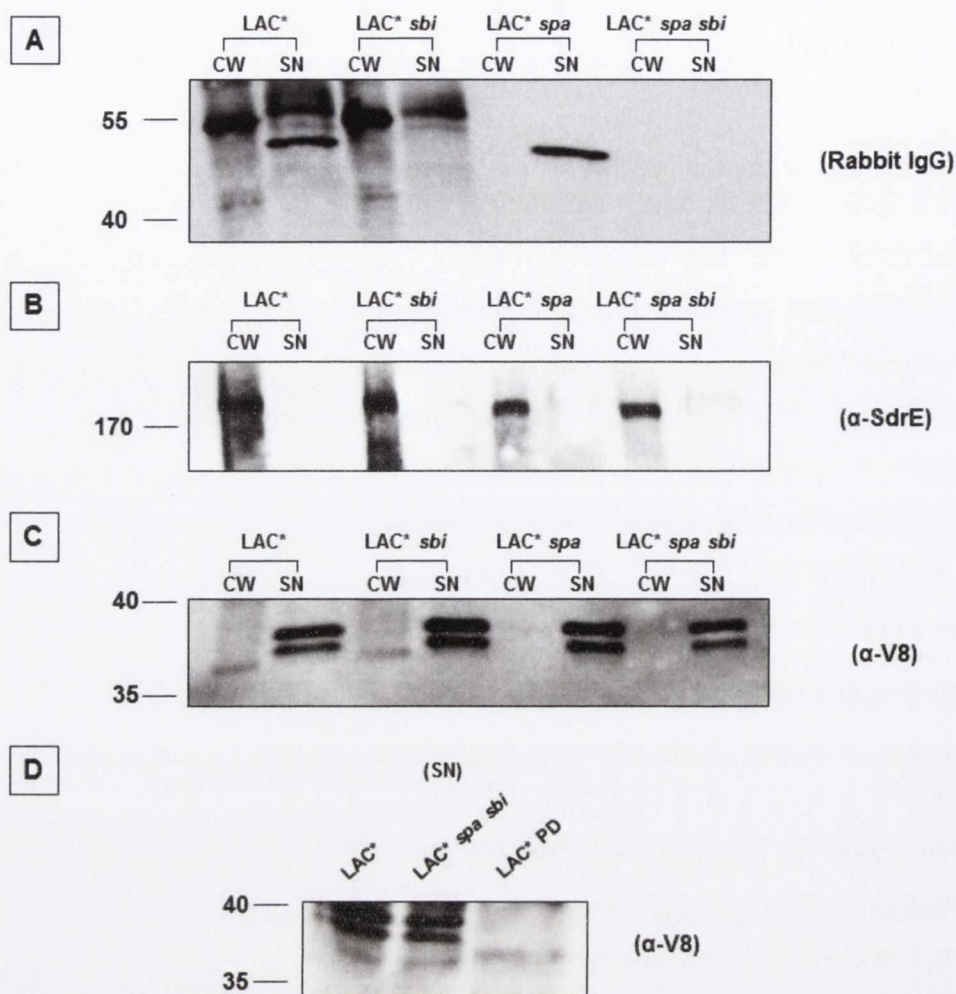


FIG. 3.1 *Staphylococcus aureus* LAC* releases SpA. (A) *S. aureus* was grown to an OD₆₀₀ of 1.2. Cell wall-extracts (CW) were diluted 1:5 prior to loading on the gel and supernatant samples (SN) were not diluted. Protein A was detected using HRP-conjugated rabbit IgG. The same strains were also investigated for the presence of SdrE (B) and V8 (C) to ensure purity of samples and equal loading of protein. The presence of V8 in the supernatant of LAC*, LAC* *spa sbi* and LAC* PD was also investigated (D). SN was concentrated 20-fold prior to loading, CW samples were unaltered. Size markers are indicated (kDa).

supernatants of LAC* and LAC* *spa sbi* (Fig. 3.1D), while no bands were detected in LAC* PD supernatants (Fig. 3.1D). This suggests that at least one of the bands identified is V8 and the second reactive band could be either one of the other extracellular proteases not present in the LAC* PD strain, or another form/breakdown product of V8. V8 is synthesised as a preproenzyme which is proteolytically cleaved into its active mature form by aureolysin (Drapeau, 1978), therefore the upper bands could represent V8 as the preproenzyme and the lower band the mature protein.

In order to determine the proportion of total SpA that is released during growth of LAC* *sbi* in brain heart infusion broth, bands on a Western blot corresponding to SpA in cell wall extracts and supernatants were quantified using densitometry (Fig. 3.2). Released SpA was expressed as a percentage of total SpA associated with the cell wall and supernatant. Released SpA represented 6.5% of total protein A from cultures grown to early exponential phase ($OD_{600} = 0.3$, Fig. 3.2A) and 7.3% of total protein A from cultures grown to an OD_{600} of 1.2 (Fig. 3.2B).

3.2.2 The role of staphylococcal extracellular proteases in the generation of released SpA in LAC*.

In order to determine if the generation of extracellular SpA in LAC* requires the activity of proteases, the amount of SpA on the surface of LAC* and LAC* PD was compared. Surface-displayed SpA was detected with FITC-labelled IgG using flow cytometry. The amount of SpA on the bacterial surface did not differ between LAC* and LAC* PD at any of the growth phases tested (Fig. 3.3A). Cell wall extracts were prepared from the same cultures and analysed by Western immunoblotting. A band corresponding to SpA was detected in the cell wall of all cultures (Fig. 3.3B).

In order to study the generation of released SpA by LAC* and LAC* PD, culture supernatants were examined by Western immunoblotting. Released SpA was detected in the culture supernatants of bacteria at all stages of growth (Fig. 3.3C). No major alterations in the level of released SpA was observed when supernatants from LAC* were compared to LAC* PD, indicating that extracellular proteases of *S. aureus* are not responsible for the generation of released SpA.

Karlsson *et al.* (2001) implicated the *S. aureus* serine protease V8 in the cleavage of SpA from the surface of the bacterium. However McGavin *et al.* (1997) could not replicate these findings. Since LAC* PD is deficient in the *sspA* gene encoding V8 it seemed unlikely that V8 protease was responsible for the generation of released SpA by LAC*. In order to

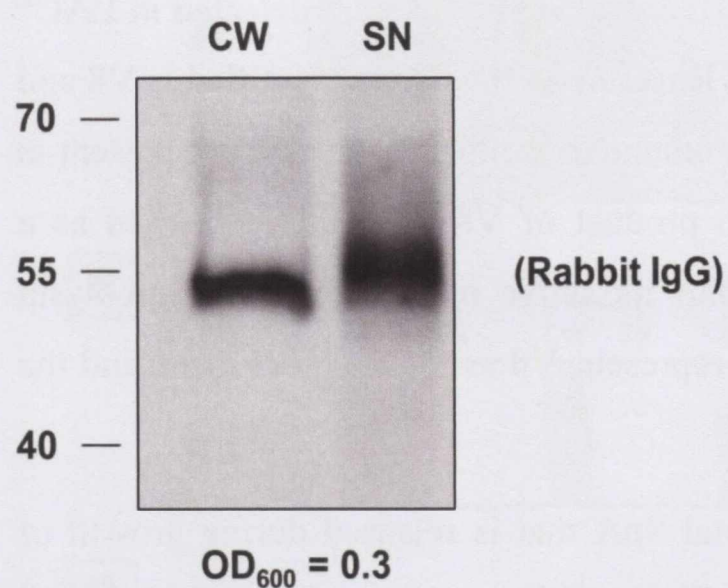
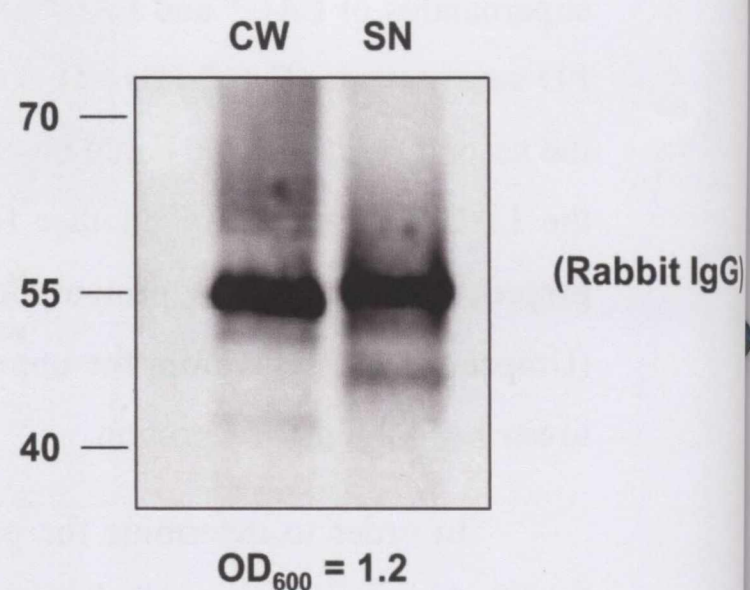
A**B**

FIG. 3.2 Comparing the levels of cell wall-associated and released SpA from LAC*. LAC* *sbi* was grown to an OD_{600} of 0.3 (A) or 1.2 (B). Cell wall-extracts (CW) were diluted 1:20 prior to loading on the gel and supernatant samples (SN) were not diluted. Protein A was detected using HRP-conjugated rabbit IgG. Size markers are indicated (kDa).

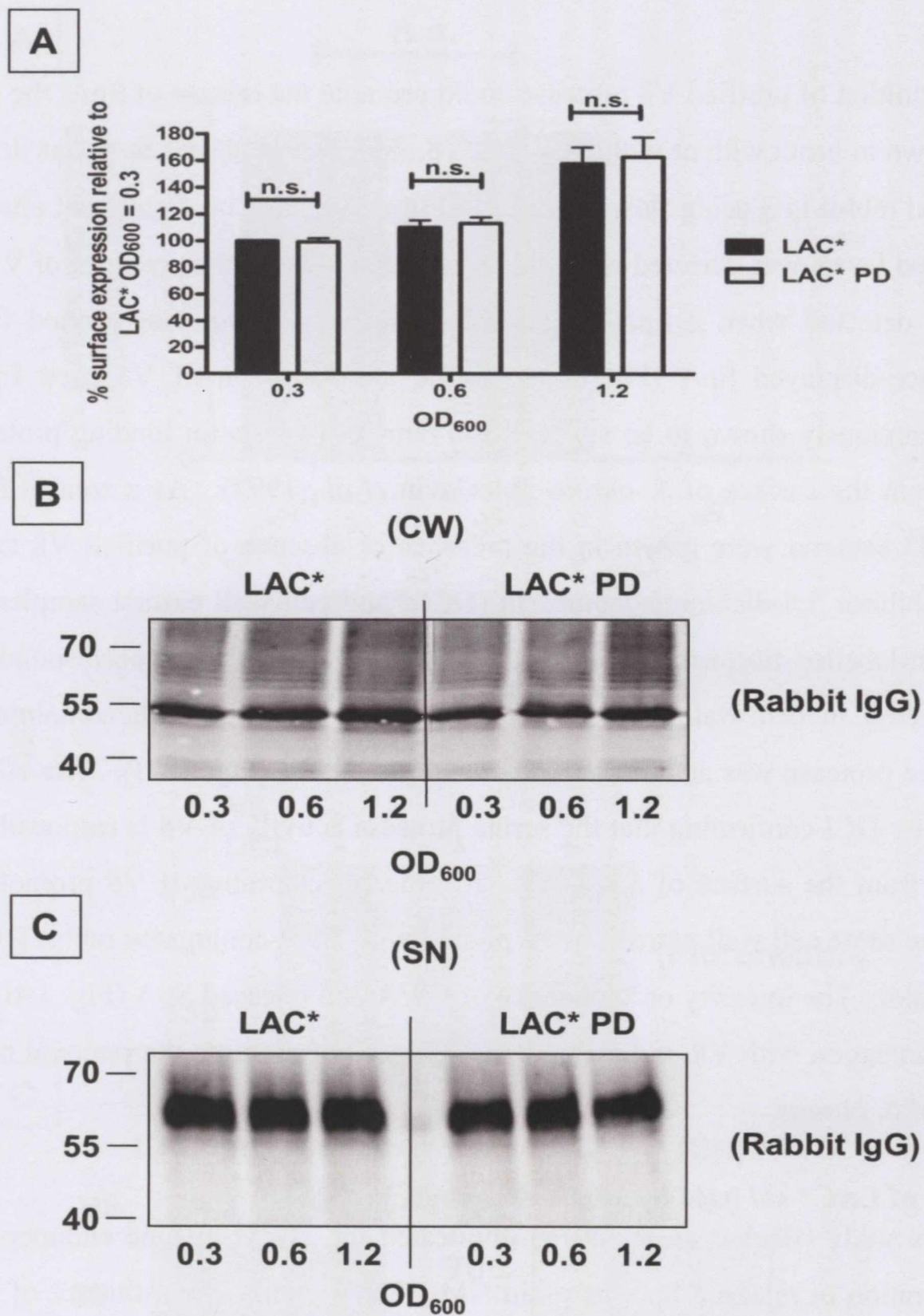


FIG. 3.3 Extracellular proteases of LAC* are not required for the release of protein A. (A) Protein A on the surface of LAC* and LAC* PD was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured by flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for LAC* harvested at OD₆₀₀ = 0.3. Bars represent the mean values and error bars indicate the SEM of three independent experiments. n.s. = not significant, $p > 0.05$. Cell wall extracts (CW) (B) and culture supernatants (SN) (C) from LAC* and LAC* PD grown to the OD₆₀₀ indicated were probed with HRP-labelled rabbit IgG in a Western immunoblot. Supernatants harvested at OD₆₀₀ = 0.3 were concentrated 8-fold, at OD₆₀₀ = 0.6 concentrated 4-fold, and at OD₆₀₀ = 1.2 concentrated 2-fold before loading on a gel. Size markers are indicated (kDa).

determine if the addition of purified V8 protease could promote the release of SpA, the LAC* PD strain was grown in broth with or without added V8. Surface-displayed SpA was detected with FITC-labelled rabbit IgG using flow cytometry (Fig. 3.4A), and no significant alteration in surface-displayed levels was detected when bacteria were grown in the presence of V8. No fluorescence was detected when a SpA-deficient mutant (LAC* *spa*) was probed for the presence of surface-displayed SpA (Fig. 3.4A). The concentration of V8 used for this experiment was previously shown to be sufficient to remove fibronectin binding proteins A and B (FnBPs) from the surface of *S. aureus* (McGavin *et al.*, 1997). As a control for V8 activity, LAC* PD bacteria were grown in the presence or absence of purified V8 and the serine protease inhibitor 3,4-dichloroisocoumarin (DCI), and cell wall extract samples were probed with biotin-labelled fibronectin to detect FnBPs. Very faint bands corresponding to FnBPs were detected in cell wall extracts from bacteria grown in broth containing V8 confirming that the protease was active under the conditions used (Fig. 3.4B). The effect of V8 was inhibited by DCI confirming that the serine protease activity of V8 is responsible for removing FnBPs from the surface of *S. aureus*. In order to determine if V8 promotes the release of SpA, the same cell wall extracts were probed with HRP-conjugated rabbit IgG in a Western immunoblot. The integrity or abundance of CWA and released SpA (Fig. 3.4C) was not affected by incubation with V8, indicating that V8 does not promote the removal of SpA from the surface of *S. aureus*.

3.2.3 Generation of LAC* *sbi* *lytM* by allelic exchange.

A previous study (Becker *et al.*, 2014) implicated the glycyl-glycine endopeptidase LytM in the generation of released SpA in strain NM. To determine the influence of LytM on SpA release in LAC it was necessary to generate a LytM-deficient mutant of LAC* *sbi*. This was achieved using the temperature-sensitive plasmid pIMAY (see section 1.7.3.1.2). Plasmid pIMAY:: Δ *lytM* carries 509 bp of DNA from upstream (US) and 512 bp of DNA from downstream (DS) of the *lytM* gene of LAC (Fig. 3.5). The resulting plasmid (pIMAY:: Δ *lytM*) was transformed into *E.coli* DC10B (Monk *et al.*, 2012) and verified by DNA sequencing. The plasmid was transformed into *S. aureus* LAC* *sbi* made electrocompetent and deletion of the *lytM* gene was achieved by allelic exchange as previously described (see section 1.7.2) (Monk *et al.*, 2012). Briefly, putative transformants grown at 28°C on agar containing chloramphenicol were screened by PCR for the presence of replicating plasmid. Colonies possessing replicating pIMAY:: Δ *lytM* yielded a band of approximately 1.2 kb using primers which anneal approximately 100 bp upstream and

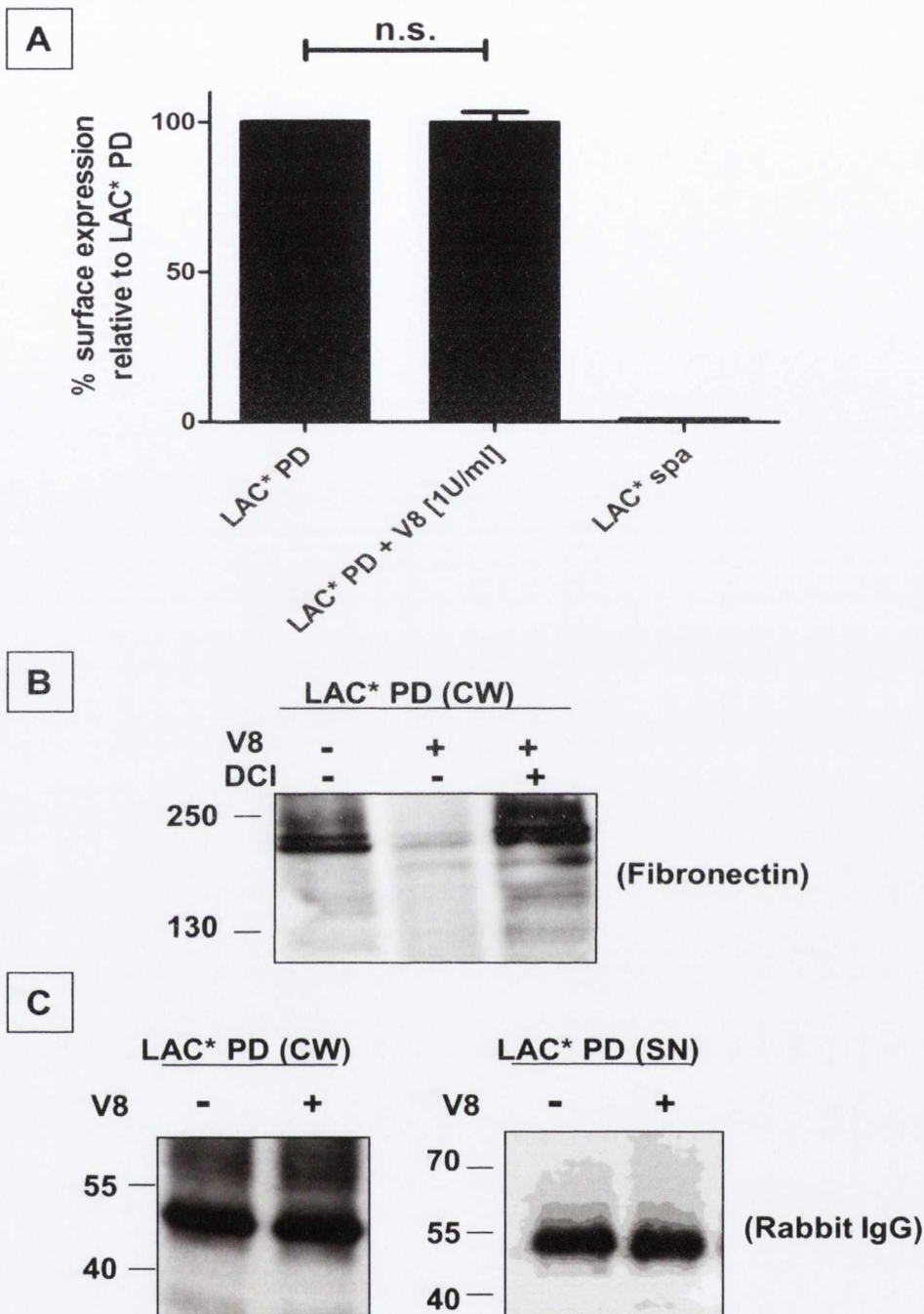


FIG. 3.4 V8 is not involved of the generation of released SpA in LAC*. (A) LAC* PD in the presence or absence of purified V8 (1U/ml) and LAC* *spa* were grown to an $OD_{600} = 1.0$. Surface-displayed SpA was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured by flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for LAC* PD in unsupplemented broth. Bars represent the mean values and error bars indicate the SEM of three independent experiments. n.s. = not significant, $p > 0.05$. (B) LAC* PD was grown to an $OD_{600} = 0.8$ in broth alone or in broth supplemented with V8 (1U/ml) and DCI (200 μ M) and cell wall extracts (CW) were probed with biotin-labelled fibronectin in a ligand affinity blot. Bound fibronectin was detected using streptavidin-HRP. (C) The same cell wall extracts were probed with HRP-conjugated rabbit IgG to detect SpA. Supernatants (SN) from the same cultures were probed with HRP-labelled rabbit IgG. Size markers are indicated (kDa).

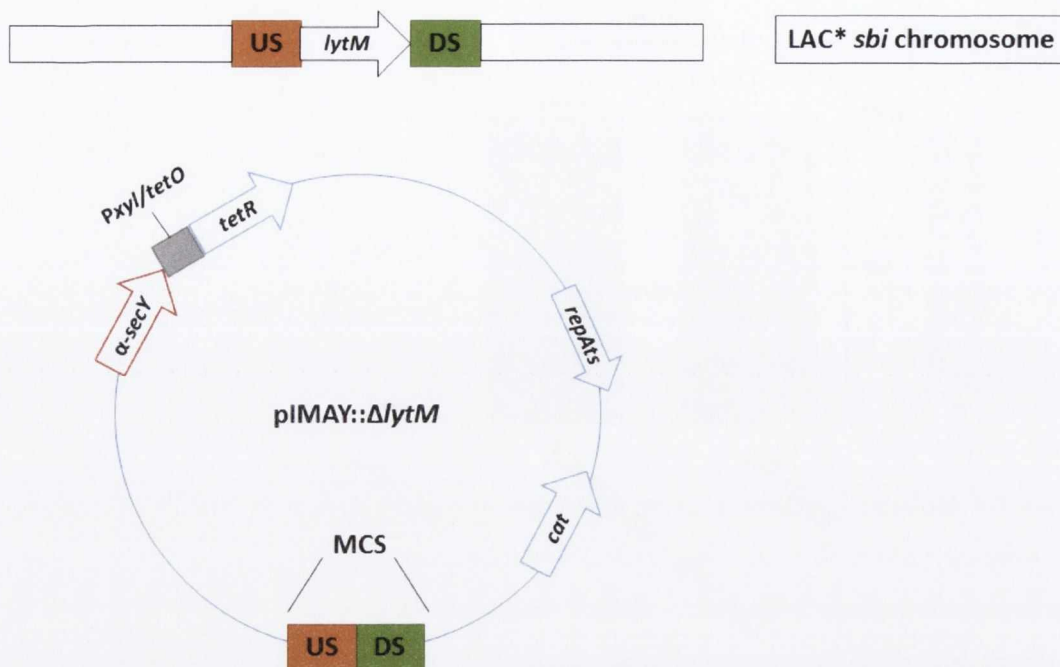


FIG. 3.5 Schematic representation of pIMAY::ΔlytM plasmid. Plasmid pIMAY::ΔlytM carries 509 bp of DNA upstream (US) and 512 bp of DNA downstream (DS) of the *lytM* gene amplified from LAC. pIMAY possesses the temperature-sensitive *repAIs* replicon from the plasmid pVE6007, which is restrictive for plasmid replication at 37°C. It also encodes a *secY* antisense RNA fragment (α -*secY*) under the tight control of a tetracycline-regulator (*tetR*) repressor. Expression of anti-*secY* is controlled by the anhydrotetracycline-inducible promoter (*Pxyl/tetO*) which allows for selection of bacteria that have lost replicating and integrated pIMAY during plasmid excision. This vector also possess a chloramphenicol resistance gene (*cat*) which allows for selection of bacteria possessing replicating, or integrated plasmid.

downstream of the pIMAY MCS (Fig. 3.6A). Positive clones (Fig. 3.6A) were grown at 37°C, which is not permissive for plasmid replication, in the presence of the chloramphenicol to select for integrants. Colonies were once again screened by PCR, this time to confirm loss of replicating plasmid (Fig. 3.6B). Integration may occur either upstream or downstream of the gene of interest. Figure 3.7A demonstrates upstream integration of pIMAY:: Δ lytM into the LAC* *sbi* chromosome. The side of integration (SOI) of pIMAY:: Δ lytM was assessed by PCR using a combination of primers internal to the *lytM* gene (InF and InR) and external primers which anneal to regions of the chromosome upstream and downstream of the *lytM* gene (OutF and OutR, Fig. 3.7). Colonies with integrated plasmid e.g. colonies c and d (Fig. 3.6B) were assessed for SOI (Fig. 3.7). Confirmed integrants were plated on agar containing anhydrotetracycline (ATc, 1 µg/ml) and incubated at 28°C to induce the expression of the anti-*secY* antisense RNA (see section 1.5.2). The *secY* gene product is essential in *S. aureus* so growth of bacteria carrying pIMAY is suppressed, selecting for excisants. Following plasmid excision, depending on where the double cross-over event occurred, either a wildtype or mutant gene product is obtained (Fig. 3.8A). Colonies that grew in the presence of ATc and were sensitive to chloramphenicol were analysed by PCR using the OutF and OutR primers. Bacteria that retained the full-length gene, e.g. colonies b and d (Fig. 3.8B) yielded a product of approximately 2 kb, whereas mutants lacking the *lytM* gene, e.g. colonies a and c (Fig. 3.8B), yielded a product of approximately 1.1 kb. Putative LAC* *sbi* *lytM* mutants were confirmed by DNA sequencing of a PCR product generated using the OutF and OutR primers. No alterations in growth rate were observed between LAC* *sbi* and LAC* *sbi* *lytM* in the following analyses.

3.2.4 The influence of the endopeptidase LytM on the generation of SpA in LAC*.

Previously Becker *et al.* (2014) reported that the *S. aureus* endopeptidase LytM promotes the release of SpA by cleaving within the pentaglycine crossbridge of peptidoglycan. The amount of SpA released into culture supernatants was greatly reduced in a LytM-deficient mutant of strain NM (Becker *et al.*, 2014). In order to determine if release of SpA by LAC* is promoted by LytM, the amount of SpA on the bacterial surface and in culture supernatants of a LytM-deficient mutant (LAC* *sbi* *lytM*) was quantified and compared to the levels of SpA produced by LAC* *sbi* (Fig. 3.9, 3.10). Cell wall extracts were analyzed by Western immunoblotting, probing with HRP-conjugated rabbit IgG (Fig. 3.9A). Bacteria were also incubated with FITC-labelled rabbit IgG to quantify the levels of surface-displayed SpA by flow cytometry (Fig. 3.9B). A decrease in band intensity was

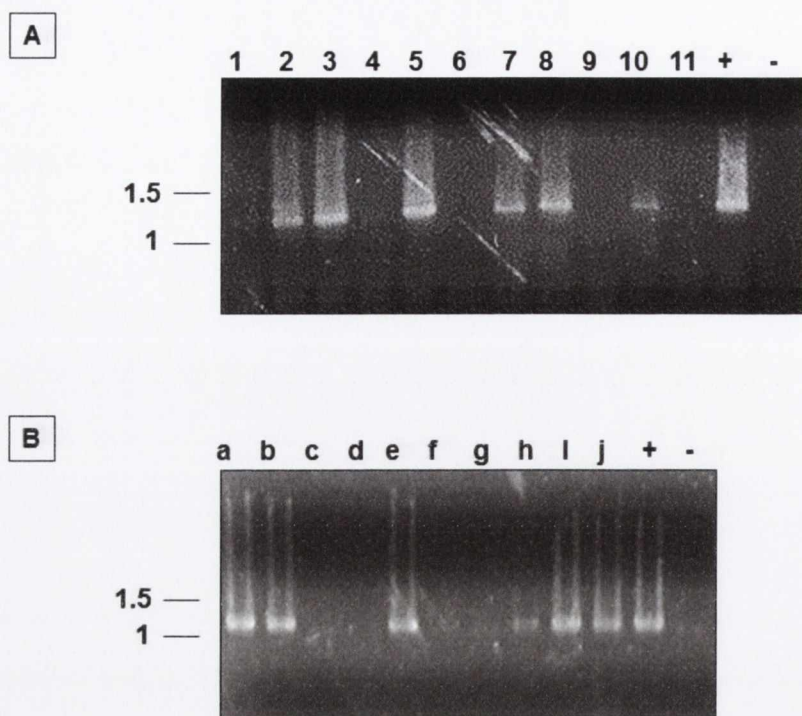


FIG. 3.6 Transformation and integration of pIMAY:: Δ lytM into LAC* *sbi*. (A) pIMAY:: Δ lytM was transformed into LAC* *sbi* and after 48 hours of growth at 28°C in the presence of chloramphenicol resulting colonies were screened by PCR for replicating plasmid using primers which anneal approximately 100 bp up- and downstream of the multiple cloning site. A colony containing replicating pIMAY with insert would produce a band of approximately 1.2 kb, e.g. colonies 2 and 3. (B) Integration of pIMAY:: Δ lytM into the chromosome of LAC* *sbi* was stimulated by growth at 37°C in the presence of chloramphenicol. After 24 hours colonies are once again screened using the MCS primers. Successful integration results in the absence of a band, e.g. colonies c and d, presence of a 1.2 kb band indicates the retention of replicating pIMAY. In both cases a positive control of *S. aureus* containing replicating pIMAY possessing an insert (+), and a negative control of *S. aureus* without plasmid (-) was included. Size markers indicated (kb).

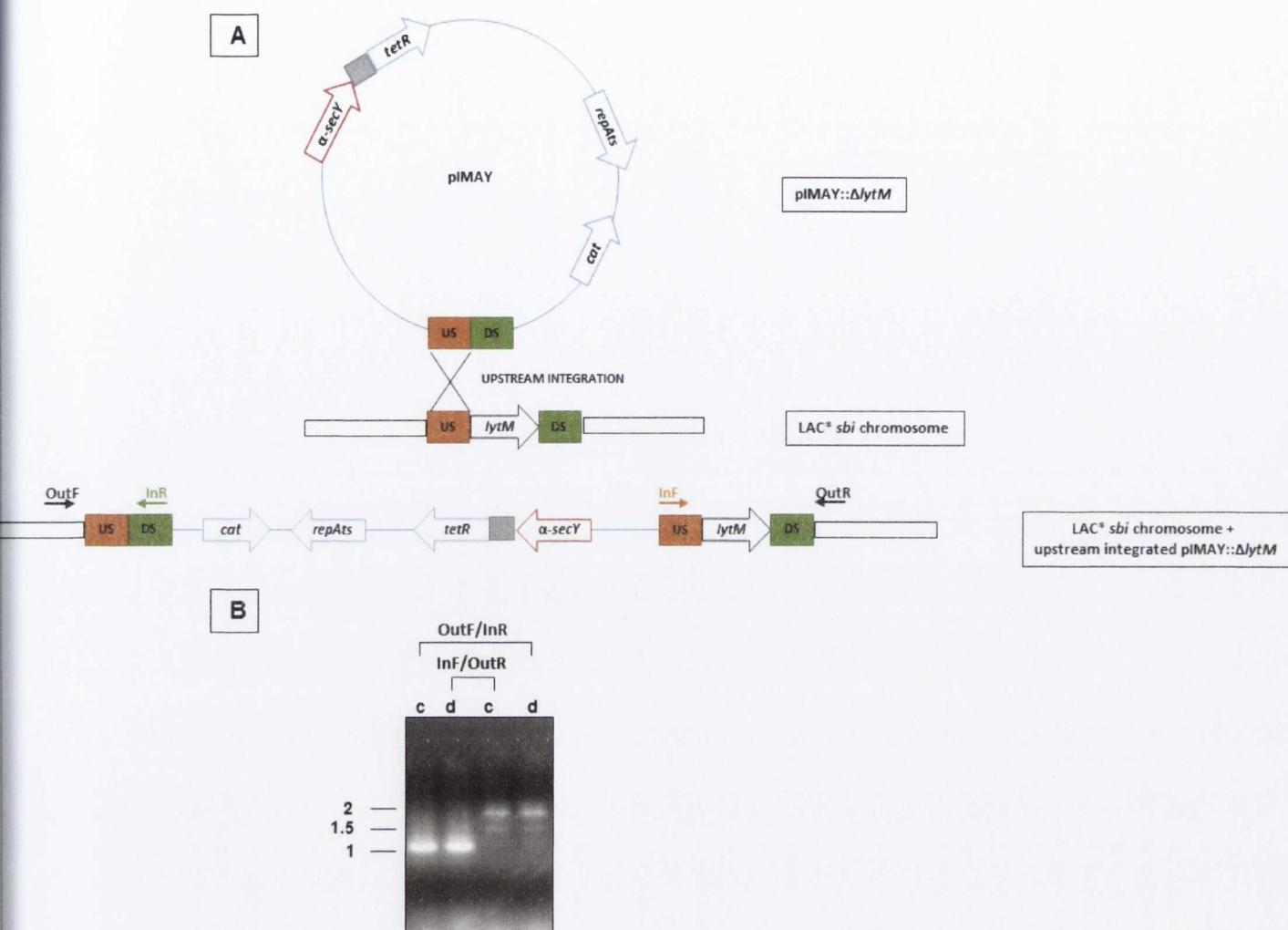


FIG. 3.7 Investigating the side of integration of pIMAY:: Δ lytM into the LAC* *sbi* chromosome. (A) Schematic representation of upstream integration of pIMAY:: Δ lytM. Via homologous recombination pIMAY:: Δ lytM can integrate into the LAC* *sbi* chromosome either upstream (US) or downstream (DS) of the *lytM* gene. (B) To determine the side of plasmid integration colonies are screened by PCR using a combination of primers. A combination of OutF and InR is used to confirm upstream integration. A positive integrant would produce a band at approximately 1.1 kb (colony c), whereas a region lacking integrated plasmid produces a product of approximately 2 kb (colony d). A combination of InF and OutR is used to confirm downstream integration. A positive integrant produces a 1.1 kb product (colony d), whereas a region lacking integrated plasmid produces a 2 kb product (colony c). Size markers are indicated (kb).

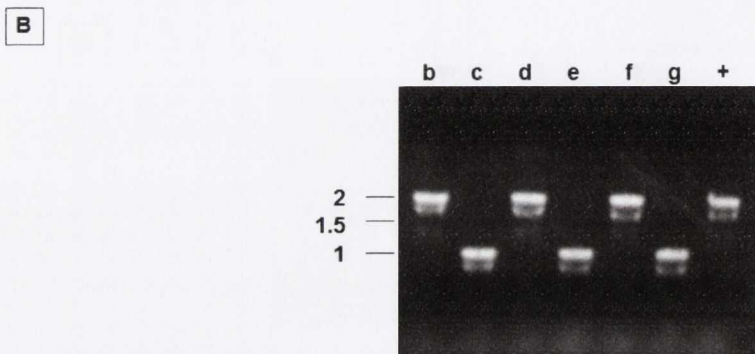
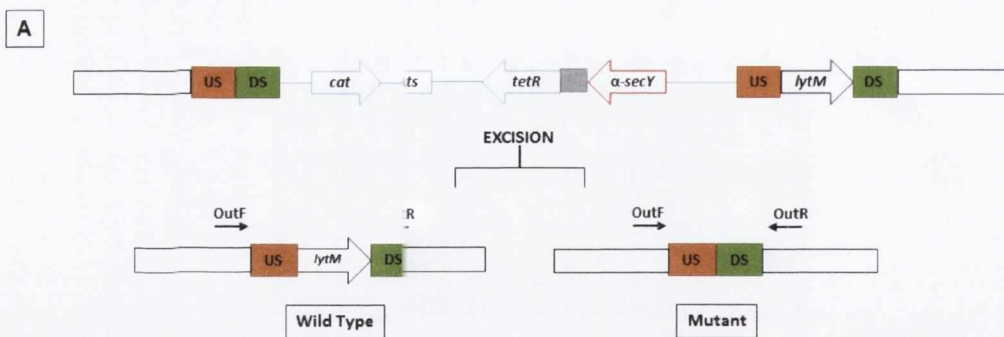


FIG. 3.8 Excision of pIMAY:: Δ lytM from the LAC* *sbi* chromosome. (A) Colonies harbouring integrated pIMAY:: Δ lytM arated on agar containing anhydrotetracycline which induces the expression of the α -secY antise RNA. Following this one of two events may occur, either plasmid excision occurs leaving behind the wildtype *lytM* gene, or it can result in the co-excision of the wildtype *lytM* gene leaving behind *lytM* deletion fragment comprised of the upstream (US) and downstream (DS) region creating a clean *lytM* deletion. (B) The outcome of this excision event is determined by PCR using primers that anneal up- and downstream of the US and DS fragments (OutF and OutR). The wild-type gene will generate a product of approximately 2 kb, e.g. colonies b and d, whereas colonies possessing the *lytM* deletion fragment will generate a band of approximately 1.1 kb, e.g. colonies a and c. LAC* *sbi* was included as a positive control (+) to indicate presence of the wild *lytM* gene. Size markers are indicated (kb).

observed for cell wall extracts of LAC* *sbi* *lytM* at both an $OD_{600} = 0.3$ (25% decrease as calculated by densitometric analysis) and 1.2 (18% decrease calculated by densitometric analysis) compared to LAC* *sbi* (Fig. 3.9A), indicating a decrease in CWA SpA levels when *lytM* is not present. However, measuring the levels of surface-displayed SpA using flow cytometry indicates that there is no significant difference in the amount of SpA displayed on the surface of LAC* *sbi* and LAC* *sbi* *lytM* grown to the same cell density (Fig. 3.9B).

In order to determine if release of SpA by LAC* is promoted by *lytM*, the amount of SpA in culture supernatants of LAC* *sbi* *lytM* was quantified using Western immunoblotting and ELISA (Fig. 3.10). Culture supernatants (SN) of LAC* *sbi* and LAC* *sbi* *lytM* grown to an OD_{600} of 0.3 and 1.2 were analyzed by Western immunoblotting, probing with HRP-conjugated rabbit IgG (Fig. 3.10A). A decrease in band intensity was observed in culture supernatants of LAC* *sbi* *lytM* in comparison to LAC* *sbi* at both stages of growth (Fig. 3.10A). This decrease in released SpA was then quantified by ELISA. SpA was captured from culture supernatants using immobilised chicken anti-SpA monoclonal IgY. Bound SpA was detected using biotin-labelled mouse monoclonal anti-SpA IgG and HRP-conjugated streptavidin. There was a 34% reduction in the amount of SpA released by LAC* *sbi* *lytM* compared to LAC* *sbi* when supernatants were harvested from bacteria grown to an OD_{600} of 0.3 (Fig. 3.10B). LAC* *sbi* *lytM* grown to an OD_{600} of 1.2 released 11% less SpA than LAC* *sbi* grown to the same optical density (Fig. 3.10B). These results show that *lytM* contributes to the release of SpA by LAC* as previously demonstrated in strain NM (Becker *et al.*, 2014), but does not alter the levels of surface-displayed SpA.

3.2.5 The influence of the endopeptidase *lytM* on the generation of SpA in NM.

Becker *et al.*, (2014) reported that *lytM* can influence the release of SpA from *S. aureus* in strain NM. Levels of SpA release were measured by densitometry analysis comparing cell wall extracts and supernatant samples probed for SpA in Western immunoblots. A significant decrease in SpA release, an approximate 75% reduction, was observed for NM *lytM* in comparison to NM (Becker *et al.*, 2014). As demonstrated above, *lytM* does influence the release of SpA from strain LAC* (Fig. 3.10), but not to the same extent as has been reported by Becker *et al.*, (2014) using strain NM.

A *lytM*-deficient mutant of NM *sbi* was generated in the same manner as described for LAC* *sbi*. The amount of SpA on the bacterial surface and culture supernatants of NM *sbi* *lytM* was quantified and compared to the levels of SpA produced by NM *sbi* (Fig. 3.11). Bacteria were incubated with FITC-labelled rabbit IgG to quantify the levels of surface-

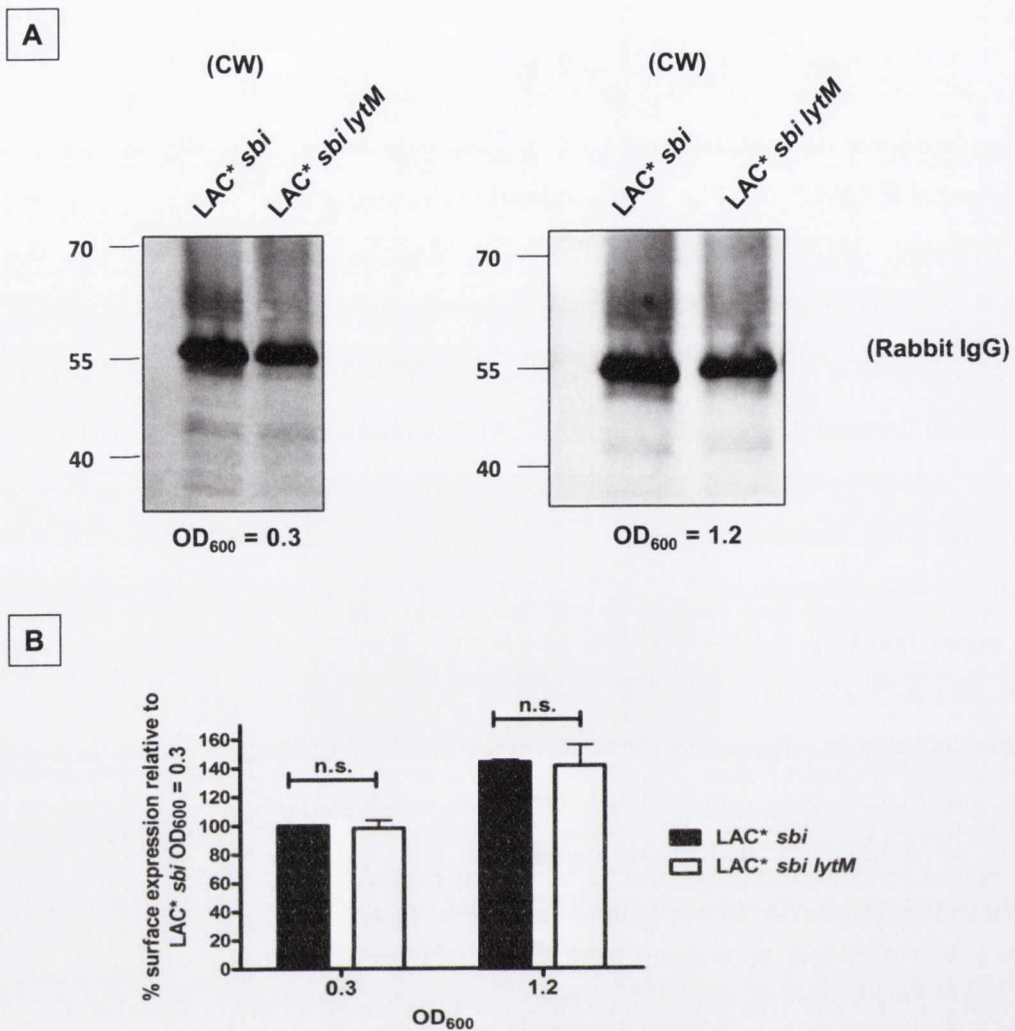


FIG. 3.9 Comparing the levels of cell wall-associated and surface-displayed SpA between LAC⁺ and a LytM-deficient mutant. (A) *S. aureus* was grown to an OD₆₀₀ of 0.3 or 1.2. Cell wall-extracts (CW) were probed for the presence of SpA using HRP-conjugated rabbit IgG. Size markers are indicated (kDa). (B) SpA on the surface of LAC⁺ *sbi* and LAC⁺ *sbi lytM* was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured by flow cytometry. Values are plotted as a percentage of the mean fluorescence intensity measured for LAC⁺ *sbi* grown to an OD₆₀₀ of 0.3. Bars represent the mean values and error bars indicate the SEM of three independent experiments, n.s. = not significant, $p > 0.05$.

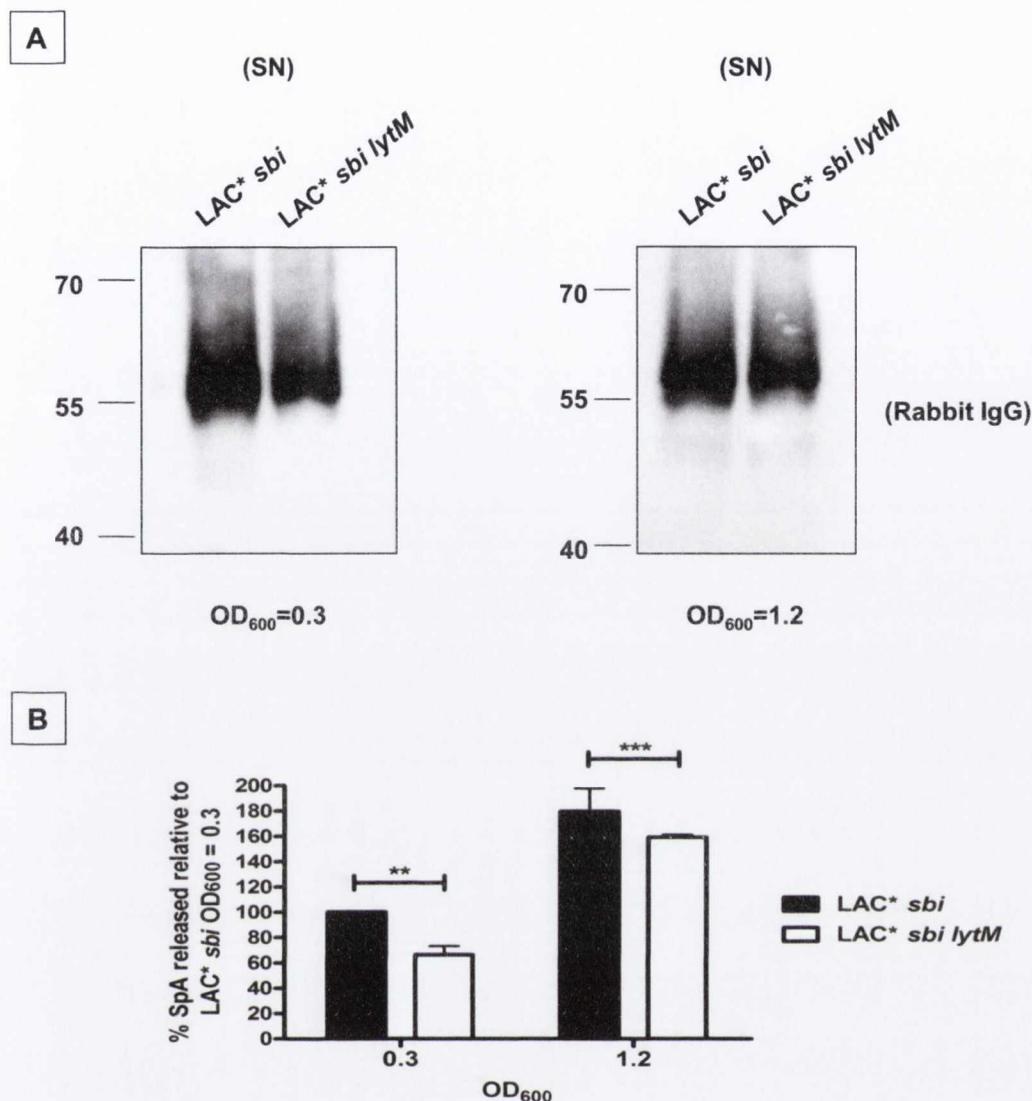


FIG. 3.10 Comparing the levels of released SpA generated by LAC* *sbi* and LAC* *sbi lytM*. (A) Culture supernatants from LAC* *sbi* and LAC* *sbi lytM* grown to the OD₆₀₀ indicated were probed with HRP-labelled rabbit IgG in a Western immunoblot. Supernatants harvested at OD₆₀₀ = 0.3 were concentrated 8-fold and supernatants harvested at OD₆₀₀ = 1.2 concentrated 2-fold before loading on a gel. Size markers are indicated (kDa). (B) Protein was captured from culture supernatants using chicken anti-SpA polyclonal IgY and detected using biotin-conjugated mouse monoclonal anti-SpA IgG followed by streptavidin-HRP in an ELISA. Values are expressed as a percentage of total released SpA measured for LAC* *sbi* grown to an OD₆₀₀ = 0.3. Bars represent the mean percentage release from four independent experiments. Error bars represent the SEM. ** $p = 0.006$, *** $p = 0.0003$.

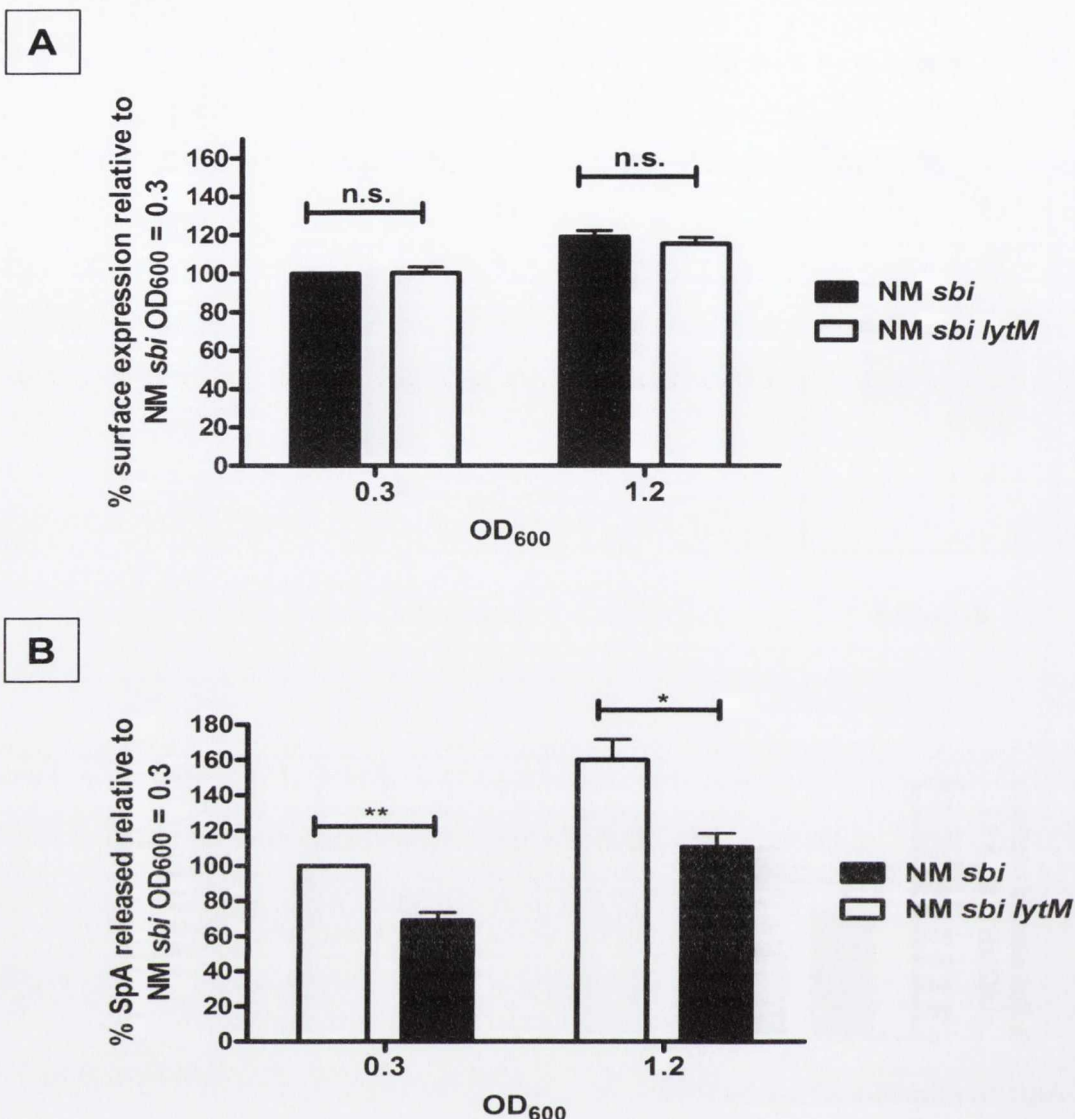


FIG. 3.11 LytM-mediated release of SpA by *S. aureus* Newman (NM). (A) SpA on the surface of Newman *sbi* and Newman *sbi lytM* was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured by flow cytometry. Values are plotted as a percentage of the mean fluorescence intensity measured for NM *sbi* grown to an OD₆₀₀ of 0.3. Bars represent the mean values and error bars indicate the SEM of three independent experiments. (B) Protein was captured from culture supernatants using chicken anti-SpA polyclonal IgY and detected using biotin-conjugated mouse monoclonal anti-SpA IgG followed by streptavidin-HRP in an ELISA. Values are expressed as a percentage of total released SpA measured for Newman *sbi* grown to an OD₆₀₀ = 0.3. Bars represent the mean percentage release from four independent experiments. Error bars represent the SEM. ** $p = 0.003$, * $p = 0.014$, n.s. = not significant, $p > 0.05$.

displayed SpA by flow cytometry and no difference in SpA levels was observed between bacteria grown to the same optical density ($OD_{600} = 0.3$ and 1.2 , Fig. 3.11A). As before, levels of released SpA from the same cultures were analysed by ELISA. There was a 34% reduction in the amount of SpA released by NM *sbi* *lytM* compared to NM *sbi* when supernatants were harvested from bacteria grown to an OD_{600} of 0.3 (Fig. 3.11B). NM *sbi* *lytM* grown to an OD_{600} of 1.2 released 36% less SpA than NM *sbi* grown to the same optical density (Fig. 3.11B). These results show that LytM contributes to the release of SpA by NM, as previously shown by Becker *et al.*, (2014).

3.3 Discussion

SpA is a potent virulence factor of *S. aureus* that is both covalently anchored to the cell wall and released into the extracellular medium. Recently research focused on the release of SpA from *S. aureus* (Becker *et al.*, 2014) and its role in invasive infection (Falugi *et al.*, 2013; Kim *et al.*, 2015) has been carried out using the common lab strain Newman (NM). Here the release of SpA from the USA300 isolate LAC* was investigated. Analysis of released SpA by Western blotting demonstrated that up to 7% of total SpA produced by LAC* is present in the extracellular medium (Fig. 3.2). Whereas 7% of total produced SpA may seem like a relatively small amount of extracellular protein, when put into context it represents a considerable level of protein release in comparison to other CWA proteins. Proteomic analysis of spent supernatant of *S. aureus* UAMS-1 demonstrated a five-fold increased abundance of SpA present in the extracellular medium after 3 hours of growth in comparison to other CWA proteins such as clumping factor B (ClfB) and fibronectin-binding protein A (FnBPA) (Jones *et al.*, 2008). Similarly, here we show that while SpA could be detected both in the cell wall and supernatant fractions by Western immunoblot (Fig. 3.1A), SdrE, another CWA protein, could only be detected associated with the cell wall (Fig. 3.1B).

Previous studies have suggested that released SpA is generated by enzymatic cleavage of CWA SpA by *S. aureus* extracellular proteases (Kolar *et al.*, 2013). Specifically, Karlsson *et al.*, (2001) implicated the staphylococcal secreted serine protease V8 as the protease cleaving SpA from the surface of *S. aureus*. This work however could not be replicated by McGavin *et al.*, (1997) who showed the removal of surface-displayed FnBPs by V8, but observed no effect on surface-displayed SpA. Due to these conflicting studies the role of extracellular proteases in the generation of released SpA in LAC* was addressed. Analysis of the levels of SpA present on the surface of LAC* PD and released into the extracellular medium (Fig. 3.3) demonstrated that extracellular proteases are not involved in SpA release. These observations were further enforced by the demonstration that no alterations in the levels of surface-displayed or released SpA were noted when a culture of LAC* PD was grown in broth supplemented with purified V8 (Fig. 3.4).

Becker *et al.*, (2014) showed that NM released greater amounts of SpA per CFU during logarithmic growth in comparison to later growth stages, i.e. stationary phase. This would suggest that the release of SpA is growth dependent and is an important factor during early stages of *S. aureus* infection. This would also suggest that staphylococcal factors directly affecting SpA release would be predominantly expressed during logarithmic growth.

LytM has been reported to facilitate the release of CWA SpA from NM (Becker *et al.*, 2014). In contrast to the extracellular proteases, which are positively regulated by *agr* (Janzon *et al.*, 1986), LytM is expressed throughout logarithmic growth and similar to SpA (Huntzinger *et al.*, 2005), is repressed by RNAIII-mediated inhibition of translation (Chunhua *et al.*, 2012). Here, we assessed the levels of CWA SpA produced in a LytM-deficient mutant of LAC* *sbi*. CW extracts of LAC* *sbi* and LAC* *sbi* *lytM* were probed for SpA in a Western immunoblot. Band quantification by densitometry showed a 18 – 25% decrease, dependent on growth phase, in CWA SpA for LAC* *sbi* *lytM* (Fig. 3.9A). This result was surprising as no alteration in the levels of CWA SpA in a LytM-deficient mutant of NM were previously reported (Becker *et al.*, 2014), although the same techniques were utilised. This result could highlight a variation in the activity of LytM between strains, or may be due to an inherent problem in the technique used. To further investigate this observation, flow cytometry was used to accurately compare the levels of surface-displayed, physiologically-active SpA on whole cells of LAC* *sbi* and LAC* *sbi* *lytM*. In contrast to the results observed by Western immunoblotting (Fig. 3.9A), no significant decrease in surface-displayed SpA was measured for LAC* *sbi* *lytM* at any phase of growth tested (Fig. 3.9B).

These contrasting results could have a number of explanations. Assuming these differing phenotypes are unique to LAC*, as Becker *et al.*, (2014) did not report any decrease in CWA SpA in NM, the different techniques used to measure SpA levels must be addressed. To prepare cell wall extracts for Western blotting, the peptidoglycan of the cells is degraded using the endopeptidase lysostaphin in the presence of a buffer that allows protoplast stability, and the soluble material is retained to prepare the final sample. LytM is a glycyl-glycine endopeptidase that cleaves peptidoglycan at the same site as lysostaphin (Bochtler *et al.*, 2004), and as all phenotypes of a LytM-deficient mutant of *S aureus* have not yet been characterised it could be possible that an important process in peptidoglycan turnover or the availability of pentaglycine cleavage sites may be affected. Therefore it is possible that lysostaphin treatment may not be fully optimal on the cell wall of this mutant, leading to less SpA being obtained from the procedure (Fig. 3.9A). In comparison, analysis by flow cytometry involves the labelling of surface-expressed SpA on whole cells that have not undergone any enzymatic lysis, thus avoiding any possible complications and providing a more accurate comparison between strains.

Alternatively, the disparity between the results obtained from the different methods could highlight an alternative phenotype of LytM. In a study comparing the proteome of

various vancomycin-intermediate *S. aureus* (VISA) isolates it was observed that the levels of LytM were 2-3 fold higher in the VISA isolate with the highest resistance to vancomycin (Gatlin *et al.*, 2006). The authors suggested that increased LytM activity may cause decreased crosslinking of peptidoglycan, as this is the most frequently observed molecular change in the CW of VISA isolates (Sieradzki & Tomasz, 1999). Gatlin *et al.*, (2006) also reported a 3-6 fold reduction in the abundance of SpA in the same VISA strain, indicating that perhaps rather than solely being responsible for the release of peptidoglycan-bound SpA, LytM could also indirectly affect SpA levels within the cell. SpA expression may be downregulated to compensate for a reduction in available pentaglycine crossbridge sites to facilitate transpeptidation of surface proteins, and whereas the abundance of SpA on the cell surface would not be altered, there would be less total SpA associated with the cellular peptidoglycan. To investigate this, *spa* transcript levels could be measured by quantitative PCR (qPCR) to determine if *spa* transcription is decreased in the absence of LytM.

As the surface-displayed levels of SpA are not altered in a LytM-deficient variant of LAC* (Fig. 3.9B) the levels of released SpA from culture supernatants could be directly compared. Comparison by both Western immunoblot and ELISA demonstrated a decrease of SpA release at both early exponential (34% reduction) and early stationary phase (11% reduction) in the absence of LytM (Fig. 3.10). Whereas it may be counter-intuitive to state that there is a reduction in the amount of released SpA while the levels of SpA present on the cell surface remain unchanged, this could possibly be explained by addressing the limitations of the techniques used. Given that released SpA constitutes 7% of total SpA (Fig. 3.2), a 34% reduction in release would result in an undetectable change (~2%) in the amount of surface-displayed SpA. Therefore while no statistical alteration in levels of surface displayed SpA were measured by flow cytometry it is likely that an overall change this small is beyond the detectable limitations of this approach.

While these results are in agreement with the study by Becker and colleagues in that there is a clear reduction in SpA release from *S. aureus* when LytM is absent, there appears to be a distinct variation in the activity of LytM between strains, as a LytM-deficient mutant of NM reportedly releases 75% less SpA (Becker *et al.*, 2014). This may highlight an alteration in the activity of LytM between strains in that LytM may have increased expression in NM compared to LAC*. Although it is likely that the influence of LytM on SpA release does differ between strains it is important to note that the results demonstrated here for LAC* are not directly comparable to the results reported by Becker *et al.*, (2014). The levels of

surface-displayed and released SpA in LAC* were quantified by flow cytometry (Fig. 3.9B) and ELISA (Fig. 3.10) respectively, whereas levels of released SpA in NM and NM *lytM* were quantified as a percentage of total produced SpA by Western blot using densitometry (Becker *et al.*, 2014). Furthermore, Becker *et al.*, (2014) did not report what growth phase was used to generate the CW and supernatant samples for Western immunoblotting and subsequent quantification, thus it was not possible to replicate the conditions applied in their study.

For these reasons strain NM *sbi lytM* was generated to accurately compare the effects of LytM on the release of SpA from NM and LAC*. Similar to the results reported for LAC*, no alteration in the levels of surface-displayed SpA were observed for NM *sbi lytM* at either early exponential or stationary phase (Fig. 3.11A). As measured by ELISA Newman *sbi lytM* produced 34% less released SpA when bacteria were grown to early exponential phase, and 36% less released SpA at early stationary phase (Fig. 3.11B). Comparing these results to what was observed in LAC* shows that during early stages of logarithmic growth there is no measurable difference in the influence of LytM in SpA release, however upon entering stationary phase LytM appears to exert a greater influence on SpA release in NM compared to LAC*. Thus it appears that there is no dramatic alteration in the influence of LytM between both strains and this would suggest LytM may have a conserved function in *S. aureus* to influence directly, or indirectly the release of SpA.

Overall, this chapter demonstrates that LAC* releases SpA during logarithmic growth, and this is not influenced by extracellular proteases; release is reduced in the absence of the endopeptidase LytM in both LAC* and NM, but a large percentage of SpA is still released under these conditions. Thus, it can be concluded that LytM does influence the release of SpA from *S. aureus* but it is not the only mechanism involved in this process.

3.4 Summary.

The major results and findings described in this chapter are as follows:

- The community-associated MRSA strain LAC produces both cell wall-associated and released SpA.
- The *S. aureus* extracellular proteases do not contribute to the release of SpA from LAC.
- The glycylglycine endopeptidase LytM accounts for up to 34% of SpA release from strain LAC, and up to 36% of SpA release from strain Newman under the conditions tested.

Chapter 4

Protein A is released into the *S. aureus* culture supernatant with an unprocessed sorting signal

4.1 Introduction.

Protein A is synthesized as a precursor with an N-terminal signal sequence and C-terminal sorting signal (SS). The signal sequence is cleaved by the signal peptidase SpsB during translocation of the precursor across the cytoplasmic membrane by the general secretory (Sec) pathway (Sibbald *et al.*, 2006). SpA is unevenly deposited on the staphylococcal cell surface at two to four discrete foci that are arranged in a ring-like structure in close proximity to sites of *de novo* cell wall synthesis in the septum of dividing daughter cells (DeDent *et al.*, 2007). The N-terminal signal sequence is essential for the directed secretion of bacterial surface proteins (Carlsson *et al.*, 2006). The signal sequence of SpA and other surface proteins that are deposited on the cell surface in a ring-like structure, such as ClfA and FnBPB, possess a YSIRK/GS motif (Bae & Schneewind, 2003), which was first identified in the signal sequence of staphylococcal lipases (Rosenstein & Gotz, 2000). Bae & Schneewind (2003) showed that deletion or modification of the YSIRK/GS motif of SpA significantly reduced signal peptide processing and translocation across the cytoplasmic membrane, however anchoring of SpA to the cell wall by sortase A was not affected. The YSIRK/GS motif within the ClfA signal sequence is essential for trafficking of the preprotein to the septum of dividing cells (DeDent *et al.*, 2008). Replacement of the ClfA signal sequence with the signal sequence from the surface protein SasF, which does not possess the YSIRK/GS motif, resulted in the deposition of ClfA at alternative sites in the cell (DeDent *et al.*, 2008). However mutation of the YSIRK/GS motif alone was not sufficient to abolish localisation of ClfA to the cross wall.

All known sortase enzymes of Gram-positive bacteria function as cysteine transpeptidases that recognise and process proteins with a C-terminal SS and attach them to an amino group associated with the cell wall (Clancy *et al.*, 2010; Marraffini & Schneewind, 2006). The gene encoding the *S. aureus* sortase A (*srtA*) enzyme was first identified by Schneewind and colleagues in 1999 (Mazmanian *et al.*, 1999). Here, using large scale mutagenesis the authors identified a mutant of *S. aureus* deficient in the cell wall anchoring of surface proteins which could be attributed to a mutation in the *srtA* gene. Based on this work Ton-That *et al.*, (1999) purified recombinant sortase A and confirmed *in vitro* that it is the enzyme responsible for catalysing the covalent linkage of cell wall-anchored (CWA) proteins to the cell wall peptidoglycan of *S. aureus*. Deletion of *srtA* in strain Newman (NM) resulted in the release of the majority of SpA from the staphylococcal cell and the remaining SpA was localised in the cytoplasm, cytoplasmic membrane and non-covalently associated with the cell wall (DeDent *et al.*, 2007). The deposition of SpA on the staphylococcal cell

surface requires sortase A and the SpA C-terminal SS (DeDent *et al.*, 2007; Schneewind *et al.*, 1993). The SS comprises an LPETG motif, a hydrophobic membrane-spanning domain and, at the extreme C-terminus, a stretch of positively charged residues (Schneewind *et al.*, 1993). The last two elements delay secretion across the membrane and facilitate recognition and cleavage by membrane-bound sortase A (Schneewind *et al.*, 1993). Sortase A cleaves between threonine and glycine of the LPETG motif forming an acyl-enzyme intermediate capturing the C-terminal carboxyl group of the protein with its active site cysteine thiol (Mazmanian *et al.*, 1999). Acyl intermediates are relieved by the nucleophilic attack of the amino group of the pentaglycine crossbridge of lipid II (Perry *et al.*, 2002). Following transglycosylation and transpeptidation, SpA becomes covalently anchored to peptidoglycan and is displayed on the surface of the bacterium (Fig. 4.1) (Mazmanian *et al.*, 2001).

The SS is essential for the efficient sorting of CWA proteins, such as SpA, harbouring LPXTG motifs to the cell wall peptidoglycan (Schneewind *et al.*, 1992; Schneewind *et al.*, 1993). Schneewind and co-workers evaluated the different features of the SpA SS and their importance in the anchoring of SpA to the staphylococcal cell wall. Site-directed mutagenesis and deletion of the SpA SS showed that deletion of either the hydrophobic membrane-spanning region or the positively-charged C-terminus of SpA resulted in the complete release of the unsorted precursor from *S. aureus* (Schneewind *et al.*, 1992). In contrast, deletion of the LPETG motif resulted in the deposition of SpA in all cellular compartments. No SpA was detected on the surface of any of these mutants. All three regions of the SpA SS were essential for the anchoring of a hybrid reporter protein to the staphylococcal cell wall (Schneewind *et al.*, 1992).

A reporter protein fused to the SpA SS comprising a shorter hydrophobic membrane-spanning region was sorted less efficiently to the cell wall (Schneewind *et al.*, 1993). The positively-charged C-terminus of the SpA SS possess three positively-charged residues, three consecutive arginines, and site-directed mutagenesis of these residues determined that the SpA sorting signal must possess at least two positively-charged residues for efficient sorting of SpA (Schneewind *et al.*, 1993). From these studies Schneewind and colleagues determined that a sorting signal must possess an LPXTG motif, hydrophobic membrane-spanning region and C-terminal positively charged region. They also proposed that a longer membrane-spanning region and an increased positive charge at the extreme C-terminus are the most important characteristics in the formation of an optimal SS for recognition by sortase A.

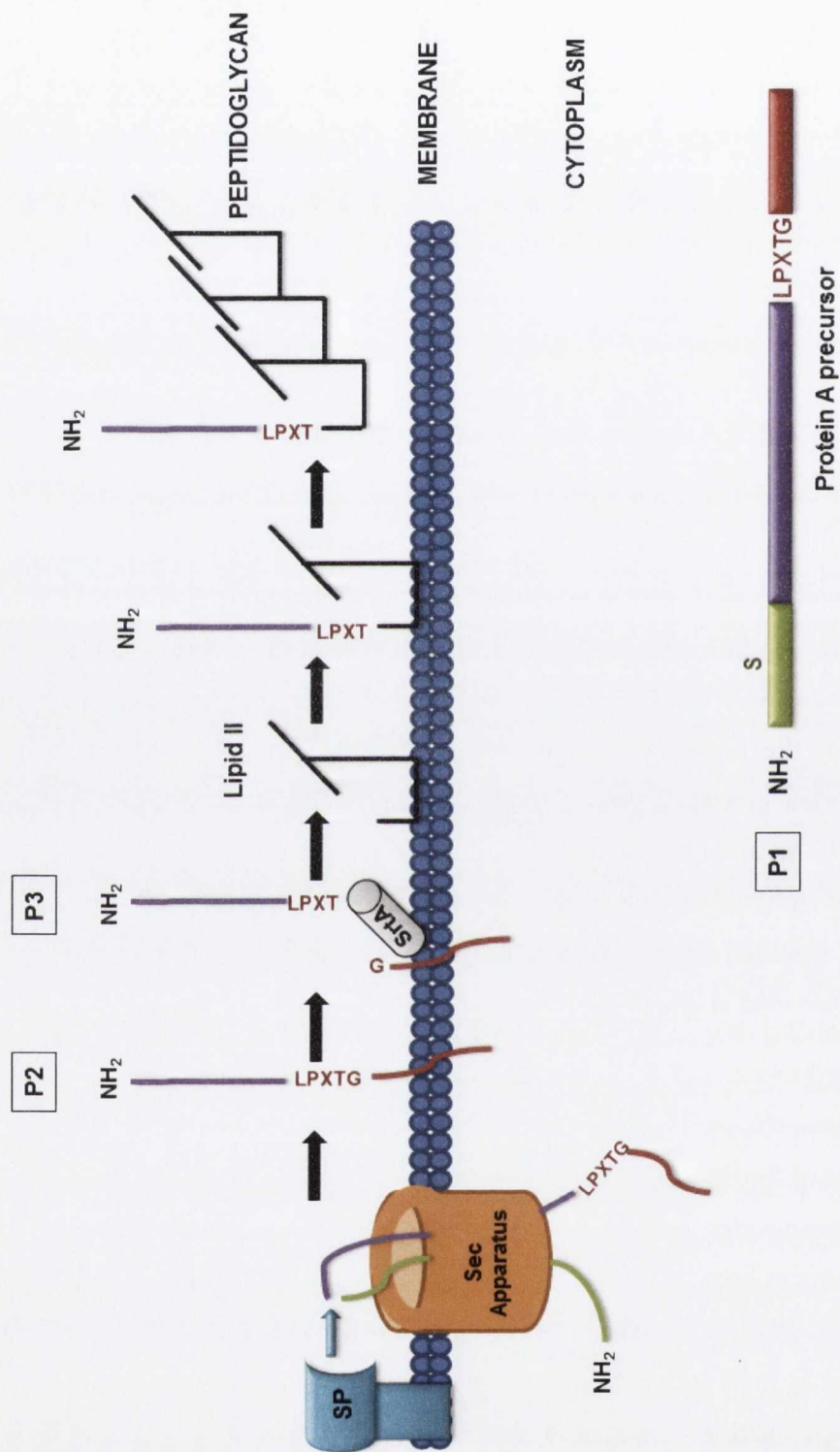


Fig. 4.1 Schematic representation of the anchoring of protein A to cell wall peptidoglycan by the sortase A enzyme. Upon directing the protein precursor 1 (P1) to the secretion (Sec) apparatus the secretion signal (S) is cleaved by the signal peptidase (SP). Precursor 2 (P2) is then recognised by the sortase A (SrtA) enzyme which cleaves between the threonine and glycine of the LPXTG motif to create an acyl-enzyme intermediate (P3). This intermediate is recognised by Lipid II, which incorporates the C-terminal threonine into its pentaglycine crossbridge. The lipid II-protein A intermediate is then incorporated into the cell wall peptidoglycan via a transglycosylation reaction.

Mass spectrometry (MS) is a widely used analytical chemistry technique which can accurately predict the identity and mass of chemicals present in a sample by measuring the mass-to-charge ratio (m/z). One of the most widely used methods for protein identification by mass spectrometry is liquid-chromatography tandem mass spectrometry (LC-MS/MS) based peptide sequencing (Hill *et al.*, 1988; Hunt *et al.*, 1986). Using this technique the protein sample is first subjected to proteolytic digestion, usually using trypsin. Following enzymatic digestion the protein sample is separated using high-performance liquid chromatography (HPLC). A MS/MS spectra and m/z value is acquired for each individual peptide, which is then used to search a chosen protein database for peptide hits and a protein hit is often identified using data obtained from sequencing multiple peptides of the same protein.

Whereas matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) based sequencing is often the method of choice for protein sequencing due its relative ease of use and quick turnover time, LC-MS/MS is recognised as a more reliable technique for accurate peptide identification, especially for samples containing a high concentration of different proteins. By analysing each peptide individually and often being able to use multiple peptide fragments of the same protein LC-MS/MS is a much more reliable method for precise and accurate protein identification (Yates *et al.*, 1997).

The work described in this chapter demonstrates the importance of the sequence of the SpA SS for the release of protein A from *S. aureus*.

4.2 Results

4.2.1 Identification of a protein A precursor with an unprocessed sorting signal in *S. aureus* culture supernatants.

Extracellular proteases are not involved in the release of SpA by *S. aureus* LAC* (Fig. 3.3) while the glycyl-glycine endopeptidase LytM promotes some SpA release (Fig. 3.10). Since the release of SpA was not completely inhibited in a LytM-deficient mutant (Fig. 3.10, (Becker *et al.*, 2014)) another mechanism of release must exist. In order to identify additional factors involved in SpA release, extracellular SpA was purified from the culture supernatant (SN) of strain LAC* *sbi* using affinity chromatography on IgG-sepharose. To ensure purity of the protein and the retention of IgG-binding ability post-purification, dilutions of purified SpA were analysed by Western immunoblotting probing with HRP-conjugated rabbit IgG (Fig. 4.2). Filtered SN from an overnight culture of LAC* *sbi* was run alongside purified SpA to indicate typical migration of released SpA (Fig. 4.2). Purified SpA migrated as a diffuse band and retained its ability to bind to IgG in a Western immunoblot (Fig. 4.2). The purified protein was analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS). The sequence of eight of the peptides identified mapped to the extreme C-terminus of SpA (Table 4.1). One peptide terminated with four successive glycines following the sequence 'LPET'. This was consistent with it originating from the cell wall and being released by LytM cleavage (Becker *et al.*, 2014). Interestingly, seven unique peptides with an intact LPETG motif were identified (Table 4.1) indicating that the preprotein had not been processed by sortase A. This suggested that the protein did not derive from the cell wall and was not released by LytM-mediated cleavage of the pentaglycine crossbridge of peptidoglycan, implying that SpA can be released by *S. aureus* prior to becoming covalently anchored to the peptidoglycan and that the SS remains intact. No peptides identified during LC-MS/MS corresponded to the N-terminal signal sequence (Fig. 4.3), suggesting that the signal sequence was removed by the signal peptidase during secretion.

To further confirm this, purified released SpA was analysed by N-terminal sequencing. The five N-terminal residues of purified extracellular SpA were identified as ₃₇AQHDE₄₁ confirming that the signal peptide (residues 1-36) had been removed by signal peptidase. Thus all extracellular SpA is processed by signal peptidase so that the N-terminal signal sequence is removed. A portion of extracellular SpA is not processed by sortase A. Unprocessed SpA with an intact sorting signal has a predicted molecular weight of 51,928.9 while SpA released from the cell wall following cleavage by LytM is linked to fragments of peptidoglycan of various lengths, with the most abundant forms having predicted molecular

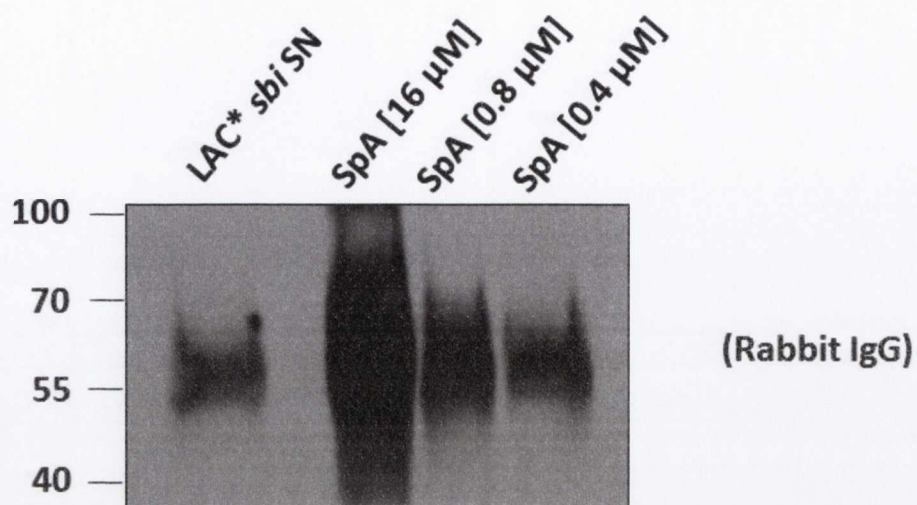


Fig. 4.2 Released SpA purified from culture supernatant of LAC* *sbi*. *S. aureus* LAC* was grown to stationary phase and SpA was purified from the culture supernatant by IgG affinity chromatography. Purified released SpA (SpA, concentrations indicated), and unprocessed culture filtrates (LAC* *sbi* SN) were probed with HRP-conjugated rabbit IgG in a Western immunoblot. Size markers are indicated (kDa).

Table 4.1 Peptides identified by liquid chromatography tandem mass spectrometry.

Peptide sequence	Number of peptides identified	Peaks peptide score -10lgP	Mass (Da)	m/z	Residue numbers
<u>KAQALPETGEENPFI</u>	1	44.48	1401.6411	701.8262	470-484
<u>KAQALPETGEENPFIGTT</u>	1	40.83	1704.7842	853.3968	470-487
<u>KAQALPETGEENPFIGTTVFG</u>	1	39.57	2019.9789	1010.9971	470-490
<u>KAQALPETGEENPFIGTTVFGG</u>	1	40.09	2077.0002	1039.5046	470-491
<u>KAQALPETGEENPFIGTTVFGGL</u>	1	36.39	2151.0483	1076.5337	470-492
<u>KAQALPETGEENPFIGTTVFGGLSL</u>	2	47.19	2351.1646	1176.5889	470-494
		47.76	2372.0847	791.7029	470-494
<u>KAQALPETGGGG</u>	1	47.56	956.4563	479.2358	470-478

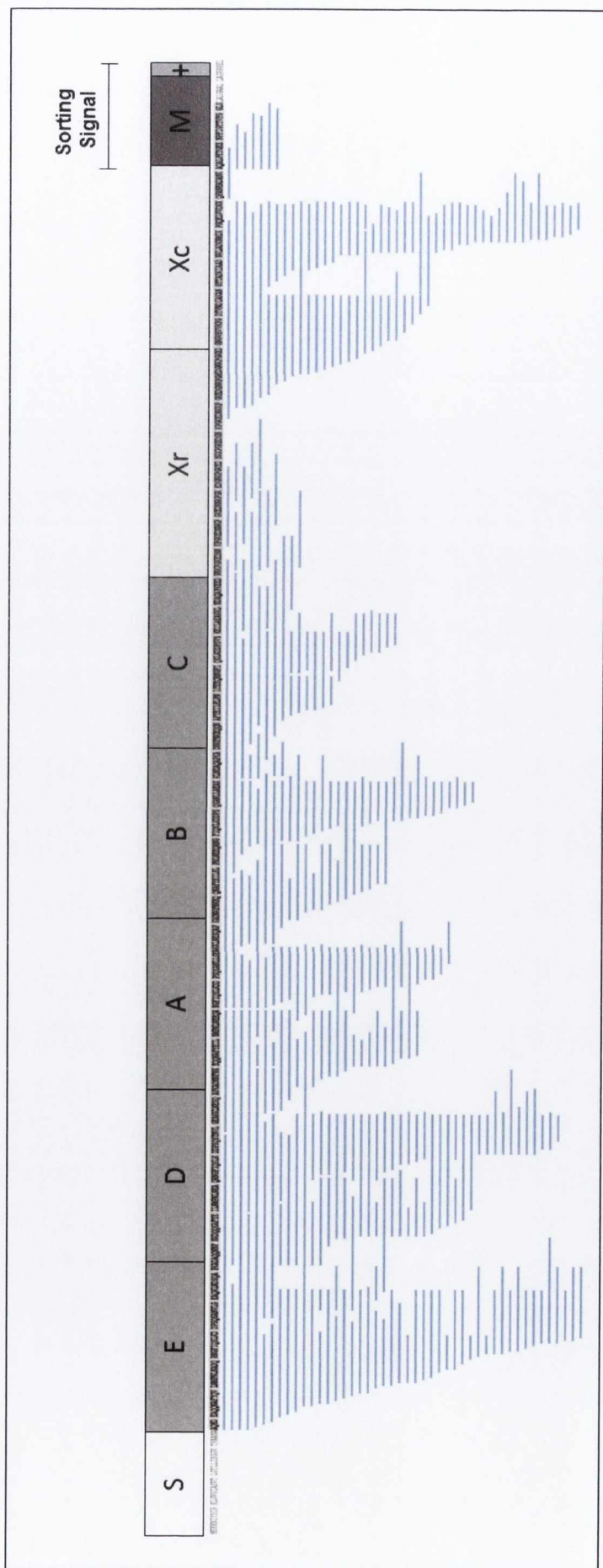


Fig. 4.3 Alignment of peptides identified by LC-MS/MS to the amino acid sequence of SpA. Domain organisation is displayed above the amino acid sequence of SpA. Regions corresponding to the C-terminal sorting signal are indicated. Peptide sequences (blue) identified by LC-MS/MS of purified released SpA are aligned to the amino acid sequence of SpA. Peptide sequences were identified using PEAKS studio software.

masses of between 52,555.4 and 54,151 (4). These different forms of extracellular SpA cannot be distinguished since they comigrate on an SDS-PAGE gel (Fig. 4.2).

4.2.2 Released SpA does not become anchored to the cell wall of *S. aureus*.

Soluble peptides that have been engineered to possess a C-terminal LPXTG motif can be incorporated into the cell wall of growing *S. aureus* in a sortase A-mediated reaction, and are detected displayed on the *S. aureus* cell surface following this reaction (Hansenova Manaskova *et al.*, 2014). As a portion of released SpA possesses an intact LPETG motif (Table 4.1, Fig. 4.3) it is possible that released SpA can become anchored to cell wall peptidoglycan of *S. aureus* by sortase A. Cultures of LAC* *sbi* were grown with and without the addition of filtered SN from an overnight culture of LAC* *spa sbi*, similarly cultures of LAC* *spa sbi* was grown with and without the addition of filtered SN from an overnight culture of LAC* *sbi* (Fig. 4.4). Levels of surface-displayed SpA were measured at different points throughout growth by flow cytometry (OD₆₀₀ = 0.3, 0.6, and 1.2) using FITC-labelled rabbit IgG to detect SpA activity. Increasing levels of SpA were detected on the surface of LAC* *sbi* bacteria grown in the presence or absence of LAC* *spa sbi* SN (Fig. 4.4). In contrast, no SpA was detected on the surface of LAC* *spa sbi* bacteria grown in the presence or absence of LAC* *sbi* SN (Fig. 4.4). This indicates that released SpA is not anchored to the *S. aureus* cell wall under the conditions used here when released SpA is at concentrations in an overnight culture of LAC* *sbi*.

4.2.3 The vector pRMC2 allows inducible expression of SpA in *S. aureus*.

The identification of SpA with an intact sorting signal in LAC* culture supernatants indicated that SpA can be released from the bacterium without being sorted to the cell wall. This strongly suggested a release mechanism independent of LytM activity. The sorting signal of cell wall anchored surface proteins is essential for covalent linkage to cell wall peptidoglycan (Schneewind *et al.*, 1992; Schneewind *et al.*, 1993). Since some released SpA harbours an unprocessed sorting signal (Table 4.1) we set out to determine if altering the sorting signal of SpA might influence release of the protein. In order to facilitate the manipulation of *spa*, the full-length *spa* gene was cloned into the anhydrotetracycline (ATc)-inducible expression vector pRMC2 (Fig. 4.5), which exhibits tight repression of gene expression in the absence of inducer (Corrigan & Foster, 2009), to generate plasmid pSpA (Fig. 4.6).

Briefly, primers were designed to amplify outwards from the multiple cloning site (MCS) of pRMC2 to linearise the vector (Fig. 4.6A). The full-length *spa* gene, including the

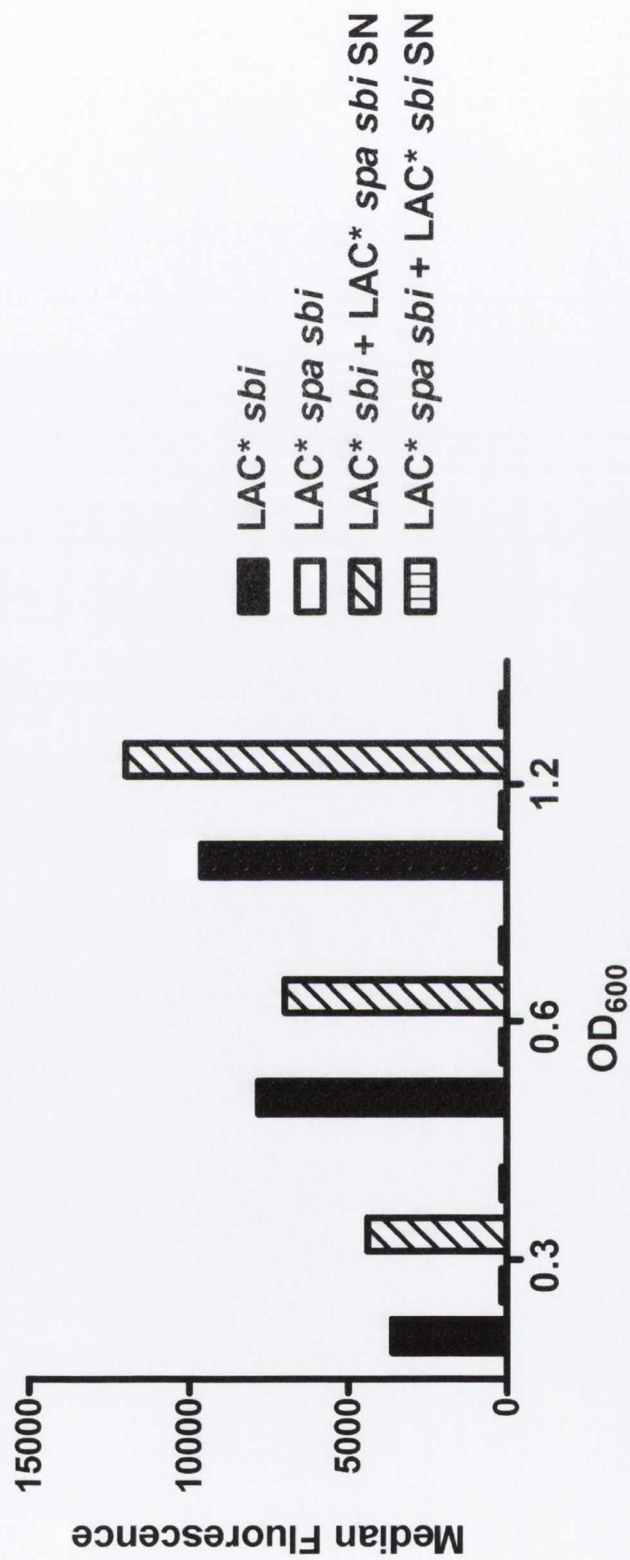


Fig. 4.4 Released SpA from LAC* is not anchored to the cell wall of *S. aureus*. Cultures of LAC* *sbi* and LAC* *spa sbi* were grown in the presence or absence of filtered supernatant (SN) from an overnight culture of LAC* *spa sbi* or LAC* *sbi* as indicated. Levels of surface-displayed SpA were detected at the OD₆₀₀ indicated using FITC-labelled rabbit IgG. Bars represent the median fluorescent values of one experiment.



Fig. 4.5 Genetic map of pRMC2. pRMC2 comprises a tetracycline-inducible promoter ($P_{xyl/tetO}$) that is derived from *Bacillus subtilis* and the *tet* regulatory (*tetR*) elements originate from transposon Tn10. The *cat* gene confers resistance to chloramphenicol in *S. aureus*. The multiple cloning site (MCS) is comprised of six unique restriction sites: KpnI, HpaI, BglII, SacI/BanII, and EcoRI. Plasmid coordinates for each element are indicated (bp). Plasmid map created using ApE software.

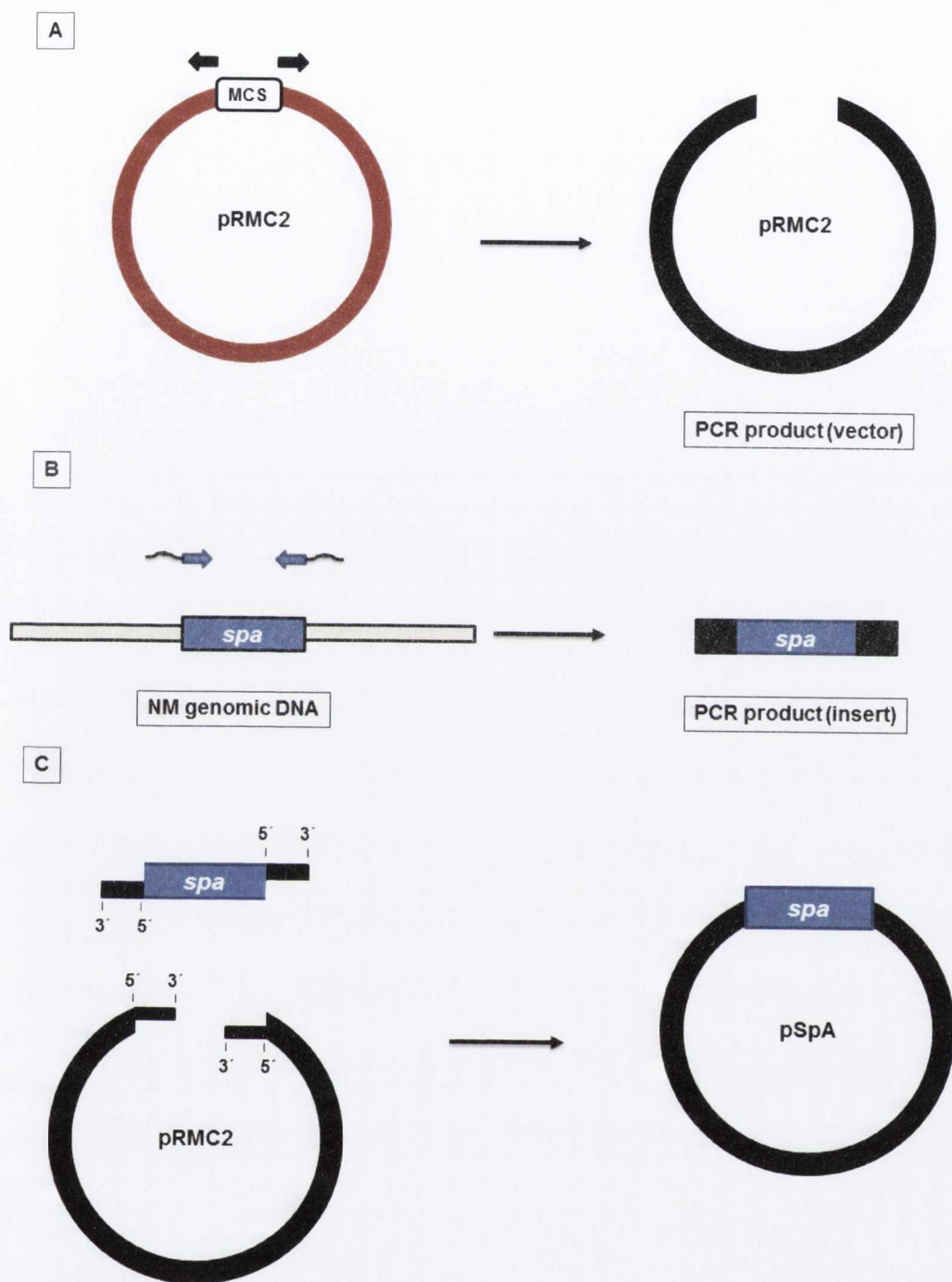


Fig. 4.6 Generation of pSpA by Sequence and Ligation Independent Cloning (SLIC). (A) pRMC2 was linearised by PCR using primers designed to amplify outwards from the multiple cloning site (MCS). (B) The full-length *spa* gene was amplified from NM genomic DNA using primers containing 5' extensions of 24 bp with homology to regions of DNA up- or downstream of MCS of pRMC2 (black). (C) Both products were treated with T4 DNA polymerase which generates 3' single-stranded homologous DNA. Insert and vector were incubated together under conditions that promote annealing to generate the plasmid pRMC2::*spa* (pSpA). The *spa* gene is cloned between the *SacI* and *EcoRI* sites of pRMC2.

ribosome binding site, was amplified from *S. aureus* NM genomic DNA using primers with 5' extensions homologous to regions directly upstream and downstream of the MCS of pRMC2 (Fig. 4.6B). Both products were treated with T4 DNA polymerase which has 3'-5' exonuclease activity, which creates single-stranded ends. The single-stranded products were incubated together under conditions which promote annealing to generate the plasmid pRMC2::*spa* (pSpA, Fig. 4.6C), which was transformed into *E. coli* strain DC10B made electrocompetent. Successful cloning of the *spa* gene into pRMC2 was confirmed by DNA sequencing. Plasmid pSpA was transformed into *S. aureus* strain LAC* *spa sbi*.

Flow cytometry was used to measure the amount of SpA on the surface of LAC* *spa sbi* carrying pSpA. Increasing concentrations of the inducer ATc were added to the growth medium (Fig. 4.7). Bacteria were harvested when an OD₆₀₀ of 1.2 was reached and surface-displayed SpA was detected using FITC-labelled rabbit IgG and fluorescence-intensity was measured by flow cytometry. No fluorescence was detected for LAC* *spa sbi* bacteria harbouring empty pRMC2 induced with ATc (312.5 ng/ml) indicating that any fluorescence detected was specific for SpA. Fluorescence was not detected when LAC* *spa sbi* (pSpA) was grown in the absence of inducer or when induced with 9.77 ng of ATc per ml (Fig. 4.7), indicating tight repression of *spa* expression in the absence of ATc or at low concentrations. The amount of SpA on the surface of LAC* *spa sbi* (pSpA) increased in a dose-dependent manner with increasing amounts of inducer (19.53 – 312.5 ng/ml, Fig. 4.7). LAC* *spa sbi* (pSpA) bacteria grown in the presence of 312.5 ng/ml ATc displayed levels of surface-displayed SpA similar to that measured for LAC* *sbi* grown under the same conditions (Fig. 4.7). The amount of SpA on the surface of LAC* *spa sbi* (pSpA) induced with 312.5 ng of ATc per ml and 625 ng of ATc per ml was similar (Fig. 4.7).

4.2.4 Replacing the SpA sorting signal with the sorting signal of SdrE reduces release of SpA.

Plasmid pSpA (Fig. 4.6C) was used to generate a variant plasmid where DNA encoding the SpA sorting signal (SS) was replaced with DNA encoding the SS from SdrE (pSpAΩSdrESS) using sequence and ligation independent cloning (SLIC). The SdrE protein is located exclusively in the cell wall fraction of *S. aureus* (Fig. 3.1A). However unlike SpA it was not detected in culture supernatants from LAC* (Fig. 3.1B). The pSpAΩSdrESS plasmid was introduced into the LAC* *spa sbi* strain. The amount of SpA displayed on the surface of LAC* *spa sbi* (pSpA) and LAC* *spa sbi* (pSpAΩSdrESS) grown in the presence or absence of inducer (ATc) was detected using FITC-labelled rabbit IgG and fluorescence

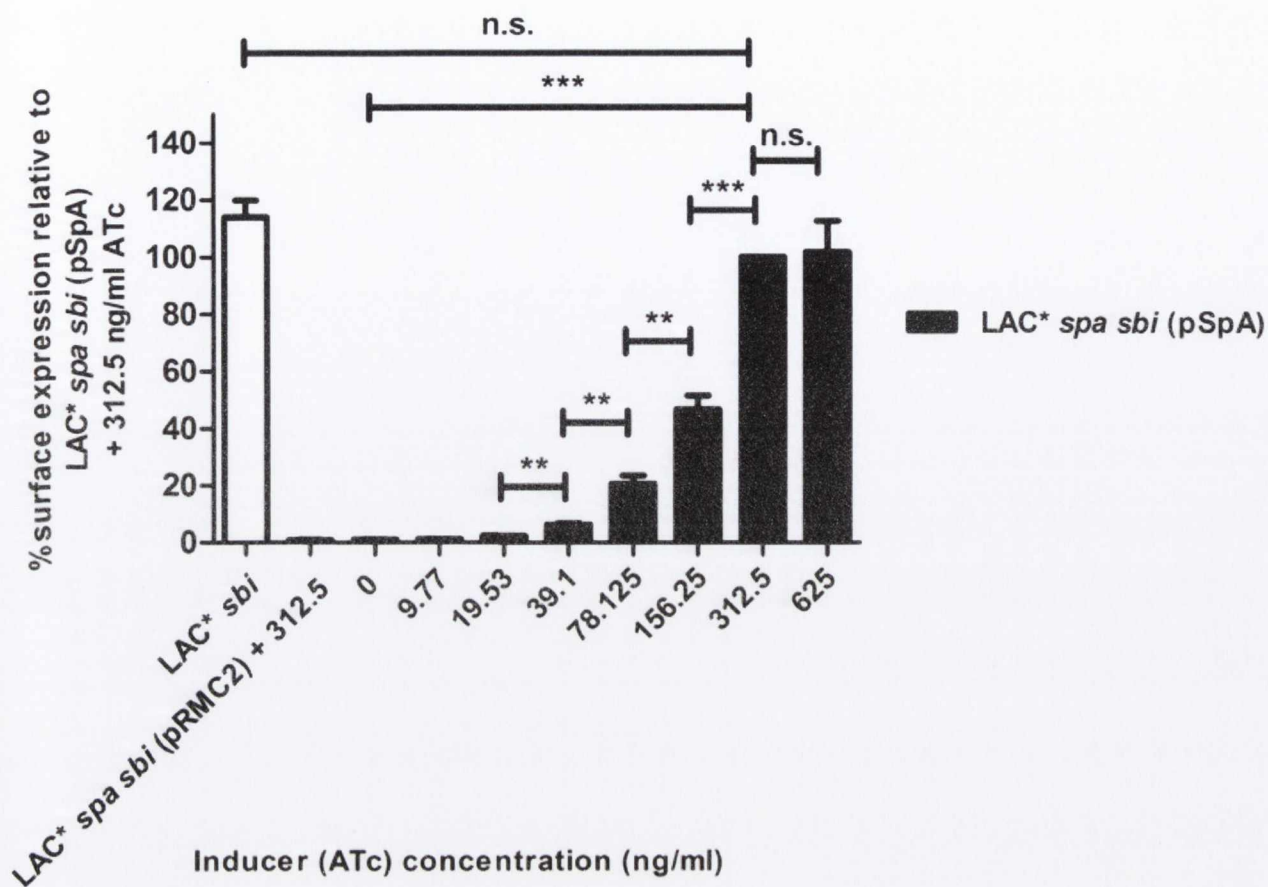


Fig. 4.7 Measuring the levels of surface-displayed SpA by LAC* *spa sbi* (pSpA). *S. aureus* was grown to an $OD_{600} = 1.2$ in the absence or presence of ATc, as indicated. Levels of surface-displayed SpA were detected using FITC-conjugated rabbit IgG and fluorescence was measured using flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for LAC* *spa sbi* (pSpA) grown in broth supplemented with ATc (312.5 ng/ml). Bars represent the mean of three independent experiments. Error bars represent the SEM. ** $p < 0.001$, *** $p < 0.0001$, n.s. = not significant, $p > 0.05$.

was measured using flow cytometry. Similarly to bacteria harbouring pSpA, SpA was detected on the surface of LAC* *spa sbi* (pSpA Ω SdrESS) when bacteria were grown in broth supplemented with ATc (Fig. 4.8A). Fluorescence was not detected in the absence of ATc and the amount of SpA displayed on the surface of LAC* *spa sbi* (pSpA Ω SdrESS) increased with increasing inducer concentration (Fig. 4.8A). The amount of surface-displayed SpA produced by *S. aureus* carrying plasmid pSpA was similar to *S. aureus* carrying pSpA Ω SdrESS at each ATc concentration tested (Fig. 4.8A), indicating that replacing the SpA SS with the SS from SdrE does not significantly alter the levels of SpA displayed on the surface of *S. aureus*.

To study released SpA, culture supernatants from LAC* *spa sbi* (pSpA) and LAC* *spa sbi* (pSpA Ω SdrESS) grown in broth supplemented with ATc (312.5 ng/ml) were examined by Western immunoblotting, SpA was detected using HRP-conjugated rabbit IgG (Fig. 4.8B). Densitometric analysis of relative band intensity indicated that LAC* *spa sbi* carrying plasmid pSpA Ω SdrESS released less SpA, approximately 65% as much as LAC* *spa sbi* carrying plasmid pSpA (Fig. 4.8B). The relative amount of released SpA in culture supernatants was then quantified by ELISA (Fig. 4.8C). SpA was captured from culture supernatants using immobilised chicken anti-SpA polyclonal IgY. Captured SpA was detected using biotin-labelled mouse monoclonal anti-SpA IgG and HRP-conjugated streptavidin. The amount of SpA released by bacteria carrying the plasmid pSpA Ω SdrESS was reduced to approximately 62% of the amount released by bacteria carrying pSpA (Fig. 4.8C). These data indicate that the release of SpA by *S. aureus* is reduced when the SpA sorting signal is replaced with the sorting signal of the wall-associated protein SdrE.

4.2.5 The C-terminal sorting signal of SpA allows release of cell wall-anchored proteins.

The C-terminal SS comprises an LPXTG motif followed by a hydrophobic domain and a positively charged tail (Fig. 1.3A) and is essential for efficient anchoring of proteins to peptidoglycan (Schneewind *et al.*, 1992). Since the release of SpA was reduced when the SpA SS was replaced with the SS of the wall-associated protein SdrE (Fig. 4.8B, C), it was possible that the sequence of the SS might influence the release of SpA. Rather than replacing the native SdrE SS with the SS from SpA we instead generated chimeric proteins where the SpA or SdrE SS was linked to a reporter protein. Chimeric proteins were generated where the D3D4 domains of Sbi were linked to the SS of SpA or SdrE (Fig. 4.9A). Sbi is an envelope associated protein which does not become anchored to cell wall

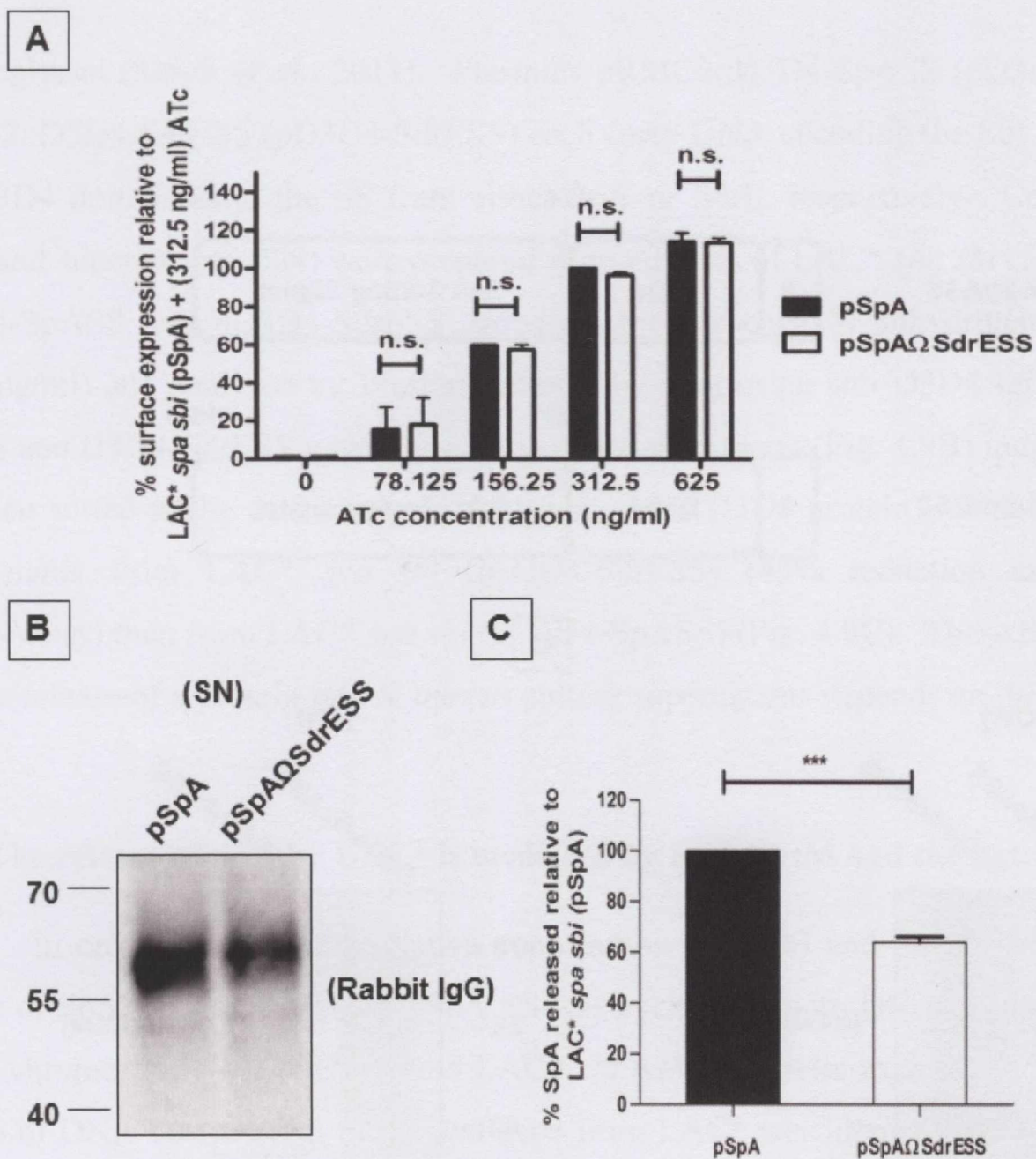


Fig. 4.8 Release of protein A into *S. aureus* culture supernatants can be reduced by altering the sorting signal. (A) Protein A on the surface of LAC* *spa sbi* (pSpA) (black bars) and LAC* *spa sbi* (pSpA Δ SdrESS) (white bars) was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured using flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for LAC* *spa sbi* (pSpA) grown in broth supplemented with ATc (312.5 ng/ml). Bars represent the mean of three independent experiments. Error bars represent the SEM. n.s. = not significant, *** $p < 0.001$. (B) Culture supernatants from LAC* *spa sbi* (pSpA) and LAC* *spa sbi* (pSpA Δ SdrESS) induced with ATc (312.5 ng/ml) were probed with HRP-conjugated rabbit IgG. Size markers are indicated (kDa). (C) Quantification of SpA in culture supernatants of LAC* *spa sbi* (pSpA) and LAC* *spa sbi* (pSpA Δ SdrESS) by ELISA. Bacteria were grown in broth supplemented with ATc (312.5 ng/ml) and SpA was captured from culture supernatants using chicken anti-SpA polyclonal IgY. Bound SpA was detected using biotin-labelled mouse monoclonal anti-SpA IgG and HRP-conjugated streptavidin in an ELISA. The absorbance at 450 nm was measured and readings from wells incubated with culture supernatants from LAC* *spa sbi* (pRMC2) were subtracted from the mean readings for LAC* *spa sbi* (pSpA) and LAC* *spa sbi* (pSpA Δ SdrESS) to account for background. Values for LAC* *spa sbi* (pSpA Δ SdrESS) are expressed as a percentage of the values measured for LAC* *spa sbi* (pSpA). Bars represent the mean of three independent experiments and error bars represent the SEM. *** $p < 0.0001$.

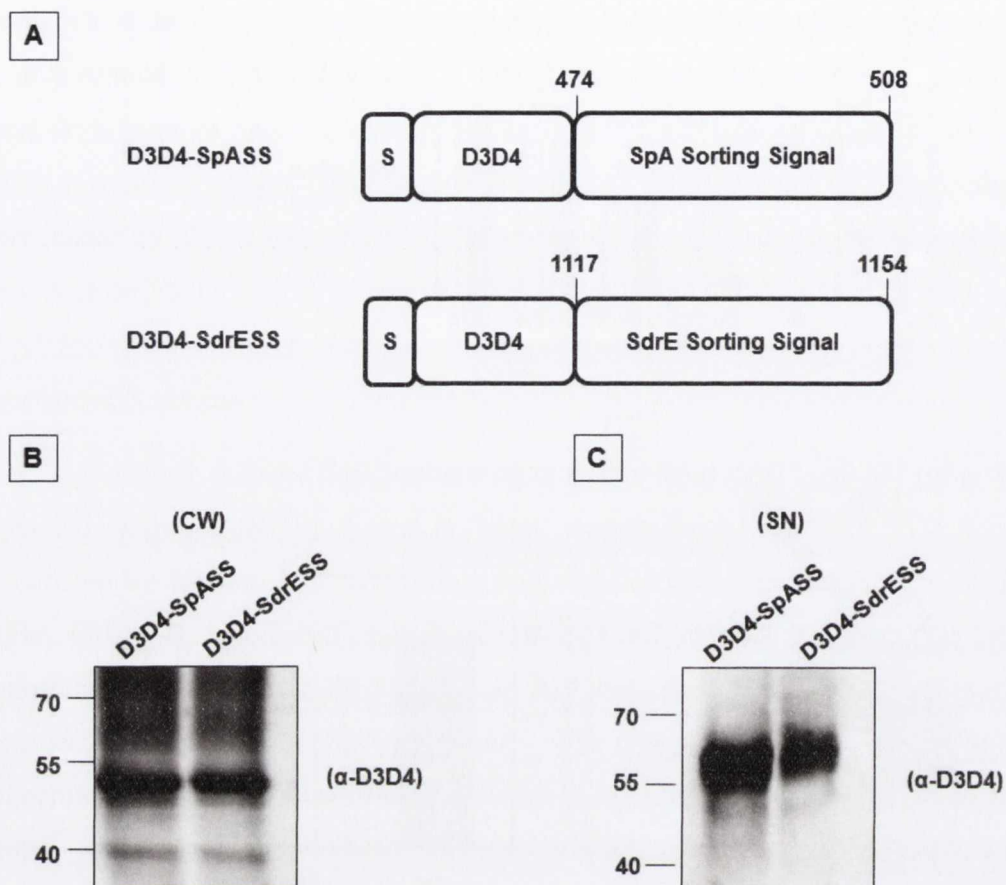


Fig. 4.9 Release of D3D4 reporter protein into *S. aureus* culture supernatants can be reduced by altering the sorting signal. (A) Schematic representation of chimeric D3D4 proteins with native Sbi signal sequence (S). Residue coordinates of the sorting signals from the full length proteins are indicated. Cell wall extracts (B) and culture supernatants (C) from LAC* *spa sbi* (pD3D4-SpASS) and LAC* *spa sbi* (pD3D4-SdrESS) following growth in growth supplemented with ATc (312.5 ng/ml) were probed with rabbit anti-D3D4 IgG. Bound antibody was detected using HRP-conjugated protein A. Size markers are indicated (kDa).

peptidoglycan (Smith *et al.*, 2011). Plasmids pRMC2::D3D4-SpASS (pD3D4-SpASS) and pRMC2::D3D4-SdrESS (pD3D4-SdrESS) each carry DNA encoding the Sbi signal sequence and D3D4 domains and the SS from either SpA or SdrE, respectively. Cell wall extracts (CW) and supernatants (SN) were prepared from cultures of LAC* *spa sbi* carrying plasmids pD3D4-SpASS and pD3D4-SdrESS grown in the presence of anhydrotetracycline (ATc; 312.5 ng/ml) and analysed by Western immunoblotting using anti-D3D4 IgG. Both D3D4-SpASS and D3D4-SdrESS were detected in cell wall extracts (Fig. 4.9B) indicating that they had been sorted to the cell wall peptidoglycan. Less D3D4 protein was detected in culture supernatants from LAC* *spa sbi* (pD3D4-SdrESS) (43% reduction as estimated by densitometry) than from LAC* *spa sbi* (pD3D4-SpASS) (Fig. 4.9C). These data demonstrate that the release of a protein into *S. aureus* culture supernatants depends on the sequence of its SS.

4.2.6 The release of SpA by LAC* is mediated by both LytM and the native SpA sorting signal.

In order to assess the relative contribution of LytM and the SpA sorting signal to release of SpA DNA encoding the SpA SS was exchanged with DNA encoding the SdrE SS on the chromosome of LAC* *sbi* and LAC* *sbi lytM* by allelic exchange. To facilitate this 500 bp of DNA downstream of the *spa* gene from LAC* was cloned directly downstream of DNA encoding the SdrE SS of plasmid pSpA Ω SdrESS (Fig. 4.10A). This plasmid was used as template to amplify DNA encoding the SdrE SS with 500 bp up- and downstream fragments of DNA. (Fig. 4.10B). This DNA was cloned into pIMAY to generate plasmid pIMAY::SdrESS (Fig. 4.10B) which was used to generate strains LAC* *sbi* [SpA Ω SdrESS] and LAC* *sbi lytM* [SpA Ω SdrESS] by allelic exchange.

These strains were used to assess the relative contribution of LytM activity and the SpA SS to SpA release. SpA displayed on the surface of bacteria was detected using FITC-labelled rabbit IgG. The amount of surface-displayed SpA expressed by LAC* *sbi* [SpA Ω SdrESS] and LAC* *sbi lytM* [SpA Ω SdrESS] was identical to the amount expressed by LAC* *sbi* at each stage of growth tested (OD₆₀₀ = 0.3 and 1.2, Fig. 4.11A). The relative amount of released SpA in culture supernatants was quantified by ELISA. The amount of SpA released by *S. aureus* LAC* *sbi* [SpA Ω SdrESS] grown to an OD₆₀₀ of 0.3 was 21% less than the amount released by LAC* *sbi* grown to an equal density (Fig. 4.11B). At an OD₆₀₀ of 1.2, LAC* *sbi* [SpA Ω SdrESS] released 42% less SpA than LAC* *sbi* Fig. 4.11B). When the sorting signal of SdrE replaced the SpA sorting signal in a LytM-deficient mutant (LAC*

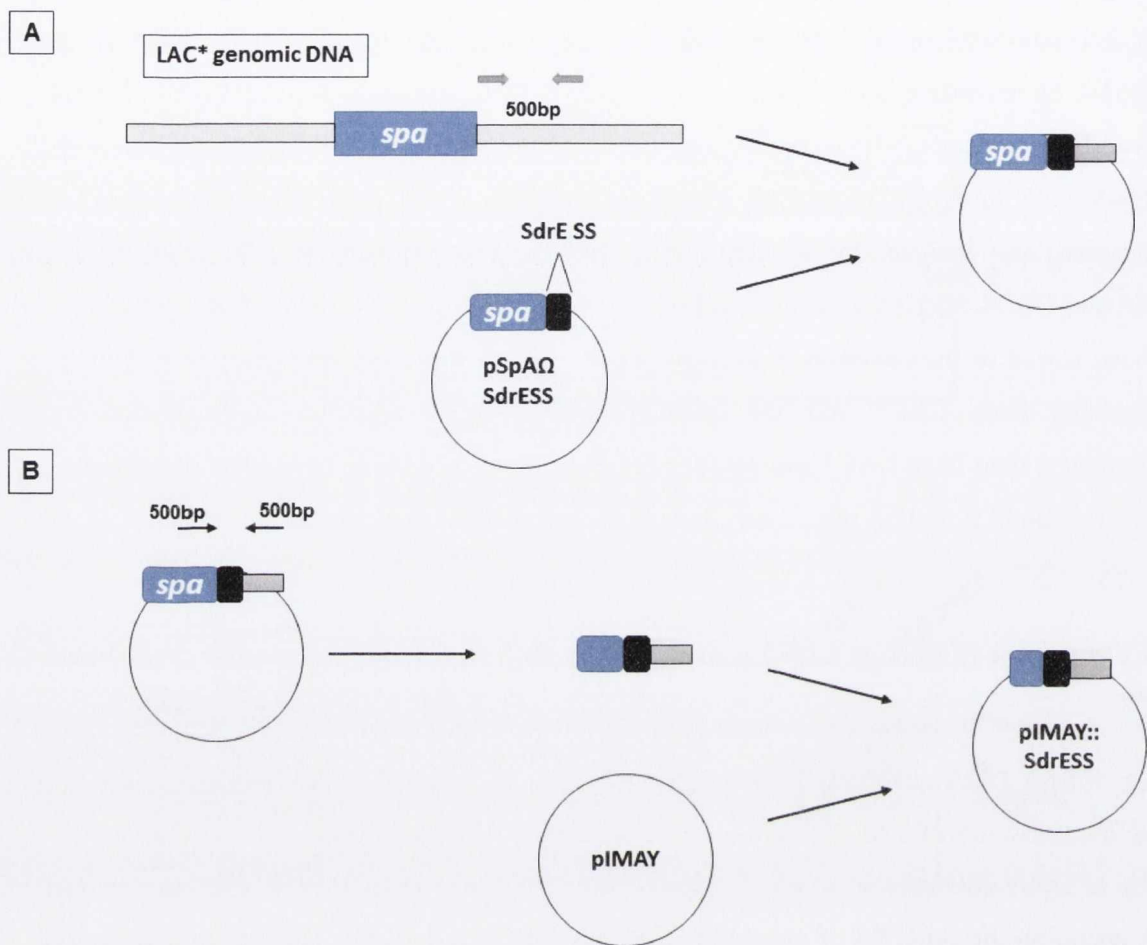


Fig. 4.10 Construction of pIMAY::SdrESS. (A) Primers were designed to amplify the 500 bp region directly downstream of the *spa* gene on the chromosome of LAC* (blue). This fragment was inserted directly downstream of the SdrE sorting signal in plasmid pSpAΩSdrESS, SdrESS indicated in black, using SLIC. (B) The resulting plasmid was used as template to amplify the SdrE sorting signal with 500 bp up- and downstream fragments of DNA. This fragment was inserted into plasmid pIMAY using SLIC to generate pIMAY::SdrESS.

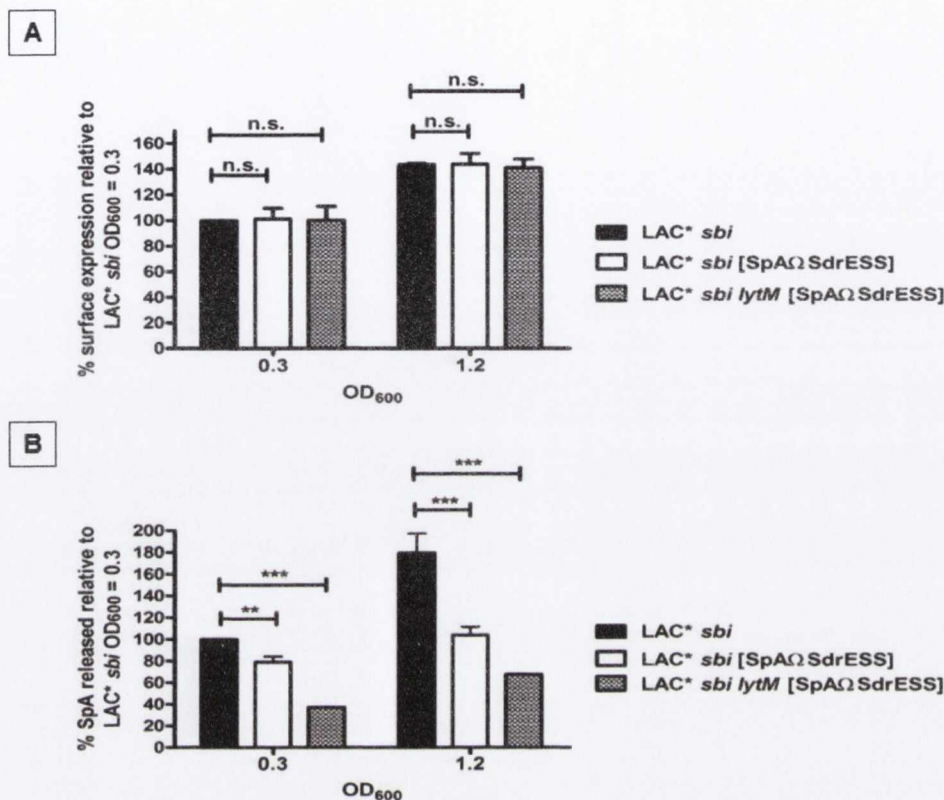


Fig. 4.11 Replacing the SpA sorting signal with the sorting signal from SdrE on the chromosome of LAC* reduces release of SpA. (A) Protein A on the surface of LAC* *sbi*, LAC* *sbi* [SpAΩSdrESS] and LAC* *sbi* *lytM* [SpAΩSdrESS] was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured using flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for LAC* *sbi* grown to an OD₆₀₀ of 0.3. Bars represent the mean values and error bars indicate the SEM of three independent experiments. (B) Protein A was captured from culture supernatants using chicken anti-SpA polyclonal IgY and detected using biotin-conjugated mouse monoclonal anti-SpA IgG followed by streptavidin-HRP in an ELISA. The absorbance at 450 nm was measured and the mean reading from wells incubated with culture supernatants from LAC* *spa sbi* were subtracted from the readings for all other wells to account for background. Values are expressed as a percentage of total released SpA measured for LAC* *sbi* grown to an OD₆₀₀ = 0.3. Bars represent the mean of four independent experiments. Error bars represent the SEM. ** $p < 0.001$, *** $p < 0.0001$, n.s. = not significant, $p > 0.05$.

sbi lytM [SpA Ω SdrESS]) the amount of SpA release was reduced by 63% and 62% for bacteria grown to an OD₆₀₀ of 0.3 and 1.2, respectively compared to LAC* *sbi* grown to the same optical density (Fig. 4.11B). These data demonstrate that the release of SpA by *S. aureus* is influenced by both the SS and LytM activity, but as not all SpA release was inhibited in LAC* *sbi lytM* [SpA Ω SdrESS] it is possible that LAC* possesses an alternative mechanism(s) to allow release of SpA.

4.2.7 Exchanging the SpA sorting signal for the sorting signal of SdrE on the chromosome of NM reduces the release of SpA.

Becker *et al.*, (2014) demonstrated a role for LytM in the release of SpA from strain NM, a commonly used MSSA lab strain of the same clonal complex (CC) as LAC* (CC8). To investigate the influence of the native SpA sorting signal on the release of SpA strain NM *sbi* [SpA Ω SdrESS] was generated by allelic exchange, as described previously, using plasmid pIMAY::SdrESS (Fig. 4.10). The amount of SpA on the bacterial surface and in culture supernatants of NM *sbi* [SpA Ω SdrESS] was quantified (Fig. 4.12). There was no significant difference in the amount of SpA displayed on the surface of NM *sbi* and NM *sbi* [SpA Ω SdrESS] grown to the same optical density (OD₆₀₀ = 0.3 or OD₆₀₀ = 1.2, Fig. 4.12) as measured by flow cytometry. There was a 32% reduction in the amount of SpA released by NM *sbi* [SpA Ω SdrESS] compared to NM *sbi* when supernatants were harvested from bacteria grown to an OD₆₀₀ of 0.3 (Fig. 4.12). NM *sbi* [SpA Ω SdrESS] grown to an OD₆₀₀ of 1.2 released 60% less SpA than NM *sbi* grown to the same optical density (Fig. 4.12). Overall it appears that at early stages of logarithmic growth (OD₆₀₀ = 0.3) LytM-deficient mutants of strains LAC* *sbi* and NM *sbi* demonstrate a similar percentage reduction in SpA release (34% decrease) whereas LytM appears to exert greater influence on the release of SpA from NM *sbi* at later stages of growth (OD₆₀₀ = 1.2), with a 36% reduction in SpA release from NM *sbi lytM* in comparison to a 11% decrease from LAC* *sbi lytM* (Fig. 3.10, Fig. 3.11). Altering the SpA SS appears to have a similar influence on the release of SpA from both LAC* *sbi* (Fig. 4.11) and NM *sbi* (Fig. 4.12).

Based on these data it can be concluded that both LytM and the native SpA SS have a greater influence on the release of SpA from NM. However, both mechanisms do not account for all SpA release, therefore it is possible, as suggested for LAC* (section 4.2.5) that NM possesses another mechanism for SpA release.

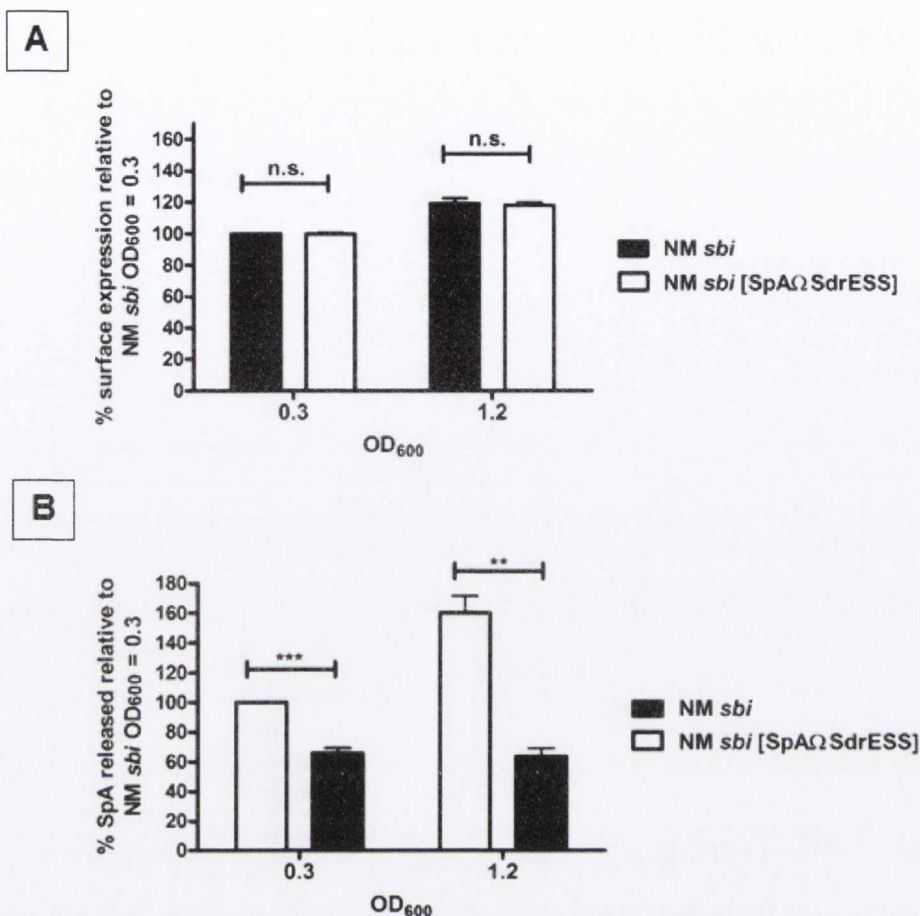


Fig. 4.12 Replacing the SpA sorting signal with the sorting signal from SdrE on the chromosome of Newman reduces release of SpA. (A) Protein A on the surface of Newman (NM) *sbi* and NM *sbi* [SpAΩSdrESS] was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured by flow cytometry. Values are plotted as a percentage of the mean fluorescence intensity measured for NM *sbi* grown to an OD₆₀₀ of 0.3. Bars represent the mean values and error bars indicate the SEM of three independent experiments. (B) Protein was captured from culture supernatants using chicken anti-SpA polyclonal IgY and detected using biotin-conjugated mouse monoclonal anti-SpA IgG followed by streptavidin-HRP in an ELISA. Values are expressed as a percentage of total released SpA measured for NM *sbi* grown to an OD₆₀₀ = 0.3. Bars represent the mean percentage release from four independent experiments. Error bars represent the SEM. *** $p = <0.001$, ** $p = 0.02$, n.s. = not significant, $p > 0.05$.

4.2.8 Amino acid substitutions in the SpA sorting signal reduce SpA release.

Schneewind *et al.*, (1992) reported that the C-terminal SS is essential for efficient anchoring of LPXTG motif-containing proteins to the peptidoglycan. The identification of an unsorted form of released SpA in the SN of LAC* (Table 4.1) and the finding that SpA release can be reduced by replacing the SpA SS with the SS from the CWA protein SdrE (Fig. 4.11B), raised the possibility that SpA does not possess an optimal SS. The hydrophobic membrane-spanning domain and the C-terminal positively charged region of the SS are important for retaining CWA proteins in the cytoplasmic membrane prior to processing by sortase A (Schneewind *et al.*, 1993). Comparing the SS of SpA and SdrE show the SdrE SS to have both a longer hydrophobic membrane-spanning region, 26 residues in comparison to 25, and more positively charged residues, 5 in comparison to 3, at the C-terminus than the SpA SS (Fig. 4.13A). Both of these characteristics have been previously shown to be important for an optimal SS (Schneewind *et al.*, 1993). Comparing the amino acid sequences of the SpA and SdrE SS highlighted a number of residues with dissimilar biochemical properties (Fig. 4.13A). We hypothesised that some of these dissimilar residues in the SpA SS may contribute to SpA release. The residues A499 and A502 were of particular interest to us as these residues reside in a region of the SS that was not detected by LC-MS/MS analysis of LAC* *sbi* released SpA (Table 4.1, Fig. 4.3). These residues, and the C-terminal positively charged region of SpA were assessed for their role in SpA release. To facilitate this a number of plasmids were created by mutagenesis of the SpA SS in the plasmid pSpA to create constructs with sorting signals that are increasingly more "SdrE-like". First, the entire positively charged C-terminus of pSpA was replaced with the corresponding region of SdrE to generate pSpA-KKQNK. Two additional amino acid substitutions were introduced individually to generate plasmids pSpA-KKQNK+A502F and pSpA-KKQNK+A502F/A499L (Fig. 4.13B). All plasmids were introduced into strain LAC* *spa sbi*.

To study the release of SpA, culture supernatant (SN) from LAC* *spa sbi* carrying plasmids pSpA, pSpA Ω SdrESS, pSpA-KKQNK, pSpA-KKQNK+A502F and pSpA-KKQNK+A502F/A499L grown until an OD₆₀₀ of 1.2 was reached in broth supplemented with ATc (312.5 ng/ml) were examined by Western immunoblotting. SpA was detected using HRP-conjugated rabbit IgG. Supernatants (SN) were concentrated 10-fold (10x, Fig. 4.13C) and 20-fold (20x, Fig. 4.13D). Bacteria carrying the plasmid pSpA Ω SdrESS demonstrated the greatest reduction in SpA release in comparison to bacteria harbouring plasmid pSpA (Fig. 4.13C), a 60% reduction as estimated by densitometry, similar to what was measured



Fig. 4.13 Clustal alignment of SpA and SdrE sorting signals (SS) and modifications of the SpA SS to become more “SdrE-like” and their effect on SpA release. (A) Sorting signals have 57% amino acid sequence identity. Membrane-spanning regions are underlined and positive residues at the C-termini are in bold. * indicates positions which have a single, fully conserved residue, : indicates conservation between residues of strongly similar properties, . indicates conservation between residues of weakly similar properties. Residue coordinates of original sequence are indicated. Regions/residues chosen for mutagenesis are highlighted in colour. (B) SpA SS modifications generated by site-directed mutagenesis. SpA-KKQNK: positively charged C-terminus (yellow) of SpA exchanged for positively-charged C-terminus of SdrE, SpA-KKQNK+A502F (green) made using SpA-KKQNK as template, SpA-KKQNK+A502F/A499L (blue) made using SpA-KKQNK+A502F as template. All constructs were created in the anhydrotetracycline-inducible plasmid pRMC2. Levels of SpA released from LAC* *spa sbi* carrying plasmids pSpA, pSpAΩSdrESS, pSpA-KKQNK, pSpA-KKQNK+A502F, and pSpA-KKQNK+A502F/A499L after induction with ATc (312.5 ng/ml) were examined by Western immunoblotting. Supernatants (SN) from cultures were filtered and concentrated either (C) 10-fold or (D) 20-fold. SpA was detected using HRP-conjugated rabbit IgG. Size markers are indicated (kDa). Data is representative of two independent experiments.

previously (FIG. 3.10). Less SpA (30% reduction) was detected in SN from LAC* *spa sbi* (pSpA-KKQNK) compared to bacteria carrying plasmid pSpA as estimated by densitometry (Fig. 4.13C). An increased reduction (35%) was measured in SpA release for LAC* *spa sbi* (pSpA-KKQNK+A502) in comparison to LAC* *spa sbi* (pSpA) (Fig. 4.13C, 4.13D). Finally, a greater reduction in SpA release was measured by densitometry for LAC* *spa sbi* (pSpA-KKQNK+A502499L), estimated as a 48% reduction in released SpA in comparison to LAC* *spa sbi* pSpA (Fig. 4.13C, 4.14D). These data demonstrate that, as described previously by Schneewind and co-workers (Schneewind *et al.*, 1992; Schneewind *et al.*, 1993), increasing the number of positively charged residues at the C-terminus of the SS can create a more optimal SS, as only replacing this region of the SpA SS with the corresponding region of SdrE (SpA-KKQNK) resulted in an estimated reduction in released SpA equivalent to 50% of the reduction measured in the SN of LAC* *spa sbi* pSpA Ω SdrESS under the same conditions (Fig. 4.13D). A further reduction in SpA release was achieved when two additional amino acid substitutions (A502F/A499L) within the predicted membrane-spanning region were introduced into the plasmid pSpA-KKQNK.

4.2.9 Increasing the number of positively-charged amino acids at the C-terminus does not influence release of A.

Increasing the number of positively charged residues at the C-terminus of the SpA SS reduced SpA release (an estimated 30% (Fig. 4.13C)). Based on this observation the influence of the positively charged C-terminus of the SS on SpA release was further studied. The CWA protein clumping factor A (ClfA) possesses a SS which harbours seven positively-charged residues at the extreme C-terminus (**RRKKENKDKK**), in comparison to the five positively-charged residues of the SdrE SS (**RRKKQNK**, Fig. 4.13A). To determine if altering the positively-charged C-terminus of the SdrE SS could generate a more optimal SS, DNA encoding the final 3 amino acids of the SdrE SS in plasmid pSpA Ω SdrESS was replaced with DNA encoding the final 6 amino acids of the ClfA SS to generate the plasmid pSpA Ω SdrESS-ENKDKK (Fig. 4.14A). Culture supernatants from LAC* *spa sbi* carrying plasmids pSpA Ω SdrESS and pSpA Ω SdrESS-ENKDKK grown until an OD₆₀₀ of 1.2 was reached in broth supplemented with ATc (312.5 ng/ml) were examined by probing with HRP-conjugated rabbit IgG in Western immunoblot. Supernatant (SN) were concentrated 20-fold (20x) and 10-fold (10x) (Fig. 4.14B). An estimated 55% reduction in SpA release was measured for LAC* *spa sbi* (pSpA Ω SdrESS-ENKDKK) in comparison to LAC* *spa sbi* (pSpA) as measured by densitometry (Fig. 4.14B). LAC* *spa sbi* (pSpA Ω SdrESS) released

an estimated 60% less SpA in comparison to LAC* *spa sbi* (pSpA) under the same conditions (Fig. 4.13C, 4.14B). Therefore it does not appear that increasing number of positively-charged amino acids at the C-terminus of the SdrE SS influences ϕ release.

A

pSpA Ω SdrESS-ENKDKK: LPETGSENNGSNNATLFGGLFAALGSLLLFGRRKKENKDKK

B



Fig. 4.14 Increasing the number of positively charged residues at the extreme C-terminus of SpA Ω SdrESS does not influence SpA release. (A) Amino acid sequence of the SS of pSpA Ω SdrESS-ENKDKK. Residues corresponding to ClfA SS are highlighted (yellow). (B) Levels of SpA released from LAC* *spa sbi* carrying plasmids pSpA, pSpA Ω SdrESS, and pSpA Ω SdrESS-ENKDKK were examined by Western immunoblotting. Supernatant from cultures induced with ATc (312.5 ng/ml) were filtered and concentrated either 20-fold or 10-fold. SpA was detected using HRP-conjugated rabbit IgG. Size markers are indicated (kDa). Data is representative of two independent experiments.

4.3 Discussion.

This chapter focused on the role of the SpA C-terminal sorting signal (SS) in the release of SpA from *S. aureus*. Released SpA was purified from the supernatant of a LAC* *sbi* culture and analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS). This showed that a portion of SpA is released from LAC* with an unprocessed SS (Table 4.1), indicating that it did not originate from the cell surface, unlike LytM-processed released SpA which is enzymatically released from cell wall peptidoglycan (Becker *et al.*, 2014). No identified peptides corresponding to the N-terminal signal peptide were identified by LC-MS/MS (Fig. 4.3). Similarly, N-terminal sequencing of the purified protein indicated that the signal peptide (residues 1-36) had been removed by the signal peptidase SspB. A small number of peptides were identified with an unprocessed LPETG motif followed by a number of residues corresponding to the SS (Table 4.1), however there was poor coverage within the SS (Fig. 4.3). No peptides corresponding to residues ₄₉₅ALGAALLAGRREL₅₀₈ were identified (Fig. 4.3) Prior to analysis by LC-MS/MS purified SpA was first digested into peptides using trypsin, which has high cleavage specificity for lysine (K) and arginine (R) (Olsen *et al.*, 2004), and whereas missed cleavage events for lysine residues is not uncommon they are comparatively rare in the case of arginine (Olsen *et al.*, 2004). The SpA SS does not possess any lysine residues and the only arginines are in close proximity to the extreme C-terminus (₅₀₄RRR₅₀₆, Fig. 4.13A), therefore if the entire SS was intact following SpA release theoretically a peptide corresponding to residues A₄₇₁ to G₅₀₃ should have been identified. Based on this information it may be possible that the C-terminal region of the SpA SS is processed by a staphylococcal protease, either membrane-associated or extracellular, prior to, or following SpA release. If this were to happen intracellularly, prior to secretion of the preprotein, this form of SpA would not be retained in the cytoplasmic membrane and would be released. However, it is also possible that these “incomplete” SS peptides are results of missed cleavages during trypsin digestion prior to MS analysis.

Becker *et al.*, (2014) used mass spectrometry to study released SpA in strain NM. Released SpA was purified from culture supernatants and subsequently analysed by MALDI-TOF (matrix-assisted laser desorption/ionization – time of flight) mass spectrometry. However, in their analysis they failed to identify the unsorted form which is described in this chapter (Table 4.1). The reason for this can be explained by the different mass spectrometry (MS) techniques used to analyse the purified proteins. MALDI-TOF MS analysis produces a single MS spectrum with each peptide hit registered as a peak based on its calculated mass to

charge ratio (m/z) value (Bafna & Edwards, 2001). Based on the low specificity of this technique multiple peptides with very similar m/z values can be identified leading to uncertainty of the genuine peptide sequence. Becker *et al.*, (2014) used their knowledge of peptidoglycan architecture and cleavage specificities of LytM to design theoretical variant SpA models with various C-terminally linked peptidoglycan molecules with similar predicted m/z values to match the peak scores identified through their MS analysis. In this way they could confidently assign predicted peptide sequences to specific discovered m/z values. The drawback of this approach however is that only peptide sequences with matching m/z values to the predicted protein models can be accurately identified and any unknown SpA variant present in their sample, e.g. unsorted SpA, may go unnoticed due its very similar molecular weight. Unprocessed SpA with an intact sorting signal has a predicted molecular weight of 51,928.9 while SpA released from the cell wall by LytM is linked to fragments of peptidoglycan of different lengths, with the most abundant forms having predicted molecular masses of between 52,555.4 and 54,151 (Becker *et al.*, 2014). For this reason the different variants of released SpA are indistinguishable by Western immunoblot as they co-migrate on an SDS-PAGE gel (Fig. 3.1A).

In contrast, LC-MS/MS analyses each individual peptide, instead of the entire protein sample, and produces an MS spectrum for each peptide, often analysing multiple fragments of the same peptide (Lim *et al.*, 2003). Due to this, an amino acid sequence can be predicted with high probability. This facilitated the discovery of the unsorted form of released SpA, which is reported in this chapter (Table 4.1). These findings indicate that the release of SpA from *S. aureus* can be influenced by both the LytM endopeptidase and the sequence of the SpA C-terminal SS.

Following this, the influence of the SpA SS on SpA release was studied in greater detail. The SdrE protein is detected exclusively in the cell wall fraction of *S. aureus* and, in contrast to SpA, it cannot be detected in culture supernatants from LAC* (Fig. 3.1B). Thus we wondered if the SdrE SS can influence protein release. The SpA SS was exchanged for the corresponding region of SdrE on the chromosome of LAC* *sbi* to generate the strain LAC* *sbi* [SpA Ω SdrESS]. Whereas no alteration in the levels of surface-displayed SpA was measured there was an approximate 42% decrease in the amount of released SpA generated by LAC* *sbi* [SpA Ω SdrESS] (Fig. 4.11), indicating that the sequence of the SpA SS is an important determinant in SpA release. A LytM-deficient mutant of LAC* *sbi* displayed a reduction in SpA release by up to 34% (Fig. 3.10B), and exchanging the SpA SS for the SdrE

SS on the chromosome of LAC* *sbi* *lytM* resulted in a 63% reduction in SpA release, with no alteration to the levels of surface-displayed SpA (Fig. 4.11). These data demonstrate that while *LytM* and the characteristics of the SpA SS can account for the majority of SpA release in LAC*, at least one other factor could be involved, perhaps another autolysin as suggested by Becker *et al.*, (2014), or that the SdrE SS is not fully optimal. No statistical reduction in surface-displayed levels was reported for these strains. This could be explained by the fact that released SpA only accounts for up to 7% of total SpA in LAC* *sbi* (Fig. 3.2), and a 63% reduction in released SpA would only equate to a 4% reduction in surface-displayed SpA, which is beyond the detectable limits of flow cytometry.

Becker *et al.*, (2014) showed that a *LytM*-deficient mutant of NM releases 75% less SpA than wild-type NM. However, here it was shown that *LytM* only accounts for up to 36% of released SpA under the conditions tested (Fig. 3.11). To investigate if the SpA SS can influence SpA release in a strain other than LAC* the strain NM *sbi* [SpA Ω SdrESS] was created. As seen in LAC* no alteration in surface-displayed levels were observed. Quantification of released SpA by ELISA revealed that SpA release is reduced by up to 60% when the SpA SS is replaced with the SS from SdrE in the NM background (Fig. 4.11). These results indicate that the properties of the SpA SS are an important factor in the release of SpA from *S. aureus* and this phenotype is not unique to LAC*.

Overexpression of the sortase A enzyme in *S. aureus* reduces the amount of P2 precursor (Fig. 4.1) of a SS-linked reporter protein within the cell (Mazmanian *et al.*, 1999). This may indicate that the ratio of SpA to sortase A within a cell can influence the release of SpA perhaps by overwhelming the sorting machinery and allows “leakage” of unprocessed SpA. However, this hypothesis does not explain the reduction in released SpA measured in LAC* when the SpA SS was replaced with the SS of SdrE and the levels of SpA production were controlled for using the inducible expression vector pRMC2 (Fig. 4.8). The SdrE SS has both a longer membrane-spanning region and possesses more positively charged residues at the C-terminus in comparison to SpA (Fig. 4.13A), two characteristics, as described by Schneewind and colleagues (Schneewind *et al.*, 1992; Schneewind *et al.*, 1993), that constitute a more optimal SS. This hypothesis was reinforced by the observation that the SdrE SS can reduce the release of SpA from LAC* (Fig. 4.11), and based on this we endeavoured to design a more optimal SS to determine if SpA release could be reduced further. Replacing the positively charged C-terminus of SpA Ω SdrESS with the corresponding region from the CW-associated protein ClfA, which contains more positively-charged

residues, however did not further reduce the release of SpA (Fig. 4.14B). This may indicate that the influence of the positively charged region of the SS on the release of SpA is minimal, or that perhaps the SdrE SS is fully optimal and is sufficient to completely inhibit the release of unsorted SpA.

The presence of the SdrE SS could reduce the release of unsorted SpA by retaining SpA in the cell membrane more efficiently and thus possibly reducing “leakage” of SpA. The SpA and SdrE sorting signals may interact differently with the cytoplasmic membrane leading to differences in retention of these proteins. This possibility could be investigated by purifying released SpA from the supernatant of strain LAC* *sbi* [SpA Ω SdrESS] and using LC-MS/MS to determine the amino acid sequence of released SpA in this strain.

In an attempt to highlight the important regions of the SdrE SS that can reduce SpA release the SpA SS was modified to stepwise become increasingly more “SdrE-like” using plasmid pSpA as template. Replacing the positively charged C-terminus of the SpA SS with the corresponding region of SdrE (SpA-KKQNK, Fig. 4.13B) reduced the release of SpA from LAC* by approximately 30% (Fig. 4.13C). Comparing the amino acid sequence of both sorting signals using Clustal Ω highlighted two dissimilar residues in close proximity to the positively charged C-terminus (A502 of SpA and F1146 of SdrE; A499 of SpA and L1143 of SdrE, Fig. 4.13A). The two amino acid substitutions were introduced individually into plasmid pSpA-KKQNK (Fig. 4.13B). The addition of the A502F substitution resulted in an estimated 5% decrease in SpA release in comparison to LAC* *spa sbi* (pSpA-KKQNK), and a further 13% decrease was measured following the introduction of the A499L substitution (Fig. 4.13C, 4.13D). These observations indicate that while the number of positively charged residues at the extreme C-terminus of the SpA SS, in agreement with previous work (Schneewind *et al.*, 1993), is an important feature of an optimal SS, a single amino acid substitution (A499L), in combination with other alterations also reduced SpA release (Fig. 4.13C). These observations suggest that characteristics other than the length of the membrane-spanning region and number of positively charged residues at the C-terminus, such as the presence of specific residues or motifs, could be important features of an optimal SS.

The host chemokines CXCL9 and CXCL10 have been shown to induce the release of SpA from LAC, however a mechanism for this process was not reported (Yung *et al.*, 2011). CXCL10 was shown to bind to both the LAC cell wall and membrane, whereas CXCL9 only bound to the cell membrane (Yung *et al.*, 2011). It's possible that these chemokines could

increase SpA release by up-regulating either *spa* or *lytM* expression, or perhaps the interaction with the cell membrane could interfere with the actions of sortase A, or disrupt the membrane itself therefore increasing the “leakage” of unsorted SpA into the extracellular milieu. It is also possible that the interaction of CXCL9 and CXCL10 with LAC induces a so far undescribed mechanism of SpA release which would further highlight the importance of released SpA to *S. aureus* during infection.

Overall this chapter provides new insights into the release of SpA by *S. aureus*, including a detailed analysis of the importance of the SpA SS in this process, and advances our knowledge of the properties of an optimal SS in *S. aureus*.

4.4 Summary.

The major results and findings described in this chapter are as follows:

- A portion of SpA is released into the culture supernatant of LAC* *sbi* with a C-terminal sorting signal unprocessed by sortase A.
- Replacing the sorting signal of SpA with the corresponding region of the cell wall-associated protein SdrE reduces release of SpA by up to 42% from strain LAC* *sbi* and up to 60% from strain Newman *sbi*.
- The influence of LytM and the C-terminal SpA sorting signal accounts for up to 63% of SpA release from *S. aureus* under the conditions tested.
- Increasing the net positive charge at the extreme C-terminus of the SpA sorting signal reduces the release of SpA from LAC*.
- A single amino acid substitution in the SpA sorting signal (A499L) was sufficient to reduce the release of SpA from LAC*.

Chapter 5

Released SpA promotes the survival and growth of *S. aureus* in human blood

5.1 Introduction.

Bacteraemia is one of the most common types of infection caused by MRSA in hospitals. Klevens *et al.*, (2007) reported that 75.2% of all reported invasive MRSA infections in the US in 2005 were bacteraemia. The treatment of staphylococcal infections with antibiotics has become increasingly difficult due to the emergence of strains harbouring resistance to vancomycin (Weigel *et al.*, 2003), the last drug with universal efficacy against MRSA. As well as increasing antibiotic resistance *S. aureus* is a successful human pathogen because of numerous strategies for adeptly evading detection and clearance by the immune system. Many *S. aureus* virulence factors are directed towards elements of the human innate immune system, including the major professional phagocyte, the neutrophil (Rooijackers & van Strijp, 2007; Spaan *et al.*, 2013b). Neutrophils make up 60% of the leukocyte population in human blood and are the most important phagocytic cells for clearing *S. aureus*. Patients that suffer from neutropenia or other congenital neutrophil deficiencies are at severe risk of bacterial infections that can often be fatal (Amulic *et al.*, 2012), and frequently develop *S. aureus* infections (Bogomolski-Yahalom & Matzner, 1995). From these observations it has been concluded that the neutrophil is the primary innate immune cell in battling *S. aureus* infection.

Neutrophils are fast-response, short-lived granulocytes derived from pluripotent hematopoietic stem cells in the bone marrow (Weissman *et al.*, 2001). Unlike other phagocytic cells they are fully equipped to kill Gram-positive bacteria, such as staphylococci (Amulic *et al.*, 2012). For specific, and effective initiation of phagocytosis neutrophils require the decoration of the bacterial surface with opsonins that have specific receptors on the neutrophil cell surface. The two major opsonin components of human serum are immunoglobulins (Ig) and complement. As most humans from early life have been exposed to *S. aureus*, commonly without the development of infection or disease, we have developed specific IgGs which recognise *S. aureus* antigens, most of which are directed against components of the cell surface, e.g. peptidoglycan, wall teichoic acid (WTA), and cell wall-anchored (CWA) proteins. Dryla *et al.*, (2005) showed the presence of specific IgGs to a number of CWA proteins, as well as other surface-associated and secreted antigens, circulating in the blood of healthy volunteers. Neutrophils possess a number of receptors which recognise pathogen-bound IgG, specifically the Fc region which is extended out from the antigen-IgG interface, called Fc-gamma receptors (FcγRs), which induces the activation

of phagocytosis. The activation of the classical complement pathway is also dependent on the presence of specific antibodies (IgG or IgM) on the bacterial surface.

S. aureus has evolved a number of strategies to evade opsonophagocytosis by human neutrophils, and many of the effector molecules are components of the cell wall. A number of CWA proteins have anti-phagocytic properties. Clumping factor A (ClfA) can protect *S. aureus* from phagocytosis via its affinity for human fibrinogen and fibrin. Fibrinogen-coated bacteria are less likely to be killed by neutrophils and ClfA-expressing bacteria show an increased survival in a septicaemia model, which is dependent on fibrinogen binding (Flick *et al.*, 2013). ClfA, as well as SdrE, can also promote the destruction of C3b through capture of regulators of complement activation, factor I and factor H, respectively (Hair *et al.*, 2010; Sharp *et al.*, 2012). The *S. aureus* collagen-adhesin (Cna) binds the collagenous domain of C1q, interfering with the interaction with the serine protease C1r, thus interrupting the classical complement pathway (Kang *et al.*, 2013).

SpA is a potent immune evasion factor that promotes *S. aureus* virulence and survival in human blood (Smith *et al.*, 2011). SpA-deficient mutants have also been shown to have reduced virulence in mice (Falugi *et al.*, 2013; Gomez *et al.*, 2004; Kim *et al.*, 2012b; Palmqvist *et al.*, 2002; Patel *et al.*, 1987), and survived poorly in an *ex vivo* human blood survival assay (Smith *et al.*, 2011). SpA is well known for its ability to bind with high affinity to IgG. The IgG-binding domains of protein A adopt a triple helix structure which allows it to bind to both the γ region of IgG (Deisenhofer, 1981), via helices I and II, and to the Fab region of human IgM of the subclass V_H3 (Graille *et al.*, 2000), via helices II and III. Other ligands of SpA include von Willebrand factor (vWF) (Hartleib *et al.*, 2000; O'Seaghda *et al.*, 2006) and tumour necrosis factor receptor 1 (TNFR1) (Gomez *et al.*, 2004). The main function of vWF is to capture platelets and immobilise them at the site of damage in a blood vessel to stimulate clot formation. SpA mediates adherence of bacteria to vWF under low shear conditions (O'Seaghda *et al.*, 2006), and this interaction promotes adherence of circulating *S. aureus* to immobilised collagen (Mascari & Ross, 2003). Activation and regulation of the TNF α signalling cascade is critically important in the host response to infection (Aggarwal, 2003; Chen & Goeddel, 2002). SpA is a potent activator of TNFR1 (Gomez *et al.*, 2004) which is essential at sites of infection where TNF α signalling is critical, such as the lung. SpA mimics TNF α by activating the TNFR1 proinflammatory signalling cascade and stimulating shedding of TNFR1 from the surface of epithelial cells and macrophages by TNF-converting enzyme which serves to limit TNF- α signalling (Gomez

et al., 2004; Gomez *et al.*, 2006; Gomez *et al.*, 2007). In agreement with this, the interaction between SpA and TNFR1 has been shown to be essential for pathogenesis in a mouse model of *S. aureus*-mediated pneumonia (Gomez *et al.*, 2004).

SpA is a potent inhibitor of phagocytosis *in vitro* (Gemmell, 1991); CWA SpA has been shown to protect *S. aureus* from opsonophagocytosis by human neutrophils *ex vivo* (Smith *et al.*, 2011). The interaction of SpA with the Fc region of IgG is predicted to be important in this process, however it has been reported that the affinity of the IgG-binding proteins in *Streptococcus pyogenes* for IgG-Fc is greatly reduced in environments where IgG concentrations are high, such as the blood stream, and only occurs in environments where the concentration of IgG is low, such as the saliva (Nordenfelt *et al.*, 2012). The importance of the affinity of SpA for IgG-Fc however has been demonstrated experimentally as *S. aureus* expressing a variant of SpA lacking the ability to recognise IgG-Fc survives poorly in mouse blood, akin to a SpA-deficient mutant (Falugi *et al.*, 2013). It is predicted that SpA interferes with neutrophil phagocytosis by sequestering host IgG on the surface of the bacterium in the opposite orientation than is required for neutrophil recognition of pathogen-bound IgG via its Fc-receptors, and will also interfere with the interaction of IgG and C1q, thus interrupting activation of the classical complement pathway (Falugi *et al.*, 2013; Smith *et al.*, 2011). Although SpA exists as both a cell wall anchored and released protein, no role in immune evasion has been described for released SpA.

SpA can also modulate the host adaptive immune response. SpA binds specifically to the V_H3 subclass of human IgM, which is the predominant subclass in the mediation of antibody responses to infection and development of immunological memory (Graille *et al.*, 2000). The interaction of SpA with IgM Fab triggers the proliferation and depletion of B cells (Goodyear & Silverman, 2003) suppressing the development of adaptive immune responses. Thus infection with SpA-expressing bacteria does not provide protection against subsequent *S. aureus* infection (Falugi *et al.*, 2013). A study by Kim *et al.*, (2010) demonstrated that immunisation of mice with a variant of SpA unable to bind IgM facilitated the production of antibodies against other staphylococcal antigens and the development of immunological protection against subsequent challenge with highly virulent CA-MRSA. Further work has shown that purified recombinant SpA can promote crosslinking of IgM from humans and guinea pigs via high V_H3 binding activity (Kim *et al.*, 2015). This leads to significant depletion of potential antibody-secreting cells from the spleen and bone marrow

(Goodyear & Silverman, 2003) and consequently dampens the adaptive immune response to *S. aureus* infection (Falugi *et al.*, 2013).

This chapter investigates the ability of released SpA to protect LAC* from killing in human blood and proposes a model by which released SpA can interfere with efficient neutrophil phagocytosis.

5.2 Results.

5.2.1 Released SpA contributes to the survival of LAC* in whole human blood.

SpA contributes to the pathogenesis of invasive infection by protecting *S. aureus* from killing in blood (Falugi *et al.*, 2013; Smith *et al.*, 2011). CWA SpA inhibits bacterial uptake by neutrophils (Kim *et al.*, 2012b; Peterson *et al.*, 1977; Smith *et al.*, 2011) but the ability of released SpA to promote *S. aureus* survival in blood has not been examined.

Previously, Malachowa *et al.* (2011) demonstrated that transcription of the *spa* gene is highly upregulated when LAC is incubated in human blood. To investigate if SpA plays a role in protecting LAC* from killing, wildtype LAC* and an isogenic *spa*-deficient mutant were incubated in blood and viable counts were used to determine bacterial survival. The LAC* *spa* mutant had a reduced ability to grow in whole blood compared to LAC* (31% reduction in survival, Fig. 5.1A), suggesting that SpA protects *S. aureus* from phagocytic killing, in agreement with previous studies (Gemmell, 1991; Smith *et al.*, 2011).

To investigate if released SpA from LAC* can promote growth of *S. aureus* in blood, LAC* *spa* bacteria were washed and resuspended in filtered, cell-free supernatants (SN) from an overnight culture of either LAC* or LAC* *spa* (Fig. 5.1B) and incubated in whole human blood. Bacteria resuspended in SN from a LAC* overnight culture showed a greater increase in viable count after 3 hours incubation in blood than bacteria with SN from a LAC* *spa* overnight culture (Fig. 5.1B). These results suggest that released SpA can protect bacteria from killing in blood.

LAC* *spa sbi* (pSpA) expresses both CWA and released SpA (Fig 4.8) when SpA expression is induced using anhydrotetracycline (ATc). Site-directed mutagenesis was used to generate a variant of SpA lacking the C-terminal sorting signal (SS) using pSpA as template, resulting in plasmid pSpAΔSS. Following confirmation by DNA sequencing this plasmid was introduced into LAC* *spa sbi*. LAC* *spa sbi* (pSpAΔSS) produced more released SpA than LAC* *spa sbi* (pSpA) (Fig. 5.2A). The SpAΔSS protein appears to migrate faster than full-length released SpA on an SDS-PAGE gel (Fig. 5.2A), in agreement with our observation that wild-type released SpA possesses a SS that is unprocessed by sortase A (Table. 4.1). The apparent slower migration of released SpA in comparison to SpAΔSS could alternatively be attributed to linked peptidoglycan fragments following LytM-mediated release of CWA SpA (Becker *et al.*, 2014). No SpA was detected in the SN of LAC* *spa sbi* (pRMC2) grown under the same conditions (Fig. 5.2A). The amount of SpA

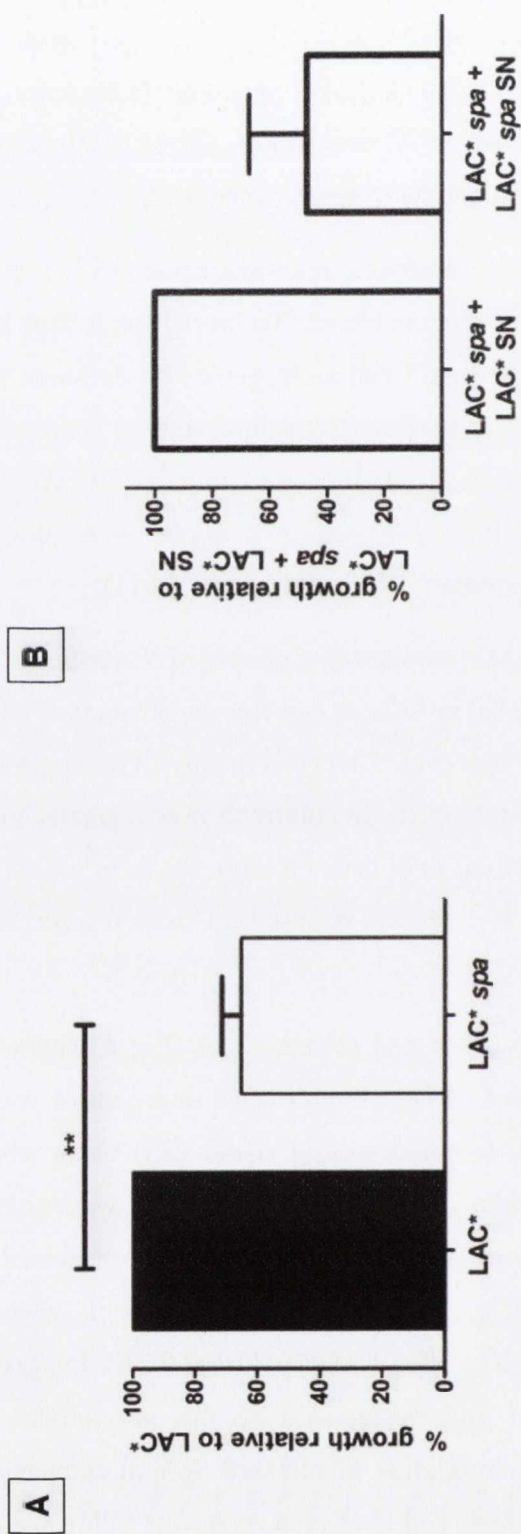


Fig. 5.1 A SpA-deficient mutant of LAC* has decreased growth in human blood compared to wild-type bacteria, and released SpA from the supernatant of a LAC* culture can increase growth. Washed bacteria were incubated in whole human blood for 3 h (A) and percentage growth was calculated by CFU counts. Values are expressed as percentage growth relative to LAC*. Bars represent the mean of 3 independent experiments. Error bars represent the SEM. (B) Prior to inoculation in whole human blood, LAC* *spa* bacteria were washed and resuspended in cell-free supernatant (SN) from overnight cultures of LAC* *spa* and LAC* *spa*, as indicated. Values are expressed as percentage growth relative to LAC* *spa* supplemented with LAC* SN. Bars are representative of mean values of 2 independent experiments. Error bars represent the SEM. ** $p < 0.001$.

displayed on the bacterial surface of these strains was detected using FITC-labelled rabbit IgG, and fluorescence was measured by flow cytometry. No SpA was detected on the surface of LAC* *spa sbi* bacteria carrying plasmids pRMC2 or pSpA Δ SS, in comparison to LAC* *spa sbi* (pSpA) (Fig. 5.2B). This is in agreement with a previous study which showed that removal of the SpA SS results in the complete release of SpA from *S. aureus* (Schneewind *et al.*, 1992) as the protein can not be recognised by sortase A.

LAC* *spa sbi* complemented with plasmid pSpA was better able to survive killing in blood compared to LAC* *spa sbi* carrying empty plasmid pRMC2 (Fig. 5.3) confirming that SpA protects LAC* from killing in blood. LAC* *spa sbi* (pSpA Δ SS) survived significantly better than bacteria carrying empty plasmid pRMC2 (Fig. 5.3). This indicates that released SpA protects *S. aureus* from killing in human blood. However bacteria producing released SpA only were not protected to the same extent as bacteria producing both surface-displayed and released SpA (Fig. 5.3) highlighting the contribution of surface-displayed SpA to protection from killing in blood. To further explore the contribution of released SpA to survival of *S. aureus* in blood, LAC* *spa sbi* (pSpA Ω SdrESS) was studied. This strain expresses similar levels of surface-displayed SpA but less released SpA (Fig. 4.8). LAC* *spa sbi* (pSpA Ω SdrESS) survived significantly better in blood than bacteria carrying empty plasmid (Fig. 5.3) but a 38% reduction in the levels of SpA release (Fig. 4.8C) did not significantly impair the ability of *S. aureus* to withstand killing in human blood (Fig. 5.3). The effect of LytM-mediated SpA release on blood survival was not examined here since LytM is proposed to contribute to the release of all proteins linked to the pentaglycine crossbridge of peptidoglycan (Becker *et al.*, 2014). Many of these proteins are important immune evasion factors and contribute to bacterial survival in blood (Smith *et al.*, 2011; Thammavongsa *et al.*, 2009) and so the effects of SpA could not be studied in isolation.

Previously, the SpA Ω SdrESS variant was introduced onto the chromosome of LAC* *sbi* to create the strain LAC* *sbi* [SpA Ω SdrESS]. This strain produces similar levels of surface-displayed SpA (Fig. 4.11A), but approximately 42% less SpA is released when bacteria are grown to an OD₆₀₀ = 1.2 (Fig. 4.11B). To determine if released SpA can influence the survival of LAC* in human blood when expressed from its native promoter on the chromosome, the ability of LAC* *sbi*, LAC* *spa sbi* and LAC* *sbi* [SpA Ω SdrESS] to grow in human blood was measured. LAC* *sbi* grew significantly better (356% of input CFU recovered) than LAC* *spa sbi* (143% of input CFU recovered (Fig. 5.4). LAC* *sbi* [SpA Ω SdrESS] also grew significantly better than LAC* *spa sbi* (227% of input CFU

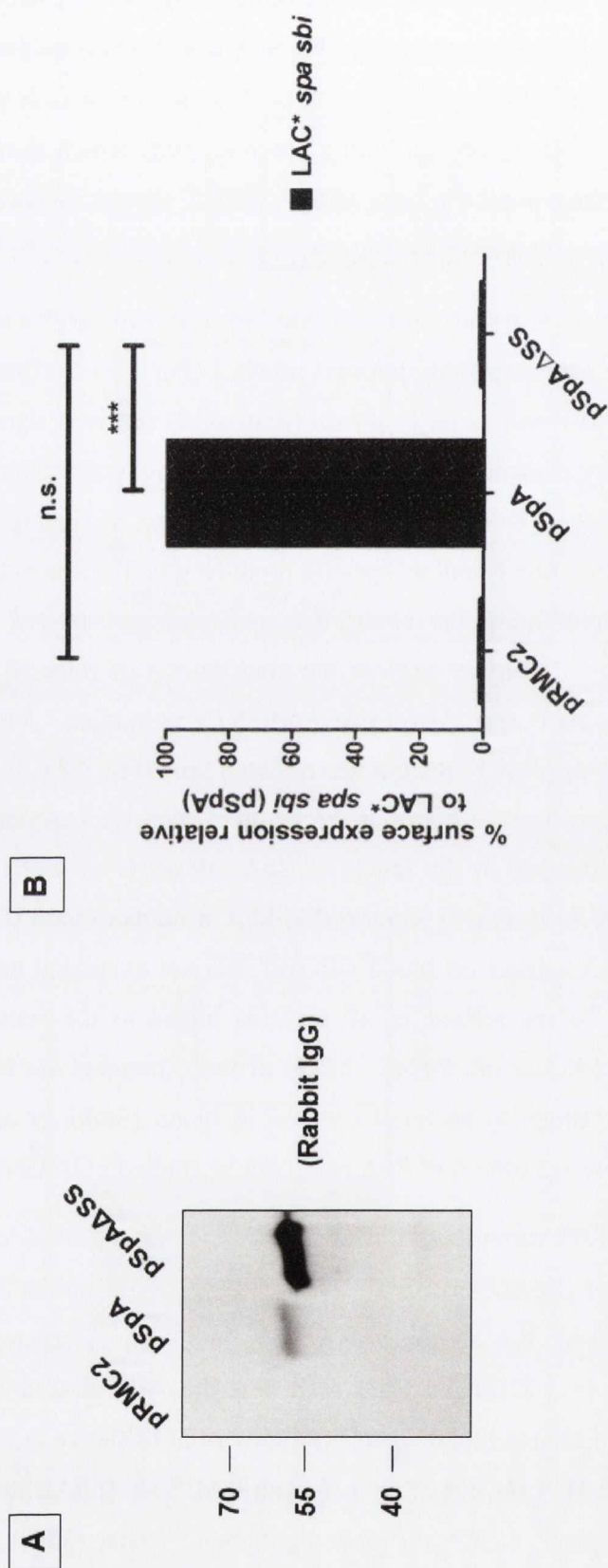


Fig. 5.2 Deletion of the Spa sorting signal (SS) increases SpA release and abrogates cell surface-display. (A) Supernatants from cultures of LAC* *spa sbi* (pRMC2), LAC* *spa sbi* (pSpA) and LAC* *spa sbi* (pSpAΔSS) induced with ATc (312.5 ng/ml) were probed with HRP-conjugated rabbit IgG to detect protein A. Size markers are indicated (kDa). (B) SpA on the surface of LAC* *spa sbi* (pRMC2), LAC* *spa sbi* (pSpA) and LAC* *spa sbi* (pSpAΔSS) induced with ATc (312.5 ng/ml) was detected using FITC-labelled rabbit IgG and the fluorescence intensity was measured using flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for LAC* *spa sbi* (pSpA). Bars represent the mean values and error bars indicate the SEM of three independent experiments. ** $p < 0.01$, n.s. = not significant.

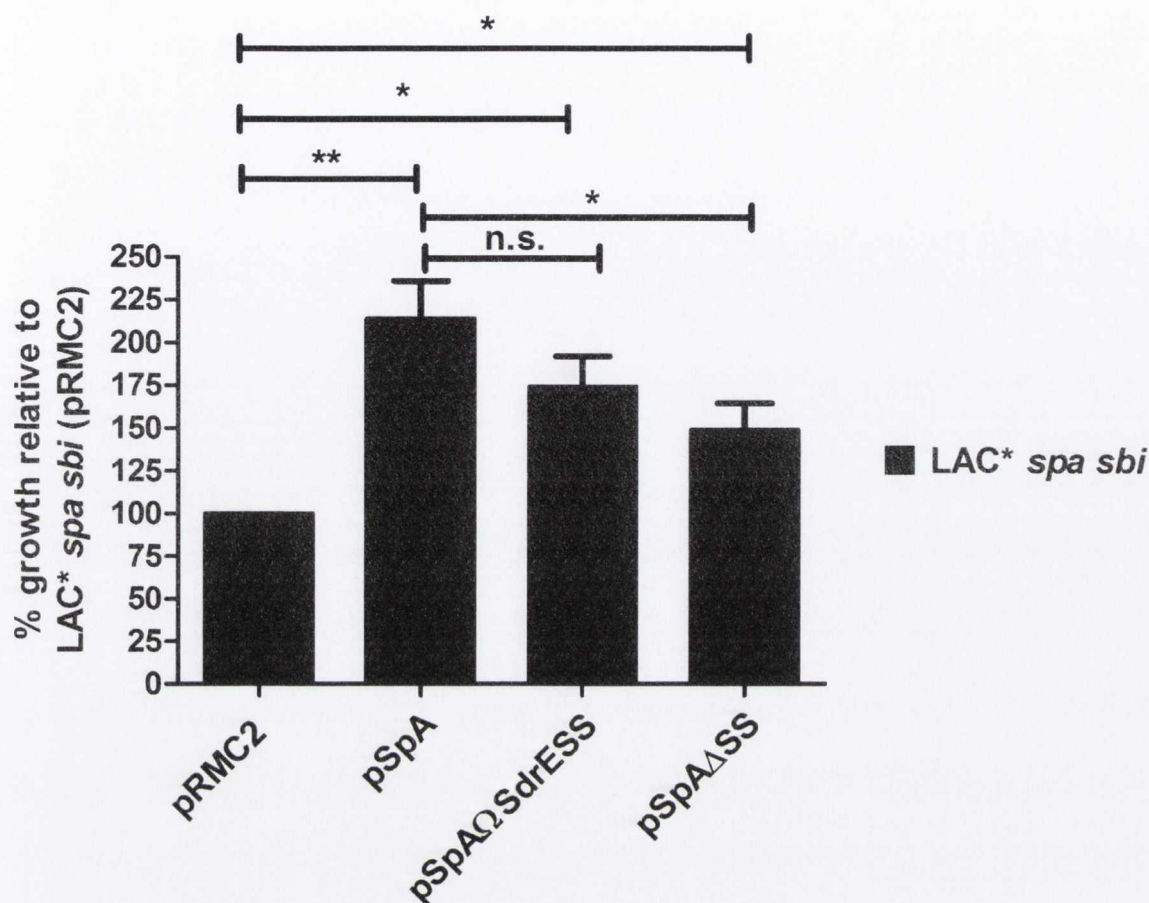


Fig. 5.3 Both cell wall-associated and released SpA protect LAC* from killing in human blood. Following growth of *S. aureus* to an $OD_{600} = 0.3$ cultures were supplemented with anhydrotetracycline (ATc; 312.5 ng/ml) and growth was allowed to continue until an $OD_{600} = 1.2$ was reached. Bacteria were washed and incubated in whole human blood supplemented with ATc (312.5 ng/ml) at 37°C with shaking (70 rpm) for 3 h. Percentage growth was calculated by viable counts. Viable counts for LAC* *spa sbi* carrying the plasmids indicated were plotted as a percentage of the mean CFU count obtained for LAC* *spa sbi* (pRMC2). Bars represent the mean from experiments conducted using four independent blood donors. Error bars indicate the SEM. *** $p = 0.0004$, ** $p = 0.0074$, * $p < 0.05$, n.s. = not significant.

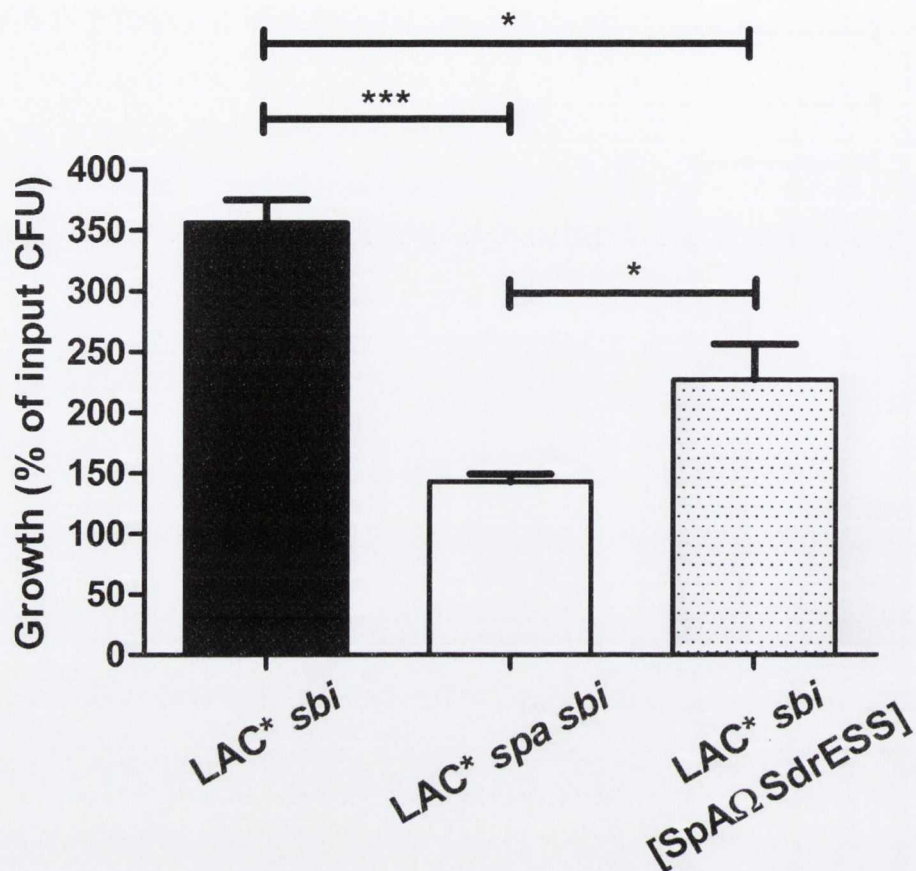


Fig. 5.4 Released SpA protects *S. aureus* from killing in human blood. Bacteria grown to an $OD_{600} = 1.2$ were washed and incubated in blood for 3 h at 37°C. Percentage bacterial growth was determined by dividing the mean CFU after 3 h by the corresponding mean input CFU. Bars represent the mean from experiments conducted using three independent blood donors. Error bars indicate the SEM. *** $p < 0.001$, * $p < 0.05$.

recovered, Fig. 5.4), demonstrating that released SpA increases the ability of *S. aureus* to survive in blood. In summary, these data indicate that released, as well as CWA SpA, protects *S. aureus* from killing in human blood.

5.2.2 Recombinant GST-SpA reduces neutrophil phagocytosis of *S. aureus* in an IgG-dependent manner.

SpA protects *S. aureus* from killing in human blood (Fig. 5.1, 5.3, 5.4), and this process is likely to be dependent on the interaction of SpA with IgG-Fc as *S. aureus* expressing a variant of SpA lacking the ability to recognise IgG-Fc survives poorly in mouse blood, akin to a SpA-deficient mutant (Falugi *et al.*, 2013). CWA SpA protects *S. aureus* from opsonophagocytosis by human neutrophils *ex vivo* (Smith *et al.*, 2011). It is believed that SpA interferes with neutrophil phagocytosis by sequestering IgG in the opposite orientation for neutrophil activation via its affinity for IgG-Fc, thus preventing classical complement fixation and recognition of IgG-Fc by neutrophil Fc receptors (Falugi *et al.*, 2013; Smith *et al.*, 2011). Released SpA contributes to the survival and growth of *S. aureus* in human blood (Fig. 5.1B, 5.3, 5.4), but the mechanism of protection is unknown. Here, the ability of soluble recombinant SpA to protect *S. aureus* from opsonophagocytosis in an IgG-dependent manner was studied (Fig. 5.5A).

S. aureus strain Wood46 was chosen for this investigation as it expresses undetectable levels of SpA (Amend *et al.*, 1984; Jongerius *et al.*, 2010b). To facilitate this work Glutathione S-transferase (GST)-tagged full-length SpA was purified from *E. coli*. GST-SpA (L17A) is a variant of GST-SpA, harbouring a single amino acid substitution of leucine 17 (L17A) in each of the five IgG binding domains, that has been shown to have reduced affinity for IgG (O'Seaghdha *et al.*, 2006). Wood46 carrying the plasmid pCM29, which constitutively expresses GFP, was pre-incubated with recombinant protein (GST-SpA, GST-SpA (L17A), or GST; at a final concentration of 1 μ M), and 10% pooled human serum. Neutrophils purified from whole human blood were added and phagocytosis was allowed to occur for 15 min. Phagocytosis was determined using flow cytometry by measuring the percentage of neutrophils bearing fluorescent bacteria. A significant reduction in phagocytosis was observed when Wood46 was incubated with GST-SpA in comparison to bacteria incubated with recombinant GST, in the presence of 10% serum (Fig. 5.5A). No significant decrease in phagocytosis was measured for bacteria incubated with GST-SpA (L17A) under the same conditions (Fig. 5.5A), suggesting that the mechanism of protection depends on the ability of SpA to bind to IgG-Fc.

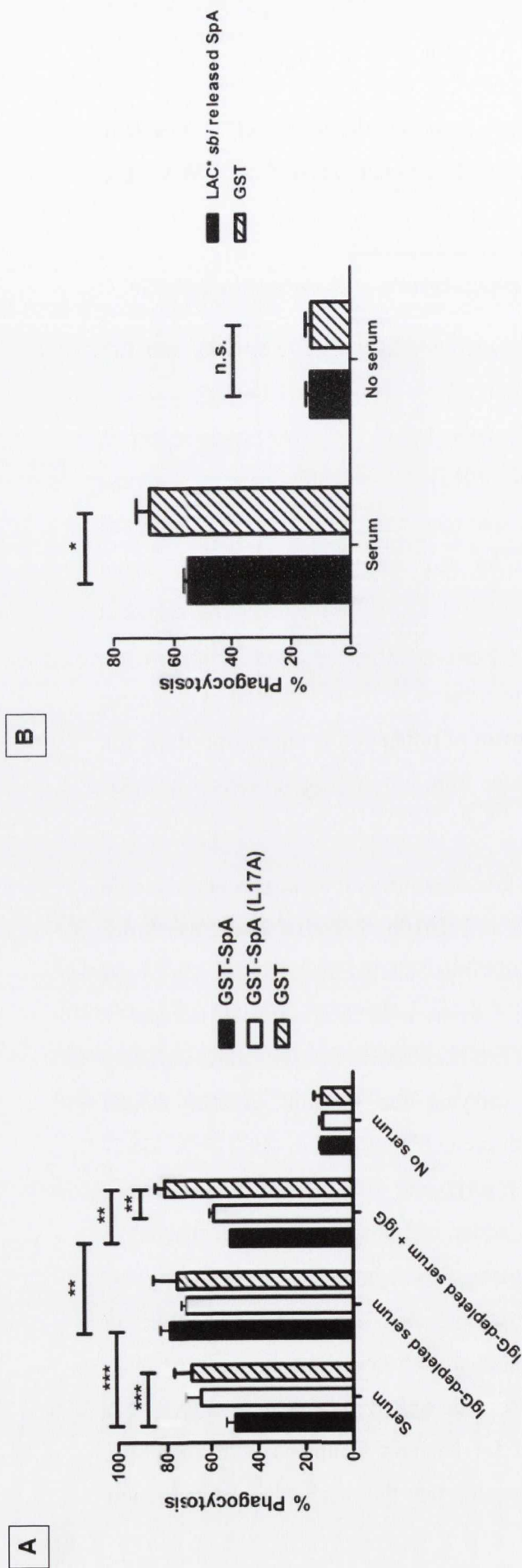


Fig. 5.5 Recombinant GST-SpA and purified released SpA from LAC* *sbi* reduce neutrophil phagocytosis of *S. aureus*. (A) *S. aureus* strain Wood46 carrying the plasmid pCM29 was incubated with recombinant proteins GST-SpA (black bars), GST-SpA (L17A) (white bars) or GST (divided bars), in 10% serum, in the absence of serum (no serum), 10% serum depleted of IgG, or 10% IgG-depleted serum supplemented with IgG (0.8 mg/ml) for 15 mins at 37°C with shaking (200 rpm). Purified human neutrophils were then added and phagocytosis was allowed to occur for 15 mins, with shaking (600 rpm) before the reaction was stopped. Percentage phagocytosis was measured by flow cytometry gating on neutrophils that are bearing fluorescent bacteria. Bars represent the mean values of 3 independent experiments, error bars represent the SEM. Difference between values were calculated as not significant unless otherwise indicated. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. (B) Wood46 pCM29 was incubated with purified released SpA from LAC* *sbi* (black bars) or GST (divided bars), in 10% serum or the absence of serum (no serum). Percentage phagocytosis was measured by flow cytometry gating on neutrophils that are bearing fluorescent bacteria. Bars represent the mean values of 3 independent experiments, error bars represent the SEM. * $p < 0.05$, n.s. = not significant. Trypan blue (20 $\mu\text{g/ml}$) was added to all samples prior to flow cytometry to quench extracellular fluorescence.

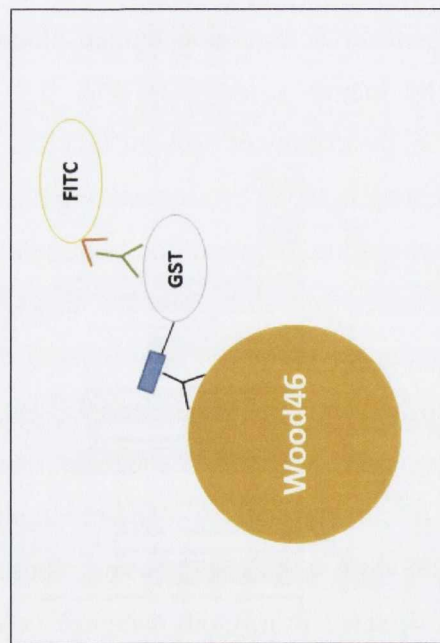
No protection from phagocytosis was observed when bacteria were incubated in 10% serum depleted of IgG (Fig. 5.5A), whereas there was significant protection from phagocytosis when bacteria were incubated with either GST-SpA or GST-SpA (L17A), but not GST, in the presence of IgG-depleted serum supplemented with purified IgG (0.8 mg/ml, Fig. 5.5A). Low amounts of phagocytosis (approximately 15% of neutrophils bearing fluorescent bacteria) was detected in samples prepared in the absence of serum (Fig. 5.5A), indicating that some opsonin-independent phagocytosis had occurred.

The ability of SpA purified from the supernatant of LAC* *sbi* (Fig. 4.2) to protect Wood46 from phagocytosis by human neutrophils was also investigated. Similar to GST-SpA (Fig. 5.5A), a significant reduction in phagocytosis was observed when *S. aureus* was incubated with purified LAC* *sbi* released SpA in the presence of 10% pooled human serum, in comparison to bacteria pre-incubated with recombinant GST under the same conditions (Fig. 5.5B). Again, low amounts of phagocytosis was detected in samples prepared in the absence of serum (Fig. 5.5B), and there was no significant difference in phagocytosis between Wood46 incubated with LAC* *sbi* released SpA or GST. These data demonstrate that soluble recombinant SpA purified from an *E. coli* host, and SpA purified from the supernatant of *S. aureus* strain LAC* *sbi* protect *S. aureus* from phagocytosis. This protection appears to be dependent on the interaction between SpA and IgG.

5.2.3 Recombinant GST-SpA binds to the surface of serum-opsonised *S. aureus*.

Released SpA contributes to the survival and growth of *S. aureus* in human blood (Fig. 5.1, 5.3, 5.4), by reducing opsonophagocytosis by human neutrophils (Fig. 5.5). Protection of *S. aureus* from phagocytosis was dependent on the affinity of SpA for IgG (Fig. 5.5A). CWA SpA is predicted to protect *S. aureus* from killing in blood by sequestering host IgG on the surface of the bacterium in the reverse orientation required for neutrophil recognition (Falugi *et al.*, 2013). Here we investigate if released SpA also protects *S. aureus* from opsonophagocytosis by inhibiting neutrophil recognition of IgG. It was wondered if released SpA can bind to the Fc region of human IgG deposited on the *S. aureus* cell surface. To investigate this *S. aureus* strain Wood46 was incubated with human serum and recombinant GST-linked SpA (GST-SpA) purified from *E. coli* (Fig. 5.6A). Any unbound IgG or GST-SpA was washed away and surface-bound GST-SpA was detected using mouse monoclonal anti (α)-GST IgG. Bound mouse IgG was detected using goat α -mouse IgG FITC-labelled Fab fragments (Fig. 5.6A). Fluorescence was measured by flow cytometry.

A



B

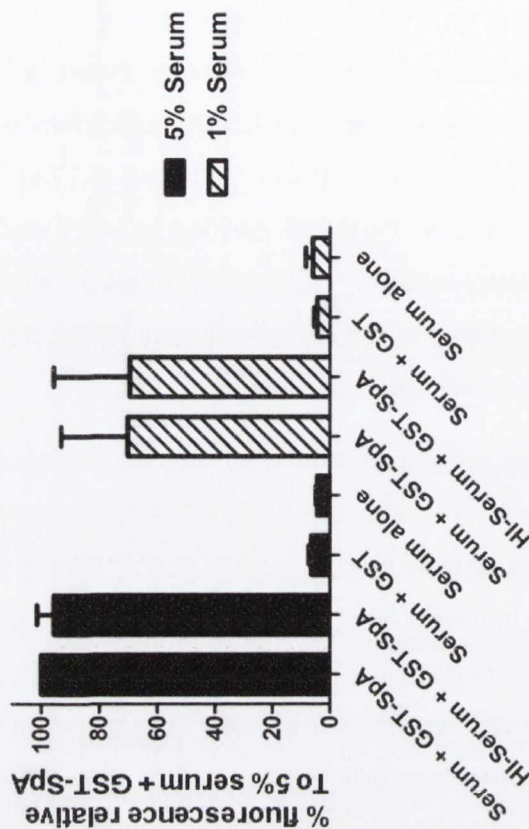


Fig. 5.6 GST-SpA can interact with the surface of serum-coated *S. aureus*. (A) *S. aureus* Wood46 is incubated with recombinant GST-SpA and human serum, which contains IgG that will recognise *S. aureus* surface antigens, at 37°C for 15 mins. Bacteria are washed and any GST-SpA associated with the surface is detected with monoclonal mouse anti (α)-GST IgG, and bound mouse IgG was detected using FITC-labelled goat α-mouse IgG F(ab)₂ fragments. Fluorescence was measured by flow cytometry. (B) Wood46 was incubated in the presence or absence of recombinant GST-SpA, or GST, in 5% or 1% pooled human serum/heat-inactivated (HI) serum, or serum alone for 15 mins at 37°C with shaking (200 rpm). Surface bound GST-SpA was detected by flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for bacteria co-incubated with GST-SpA and 5% serum. Bars represent the mean values and error bars indicate the SEM of two independent experiments.

GST-SpA bound to the surface of Wood46 when bacteria were incubated in 5% or 1% pooled human serum (Fig. 5.6B). No fluorescence was detected when bacteria were incubated with recombinant GST in the presence of 5% or 1% serum (Fig. 5.6B), indicating that fluorescence is specific to surface-bound SpA. Bacteria were incubated with GST-SpA in the presence of 5% or 1% pooled human serum that had been previously heat-treated to inactivate complement components (HI-serum). No apparent alteration in the levels of GST-SpA detected on the surface of Wood46 was recorded when bacteria were incubated in 5% or 1% HI-serum (Fig. 5.6B), indicating that the interaction was not complement-dependent. No fluorescence was detected when bacteria were incubated in serum alone (Fig. 5.6B). These data demonstrate that soluble recombinant SpA can interact with the surface of serum-opsonised *S. aureus*, and that this interaction is dependent on the presence of human serum, but not components of the complement system.

5.2.4 Binding of GST-SpA to the surface of serum-opsonised *S. aureus* is dependent on IgG.

The interaction of soluble GST-SpA with the surface of serum-coated *S. aureus* is dependent on the presence of serum, but is unlikely to be mediated by complement, as no alteration in the levels of surface-bound SpA was measured when bacteria were incubated in HI-serum (Fig. 5.6B). The protection of *S. aureus* from phagocytosis is dependent on the affinity of soluble SpA for IgG (Fig. 5.5A). Here we investigated if the interaction of GST-SpA with the surface of serum-coated *S. aureus* is dependent on IgG. GST-SpA bound to the surface of bacteria incubated in 1% human serum (Fig. 5.6B, 5.7). Similar levels of surface-bound GST-SpA were measured when bacteria were incubated in a mixture of purified IgG at a concentration of 0.08 mg/ml (Fig. 5.7), which is approximately equivalent to levels of soluble IgG in 1% serum (Gonzalez-Quintela *et al.*, 2008). This indicates that this interaction is IgG-dependent. Furthermore, there was a significant reduction in the level of GST-SpA bound to the surface of Wood46 when bacteria were incubated in 1% serum that had been depleted of IgG (Fig. 5.7). Binding of soluble GST-SpA to the surface of Wood46 was restored by supplementation of IgG-depleted serum with purified IgG (0.08 mg/ml, Fig. 5.7). No fluorescence was detected when bacteria were incubated with recombinant GST, or in the absence of serum or purified IgG (Fig. 5.7).

To investigate if the interaction of soluble recombinant SpA with surface-bound IgG is Fc-dependent, the ability of GST-SpA (L17A) to bind to the surface of opsonised Wood46 was measured. A significant decrease in fluorescence was recorded when bacteria were

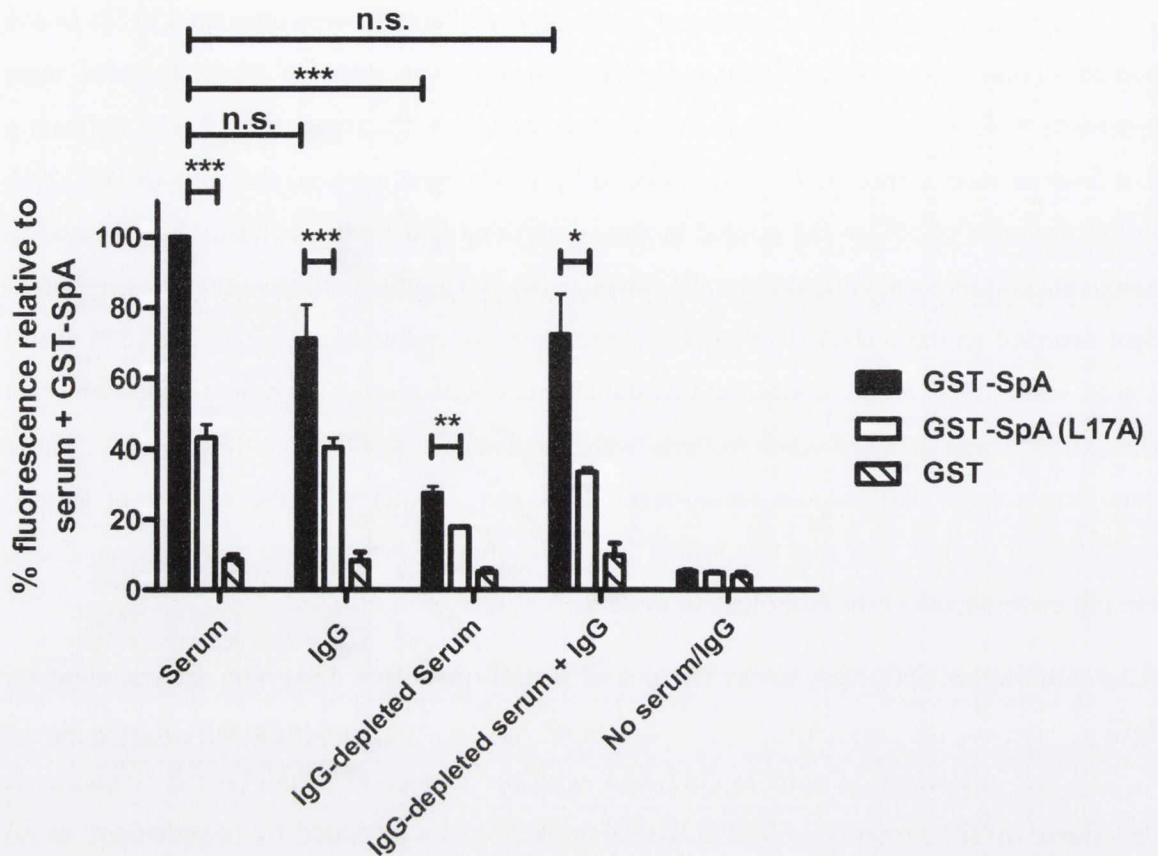


Fig. 5.7 The interaction of GST-SpA with the surface of serum-coated *S. aureus* is dependent on IgG. *S. aureus* Wood46 was incubated with recombinant proteins GST-SpA (black bars), GST-SpA (L17A) (white bars) or GST (divided bars), in 1% serum, in the absence of serum (no serum), 1% serum depleted of IgG, 1% IgG-depleted serum supplemented with IgG (0.08 mg/ml), or a solution of IgG (0.08 mg/ml) for 15 mins at 37°C with shaking (200 rpm). Bacteria were washed and surface-bound protein was probed with a mouse α -GST monoclonal antibody. Bound monoclonal antibody was detected using FITC-labelled goat α -mouse IgG F(ab)₂ fragments. Fluorescence was detected using a flow cytometer. Values are expressed as a percentage of the mean fluorescence intensity measured for bacteria co-incubated with GST-SpA and 1% serum. Bars represent the mean of three independent experiments. Error bars indicate the SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. not significant.

incubated with GST-SpA (L17A) in all conditions where IgG is present (Fig. 5.7). A small, but significant reduction in fluorescence was measured for bacteria incubated with GST-SpA (L17A) in comparison to GST-SpA in the presence of IgG-depleted serum (Fig. 5.7). This may indicate that the IgG-depletion was not complete and some IgG is still present. These data indicate that soluble recombinant SpA can bind to the surface of serum-coated *S. aureus*, and that this interaction is likely dependent on the affinity of SpA for IgG-Fc.

5.3 Discussion.

This chapter focused on the importance of released SpA in the survival of *S. aureus* from phagocytic killing in human blood. SpA has previously shown to be important for the survival of Newman in an *ex vivo* blood survival assay (Falugi *et al.*, 2013; Smith *et al.*, 2011). Protection of *S. aureus* from killing was shown to be dependent on the ability of SpA to recognise the Fc-region of IgG, as bacteria expressing a SpA variant with reduced ability to bind IgG-Fc survives poorly in mouse blood, akin to a SpA-deficient mutant (Falugi *et al.*, 2013). A Newman *spa* mutant was also more efficiently phagocytosed in comparison to wild-type Newman by purified human neutrophils (Smith *et al.*, 2011). The CA-MRSA isolate LAC of the USA300 lineage is associated with increased mortality rates in cases of *S. aureus* bacteraemia (Kempker *et al.*, 2010). It has previously been shown that LAC* can survive and proliferate in human blood (Malachowa *et al.*, 2011), and this has partially been attributed to the ability of USA300 strains to induce rapid neutrophil lysis upon phagocytosis (Kobayashi *et al.*, 2010). Malachowa *et al.* (2011) demonstrated that transcription of the *spa* gene is highly upregulated when LAC is incubated in human blood. This evidence indicates that SpA is an important virulence factor in bloodstream infections caused by USA300 isolates.

Here, the USA300 isolate LAC* and an isogenic *spa*-deficient mutant were incubated in blood and viable counts were used to determine bacterial survival. The LAC* *spa* mutant had a reduced ability to survive in whole blood compared to LAC* (31% reduction in survival, Fig. 5.1A), indicating that SpA protects LAC* from phagocytic killing. The ability of released SpA to protect LAC* from killing in blood was indirectly examined by measuring the growth of LAC* *spa* in blood when bacteria were resuspended in cell-free supernatant (SN) from cultures of either LAC* *sbi* (SpA positive) or LAC* *spa sbi* (SpA negative). Bacteria resuspended in LAC* *sbi* SN survived significantly better than bacteria in SpA-deficient SN (Fig. 5.1B) demonstrating the ability of released SpA to promote *S. aureus* growth in blood. These observations however must be considered carefully as *S. aureus* secretes a number of proteins that can both interfere with neutrophil phagocytosis, e.g. SCIN (Rooijackers *et al.*, 2005), and induce neutrophil lysis, e.g. phenol-soluble modulins (Peschel & Otto, 2013), which would significantly alter the survival and growth of *S. aureus* in blood. Although LAC* and LAC* *spa*, with the exception of a mutation in the *spa* gene, are otherwise identical, the secretome of both strains would need to be carefully analysed and

quantitatively compared before this phenotype could be attributed solely to the presence or absence of released SpA.

To study specifically the influence of released SpA on the survival of LAC* in blood a variant of SpA was created, SpA Δ SS, which lacks the C-terminal sorting signal (SS). It has previously been shown that deleting the SS inhibits sorting of SpA to the *S. aureus* cell surface (Schneewind et al., 1992). LAC* *spa sbi* (pSpA Δ SS) did not display SpA on its surface and released more SpA into culture supernatants compared to LAC* *spa sbi* (pSpA) (Fig. 5.2A). LAC* *spa sbi* (pSpA Δ SS) survived significantly better than LAC* *spa sbi* (pRMC2) following incubation in human blood (Fig. 5.2B), further indicating a role for released SpA in *S. aureus* immune evasion. Whereas these results demonstrate more convincingly a role for released SpA in promoting the survival of *S. aureus* in blood, it could be argued that the conditions here do not mimic what would be seen *in vivo*. Levels of SpA release are much higher than that produced by *S. aureus* expressing full-length SpA (Fig. 5.2A), and the expression of *spa* is under the control of an artificial inducible promoter. However, although the vast majority of *S. aureus* isolates generate both cell wall anchored and released SpA, a study by Baum *et al.*, (2009) identified a strain of *S. aureus*, isolated from a patient with bacteraemia, which possessed a premature stop-codon preceding the SS, which resulted in the production of only released SpA by this isolate. This demonstrates that, while rare, these conditions can occur naturally *in vivo*.

To further analyse the influence of released SpA, LAC* *sbi* [SpA Ω SdrESS] was assessed for its ability to survive killing in human blood. LAC* *sbi* [SpA Ω SdrESS] survived significantly better than LAC* *spa sbi* after 3 hours incubation in human blood, and a significant decrease in survival was measured in comparison to LAC* *sbi*. These results convincingly demonstrate for the first time that released SpA can protect LAC* from killing in human blood and promote growth of *S. aureus* in these conditions. Analysis of factors influencing the release of SpA from LAC* in chapter 4 demonstrated that the greatest decrease in SpA release was observed from the strain LAC* *sbi lytM* [SpA Ω SdrESS] (Fig. 4.11). The effect of LytM-mediated SpA release on blood survival was not examined here since LytM is proposed to contribute to the release of all proteins linked to the pentaglycine crossbridge of peptidoglycan (Becker *et al.*, 2014). Many of these proteins are important immune evasion factors and contribute to bacterial survival in blood (Smith *et al.*, 2011; Thammavongsa *et al.*, 2009) and so the effects of LytM-released SpA could not be studied in isolation.

Work by Falugi *et al.* (2013) demonstrated that *S. aureus* strain Newman expressing a variant of SpA unable to recognise IgG-Fc survived poorly in mouse blood in comparison to wild-type Newman. The authors hypothesised that this was due to an inability to subvert neutrophil opsonophagocytosis. The ability of CWA SpA to interfere with phagocytosis of *S. aureus* by purified human neutrophils has previously been shown (Smith *et al.*, 2011), but the ability of released SpA to interfere with this process has not been investigated. Here, we showed that incubation of serum-coated Wood46, a strain of *S. aureus* that expresses undetectable levels of SpA (Amend *et al.*, 1984), with recombinant GST-SpA (Fig. 5.5A) and released SpA from LAC* *sbi* (Fig. 5.5B) reduced phagocytosis of *S. aureus* by purified human neutrophils, demonstrating for the first time that released SpA can interfere with this process. The ability of SpA to protect *S. aureus* from phagocytosis was abolished when bacteria were incubated in serum that had been depleted of IgG, and protection was restored following addition of purified IgG (Fig. 5.5A), confirming that SpA-mediated protection is dependent on IgG. A variant of SpA with decreased affinity for IgG, GST-SpA (L17A) (Cedergren *et al.*, 1993; O'Seaghdha *et al.*, 2006), was used to assess the importance of the affinity of SpA for IgG-Fc in protecting *S. aureus* from phagocytosis. Structural analyses of SpA in complex with IgG have indicated that L17, along with 10 other residues within helices 1 and 2, mediate binding to IgG-Fc, and a recent study (Deis *et al.*, 2015) has suggested that SpA can bind multiple IgG molecules simultaneously. Protection from phagocytosis was decreased when *S. aureus* was incubated with GST-SpA (L17A) in all conditions where IgG was present (Fig. 5.5A), which strongly suggests that released SpA protects *S. aureus* from opsonophagocytosis in an IgG-Fc dependent manner.

TNFR1 is the mammalian cell surface receptor for the pro-inflammatory cytokine TNF α , which is present on the cell surface of neutrophils and upon activation triggers neutrophil apoptosis (Shalaby *et al.*, 1985). The IgG-binding domains of SpA also facilitate binding to TNFR1 (Correa *et al.*, 2004), via residues present on helices I and II, one such residue being L17 (Gomez *et al.*, 2006). Therefore it must be considered that released SpA can bind directly to TNFR1 on the surface of neutrophils, thereby triggering apoptosis and contributing to the protection of *S. aureus* from phagocytosis.

CWA SpA is predicted to inhibit opsonophagocytosis by sequestering IgG on the bacterial surface via the Fc region, thus preventing classical complement fixation and subsequent recognition by neutrophil Fc receptors (Falugi *et al.*, 2013; Smith *et al.*, 2011). Released SpA is similarly hypothesised to interfere with opsonophagocytosis by interacting

with the Fc region of soluble IgG (Kobayashi & DeLeo, 2013) and therefore inhibit IgG from binding to cell surface-antigens. Human serum contains a high concentration of IgG, approximately 8 mg/ml (Gonzalez-Quintela *et al.*, 2008). Based on this information, *S. aureus* would need to produce a very high concentration of released SpA to effectively interfere with the actions of soluble IgG, which seems unlikely. Additionally, it has been reported that the binding of the IgG Fc region by IgG-binding proteins in *Streptococcus pyogenes* is greatly reduced in environments where IgG concentrations are high, such as the blood stream, and only occurs in environments where the concentration of IgG is low, such as the saliva (Nordenfelt *et al.*, 2012). Thus it seems unlikely that released SpA can protect *S. aureus* from opsonophagocytosis by sequestering soluble IgG.

Here an alternative model of protection for released SpA is suggested whereby released SpA can recognise and bind to the Fc-region of surface antigen-bound IgG that is in the correct orientation to induce phagocytosis. It is possible that the concentration of localised surface-bound IgG equates to a less dense IgG-environment than that is present soluble in serum, which could facilitate SpA recognition of IgG-Fc based on the observations by Nordenfelt *et al.*, (2011). According to this model, similar to CWA SpA, released SpA can interrupt neutrophil recognition of pathogen-bound IgG via its Fc-receptors, and will also interfere with the interaction of IgG and C1q, thus interrupting the classical complement pathway. This mechanism was shown to be plausible as recombinant GST-SpA incubated with *S. aureus* in the presence of 5% or 1% pooled human serum could be detected in association with the cell surface by flow cytometry (Fig. 5.6). The affinity of SpA for the surface of serum-coated *S. aureus* was determined to be independent of complement components, as no alteration in the levels of surface-associated GST-SpA was measured when bacteria were incubated with serum in which complement had been inactivated via heat treatment (Fig. 5.6). This suggested, in agreement with the proposed model, that the association of soluble GST-SpA with the surface of *S. aureus* is dependent on the presence of surface-bound IgG.

The importance of the affinity of SpA for IgG-Fc in the surface-association of GST-SpA was investigated further. Less GST-SpA (L17A) was detected associated with the surface of *S. aureus* under conditions that facilitated binding of GST-SpA (Fig. 5.9), indicating that the binding of GST-SpA is dependent on its affinity for IgG-Fc. In agreement with this, GST-SpA was present on the surface of *S. aureus* incubated with a mixture of purified human IgG alone, less GST-SpA was present on the cell surface when bacteria were

incubated with IgG-depleted serum, and binding could be restored when depleted serum was supplemented with purified IgG (Fig.5.9). These observations convincingly suggest that released SpA can bind to the Fc-regions of surface-bound IgG and consequently interfere with neutrophil phagocytosis.

Further work should focus on investigating if released SpA can interfere with the recognition of surface-bound IgG by neutrophil Fc-receptors and also the interaction of surface-bound IgG and C1q. It will also be important to evaluate the significance of the protection against phagocytosis conferred by released SpA to a strain that expresses high levels cell wall-associated SpA, such as LAC. Additionally, it would be interesting to evaluate if the secreted IgG-binding protein, Sbi, could also protect *S. aureus* from phagocytosis by this mechanism. It has been suggested that SpA can bind to at least two IgG molecules simultaneously (Deis *et al.*, 2015). This function of SpA coupled with the ability to interact with the surface of serum-opsonised *S. aureus* could hint towards a number of other immune evasion strategies. Theoretically surface-bound released SpA could mask more than one surface-bound IgG molecule at once, thereby doubling the potency of a single SpA peptide. Another possibility is that a single molecule of SpA could bind to the Fc-region of two IgG molecules bound to the surface of two separate cells of *S. aureus*, thereby promoting bacterial aggregation which can protect *S. aureus* from neutrophil phagocytosis (McAdow *et al.*, 2011). Additionally, IgG-SpA complexes localised to the surface of bacteria could shield other surface antigens from opsonisation, or alternatively mask surface-bound opsonins from recognition by immune cells. Further study is essential to appreciate fully the mechanisms by which released SpA promotes the virulence of *S. aureus*.

S. aureus is vastly accomplished in its ability to evade the human immune system. SpA as a virulence factor has been the focus of many studies, and whereas the functions of CWA SpA are well understood, released SpA has been relatively ignored. This chapter provides convincing data describing the ability of released SpA to protect *S. aureus* from killing in human blood, and details a previously undescribed mechanism by which released SpA interferes with efficient neutrophil phagocytosis of *S. aureus*.

5.4 Summary.

The major results and findings described in this chapter are as follows:

- SpA increases the growth of LAC* in whole human blood.
- Released SpA significantly contributes to the survival of LAC* from phagocytic killing in whole human blood.
- Recombinant SpA reduces the phagocytic uptake of *S. aureus* by human neutrophils in an IgG-Fc dependent manner.
- Recombinant SpA was detected bound to the surface of serum-opsonised *S. aureus* in an IgG-Fc dependent manner.

Chapter 6

SasY: a novel cell wall-anchored protein that enhances the survival and growth of *S. aureus* in human blood

6.1 Introduction.

EMRSA-15, a strain of the ST22 lineage, is the dominant MRSA clone in the UK, accounting for up to 80% of hospital-associated MRSA infections (Grundmann *et al.*, 2010), and in 2007 accounted for more than 60% of bacteraemia infections (Ellington *et al.*, 2010). A study of an MRSA outbreak in a special care baby unit in the UK in 2011 used whole genome sequencing to identify the infecting strain and to track the source of infection. Analysis of MRSA strains isolated from 17 infected infants identified just one ST22 strain and 14 of the 17 infected infants harboured a new sequence type (ST2371) (Harris *et al.*, 2013). ST2371 is a single locus variant of ST22 that, unlike most ST22 strains, carries genes that encode for PVL, and a plasmid which harbours the *ermC* gene conferring erythromycin resistance as well as a number of genes conferring resistance to heavy metals (Harris *et al.*, 2013). Whole genome sequencing identified a gene encoding for a novel, previously unidentified, cell wall-anchored (CWA) protein on a second plasmid, known as pSCBU, which we have named *S. aureus* surface protein Y (SasY). The 14 ST2371 isolates were very closely related, genetically differing by 20 SNPs or less, whereas a mean of 550 SNPs between the isolates differentiated them from the ST22 strain (Harris *et al.*, 2013). EMRSA-15 is predominantly associated with healthcare-acquired infections, does not have a high transmission rate in the community setting and does not commonly cause skin and soft tissue infections (SSTIs) (Johnson *et al.*, 2001). In contrast ST2371 was found to have a high transmission rate, even among healthy adults, and a high tendency to cause SSTIs. Another characteristic was prolonged carriage, one patient developed an ST2371-mediated infection 325 days after the initial outbreak, (Harris *et al.*, 2013). These characteristics are similar to those described for community-associated (CA) MRSA clones such as USA300, which is now the predominant clone in the US. In agreement with this, a recent study reports the emergence of ST2371 in southern India, which is the first time this clone has been identified in Asia (Rajan *et al.*, 2015). Considering the importance of CWA proteins to the success of *S. aureus* as a coloniser and a pathogen this chapter focuses on the investigation of the function of SasY.

SasY from the ST2371 strain SASCBU26 comprises 389 residues which are predicted to encode an N-terminal signal sequence (residues 1-35), followed by a region comprising of nine octapeptide repeats (residues 44-116), a central alpha C (α C) domain (residues 143-299) and a C-terminal sorting signal (SS, residues 349-389), consisting of a LPNTG motif, a membrane-spanning region (M) and a terminal positively charged region (+, Fig. 6.1). The

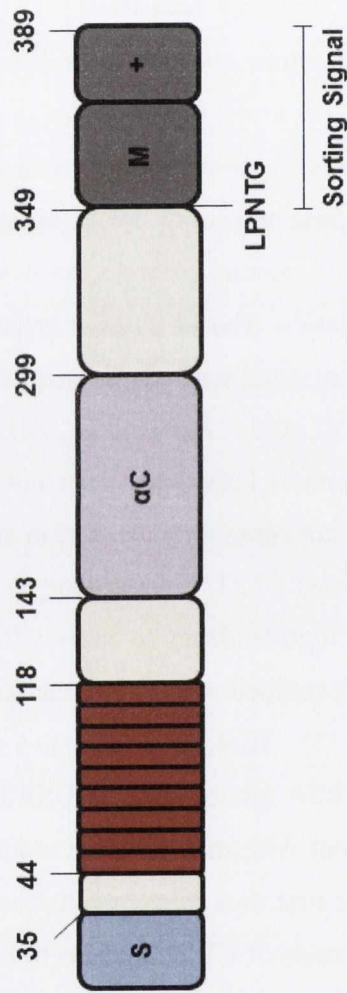


Fig. 6.1 Schematic representation of the domain organisation of SasY. SasY consists of an N-terminal signal sequence (S) followed by a region consisting of nine octapeptide repeats (residues 38-118), a central domain with predicted sequence similarity to the LPXTG-anchored alpha C protein (α CP) of Group B Streptococci (GBS) (α C; residues 143-299) and a C-terminal sorting signal (residues 349-389), consisting of a LPNTG motif, a membrane-spanning region (M) and a C-terminal positively charged region (+). Residue coordinates are indicated.

central α C domain of SasY has predicted amino acid sequence similarity and structural homology to the LPXTG-anchored alpha C protein (ACP) of Group B Streptococci (GBS). The ACP is the archetypal protein of a family of CWA proteins found in 90% of GBS clinical isolates (Lachenauer *et al.*, 2000), as well as other Gram-positive organisms (Shankar *et al.*, 1999), known as the alpha-like proteins (Alps) which are encoded by the *bca* gene. The major biological functions of the Alp family are not fully understood, but deletion of the *bca* gene attenuates the virulence of GBS in a mouse neonatal model (Li *et al.*, 1997), and α CP has been shown to promote the internalisation of GBS into human epithelial cells (Bolduc *et al.*, 2002) by binding the surface-located α 1 β 1-integrin via a conserved KTD motif within the ACP domain (Bolduc & Madoff, 2007). Protein homology modelling has shown that SasY does not possess this KTD motif, which is instead replaced by Glycine₁₇₉–Serine₁₈₀–Aspartic acid₁₈₁ (GSD) at an equivalent position (Joan A. Geoghegan; personal communication), and therefore SasY may not bind to the α 1 β 1-integrin. The ability of SasY to bind to certain host ligands has previously been investigated. SasY does not promote bacterial adherence to human loricrin, fibronectin, keratins or fibrinogen (unpublished). SasY does promote attachment of *S. aureus* to an immortalised human keratinocyte cell line (HaCaT cells). This chapter will assess if SasY protects *S. aureus* from bacterial killing in human blood.

SasY is not the only CWA protein of *S. aureus* that is encoded on a mobile genetic element. The *S. aureus* sequence type ST239 is the predominant MRSA clone in China and most of Asia (Liu *et al.*, 2009). In 2009 whole genome sequencing of a ST239 isolate identified a novel gene encoding a protein, termed SasX, encoded on a lysogenic prophage (Li *et al.*, 2012). SasX is a 15 kDa CWA protein that is associated with increased severity of ST239-mediated skin and lung infections, and also facilitates nasal colonisation (Li *et al.*, 2012). SasX has been assessed as a potential target for therapeutics and vaccines. Anti-SasX rabbit IgG promoted opsonophagocytic killing of ST239 isolates by human neutrophils (Liu *et al.*, 2015). Active immunisation with recombinant SasX and passive immunisation with anti-SasX IgG reduced nasal colonisation in mice, and reduced the size of lesions caused by *S. aureus* in a murine skin infection model (Liu *et al.*, 2015). It has been reported that the *sasX* gene is rapidly spreading among ST239 isolates and other MRSA lineages in Chinese hospitals and is considered to be an important factor in the spread and success of the ST239 lineage (Li *et al.*, 2012).

The neutrophil is the primary innate immune cell in battling *S. aureus* infection (Rigby & DeLeo, 2012). The ability of *S. aureus* to evade opsonophagocytosis is of major

importance for survival of the bacterium in human blood, and a number of CWA proteins have been identified that contribute to subverting neutrophil phagocytosis (see section 1.5.2.2). Both CWA (Smith *et al.*, 2011) and released SpA (section 5.2.1) have been shown to reduce neutrophil phagocytosis of *S. aureus*, likely by interfering with the recognition of antigen-bound IgG by neutrophil Fc-receptors (Falugi *et al.*, 2013) (section 5.2.3). ClfA protects *S. aureus* from phagocytosis via its affinity for human fibrinogen and fibrin, likely inhibiting opsonin deposition by masking the bacterial surface with a fibrinogen “coat” (Flick *et al.*, 2013; Higgins *et al.*, 2006). ClfA, as well as SdrE, can also promote the destruction of C3b (Hair *et al.*, 2010; Sharp *et al.*, 2012). The *S. aureus* collagen-adhesin (Cna) binds the collagenous domain of C1q, interfering with the interaction with the serine protease C1r, thus interrupting the classical complement pathway (Kang *et al.*, 2013). AdsA facilitates the disruption of neutrophil extracellular traps (NETs) converts adenosine to deoxyadenosine which triggers caspase-3 mediated of immune cells (Thammavongsa *et al.*, 2013). Thus *S. aureus* has evolved numerous strategies to block the functions of the human neutrophil and this is key to its success as a pathogen.

This chapter investigates if SasY can increase the survival of *S. aureus* in human blood by interfering with neutrophil function.

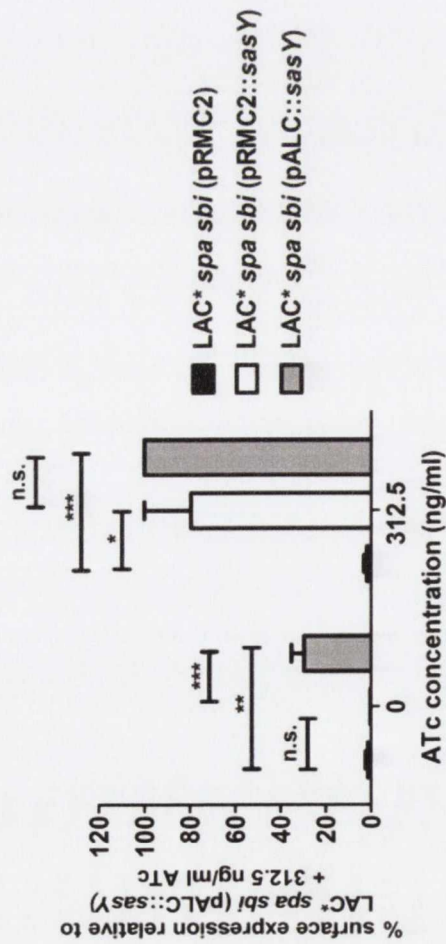
6.2 Results.

6.2.1 SasY is associated with the cell wall of *S. aureus*.

SasY harbours a C-terminal sorting signal (SS) which consists of an LPNTG motif, a hydrophobic membrane-spanning domain, and a C-terminal positively charged region (Fig. 6.1). Therefore SasY is predicted to be covalently anchored to the cell wall of *S. aureus* by sortase A. The expression profile of SasY in its native host, ST2371, has not been studied. To facilitate initial studies of SasY location and function the full-length *sasY* gene, including the native ribosome-binding site, from ST2371 was cloned into the anhydrotetracycline (ATc)-inducible expression vectors pALC2073 (Bateman *et al.*, 2001), and pRMC2 (Corrigan & Foster, 2009) to create the plasmids pALC::*sasY* (Emma J. Smith & Joan A. Geoghegan; unpublished) and pRMC2::*sasY* (Joan A. Geoghegan; unpublished), respectively. Plasmid pALC2073 is a plasmid allowing ATc-inducible gene expression. However, expression is not completely repressed in the absence of inducer. Plasmid pRMC2 possesses a *tetR* promoter with the consensus *xyl/tetO* promoter which strongly represses gene expression in the absence of inducer. Plasmid pRMC2 is a derivative of pALC2073 where the -10 box of the *tetR* promoter was changed to the *Bacillus subtilis* consensus so that repression of gene expression in the absence of inducer is complete (Corrigan & Foster, 2009). Both plasmids were introduced into strain LAC* *spa sbi*.

To investigate if SasY is displayed on the cell surface of *S. aureus*, cultures of LAC* *spa sbi* carrying plasmids pRMC2, pRMC2::*sasY*, or pALC::*sasY*, were grown until an OD₆₀₀ of 1.2 was reached in the presence, or absence of inducer (ATc). Surface-displayed SasY was detected using mouse polyclonal IgG raised against recombinant SasY, followed by FITC-labelled rabbit anti-mouse IgG (Fig. 6.2A), and fluorescence was measured using flow cytometry. No SasY was detected on the surface of LAC* *spa sbi* (pRMC2) grown in the presence of ATc, or LAC* *spa sbi* (pRMC2::*sasY*) grown in the absence of ATc (Fig. 6.2A). SasY was detected on the surface of LAC* *spa sbi* (pRMC2::*sasY*) grown in the presence of ATc, and LAC* *spa sbi* (pALC::*sasY*) grown both in the presence and absence of ATc (Fig. 6.2A). The levels of SasY on the surface of LAC* *spa sbi* bacteria carrying pRMC2::*sasY* and pALC::*sasY* were similar following growth in the presence of inducer, and an approximate 60% reduction in surface-displayed SasY was measured when LAC* *spa sbi* (pALC::*sasY*) was grown in the absence of inducer (Fig. 6.2A). These data indicate that SasY is displayed on the surface of *S. aureus* when expressed from plasmids pRMC2 and pALC2073.

A



B

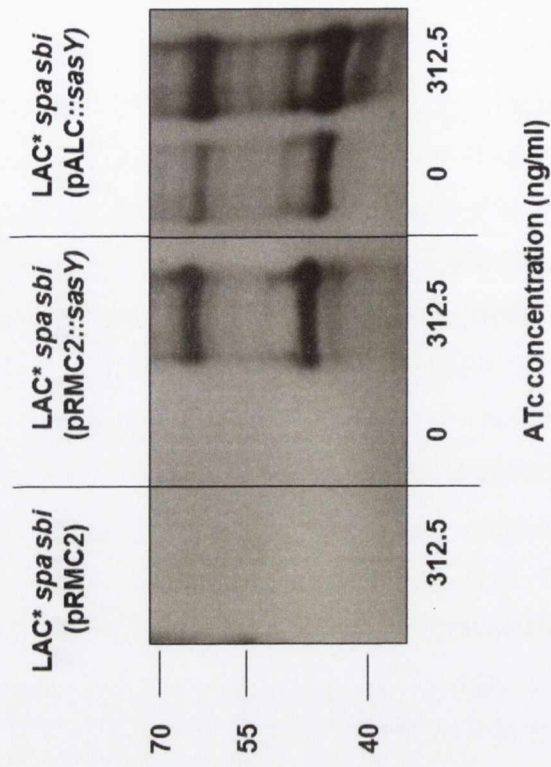


Fig. 6.2 SasY is a cell wall-associated protein that is displayed on the surface of *S. aureus*. (A) SasY on the surface of LAC* *spa sbi* carrying the plasmids pRMC2 (black bars), pRMC2::sasY (white bars), or pALC::sasY (grey bars) grown to an OD₆₀₀ = 1.2 in the presence or absence of ATc, as indicated, was detected using mouse polyclonal IgG raised against recombinant SasY, followed by FITC-labelled rabbit anti-mouse IgG. Fluorescence intensity was measured using flow cytometry. Values are expressed as a percentage of the mean fluorescence intensity measured for LAC* *spa sbi* (pALC::sasY) grown in broth supplemented with ATc (312.5 ng/ml). Bars represent the mean of three independent experiments. Error bars represent the SEM. n.s. = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (B) Cell wall extracts were prepared from the same cultures and probed with mouse polyclonal IgG raised against recombinant SasY, followed by HRP-conjugated goat anti-mouse IgG in a Western immunoblot. Size markers are indicated (kDa).

Cell wall extracts were prepared from the same cultures and analysed by Western immunoblotting. CWA SasY was detected using mouse polyclonal IgG raised against recombinant SasY, followed by HRP-conjugated goat anti-mouse IgG (Fig. 6.2B). No reactive bands were detected in cell wall extracts of LAC* *spa sbi* (pRMC2) grown in the presence of ATc, or in cell wall extracts of LAC* *spa sbi* (pRMC2::*sasY*) grown in the absence of inducer (Fig. 6.2A). Two dominant reactive bands of approximately 50 kDa and 65 kDa were detected in cell wall extracts of LAC* *spa sbi* (pRMC2::*sasY*) grown in the presence of ATc, and LAC* *spa sbi* (pALC::*sasY*) grown both in the presence and absence of ATc (Fig. 6.2B). The intensity of the band was weaker for cell wall extracts of LAC* *spa sbi* (pALC::*sasY*) (Fig. 6.2B). These data suggest that SasY is associated with the cell wall of *S. aureus*. Two bands of different molecular-weights were detected in cell wall extracts of SasY-expressing bacteria only indicating that both correspond to SasY.

6.2.2 SasY promotes the growth of *S. aureus* in human blood.

A number of CWA proteins are important virulence factors which influence the survival of *S. aureus* in human blood. To investigate if SasY can promote the survival of LAC* in blood, LAC* *spa sbi* (pRMC2), and LAC* *spa sbi* (pALC::*sasY*) bacteria grown to an OD₆₀₀ of 1.2 were washed and incubated in human blood for 3 h and viable counts were used to measure bacterial growth. LAC* *spa sbi* (pALC::*sasY*) grown in the absence of ATc demonstrated a significant increase in growth in blood (an approximate 221% increase of input CFU) in comparison to LAC* *spa sbi* (pRMC2) bacteria (an approximate 140% increase of input CFU) (Fig. 6.3A), indicating that SasY promotes growth of *S. aureus* in human blood. To determine if SasY promotes growth of LAC* in blood by protecting bacteria from phagocytic killing LAC* *spa sbi* (pRMC2) and LAC* *spa sbi* (pALC::*sasY*) grown to an OD₆₀₀ of 1.2 were washed and incubated in plasma, isolated from the same donors, for 3 h. There was no significant difference in the growth of LAC* *spa sbi* (pRMC2) and LAC* *spa sbi* (pALC::*sasY*) in human plasma (Fig. 6.3B), suggesting that SasY promotes the growth of LAC* in human blood by enhancing survival of bacteria from phagocytic killing.

ST2371 is a single locus variant of the ST22 isolate EMRSA-15 that possesses additional genes that encode for PVL and SasY, and a plasmid which harbours the *ermC* gene which confers resistance to erythromycin as well as a number of genes conferring resistance to heavy metals (Harris *et al.*, 2013). To investigate if SasY enhances the survival of EMRSA-15 in blood the plasmids pALC2073 and pALC::*sasY* were introduced into the

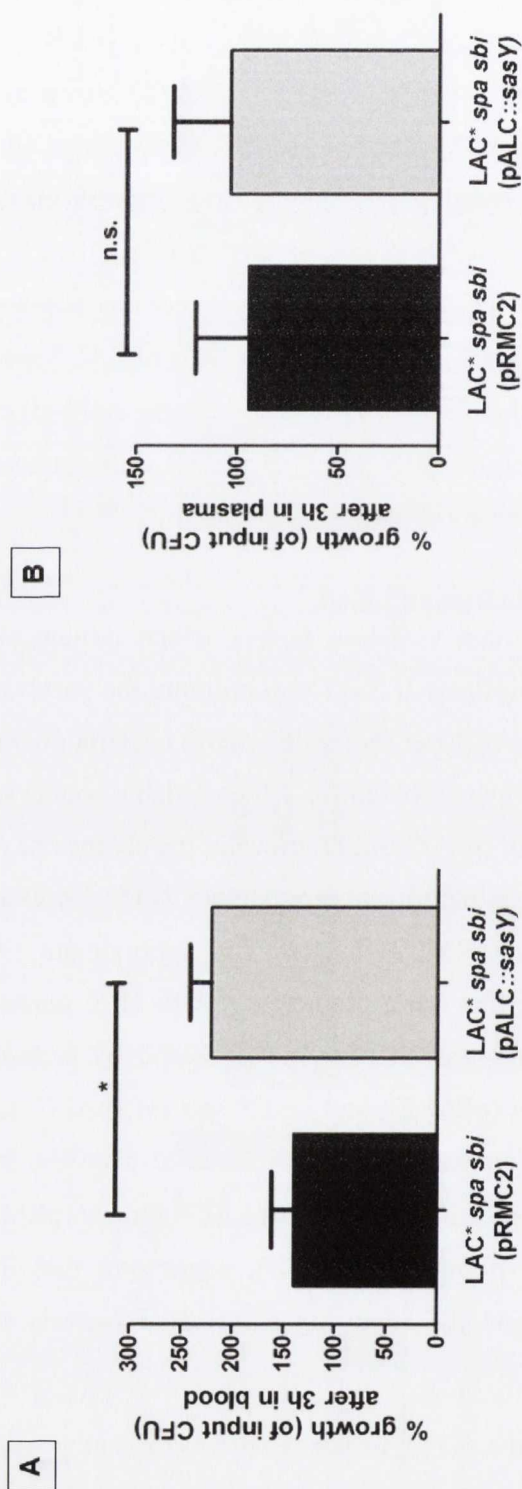


Fig. 6.3 SasY promotes the growth of LAC* *spa sbi* in human blood. (A) *S. aureus* was grown to an OD₆₀₀ of 1.2. Bacteria were washed and incubated in whole human blood (A) or plasma (B) at 37°C with shaking (200 rpm) for 3 h. Percentage bacterial growth was determined by dividing the mean CFU after 3 h by the corresponding mean input CFU. Bars represent the mean from experiments conducted using three independent blood donors. Error bars indicate the SEM. n.s. = not significant, * p < 0.05.

strain EMRSA-15. Cell wall extracts of EMRSA-15 carrying pALC2073 and pALC::*sasY* grown to an OD₆₀₀ of 1.2 in the absence of ATc were analysed by Western immunoblotting. SasY was detected using mouse polyclonal IgG raised against recombinant SasY, followed by HRP-conjugated goat anti-mouse IgG. Two bands of approximately 50 kDa and 65 kDa were detected in cell wall extracts of EMRSA-15 (pALC::*sasY*), but not EMRSA-15 (pALC) (Fig. 6.4A), confirming the expression of SasY. The ability of SasY to enhance the survival of EMRSA-15 in human blood was assessed. EMRSA-15 (pALC) and EMRSA-15 (pALC::*sasY*) were grown until an OD₆₀₀ of 1.2 was reached, washed and incubated in human blood for 3 h. Bacterial survival was determined by viable counting. No significant increase in CFU recovered was measured for EMRSA-15 (pALC::*sasY*) compared to EMRSA-15 (pALC) (Fig. 6.4B). These results suggest that the ability of SasY to promote survival and growth of *S. aureus* in blood may vary between strains. EMRSA-15 (pALC) does not readily survive phagocytic killing in human blood (approximate 42% reduction in CFU after 3 h in blood, Fig. 6.4B) whereas a variant of LAC* deficient in both SpA and Sbi can survive and proliferate in the same conditions (Fig. 6.3A).

6.2.3 Cell wall-anchored SasY reduces neutrophil phagocytosis of LAC*.

SasY enhances the survival and growth of LAC* in human blood (Fig. 6.3A). The neutrophil is the primary immune cell in host defence against *S. aureus* infection (Amulic *et al.*, 2012; Bogomolski-Yahalom & Matzner, 1995; Rigby & DeLeo, 2012), therefore it is possible that SasY can interfere with certain neutrophil functions to promote *S. aureus* survival in human blood. To investigate this, neutrophil phagocytosis of LAC* *spa sbi* (pRMC2) and LAC* *spa sbi* (pALC::*sasY*) was compared. Bacteria were grown until an OD₆₀₀ of 1.2 was reached, washed and incubated with purified human neutrophils at a multiplicity of infection (MOI) of 10 in the presence of 10% pooled human serum. Samples were incubated at 37°C with shaking (600 rpm) for 15 min to promote phagocytosis. After 15, 30 and 60 min of phagocytosis samples were plated to determine total viable bacteria (Fig. 6.5A), or treated with a lysostaphin/gentamycin solution to kill extracellular bacteria prior to plating, to determine total viable intracellular (IC) bacteria (Fig. 6.5B). An approximate 25% reduction in total viable bacteria relative to input CFU was recorded following 15 min of phagocytosis (T15) for both LAC* *spa sbi* (pRMC2) and LAC* *spa sbi* (pALC::*sasY*) (Fig. 6.5A). The differences in the levels of bacterial killing between strains was not significant. No further reduction in viable bacteria was observed following 30 min incubation with neutrophils (T30). After 60 min (T60) the total CFU increased to levels

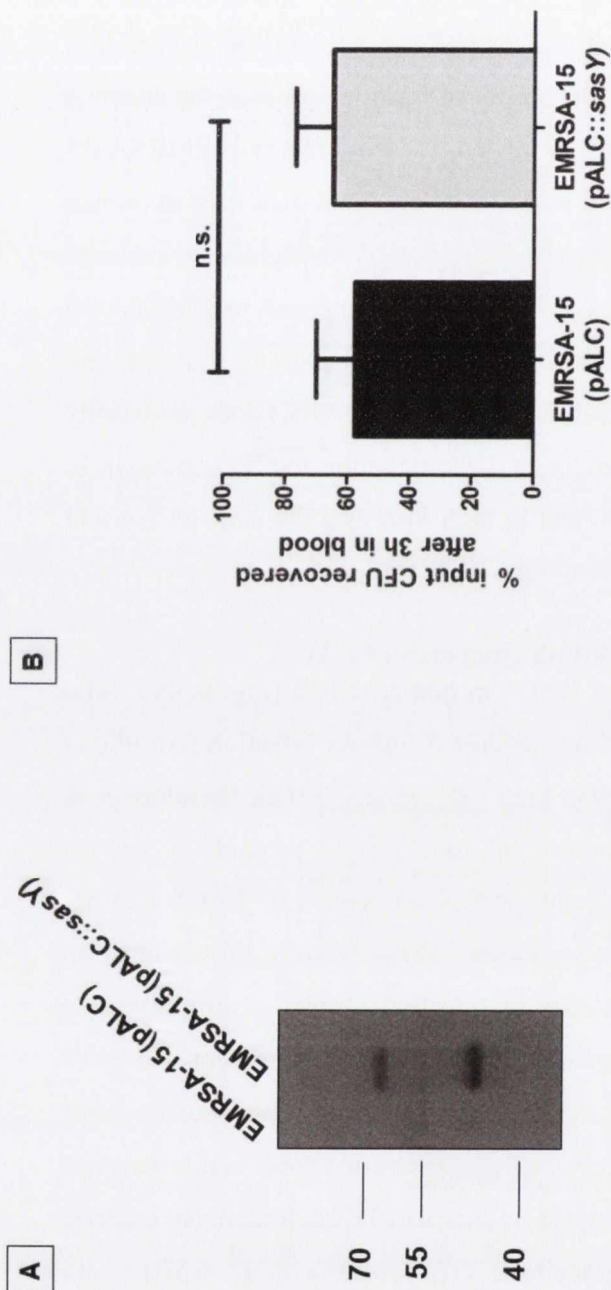


Fig. 6.4 SasY does not promote the growth of *S. aureus* EMRSA-15 in human blood. Cell wall extracts of EMRSA-15 carrying plasmids pALC or pALC::sasY grown to an OD₆₀₀ of 1.2 were probed with mouse polyclonal IgG raised against recombinant SasY, followed by HRP-conjugated goat anti-mouse IgG in a Western immunoblot. Size markers are indicated (kDa). (B) Bacteria from the same culture were washed and incubated in whole human blood at 37°C with shaking (200 rpm) for 3 h. Percentage bacterial growth was determined by dividing the mean CFU after 3 h by the corresponding mean input CFU. Bars represent the mean from experiments conducted using three independent blood donors. Error bars indicate the SEM. n.s. = not significant.

approximately equal to the initial input (Fig. 6.5A). There was no significant difference in the number of total viable bacteria between LAC* *spa sbi* (pRMC2) or LAC* *spa sbi* (pALC::*sasY*) at any of the time points (Fig. 6.5A).

The same samples were treated with a solution of gentamicin and lysostaphin to kill extracellular bacteria. Neutrophils were lysed and extracellular bacteria were enumerated by viable counting. After 15 min of phagocytosis (T15) approximately 6.8% of the initial CFU input of LAC* *spa sbi* (pRMC2) were inside neutrophils (Fig. 6.5B), whereas significantly less LAC* *spa sbi* (pALC::*sasY*) bacteria (approximately 3.8% of initial input CFU) were recovered from within neutrophils at the same time point (Fig. 6.5B). After a further 15 min incubation (T30) approximately 5% of LAC* *spa sbi* (pRMC2) bacteria relative to input inoculum were present within neutrophils, in comparison to 2.7% of intracellular (IC) LAC* *spa sbi* (pALC::*sasY*) bacteria, which represents a significant decrease of intracellular SasY-expressing bacteria (Fig. 6.5B). After 60 min (T60) incubation with human neutrophils the levels of IC LAC* *spa sbi* (pRMC2) represented 2.6% of initial input CFU in comparison to 1.8% of IC LAC* *spa sbi* (pALC::*sasY*), but this difference was not statistically significant (Fig. 6.5B). These results indicate that CWA SasY can protect LAC* *spa sbi* bacteria from phagocytosis by human neutrophils

6.2.4 Recombinant SasY does not protect *S. aureus* from phagocytosis by human neutrophils.

SasY enhances the survival and growth of LAC* in human blood, by interfering with neutrophil phagocytosis (Fig. 6.5). In the previous chapter it was shown that recombinant GST-SpA could reduce *S. aureus* phagocytosis by purified human neutrophils (Fig. 5.5A). Here the ability of recombinant hexahistidine-tagged SasY protein (HIS-SasY) to inhibit neutrophil phagocytosis of *S. aureus* was examined. HIS-SasY was incubated with *S. aureus* Wood46 (pCM29) in the presence of 10% human serum prior to addition of purified human neutrophils. Phagocytosis was allowed to occur for 15 min before the reaction was stopped. Phagocytosis was measured using flow cytometry by measuring the percentage of neutrophils bearing fluorescent bacteria. No reduction in phagocytosis was measured for bacteria incubated with HIS-SasY in comparison to bacteria incubated with GST (Fig. 6.6), whereas, as reported previously (Fig. 5.5A), a significant reduction in phagocytosis was measured when bacteria were incubated with recombinant GST-SpA (Fig. 6.6). Low level fluorescence (approximately 15% of neutrophils bearing fluorescent bacteria) was measured when serum

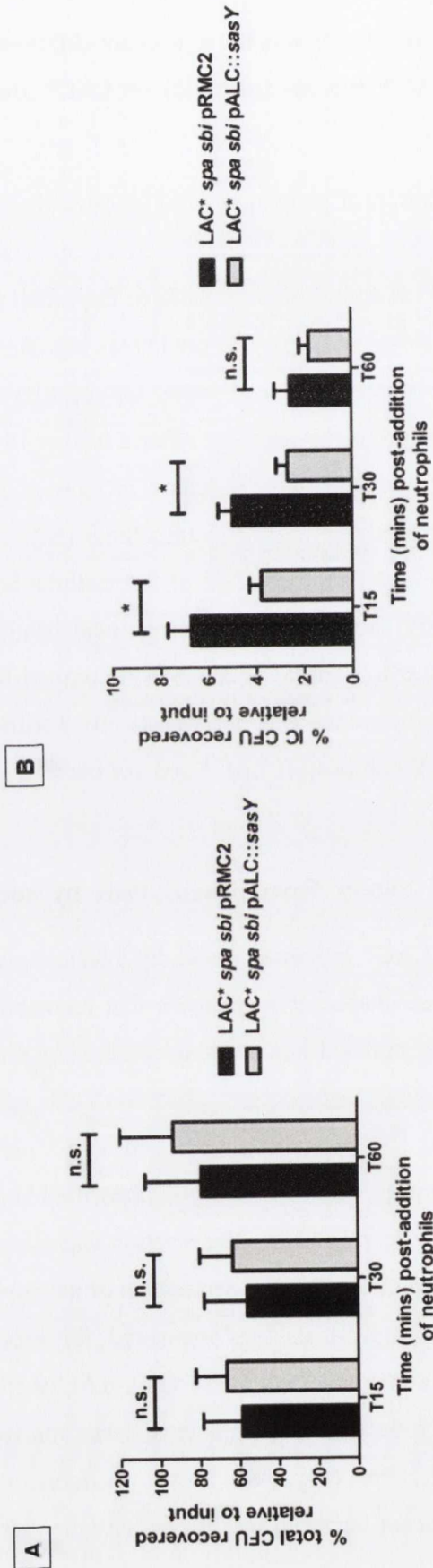


Fig. 6.5 Cell wall-associated SasY protects LAC⁺ from phagocytosis by human neutrophils. (A) Bacteria were incubated with human neutrophils in 10% human serum at a multiplicity of infection (MOI) of 10 for 15 min at 37°C with shaking (600 rpm) to encourage phagocytosis, following this shaking was reduced to 200 rpm. At time points indicated, samples were diluted in ice-cold endotoxin-free H₂O to lyse neutrophils and the number of CFU was determined by viable counts. Values are expressed as a percentage of input CFU. (B) To calculate the number viable intracellular (IC) bacteria an aliquot of each sample at the time points indicated was incubated with a solution of gentamicin/lysothaphin to kill all extracellular bacteria. Neutrophils were washed and lysed in ice-cold endotoxin-free H₂O and CFU was determined by viable counts. Values are expressed as a percentage of input CFU. Bars represent the mean values of 3 independent experiments, error bars represent the SEM. n.s. not significant, * $p < 0.05$.

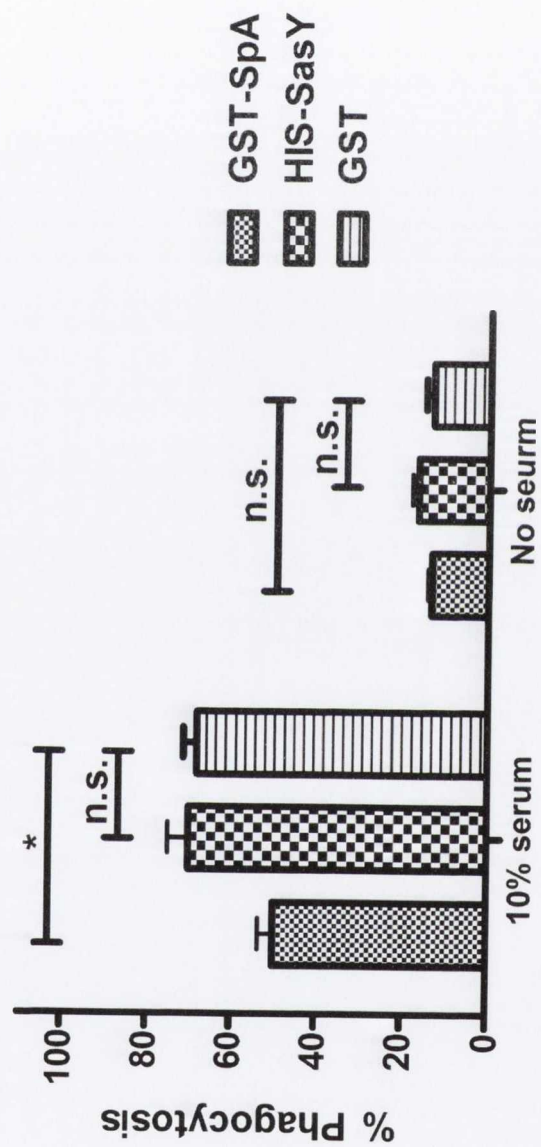


Fig. 6.6 Recombinant HIS-SasY does not reduce neutrophil phagocytosis of *S. aureus*. *S. aureus* strain Wood46 carrying the plasmid pCM29 was incubated with recombinant proteins GST-SpA (dotted bars), HIS-SasY (chequered bars) or GST (striped bars), in 10% serum, or in the absence of serum (no serum) for 15 min at 37°C with shaking (200 rpm). Purified human neutrophils were then added and phagocytosis was allowed to occur for 15 mins, with shaking (600 rpm) before the reaction was stopped. Percentage phagocytosis was measured by flow cytometry gating on neutrophils bearing fluorescent bacteria. Bars represent the mean values of 3 independent experiments, error bars represent the SEM. * $p < 0.05$, n.s. not significant.

was not added (Fig. 6.6). These results demonstrate that exogenously added recombinant SasY does not inhibit neutrophil phagocytosis of *S. aureus*.

6.2.5 SasY promotes survival of ST2371 in human blood.

The isolation of a SasY-deficient mutant of ST2371 (Emma J. Smith & Joan A. Geoghegan; unpublished) allowed the ability of SasY to protect ST2371 from killing in blood to be assessed. A 29 bp insertion was introduced into the pSCBU plasmid to generate a frame-shift mutation resulting in a premature stop codon within the *sasY* gene.

To assess the contribution of SasY to the survival of ST2371 in blood, ST2371 and ST2371 Δ *sasY* were grown to an OD₆₀₀ of 1.2, washed, and incubated in human blood for 3 h. Bacterial survival was measured by viable counts. A significant decrease (approximately 20%) in the survival of ST2371 Δ *sasY* in comparison to ST2371 was measured (Fig. 6.7A), demonstrating that SasY can enhance the survival of ST2371 in blood. As previously, the effect of SasY expression on the growth of ST2371 and ST2371 Δ *sasY* in human plasma was measured. No significant difference in the growth rate of ST2371 and ST2371 Δ *sasY* in plasma was measured (Fig. 6.7B), which is in agreement with the results obtained in the LAC* *spa sbi* background (Fig. 6.3B). These data suggest that SasY can increase the ability of ST2371 to survive and grow in human blood.

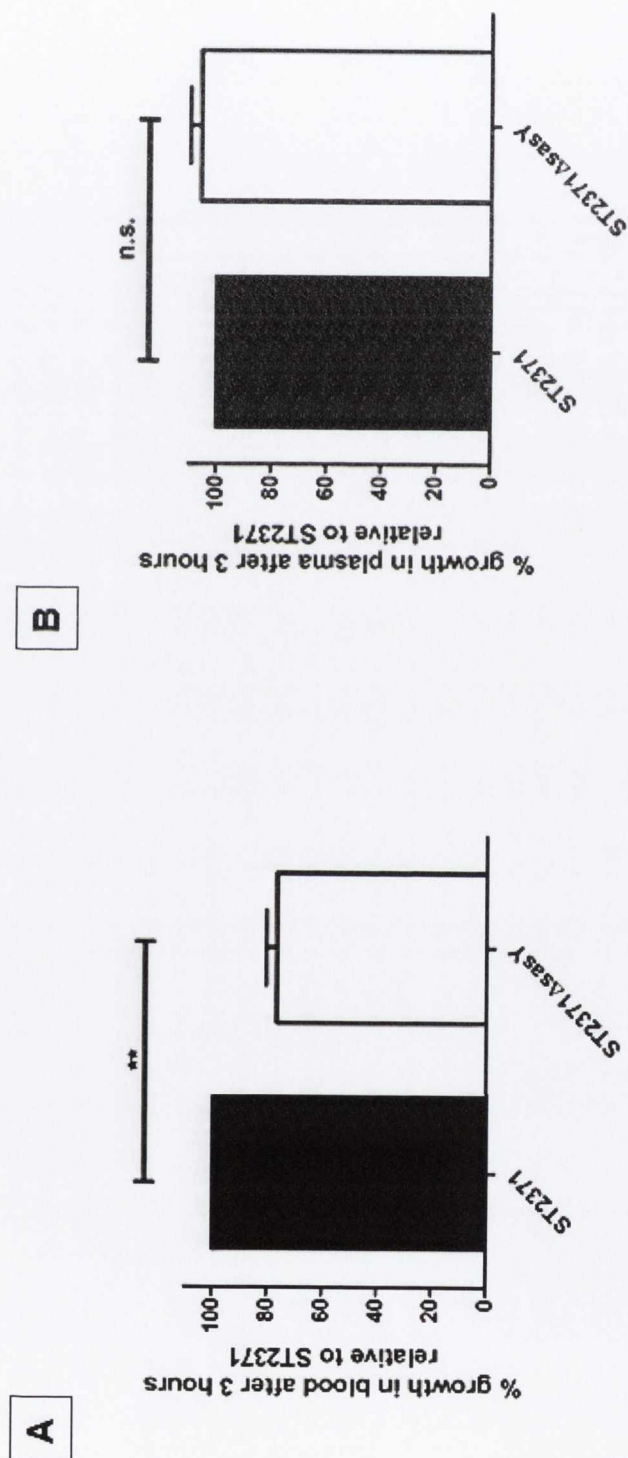


Fig. 6.8 SasY protects ST2371 from killing in human blood. Following growth in broth to an OD₆₀₀ of 1.2 washed bacteria were incubated in human blood (A) or plasma (B) for 3 h at 37°C and percentage bacterial growth was determined by dividing the mean CFU after 3 h by the corresponding mean input CFU. Viable counts for ST2371ΔsasY are plotted as a percentage of the mean CFU count obtained for ST2371. Bars are representative of mean values of 3 independent experiments. Error bars represent the SEM. ** $p < 0.01$, n.s. not significant.

6.3 Discussion.

Recently, there has been a dramatic increase in healthy individuals developing MRSA infections in the community setting (Diep *et al.*, 2006a; Seybold *et al.*, 2006). These highly transmissible and virulent strains are referred to as community-associated (CA-) MRSA. CA-MRSA of the USA300 lineage are the major cause of skin and soft tissue infections in the US (Johnson *et al.*, 2007). The success of CA-MRSA isolates has, in-part, been attributed to an increased repertoire of potent virulence factors, such as PVL and the arginine catabolic mobile element (ACME) which are present on mobile genetic elements (Diep *et al.*, 2006b; Diep *et al.*, 2008), or high expression of core genomic virulence determinants such as PSMs (Li *et al.*, 2009; Li *et al.*, 2010) or Hla (Chua *et al.*, 2014)

ST2371 is a single locus variant of the dominant HA-MRSA strain in the UK, EMRSA-15 of the ST22 lineage (Grundmann *et al.*, 2010). As well as 550 SNPs on the core genome, ST2371 possesses the gene encoding PVL, a plasmid which harbours the *ermC* gene conferring erythromycin resistance and an additional plasmid (pSCBU) which carries a gene that encodes for the novel CWA protein SasY (Harris *et al.*, 2013). ST2371 strains display an infection pattern more commonly associated with CA-MRSA strains. In an MRSA outbreak in a special care baby unit in the UK in 2011 ST2371 was the predominant strain isolated from 14 of 17 patients, was demonstrated to have a high transmission rate, even among healthy adults, prolonged carriage, and a higher tendency to cause SSTIs (Harris *et al.*, 2013). These characteristics are more comparable to the CA-MRSA clone USA300 than EMRSA-15. This raises the possibility that ST2371 could displace EMRSA-15 as the dominant MRSA clone in the UK, which is what is currently happening in Southern India (Rajan *et al.*, 2015). It would be interesting to compare the fitness of ST2371 and EMRSA-15 under *in vitro* and *in vivo* conditions.

The full-length *sasY* gene from ST2371 SASCBU26 was cloned into the expression vectors pRMC2 and pALC2073 to facilitate functional studies. Flow cytometry and Western immunoblotting demonstrated that SasY is displayed on the surface of *S. aureus* (Fig. 6.2A) and in the cell wall (Fig. 6.2B). Curiously, two bands (approximately 50 kDa and 65 kDa) were detected in cell wall extracts probed with anti-SasY IgG (Fig. 6.2B). The reason for this is not clear. The predicted molecular weight (MW) of the mature form of SasY, i.e. SasY lacking a signal peptide and sorting signal, is estimated to be 36 kDa as calculated by ExPASy ProtParam. Cross-reaction of the polyclonal antibodies with other cell wall proteins was ruled out as no reactive bands were observed in cell wall extracts of LAC* *spa sbi*

carrying plasmid pRMC2 (Fig. 6.2B). Based on the predicted MW of mature SasY (36 kDa) it is possible that the upper band (~65 kDa) in Figure 6.2B represents a dimer of SasY. Alternatively SasY may not migrate according to its MW. The lower molecular band (~50 kDa) is more puzzling. Perhaps SasY is susceptible to proteolytic degradation by staphylococcal proteases and the lower band represents a degradation product of SasY. The MW of SasY could be determined using native mass spectrometry.

A number of CWA proteins are important for the resistance of *S. aureus* to phagocytic killing in human blood, e.g. SpA (Falugi *et al.*, 2013; Smith *et al.*, 2011) and ClfA (Flick *et al.*, 2013; Smith *et al.*, 2011). SasY significantly enhanced the growth of LAC* *spa sbi* in blood (Fig. 6.3A). Additionally, no significant alteration in growth rate was measured when both strains were incubated in human plasma (Fig. 6.3B), indicating that SasY promotes growth of *S. aureus* in blood by interfering with phagocytic killing. As previously described, ST2371 and EMRSA-15 are closely related, and one of the key differences highlighted was the presence of the gene encoding *sasY* on a plasmid in ST2371 (Harris *et al.*, 2013). Therefore it was assessed if SasY could enhance the growth of EMRSA-15 in blood. Although LAC* *spa sbi* grows in blood, EMRSA-15 does not survive well and viable CFU decrease after 3 h in human blood (Fig. 6.4B). This suggests that the expression of SasY does not provide protection unless expressed in an isolate already adapted for survival and proliferation in human blood, such as the USA300 isolate LAC. In agreement with this a SasY-deficient mutant of ST2371 showed a significant decrease in growth in human blood when compared to ST2371 (Fig. 6.7A) and displayed no significant alteration in growth rate in human plasma (Fig. 6.7B). The contrasting influence of SasY for the survival of ST2371 and EMRSA-15 in human blood demonstrates the importance of assessing the role of a putative virulence factor in an appropriate host strain, and that any observed phenotype must be confirmed using the native host. These results strongly indicate that SasY enhances the survival of *S. aureus* in human blood.

The neutrophil is the most important innate immune cell in battling *S. aureus* infection (Amulic *et al.*, 2012; Bogomolski-Yahalom & Matzner, 1995). SasY enhances the growth of LAC* *spa sbi* in human blood (Fig. 6.3A) possibly by interfering with phagocytic killing as SasY did not enhance growth of bacteria in plasma (Fig. 6.3B). However SasY could enhance intracellular growth of bacteria following phagocytic uptake. Recombinant HIS-SasY did not protect *S. aureus* from phagocytosis by purified human neutrophils *in vitro* (Fig. 6.6), however, as SasY is displayed on the surface of *S. aureus* (Fig. 6.2A) a soluble

form of SasY expressed in *E. coli* may not function similarly to the staphylococcal CWA protein. LAC* *spa sbi* (pRMC2) and LAC* *spa sbi* (pALC::sasY) were incubated with purified human neutrophils and viable intracellular bacteria was measured by viable counting. There was significantly less SasY-expressing *S. aureus* within neutrophils 15- and 30-min post-phagocytosis (Fig. 6.5B), indicating that CWA SasY protects *S. aureus* from phagocytosis by human neutrophils. However, although a decrease in phagocytosis was observed this did not translate to an increase in total viable SasY-expressing bacteria, as there was no significant difference in the total viable CFU recovered for LAC* *spa sbi* (pRMC2) and LAC* *spa sbi* (pALC::sasY) 60 minutes following the addition of neutrophils (Fig. 6.5A). Perhaps a difference in total CFU would be observed after a 3 h incubation with neutrophils which would mimic the conditions used in the blood survival experiments, but this could also be a consequence of the MOI used for these experiments.

Pang *et al.* (2010) reported the ability of the *agr* locus in LAC to promote intracellular survival and lysis of neutrophils, and some phenotypes were only measurable at lower MOIs. For example a LAC *hla* mutant was shown to survive poorly within the phagosome in comparison to wild-type LAC when an MOI of 1 was used (Pang *et al.*, 2010). Similarly when measuring neutrophil lysis no significant differences between LAC and an *agr*-deficient mutant were detected at an MOI of 10, however when an MOI of 5 was used a significant decrease in neutrophil lysis was measured for LAC *agr* 60 minutes post-incubation with neutrophils (Pang *et al.*, 2010). The authors summarised that increased neutrophil lysis of LAC *agr* measured at an MOI of 10 could be due to an over-burdening of neutrophils with bacteria and that lysis was not occurring in a toxin-dependent manner. These observations highlight the importance in maintaining a proper balance of neutrophils to bacteria for true phenotypes to be observed. In accordance with this, the phagocytosis assay could be repeated using a range of MOIs, e.g. 1, 2.5 and 5, with an increased number of time points, e.g. 120 min and 180 min, to assess if a decrease in phagocytosis for LAC* *spa sbi* (pALC::sasY) correlates with an increase in total viable CFU.

CWA SasY reduces neutrophil phagocytosis of *S. aureus* (Fig. 6.5). It will be important to identify both the host target which SasY utilises to block phagocytosis, e.g. a complement component, and also the region of SasY important for this interaction. SasY possesses a central α C domain (Fig. 6.1) which has predicted sequence similarity to the alpha C protein (ACP) of group B streptococci. ACP has been shown to promote the internalisation of GBS into human epithelial cells (Bolduc *et al.*, 2002) by binding the surface-located α 1 β 1-

integrin via a conserved KTD motif within the D1 domain of ACP (Bolduc & Madoff, 2007), SasY does not possess this KTD motif. There is however a wide variety of integrin subtypes that are present on the surface of virtually all mammalian cells. Integrins are one of the two major groups of neutrophil adhesion receptors. LFA-1 and Mac-1 are members of the $\beta 2$ family of integrins present on the surface of neutrophils and are known to be important in a number of neutrophil functions such as migration and phagocytosis of opsonised bacteria (Anderson & Springer, 1987), and could be SasY targets. Indeed, *S. aureus* already encodes a protein that targets the Mac-1 integrin. The secreted cytotoxin LukAB binds to the CD11b subunit of Mac-1 on human neutrophils (DuMont *et al.*, 2013a). It would be interesting to determine if the αC domain of SasY is responsible for interfering with phagocytosis of *S. aureus*. This could be investigated by expressing the αC domain alone on the surface of bacteria, or by deleting this region of the protein.

SasX, like SasY, is a *S. aureus* CWA protein that was acquired by horizontal gene transfer on a mobile genetic element (Li *et al.*, 2012). SasX increases the severity of a number of *S. aureus* infections and is involved in nasal colonisation (Li *et al.*, 2012). Recently SasX has been assessed as a target for therapeutic and vaccine strategies against *S. aureus* infection. Anti-SasX IgG promoted phagocytosis of *S. aureus* by human neutrophils, and active and passive immunisation of mice reduced *S. aureus* disease severity (Liu *et al.*, 2015). Further study is necessary to determine the role of SasY in different *S. aureus* infections, however based on work described here it is possible that SasY could influence bacterial dissemination, increased rates of bacteraemia, or even increase the severity of SSTIs by inhibiting bacterial killing by immune cells. Thus, SasY may represent an attractive therapeutic and vaccine target for future treatment of ST2371-mediated infections.

ST2371 is a new lineage of MRSA. SasY is a novel CWA protein, encoded on a mobile genetic element, which influences the ability of ST2371 to survive in human blood. Future work to further understand the functions of SasY and the mechanism by which it protects *S. aureus* from neutrophil phagocytosis will be vital as ST2371 continues to spread, and with the potential for horizontal transfer of the pSCBU plasmid to other successful MRSA isolates.

6.4 Summary.

The major results and findings described in this chapter are as follows:

- The novel protein SasY from the ST2371 strain SASCBU26 is associated with the cell wall of *S. aureus*.
- SasY promotes the growth of LAC* in whole human blood.
- Cell wall-associated SasY reduces phagocytic uptake of *S. aureus* by human neutrophils.
- SasY increases the survival of ST2371 from phagocytic killing in whole human blood.

Chapter 7

Discussion

7.1 Discussion.

Almost all clinical isolates of *S. aureus* express the major virulence factor, staphylococcal protein A (SpA) (Votintseva *et al.*, 2014). Analysis of the surface proteome of *S. aureus* strain LAC, of the USA300 lineage, by mass spectrometry showed that SpA is the most abundant cell wall-anchored (CWA) protein displayed on the surface of exponentially growing *S. aureus* (Ventura *et al.*, 2010). SpA is located both on the surface of the bacterium and in the extracellular medium (Becker *et al.*, 2014; Lindmark *et al.*, 1977; Movitz, 1976). Becker *et al* (2014) showed that a portion of CWA SpA is shed from the cell envelope of Newman into the culture medium following cleavage of the pentaglycine crossbridge of peptidoglycan by the glycyl-glycine endopeptidase LytM (Becker *et al.*, 2014), however SpA release is not completely inhibited in a LytM-deficient mutant.

SpA is best known for its ability to bind to the Fc region (Fc γ) of mammalian IgG in a non-immune reaction (Deisenhofer, 1981), which is predicted to facilitate the evasion of *S. aureus* from opsonophagocytosis by human neutrophils (Falugi *et al.*, 2013). SpA-deficient mutants of *S. aureus* have reduced virulence in mice (Falugi *et al.*, 2013; Gomez *et al.*, 2004; Kim *et al.*, 2012b; Palmqvist *et al.*, 2002; Patel *et al.*, 1987), and survived poorly in an *ex vivo* human blood survival assay (Smith *et al.*, 2011). Similarly, SpA-specific monoclonal antibodies promote opsonophagocytic killing of *S. aureus* in murine and human blood (Kim *et al.*, 2012b). Released SpA can be detected in the skin lesions of mice infected with a USA300 strain of community-associated methicillin-resistant *S. aureus* (CA-MRSA) and in fluids recovered from patients with *S. aureus* infection (Yung *et al.*, 2011). The amount of protein A released by *S. aureus* during infection is estimated to be 5 to 10 ng per 10⁷ CFU (Kim *et al.*, 2012b). Whereas CWA SpA has been shown to reduce phagocytosis of *S. aureus* by human neutrophils (Smith *et al.*, 2011), the role of released SpA during *S. aureus* infection has not been addressed.

Work described in this thesis shows that loss of LytM in the CA-MRSA strain LAC and the MSSA strain Newman reduced SpA release by up to 36%. Analysis of SpA purified from the supernatant of an overnight culture of LAC using mass spectrometry revealed that a portion of SpA is released into the extracellular medium with an unprocessed sorting signal, indicating that it does not originate from the CW. This is the first time that this form of released SpA has been described. The importance of the C-terminal CW anchoring domain, known as the sorting signal (SS), in SpA release was investigated. Exchanging the SpA SS with the corresponding region of the CWA protein SdrE reduced SpA release from LAC and

Newman by up to 60%. The different forms of SpA generated by *S. aureus* are illustrated in Fig. 7.1A.

We report for the first time a role for released SpA in *S. aureus* infection. Released SpA increased the growth of LAC in human blood and interfered with phagocytic uptake of *S. aureus* by human neutrophils in an IgG-dependent manner. Furthermore, recombinant SpA bound to the surface of serum-opsonised *S. aureus*, and the interaction between SpA and IgG-Fc was essential in this process. This interaction may indicate a model of protection from phagocytic uptake whereby released SpA interferes with neutrophil recognition of pathogen-bound IgG via its Fc-receptors, however further study is necessary to determine if the interaction of released SpA and pathogen-bound IgG can be replicated under *in vivo* conditions. All previously reported interactions of CWA and released SpA with the human immune system, accompanied with the proposed interactions outlined in this thesis, are illustrated in Fig. 7.1B.

Experiments outlined in this thesis confirmed a role for LytM in the release of SpA from *S. aureus*, however it seems unlikely that this mechanism has evolved as a direct and targeted approach for the release of SpA. LytM is involved in the relaxation of peptidoglycan cross-linking by cleaving within the pentaglycine crossbridge which links two neighbouring glycan strands in *S. aureus* (Ramadurai *et al.*, 1999). All CWA proteins of *S. aureus* are covalently anchored to the pentaglycine crossbridge of peptidoglycan by the action of the membrane-bound sortase enzymes (Marraffini *et al.*, 2006), and therefore should be susceptible to LytM-mediated release, especially considering that LytM is deposited over the entire cell surface (Ramadurai *et al.*, 1999). Thus the role of LytM in the release of the other CWA proteins of *S. aureus* should be investigated. The fact that LytM targets the site of anchoring of all *S. aureus* CWA proteins (pentaglycine cross-bridge) suggests that LytM does not specifically target SpA for release from *S. aureus*, or that direct SpA release by LytM is minimal.

Recently it has been shown that LytM is involved in the rapid re-shaping of *S. aureus* following the separation of two daughter cells during cell division (Monteiro *et al.*, 2015). Using super-resolution microscopy the authors determined that *S. aureus* cells do not remain spherical throughout the cell cycle but rather elongate during peptidoglycan synthesis and remodelling. Remodelling of the flat division septum to generate spherical daughter cells was shown to be extremely rapid, occurring in less than 2 ms, and driven by an energetic

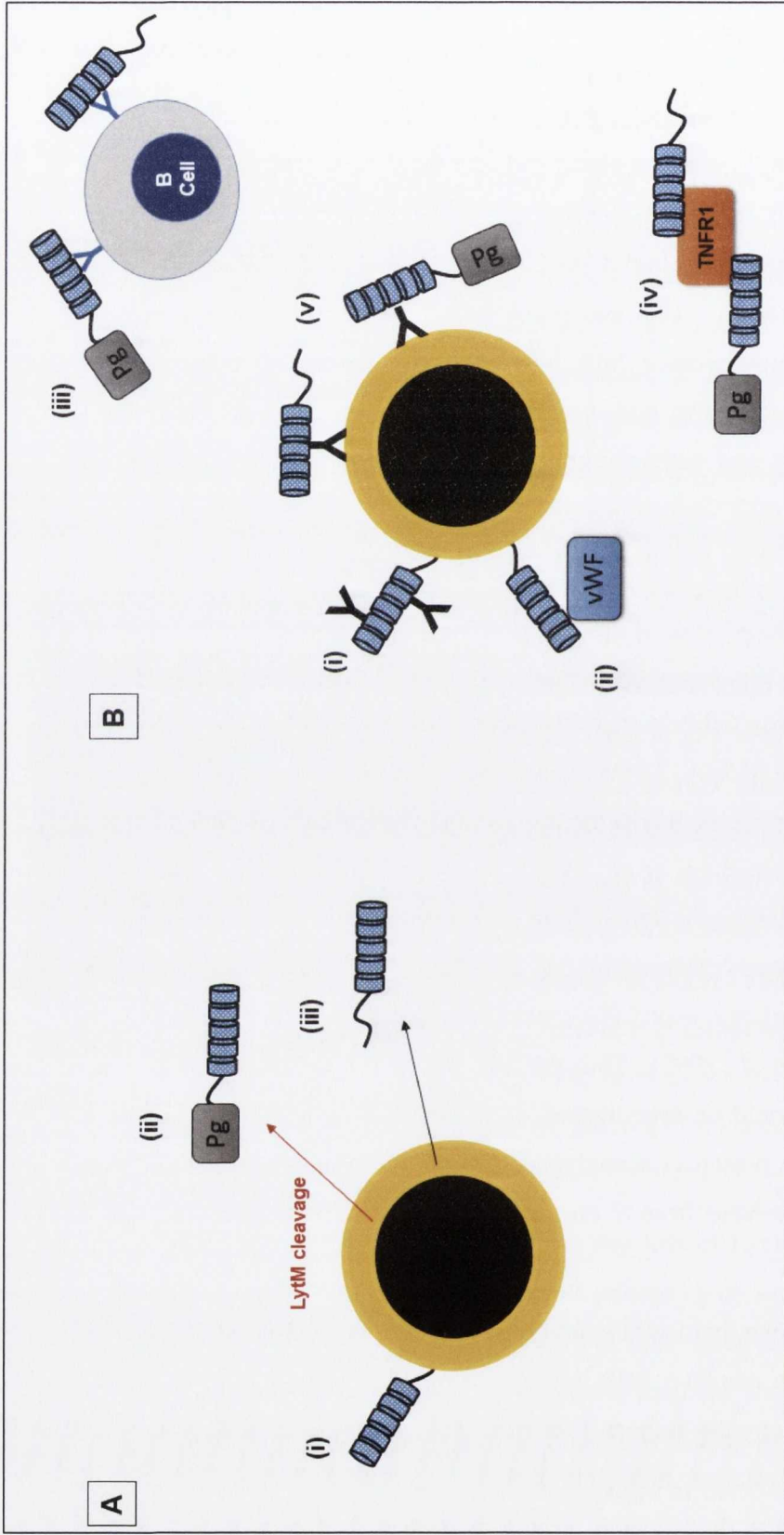


Fig. 7.1 The different forms of protein A and their roles during *S. aureus* infection. (A) Protein A exists either covalently anchored to the cell wall of *S. aureus* (i), released into the extracellular medium linked to a peptidoglycan fragment (Pg) following cleavage of the pentaglycine crossbridge by LytM (ii), or released with the C-terminal sorting signal unprocessed by sortase A (iii). (B) Cell wall-associated protein A sequesters IgG at the bacterial surface by non-specific recognition of the Fc region (i). Cell wall-associated protein A also binds to von Willebrand factor (vWF; ii). Released protein A binds to the Fab region of human IgM expressed on the surface of human B cells (iii), binds to tumour necrosis factor receptor 1 (TNFR1) on the surface of human lung epithelial cells (iv), and can bind to the Fc region of *S. aureus*-specific IgG bound to the surface of the bacterial cell (v).

“popping” process that relies on internal turgor pressure and the relief of CW elastic tension by peptidoglycan hydrolases, such as Sle1 and LytM (Monteiro *et al.*, 2015). LytM-deficient mutants of *S. aureus* displayed defects in septum remodelling with many cells failing to become spherical following daughter cell separation (Monteiro *et al.*, 2015). A number of CWA proteins, including SpA, possess a YSIRK/GS-containing N-terminal signal sequence which directs internal trafficking of proteins to the septum of growing cells (DeDent *et al.*, 2008). Covalent anchoring of SpA to cell wall peptidoglycan occurs at this location and CWA SpA is then spread across the cell surface in a ring-like structure by *de novo* peptidoglycan synthesis (DeDent *et al.*, 2007). Perhaps the reduction in SpA release measured in LytM-deficient mutants of *S. aureus* is an indirect consequence of the loss of LytM peptidoglycan-hydrolysing activity in the cell. The suboptimal sorting signal of SpA could facilitate the propulsion, or leakage, of unsorted SpA from the bacterial membrane during rapid septum remodelling, whereas CWA proteins with more optimal sorting signals are retained. In this way a LytM-deficient mutant of *S. aureus* could generate less released SpA due to the impairment in septum remodelling reported for these strains (Monteiro *et al.*, 2015). Based on this hypothesis, the localisation of SpA at the septum of dividing cells is an essential step in its release and it would be interesting to investigate if replacing the signal sequence of SpA with a non-YSIRK/GS signal sequence so that is not deposited at the septum (DeDent *et al.*, 2008) would alter the release of SpA.

An interesting feature of SpA that makes it unique among the CWA proteins of *S. aureus* is the presence of a lysin motif (LysM) domain in close proximity to the C-terminal sorting signal (Bateman & Bycroft, 2000). Many LysM domains facilitate non-covalent association with peptidoglycan (Buist *et al.*, 2008), and a number of LysM-containing proteins are peptidoglycan hydrolases (Kajimura *et al.*, 2005), e.g. Sle1, a murein hydrolase of *S. aureus*, binds to peptidoglycan in the septum of dividing cells via its LysM domain (Frankel & Schneewind, 2012). As no other CWA protein of *S. aureus* appears to require this domain for efficient sorting it is possible that it contributes to SpA release, perhaps by positioning the protein to a certain region of the septum or co-localising it with another LysM domain containing protein such as an autolysin. However the LysM domain of SpA has not been assessed for functionality and was identified based on sequence similarity to the 43 amino acid LysM domain sequence found in the *E. coli* lytic proteins LysA and LysB (Birkeland, 1994). Thus, more extensive study of this domain must be undertaken before a role in SpA anchoring, function or release can be considered.

It has been shown that incubation of *S. aureus* with the host chemokines CXCL9 and CXCL10 induces the post-translational release of SpA in the absence of *de novo* protein synthesis (Yung *et al.*, 2011), however the authors failed to identify the mechanism of SpA release. CXCL9 bound specifically to the bacterial cytoplasmic membrane while CXCL10 bound to both the membrane and CW (Yung *et al.*, 2011). Both CXCL9 and CXCL10 are known to possess antimicrobial activity (Yung & Murphy, 2012) and CXCL9 in particular has been shown to inhibit *in vitro* growth of *S. aureus* (Yung *et al.*, 2011). In accordance with this Yung and colleagues used sub-inhibitory concentrations of purified chemokines when assessing effects on SpA release. Other chemokines, such as CXCL6, have been shown to possess membrane-disrupting properties (Linge *et al.*, 2008), and CXCL6, although not evaluated to the same extent, was also reported to stimulate SpA release (Yung *et al.*, 2011). Therefore it is possible that the interaction of CXCL9 and CXCL10 with the bacterial membrane can displace membrane-bound SpA into the extracellular medium, or interfere with the activity of the membrane-bound sortases. This possibility was not addressed as the authors failed to examine the effects of incubating *S. aureus* with purified chemokines on the surface-display or release of other CWA surface proteins. Therefore, whereas release of SpA from *S. aureus* following detection of host chemokines could represent a novel mechanism of SpA release *in vivo* further work is necessary to validate this claim.

The work described in this thesis shows that that the contribution of LytM and properties of the C-terminal SS account for the majority of SpA release from LAC, however complete inhibition of SpA release was not achieved. So what might account for the remainder of released SpA detected in LAC? Perhaps, as suggested by Becker *et al.*, (2014), additional autolytic enzymes or peptidases exist in *S. aureus* that directly, or indirectly, influence the release of CWA proteins. Additionally, here we describe the SS of SpA as suboptimal in comparison to the SdrE SS, however it is possible that the SdrE SS itself is not completely effective in inhibiting the release of unsorted SpA.

Prior to this study a role for released SpA in *S. aureus* infection had not specifically been investigated but a number of studies, through use of purified recombinant SpA, have indicated roles for soluble SpA in the promotion of inflammation and modulation of the adaptive immune response. SpA is a potent activator of tumour necrosis factor α (TNF α) receptor 1 (TNFR1) signalling (Classen *et al.*, 2011; Gomez *et al.*, 2004). The interaction of SpA with TNFR1 has been demonstrated using purified recombinant SpA variants incubated with human keratinocytes (Classen *et al.*, 2011) and lung airway epithelial cells (Gomez *et*

al., 2006). The latter interaction is essential in the pathogenesis of *S. aureus*-mediated pneumonia (Gomez *et al.*, 2004). SpA also triggers inflammation via induction of type I interferon (IFN) signalling in airway epithelial cells, in particular increasing IFN- β transcription (Martin *et al.*, 2009). Purified recombinant SpA was endocytosed by airway epithelial cells and subsequently induced IFN-beta expression, JAK-STAT signalling, and IL-6 production (Martin *et al.*, 2009). Thus released SpA may contribute to the advancement of *S. aureus* infection *in vivo* by altering the host inflammatory response.

CWA SpA protects *S. aureus* from phagocytic killing by human neutrophils (Gemmell, 1991; Smith *et al.*, 2011) and work described in this thesis has shown, for the first time, that released SpA also contributes to evasion of phagocytosis and growth of *S. aureus* in human blood. The development of a successful *S. aureus* vaccine has been hindered by the fact that *S. aureus* infections do not generate protective immunity in humans or animals (Kim *et al.*, 2012a). SpA binds to the V_H3-Fab region of human B cells (Sasano *et al.*, 1993; Sasso *et al.*, 1989) which results in their activation, proliferation and subsequent apoptotic destruction (Goodyear & Silverman, 2003). It has been shown that purified recombinant SpA promotes crosslinking of IgM from humans and guinea pigs via high V_H3 binding activity (Kim *et al.*, 2015). This leads to significant depletion of potential antibody-secreting cells from the spleen and bone marrow (Goodyear & Silverman, 2003) and consequently dampens the adaptive immune response to *S. aureus* infection (Falugi *et al.*, 2013). Thus, it is likely that as well as protecting bacteria from opsonophagocytosis by human neutrophils, released SpA significantly inhibits the development of an immune memory response to *S. aureus* infection. Immunisation of mice and guinea pigs with a variant of recombinant SpA possessing amino acid substitutions in each of the IgG-binding domains that abrogate binding of SpA to IgG and IgM (SpA_{KKAA}) facilitated the production of SpA-specific neutralising antibodies which enabled the development of protective immunity against subsequent lethal MRSA challenge (Kim *et al.*, 2010; Kim *et al.*, 2015). The addition of recombinant full-length SpA_{KKAA} to a trivalent vaccine consisting of the CWA proteins ClfA, FnBPB and SdrD significantly increased the survival and delayed the time to death of mice in a *S. aureus* sepsis model in comparison to the trivalent vaccine alone (Kim *et al.*, 2011). Kim *et al.*, (2012b) have shown by ELISA that the SpA-specific monoclonal antibodies generated against full-length SpA_{KKAA}, that provide the highest level of protection against staphylococcal disease in mice, bound to each IgG-binding domain but not to peptides possessing only one or two helices of the triple-helical bundle. This indicates that antibodies

generated against SpA_{KKAA} are directed against highly conserved conformational epitopes of the helical bundle for each IgG-binding domain, suggesting that these antibodies could provide broad protection against *S. aureus* infection by genetically-distinct isolates. Based on the studies to date the development of an Ig-binding deficient variant of SpA appears to be a promising step in the development of a successful *S. aureus* vaccine. It seems likely that this vaccination strategy will provide universal protection against all strains of *S. aureus*, however, this approach should first be validated using a variety of strains corresponding to the current dominant clones of *S. aureus* worldwide.

SasY is a novel CWA protein of *S. aureus* that was identified by whole genome sequencing on a plasmid carried by a newly described sequence type (ST) of *S. aureus*, ST2371, during an MRSA outbreak in a healthcare facility in the UK in 2011 (Harris *et al.*, 2013). Similar to SpA, SasY is a relatively small (~50 kDa) protein that is expressed throughout staphylococcal growth *in vitro*. SasY possess a central “ α C” domain that has predicted sequence similarity to the CWA alpha C protein (ACP) (Lachenauer *et al.*, 2000) of Group B Streptococci (GBS). SasY is not the only CWA protein of *S. aureus* that is encoded on a mobile genetic element (MGE). The plasmin-sensitive (Pls) protein is encoded on both SCCmec type I and IV elements (Ito *et al.*, 2001; Savolainen *et al.*, 2001; Werbick *et al.*, 2007), and SasX is encoded on a lysogenic prophage (Holden *et al.*, 2010) (Li *et al.*, 2012). SasX possess high amino acid sequence similarity to the phage-encoded SesI protein from *S. epidermidis* (Li *et al.*, 2012) and it is possible that the *sasX* gene was acquired by ST239 MRSA strains by horizontal gene transfer between these two species. Perhaps SasY was acquired by a similar HGT event.

A recent study using whole genome sequencing of surveillance of MRSA at diagnostic laboratories at Cambridge University Hospitals has shown that ST2371 has persisted and spread in the community (Toleman, Reuter, Coll, Harrison and Peacock, manuscript in preparation). Furthermore, the SasY-encoding plasmid has been identified in CC22 strains causing infection in the hospital and community in Scotland and England (Coll, Peacock and Holden, personal communication). Work outlined in this thesis has shown that SasY, similar to SpA, promotes the growth of *S. aureus* in human blood and interferes with efficient phagocytic uptake by purified human neutrophils. Thus expression of SasY may contribute to the ability of *S. aureus* to establish infection and transmit in the hospital and community. The mechanism of host immune evasion has yet to be elucidated. One

possibility to investigate is if SasY can bind to components of serum or to receptors on the surface of neutrophils to interfere with phagocytosis.

The traditional animal model of choice for study of bacterial infections is the mouse, however *S. aureus* is a largely human-adapted pathogen, and a number of studies have been hampered by the selection of inappropriate animal infection models. For example, historically much controversy surrounded the role of PVL in *S. aureus* infection due to the fact that PVL does not recognise the C5a receptor on the surface of murine neutrophils (Loffler *et al.*, 2010; Spaan *et al.*, 2013a). In fact almost all of the secreted immune evasion factors of *S. aureus* are human specific. SpA is best known for its ability to bind to human IgG and IgM to subvert the innate and adaptive immune response (Kim *et al.*, 2015), however SpA does not bind to all subtypes of murine IgG and displays weak affinity towards murine IgM (Kim *et al.*, 2015). Therefore future studies must first focus on identifying the host factor targeted by SasY so that an appropriate animal model for studying the contribution of SasY to infection can be developed. ST2371, like ST239, in the majority of cases causes severe skin and soft tissue infections (Harris *et al.*, 2013; Rajan *et al.*, 2015), and SasX is associated with increased severity of ST239-mediated skin and lung infections (Li *et al.*, 2012). Further work could focus on establishing the contribution of SasY to *S. aureus* skin colonisation and infection using three dimensional organotypic human skin culture models that allow the study of *S. aureus* infection (Popov *et al.*, 2014).

Recently SasX has been assessed as a target for therapeutic and vaccine strategies against *S. aureus* infection. Anti-SasX IgG promoted opsonophagocytosis of *S. aureus* by human neutrophils, and active and passive immunisation of mice reduced *S. aureus* disease severity (Liu *et al.*, 2015). Further study is necessary to determine the role of SasY in different *S. aureus* infections. If the prevalence of ST2371 and SasY-positive CC22 continues to increase, this protein may represent an attractive therapeutic and vaccine target for the prevention of SSTIs and other types of infection in the hospital and community.

References

- Abraham NM, Jefferson KK. 2012. *Staphylococcus aureus* clumping factor B mediates biofilm formation in the absence of calcium. *Microbiology* **158**:1504-1512.
- Aggarwal BB. 2003. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* **3**:745-756.
- Akerstrom B, Brodin T, Reis K, Bjorck L. 1985. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. *J Immunol* **135**:2589-2592.
- Akerstrom B, Lindqvist A, Lindahl G. 1991. Binding properties of protein A, a bacterial IgA-receptor. *Mol Immunol* **28**:349-357.
- Alonzo F, 3rd, Benson MA, Chen J, Novick RP, Shopsin B, Torres VJ. 2012. *Staphylococcus aureus* leucocidin ED contributes to systemic infection by targeting neutrophils and promoting bacterial growth in vivo. *Mol Microbiol* **83**:423-435.
- Alonzo F, 3rd, Kozhaya L, Rawlings SA, Reyes-Robles T, DuMont AL, Myszkowski DG, Landau NR, Unutmaz D, Torres VJ. 2013. CCR5 is a receptor for *Staphylococcus aureus* leukotoxin ED. *Nature* **493**:51-55.
- Alonzo F, 3rd, Torres VJ. 2014. The bicomponent pore-forming leucocidins of *Staphylococcus aureus*. *Microbiol Mol Biol Rev* **78**:199-230.
- Amend A, Chhatwal GS, Schaeg W, Blobel H. 1984. Characterization of immunoglobulin G binding to *Staphylococcus aureus* strain Wood 46. *Zentralbl Bakteriol Mikrobiol Hyg A* **258**:472-479.
- Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. 2012. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol* **30**:459-489.
- Anderson DC, Springer TA. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu Rev Med* **38**:175-194.
- Anguera I, Del Rio A, Miro JM, Martinez-Lacasa X, Marco F, Guma JR, Quaglio G, Claramonte X, Moreno A, Mestres CA, Mauri E, Azqueta M, Benito N, Garcia-de la Maria C, Almela M, Jimenez-Exposito MJ, Sued O, De Lazzari E, Gatell JM, Hospital Clinic Endocarditis Study G. 2005. *Staphylococcus lugdunensis* infective endocarditis: description of 10 cases and analysis of native valve, prosthetic valve, and pacemaker lead endocarditis clinical profiles. *Heart* **91**:e10.
- Arcus VL, Langley R, Proft T, Fraser JD, Baker EN. 2002. The Three-dimensional structure of a superantigen-like protein, SET3, from a pathogenicity island of the *Staphylococcus aureus* genome. *J Biol Chem* **277**:32274-32281.
- Atkins KL, Burman JD, Chamberlain ES, Cooper JE, Poutrel B, Bagby S, Jenkins AT, Feil EJ, van den Elsen JM. 2008. *S. aureus* IgG-binding proteins SpA and Sbi: host specificity and mechanisms of immune complex formation. *Mol Immunol* **45**:1600-1611.
- Aziz RK, Kansal R, Aronow BJ, Taylor WL, Rowe SL, Kubal M, Chhatwal GS, Walker MJ, Kotb M. 2010. Microevolution of group A streptococci in vivo: capturing regulatory networks engaged in sociomicrobiology, niche adaptation, and hypervirulence. *PLoS One* **5**:e9798.
- Bae T, Banger AK, Wallace A, Glass EM, Aslund F, Schneewind O, Missiakas DM. 2004. *Staphylococcus aureus* virulence genes identified by *bursa aurealis* mutagenesis and nematode killing. *Proc Natl Acad Sci U S A* **101**:12312-12317.
- Bae T, Schneewind O. 2003. The YSIRK-G/S motif of staphylococcal protein A and its role in efficiency of signal peptide processing. *J Bacteriol* **185**:2910-2919.
- Bae T, Schneewind O. 2006. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* **55**:58-63.
- Bafna V, Edwards N. 2001. SCOPE: a probabilistic model for scoring tandem mass spectra against a peptide database. *Bioinformatics* **17 Suppl 1**:S13-21.

- Bagnoli F, Bertholet S, Grandi G.** 2012. Inferring reasons for the failure of *Staphylococcus aureus* vaccines in clinical trials. *Front Cell Infect Microbiol* **2**:16.
- Bally I, Rossi V, Lunardi T, Thielens NM, Gaboriaud C, Arlaud GJ.** 2009. Identification of the C1q-binding Sites of Human C1r and C1s: a refined three-dimensional model of the C1 complex of complement. *J Biol Chem* **284**:19340-19348.
- Barbu EM, Mackenzie C, Foster TJ, Hook M.** 2014. SdrC induces staphylococcal biofilm formation through a homophilic interaction. *Mol Microbiol* **94**:172-185.
- Bateman A, Bycroft M.** 2000. The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). *J Mol Biol* **299**:1113-1119.
- Bateman BT, Donegan NP, Jarry TM, Palma M, Cheung AL.** 2001. Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus* in vitro and in vivo and its application in demonstrating the role of *sigB* in microcolony formation. *Infect Immun* **69**:7851-7857.
- Baum C, Haslinger-Löffler B, Westh H, Boye K, Peters G, Neumann C, Kahl BC.** 2009. Non-*spa*-typeable clinical *Staphylococcus aureus* strains are naturally occurring protein A mutants. *J Clin Microbiol* **47**:3624-3629.
- Baur S, Rautenberg M, Faulstich M, Grau T, Severin Y, Unger C, Hoffmann WH, Rudel T, Autenrieth IB, Weidenmaier C.** 2014. A nasal epithelial receptor for *Staphylococcus aureus* WTA governs adhesion to epithelial cells and modulates nasal colonization. *PLoS Pathog* **10**:e1004089.
- Becker K, Friedrich AW, Lubritz M, Weilert G, Peters G, von Eiff C.** 2011. *Staphylococcus*, *Micrococcus*, and other catalase-positive cocci. *Manual of Clinical Microbiology*, American Society for Microbiology Washington, DC **1**:308-330.
- Becker S, Frankel MB, Schneewind O, Missiakas D.** 2014. Release of protein A from the cell wall of *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **111**:1574-1579.
- Bera A, Herbert S, Jakob A, Vollmer W, Gotz F.** 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. *Mol Microbiol* **55**:778-787.
- Berends ET, Horswill AR, Haste NM, Monestier M, Nizet V, von Kockritz-Blickwede M.** 2010. Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J Innate Immun* **2**:576-586.
- Bestebroer J, Aerts PC, Rooijackers SH, Pandey MK, Kohl J, van Strijp JA, de Haas CJ.** 2010. Functional basis for complement evasion by staphylococcal superantigen-like 7. *Cell Microbiol* **12**:1506-1516.
- Bestebroer J, Poppelier MJ, Ulfman LH, Lenting PJ, Denis CV, van Kessel KP, van Strijp JA, de Haas CJ.** 2007. Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. *Blood* **109**:2936-2943.
- Bhakdi S, Trantum-Jensen J.** 1991. Alpha-toxin of *Staphylococcus aureus*. *Microbiol Rev* **55**:733-751.
- Birkeland NK.** 1994. Cloning, molecular characterization, and expression of the genes encoding the lytic functions of lactococcal bacteriophage phi LC3: a dual lysis system of modular design. *Can J Microbiol* **40**:658-665.
- Biswas I, Gruss A, Ehrlich SD, Maguin E.** 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. *J Bacteriol* **175**:3628-3635.
- Bjorck L.** 1988. Protein L. A novel bacterial cell wall protein with affinity for Ig L chains. *J Immunol* **140**:1194-1197.
- Bjorck L, Kronvall G.** 1984. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J Immunol* **133**:969-974.

- Bloes DA, Kretschmer D, Peschel A.** 2015. Enemy attraction: bacterial agonists for leukocyte chemotaxis receptors. *Nat Rev Microbiol* **13**:95-104.
- Bochtler M, Odintsov SG, Marcyjaniak M, Sabala I.** 2004. Similar active sites in lysostaphins and D-Ala-D-Ala metallopeptidases. *Protein Sci* **13**:854-861.
- Bogomolski-Yahalom V, Matzner Y.** 1995. Disorders of neutrophil function. *Blood Rev* **9**:183-190.
- Bolduc GR, Baron MJ, Gravekamp C, Lachenauer CS, Madoff LC.** 2002. The alpha C protein mediates internalization of group B *Streptococcus* within human cervical epithelial cells. *Cell Microbiol* **4**:751-758.
- Bolduc GR, Madoff LC.** 2007. The group B streptococcal alpha C protein binds alpha1beta1-integrin through a novel KTD motif that promotes internalization of GBS within human epithelial cells. *Microbiology* **153**:4039-4049.
- Boles BR, Thoendel M, Roth AJ, Horswill AR.** 2010. Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation. *PLoS One* **5**:e10146.
- Borregaard N, Cowland JB.** 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**:3503-3521.
- Boyle-Vavra S, Li X, Alam MT, Read TD, Sieth J, Cywes-Bentley C, Dobbins G, David MZ, Kumar N, Eells SJ, Miller LG, Boxrud DJ, Chambers HF, Lynfield R, Lee JC, Daum RS.** 2015. USA300 and USA500 clonal lineages of *Staphylococcus aureus* do not produce a capsular polysaccharide due to conserved mutations in the *cap5* locus. *MBio* **6**.
- Boyle MD, Hawlitzky J, Raeder R, Podbielski A.** 1994. Analysis of genes encoding two unique type IIa immunoglobulin G-binding proteins expressed by a single group A streptococcal isolate. *Infect Immun* **62**:1336-1347.
- Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A.** 2004. Neutrophil extracellular traps kill bacteria. *Science* **303**:1532-1535.
- Brouillette E, Grondin G, Shkreta L, Lacasse P, Talbot BG.** 2003. *In vivo* and *in vitro* demonstration that *Staphylococcus aureus* is an intracellular pathogen in the presence or absence of fibronectin-binding proteins. *Microb Pathog* **35**:159-168.
- Brown S, Xia G, Luhachack LG, Campbell J, Meredith TC, Chen C, Winstel V, Gekeler C, Irazoqui JE, Peschel A, Walker S.** 2012. Methicillin resistance in *Staphylococcus aureus* requires glycosylated wall teichoic acids. *Proc Natl Acad Sci U S A* **109**:18909-18914.
- Bruckner R.** 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. *FEMS Microbiol Lett* **151**:1-8.
- Buist G, Steen A, Kok J, Kuipers OP.** 2008. LysM, a widely distributed protein motif for binding to (peptido)glycans. *Mol Microbiol* **68**:838-847.
- Cameron DR, Howden BP, Peleg AY.** 2011. The interface between antibiotic resistance and virulence in *Staphylococcus aureus* and its impact upon clinical outcomes. *Clin Infect Dis* **53**:576-582.
- Carlsson F, Stalhammar-Carlemalm M, Flardh K, Sandin C, Carlemalm E, Lindahl G.** 2006. Signal sequence directs localized secretion of bacterial surface proteins. *Nature* **442**:943-946.
- Carnes EC, Lopez DM, Donegan NP, Cheung A, Gresham H, Timmins GS, Brinker CJ.** 2010. Confinement-induced quorum sensing of individual *Staphylococcus aureus* bacteria. *Nat Chem Biol* **6**:41-45.
- Carroll MC.** 2004. The complement system in regulation of adaptive immunity. *Nat Immunol* **5**:981-986.
- Cassat JE, Hammer ND, Campbell JP, Benson MA, Perrien DS, Mrak LN, Smeltzer MS, Torres VJ, Skaar EP.** 2013. A secreted bacterial protease tailors the *Staphylococcus aureus* virulence repertoire to modulate bone remodeling during osteomyelitis. *Cell Host Microbe* **13**:759-772.

- Cedergren L, Andersson R, Jansson B, Uhlen M, Nilsson B.** 1993. Mutational analysis of the interaction between staphylococcal protein A and human IgG1. *Protein Eng* **6**:441-448.
- Chambers HF, Deleo FR.** 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol* **7**:629-641.
- Chan PF, Foster SJ.** 1998. Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*. *J Bacteriol* **180**:6232-6241.
- Chavakis T, Hussain M, Kanse SM, Peters G, Bretzel RG, Flock JI, Herrmann M, Preissner KT.** 2002. *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat Med* **8**:687-693.
- Chen G, Goeddel DV.** 2002. TNF-R1 signaling: a beautiful pathway. *Science* **296**:1634-1635.
- Cheung GY, Duong AC, Otto M.** 2012. Direct and synergistic hemolysis caused by *Staphylococcus* phenol-soluble modulins: implications for diagnosis and pathogenesis. *Microbes Infect* **14**:380-386.
- Chua KY, Monk IR, Lin YH, Seemann T, Tuck KL, Porter JL, Stepnell J, Coombs GW, Davies JK, Stinear TP, Howden BP.** 2014. Hyperexpression of alpha-hemolysin explains enhanced virulence of sequence type 93 community-associated methicillin-resistant *Staphylococcus aureus*. *BMC Microbiol* **14**:31.
- Chung MC, Wines BD, Baker H, Langley RJ, Baker EN, Fraser JD.** 2007. The crystal structure of staphylococcal superantigen-like protein 11 in complex with sialyl Lewis X reveals the mechanism for cell binding and immune inhibition. *Mol Microbiol* **66**:1342-1355.
- Chunhua M, Yu L, Yaping G, Jie D, Qiang L, Xiaorong T, Guang Y.** 2012. The expression of LytM is down-regulated by RNAIII in *Staphylococcus aureus*. *J Basic Microbiol* **52**:636-641.
- Clancy KW, Melvin JA, McCafferty DG.** 2010. Sortase transpeptidases: insights into mechanism, substrate specificity, and inhibition. *Biopolymers* **94**:385-396.
- Clarke SR, Brummell KJ, Horsburgh MJ, McDowell PW, Mohamad SA, Stapleton MR, Acevedo J, Read RC, Day NP, Peacock SJ, Mond JJ, Kokai-Kun JF, Foster SJ.** 2006. Identification of *in vivo*-expressed antigens of *Staphylococcus aureus* and their use in vaccinations for protection against nasal carriage. *J Infect Dis* **193**:1098-1108.
- Clarke SR, Foster SJ.** 2008. IsdA protects *Staphylococcus aureus* against the bactericidal protease activity of apolactoferrin. *Infect Immun* **76**:1518-1526.
- Claro T, Widaa A, McDonnell C, Foster TJ, O'Brien FJ, Kerrigan SW.** 2013. *Staphylococcus aureus* protein A binding to osteoblast tumour necrosis factor receptor 1 results in activation of nuclear factor kappa B and release of interleukin-6 in bone infection. *Microbiology* **159**:147-154.
- Claro T, Widaa A, O'Seaghdha M, Miajlovic H, Foster TJ, O'Brien FJ, Kerrigan SW.** 2011. *Staphylococcus aureus* Protein A Binds to Osteoblasts and Triggers Signals That Weaken Bone in Osteomyelitis. *PLoS ONE* **6**:e18748.
- Classen A, Kalali BN, Schnopp C, Andres C, Aguilar-Pimentel JA, Ring J, Ollert M, Mempel M.** 2011. TNF receptor I on human keratinocytes is a binding partner for staphylococcal protein A resulting in the activation of NF kappa B, AP-1, and downstream gene transcription. *Exp Dermatol* **20**:48-52.
- Clement S, Vaudaux P, Francois P, Schrenzel J, Huggler E, Kampf S, Chaponnier C, Lew D, Lacroix JS.** 2005. Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent *Staphylococcus aureus* rhinosinusitis. *J Infect Dis* **192**:1023-1028.
- Cole AM, Tahk S, Oren A, Yoshioka D, Kim YH, Park A, Ganz T.** 2001. Determinants of *Staphylococcus aureus* nasal carriage. *Clin Diagn Lab Immunol* **8**:1064-1069.
- Coley J, Archibald AR, Baddiley J.** 1976. A linkage unit joining peptidoglycan to teichoic acid in *Staphylococcus aureus* H. *FEBS Lett* **61**:240-242.

- Collen D.** 1998. Staphylokinase: a potent, uniquely fibrin-selective thrombolytic agent. *Nat Med* **4**:279-284.
- Collins J, Rudkin J, Recker M, Pozzi C, O'Gara JP, Massey RC.** 2010. Offsetting virulence and antibiotic resistance costs by MRSA. *ISME J* **4**:577-584.
- Collins LV, Kristian SA, Weidenmaier C, Faigle M, Van Kessel KP, Van Strijp JA, Gotz F, Neumeister B, Peschel A.** 2002. *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *J Infect Dis* **186**:214-219.
- Conrady DG, Wilson JJ, Herr AB.** 2013. Structural basis for Zn²⁺-dependent intercellular adhesion in staphylococcal biofilms. *Proc Natl Acad Sci U S A* **110**:E202-211.
- Correa PA, Gomez LM, Anaya JM.** 2004. [Polymorphism of TNF-alpha in autoimmunity and tuberculosis]. *Biomedica* **24 Supp 1**:43-51.
- Corrigan RM, Abbott JC, Burhenne H, Kaeffer V, Grundling A.** 2011. c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS Pathog* **7**:e1002217.
- Corrigan RM, Foster TJ.** 2009. An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. *Plasmid* **61**:126-129.
- Corrigan RM, Miajlovic H, Foster TJ.** 2009. Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. *BMC Microbiol* **9**:22.
- Corrigan RM, Rigby D, Handley P, Foster TJ.** 2007. The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. *Microbiology* **153**:2435-2446.
- Corvaglia AR, Francois P, Hernandez D, Perron K, Linder P, Schrenzel J.** 2010. A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical *Staphylococcus aureus* strains. *Proc Natl Acad Sci U S A* **107**:11954-11958.
- Clegg KM, Wilding I, Black MT.** 1996. Molecular cloning and expression of the *spsB* gene encoding an essential type I signal peptidase from *Staphylococcus aureus*. *J Bacteriol* **178**:5712-5718.
- Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR.** 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol* **183**:2888-2896.
- David MZ, Daum RS.** 2010. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. *Clin Microbiol Rev* **23**:616-687.
- DeDent A, Bae T, Missiakas DM, Schneewind O.** 2008. Signal peptides direct surface proteins to two distinct envelope locations of *Staphylococcus aureus*. *EMBO J* **27**:2656-2668.
- DeDent AC, McAdow M, Schneewind O.** 2007. Distribution of protein A on the surface of *Staphylococcus aureus*. *J Bacteriol* **189**:4473-4484.
- Deis LN, Wu Q, Wang Y, Qi Y, Daniels KG, Zhou P, Oas TG.** 2015. Suppression of conformational heterogeneity at a protein-protein interface. *Proc Natl Acad Sci U S A* **112**:9028-9033.
- Deisenhofer J.** 1981. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-A resolution. *Biochemistry* **20**:2361-2370.
- Deivanayagam CC, Rich RL, Carson M, Owens RT, Danthuluri S, Bice T, Hook M, Narayana SV.** 2000. Novel fold and assembly of the repetitive B region of the *Staphylococcus aureus* collagen-binding surface protein. *Structure* **8**:67-78.
- Deivanayagam CC, Wann ER, Chen W, Carson M, Rajashankar KR, Hook M, Narayana SV.** 2002. A novel variant of the immunoglobulin fold in surface adhesins of *Staphylococcus*

- aureus*: crystal structure of the fibrinogen-binding MSCRAMM, clumping factor A. *EMBO J* **21**:6660-6672.
- Deshaies RJ, Schekman R.** 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J Cell Biol* **105**:633-645.
- Diep B, Gill S, Chang R, Phan T, Chen J, Davidson M, Lin F, Lin J, Carleton H, Mongodin E, Sensabaugh G, Perdreau-Remington F.** 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* **367**:731-739.
- Diep BA, Chan L, Tattevin P, Kajikawa O, Martin TR, Basuino L, Mai TT, Marbach H, Braughton KR, Whitney AR, Gardner DJ, Fan X, Tseng CW, Liu GY, Badiou C, Etienne J, Lina G, Matthay MA, DeLeo FR, Chambers HF.** 2010. Polymorphonuclear leukocytes mediate *Staphylococcus aureus* Panton-Valentine leukocidin-induced lung inflammation and injury. *Proc Natl Acad Sci U S A* **107**:5587-5592.
- Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-Remington F.** 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* **367**:731-739.
- Diep BA, Otto M.** 2008. The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol* **16**:361-369.
- Diep BA, Stone GG, Basuino L, Graber CJ, Miller A, des Etages SA, Jones A, Palazzolo-Ballance AM, Perdreau-Remington F, Sensabaugh GF, DeLeo FR, Chambers HF.** 2008. The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* **197**:1523-1530.
- Drapeau GR.** 1978. Role of metalloprotease in activation of the precursor of staphylococcal protease. *J Bacteriol* **136**:607-613.
- Dryla A, Prustomersky S, Gelbmann D, Hanner M, Bettinger E, Kocsis B, Kustos T, Henics T, Meinke A, Nagy E.** 2005. Comparison of antibody repertoires against *Staphylococcus aureus* in healthy individuals and in acutely infected patients. *Clin Diagn Lab Immunol* **12**:387-398.
- Dumont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, Kreiswirth BN, Shopsin B, Unutmaz D, Voyich JM, Torres VJ.** 2011. Characterization of a new cytotoxin that contributes to *Staphylococcus aureus* pathogenesis. *Mol Microbiol* **79**:814-825.
- DuMont AL, Yoong P, Day CJ, Alonzo F, 3rd, McDonald WH, Jennings MP, Torres VJ.** 2013. *Staphylococcus aureus* LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc Natl Acad Sci U S A* **110**:10794-10799.
- DuMont AL, Yoong P, Liu X, Day CJ, Chumblor NM, James DB, Alonzo F, 3rd, Bode NJ, Lacy DB, Jennings MP, Torres VJ.** 2014. Identification of a crucial residue required for *Staphylococcus aureus* LukAB cytotoxicity and receptor recognition. *Infect Immun* **82**:1268-1276.
- DuMont AL, Yoong P, Surewaard BG, Benson MA, Nijland R, van Strijp JA, Torres VJ.** 2013. *Staphylococcus aureus* elaborates leukocidin AB to mediate escape from within human neutrophils. *Infect Immun* **81**:1830-1841.
- Duncan AR, Winter G.** 1988. The binding site for C1q on IgG. *Nature* **332**:738-740.
- Duong F, Wickner W.** 1997. The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. *EMBO J* **16**:4871-4879.
- Dziewanowska K, Patti JM, Deobald CF, Bayles KW, Trumble WR, Bohach GA.** 1999. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect Immun* **67**:4673-4678.

- Edwards AM, Potts JR, Josefsson E, Massey RC. 2010. *Staphylococcus aureus* host cell invasion and virulence in sepsis is facilitated by the multiple repeats within FnBPA. PLoS Pathog 6:e1000964.
- Ellington MJ, Hope R, Livermore DM, Kearns AM, Henderson K, Cookson BD, Pearson A, Johnson AP. 2010. Decline of EMRSA-16 amongst methicillin-resistant *Staphylococcus aureus* causing bacteraemias in the UK between 2001 and 2007. J Antimicrob Chemother 65:446-448.
- Emr SD, Hanley-Way S, Silhavy TJ. 1981. Suppressor mutations that restore export of a protein with a defective signal sequence. Cell 23:79-88.
- Falugi F, Kim HK, Missiakas DM, Schneewind O. 2013. Role of protein A in the evasion of host adaptive immune responses by *Staphylococcus aureus*. MBio 4:e00575-00513.
- Fattom A, Fuller S, Propst M, Winston S, Muenz L, He D, Naso R, Horwith G. 2004. Safety and immunogenicity of a booster dose of *Staphylococcus aureus* types 5 and 8 capsular polysaccharide conjugate vaccine (StaphVAX) in hemodialysis patients. Vaccine 23:656-663.
- Fevre C, Bestebroer J, Mebius MM, de Haas CJ, van Strijp JA, Fitzgerald JR, Haas PJ. 2014. *Staphylococcus aureus* proteins SSL6 and SEIX interact with neutrophil receptors as identified using secretome phage display. Cell Microbiol 16:1646-1665.
- Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, Bayles KW. 2013. A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. MBio 4:e00537-00512.
- Fischer W, Mannsfeld T, Hagen G. 1990. On the basic structure of poly(glycerophosphate) lipoteichoic acids. Biochem Cell Biol 68:33-43.
- Flick MJ, Du X, Prasad JM, Raghu H, Palumbo JS, Smeds E, Hook M, Degen JL. 2013. Genetic elimination of the binding motif on fibrinogen for the *S. aureus* virulence factor ClfA improves host survival in septicemia. Blood 121:1783-1794.
- Forsgren A, Nordstrom K. 1974. Protein A from *Staphylococcus aureus*: the biological significance of its reaction with IgG. Ann N Y Acad Sci 236:252-266.
- Forsman H, Christenson K, Bylund J, Dahlgren C. 2012. Receptor-dependent and -independent immunomodulatory effects of phenol-soluble modulins from *Staphylococcus aureus* on human neutrophils are abrogated through peptide inactivation by reactive oxygen species. Infect Immun 80:1987-1995.
- Foster TJ, Geoghegan JA, Ganesh VK, Hook M. 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat Rev Microbiol 12:49-62.
- Foster TJ, Hook M. 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol 6:484-488.
- Frankel MB, Schneewind O. 2012. Determinants of murein hydrolase targeting to cross-wall of *Staphylococcus aureus* peptidoglycan. J Biol Chem 287:10460-10471.
- Frick IM, Akesson P, Cooney J, Sjobring U, Schmidt KH, Gomi H, Hattori S, Tagawa C, Kishimoto F, Bjorck L. 1994. Protein H--a surface protein of *Streptococcus pyogenes* with separate binding sites for IgG and albumin. Mol Microbiol 12:143-151.
- Frick IM, Wikstrom M, Forsen S, Drakenberg T, Gomi H, Sjobring U, Bjorck L. 1992. Convergent evolution among immunoglobulin G-binding bacterial proteins. Proc Natl Acad Sci U S A 89:8532-8536.
- Friedrich R, Panizzi P, Fuentes-Prior P, Richter K, Verhamme I, Anderson PJ, Kawabata S, Huber R, Bode W, Bock PE. 2003. Staphylocoagulase is a prototype for the mechanism of cofactor-induced zymogen activation. Nature 425:535-539.
- Fujita T, Matsushita M, Endo Y. 2004. The lectin-complement pathway--its role in innate immunity and evolution. Immunol Rev 198:185-202.

- Ganesh VK, Barbu EM, Deivanayagam CC, Le B, Anderson AS, Matsuka YV, Lin SL, Foster TJ, Narayana SV, Hook M.** 2011. Structural and biochemical characterization of *Staphylococcus aureus* clumping factor B/ligand interactions. *J Biol Chem* **286**:25963-25972.
- Ganesh VK, Rivera JJ, Smeds E, Ko YP, Bowden MG, Wann ER, Gurusiddappa S, Fitzgerald JR, Hook M.** 2008. A structural model of the *Staphylococcus aureus* ClfA-fibrinogen interaction opens new avenues for the design of anti-staphylococcal therapeutics. *PLoS Pathog* **4**:e1000226.
- Gardel C, Benson S, Hunt J, Michaelis S, Beckwith J.** 1987. *secD*, a new gene involved in protein export in *Escherichia coli*. *J Bacteriol* **169**:1286-1290.
- Gatlin CL, Pieper R, Huang ST, Mongodin E, Gebregeorgis E, Parmar PP, Clark DJ, Alami H, Papazisi L, Fleischmann RD, Gill SR, Peterson SN.** 2006. Proteomic profiling of cell envelope-associated proteins from *Staphylococcus aureus*. *Proteomics* **6**:1530-1549.
- Gemmell CG.** 1991. Antibiotics and expression of microbial virulence factors: implications for host defense. *J Chemother* **3 Suppl 1**:105-111.
- Geoghegan JA, Corrigan RM, Gruszka DT, Speziale P, O'Gara JP, Potts JR, Foster TJ.** 2010. Role of surface protein SasG in biofilm formation by *Staphylococcus aureus*. *J Bacteriol* **192**:5663-5673.
- Geoghegan JA, Monk IR, O'Gara JP, Foster TJ.** 2013. Subdomains N2N3 of fibronectin binding protein A mediate *Staphylococcus aureus* biofilm formation and adherence to fibrinogen using distinct mechanisms. *J Bacteriol* **195**:2675-2683.
- Ghuysen JM, Strominger JL.** 1963. Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. Ii. Separation and Structure of Disaccharides. *Biochemistry* **2**:1119-1125.
- Ghuysen JM, Tipper DJ, Strominger JL.** 1965. Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. Iv. The Teichoic Acid-Glycopeptide Complex. *Biochemistry* **4**:474-485.
- Godehardt AW, Hammerschmidt S, Frank R, Chhatwal GS.** 2004. Binding of alpha2-macroglobulin to GRAB (Protein G-related alpha2-macroglobulin-binding protein), an important virulence factor of group A streptococci, is mediated by two charged motifs in the DeltaA region. *Biochem J* **381**:877-885.
- Gomez MI, Lee A, Reddy B, Muir A, Soong G, Pitt A, Cheung A, Prince A.** 2004. *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat Med* **10**:842-848.
- Gomez MI, O'Seaghdha M, Magargee M, Foster TJ, Prince AS.** 2006. *Staphylococcus aureus* protein A activates TNFR1 signaling through conserved IgG binding domains. *J Biol Chem* **281**:20190-20196.
- Gomez MI, Seaghdha MO, Prince AS.** 2007. *Staphylococcus aureus* protein A activates TACE through EGFR-dependent signaling. *EMBO J* **26**:701-709.
- Gonzalez-Quintela A, Alende R, Gude F, Campos J, Rey J, Meijide LM, Fernandez-Merino C, Vidal C.** 2008. Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clinical and Experimental Immunology* **151**:42-50.
- Goodyear CS, Silverman GJ.** 2003. Death by a B cell superantigen: In vivo VH-targeted apoptotic supraclonal B cell deletion by a Staphylococcal Toxin. *J Exp Med* **197**:1125-1139.
- Gouda H, Torigoe H, Saito A, Sato M, Arata Y, Shimada I.** 1992. Three-dimensional solution structure of the B domain of staphylococcal protein A: comparisons of the solution and crystal structures. *Biochemistry* **31**:9665-9672.
- Graille M, Stura EA, Corper AL, Sutton BJ, Taussig MJ, Charbonnier JB, Silverman GJ.** 2000. Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab

- fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. *Proc Natl Acad Sci U S A* **97**:5399-5404.
- Grigg JC, Ukpabi G, Gaudin CF, Murphy ME.** 2010. Structural biology of heme binding in the *Staphylococcus aureus* Isd system. *J Inorg Biochem* **104**:341-348.
- Grigg JC, Vermeiren CL, Heinrichs DE, Murphy ME.** 2007. Heme coordination by *Staphylococcus aureus* IsdE. *J Biol Chem* **282**:28815-28822.
- Grundmann H, Aanensen DM, van den Wijngaard CC, Spratt BG, Harmsen D, Friedrich AW, European Staphylococcal Reference Laboratory Working G.** 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. *PLoS Med* **7**:e1000215.
- Gruszka DT, Whelan F, Farrance OE, Fung HK, Paci E, Jeffries CM, Svergun DI, Baldock C, Baumann CG, Brockwell DJ, Potts JR, Clarke J.** 2015. Cooperative folding of intrinsically disordered domains drives assembly of a strong elongated protein. *Nat Commun* **6**:7271.
- Gruszka DT, Wojdyla JA, Bingham RJ, Turkenburg JP, Manfield IW, Steward A, Leech AP, Geoghegan JA, Foster TJ, Clarke J, Potts JR.** 2012. Staphylococcal biofilm-forming protein has a contiguous rod-like structure. *Proc Natl Acad Sci U S A* **109**:E1011-1018.
- Guss B, Eliasson M, Olsson A, Uhlen M, Frej AK, Jornvall H, Flock JI, Lindberg M.** 1986. Structure of the IgG-binding regions of streptococcal protein G. *EMBO J* **5**:1567-1575.
- Hair PS, Echague CG, Sholl AM, Watkins JA, Geoghegan JA, Foster TJ, Cunnion KM.** 2010. Clumping factor A interaction with complement factor I increases C3b cleavage on the bacterial surface of *Staphylococcus aureus* and decreases complement-mediated phagocytosis. *Infect Immun* **78**:1717-1727.
- Hair PS, Foley CK, Krishna NK, Nyalwidhe JO, Geoghegan JA, Foster TJ, Cunnion KM.** 2013. Complement regulator C4BP binds to *Staphylococcus aureus* surface proteins SdrE and Bbp inhibiting bacterial opsonization and killing. *Results Immunol* **3**:114-121.
- Hair PS, Ward MD, Semmes OJ, Foster TJ, Cunnion KM.** 2008. *Staphylococcus aureus* clumping factor A binds to complement regulator factor I and increases factor I cleavage of C3b. *J Infect Dis* **198**:125-133.
- Hammel M, Sfyroera G, Ricklin D, Magotti P, Lambris JD, Geisbrecht BV.** 2007. A structural basis for complement inhibition by *Staphylococcus aureus*. *Nat Immunol* **8**:430-437.
- Hansenova Manaskova S, Nazmi K, van Belkum A, Bikker FJ, van Wamel WJ, Veerman EC.** 2014. Synthetic LPETG-containing peptide incorporation in the *Staphylococcus aureus* cell-wall in a sortase A- and growth phase-dependent manner. *PLoS One* **9**:e89260.
- Harris SR, Cartwright EJ, Torok ME, Holden MT, Brown NM, Ogilvy-Stuart AL, Ellington MJ, Quail MA, Bentley SD, Parkhill J, Peacock SJ.** 2013. Whole-genome sequencing for analysis of an outbreak of methicillin-resistant *Staphylococcus aureus*: a descriptive study. *Lancet Infect Dis* **13**:130-136.
- Hartford O, Francois P, Vaudaux P, Foster TJ.** 1997. The dipeptide repeat region of the fibrinogen-binding protein (clumping factor) is required for functional expression of the fibrinogen-binding domain on the *Staphylococcus aureus* cell surface. *Mol Microbiol* **25**:1065-1076.
- Hartleib J, Kohler N, Dickinson RB, Chhatwal GS, Sixma JJ, Hartford OM, Foster TJ, Peters G, Kehrel BE, Herrmann M.** 2000. Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *Blood* **96**:2149-2156.
- Haupt K, Reuter M, van den Elsen J, Burman J, Halbich S, Richter J, Skerka C, Zipfel PF.** 2008. The *Staphylococcus aureus* protein Sbi acts as a complement inhibitor and forms a tripartite complex with host complement Factor H and C3b. *PLoS Pathog* **4**:e1000250.

- Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D, Gotz F.** 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol Microbiol* **20**:1083-1091.
- Herbert S, Ziebandt AK, Ohlsen K, Schafer T, Hecker M, Albrecht D, Novick R, Gotz F.** 2010. Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. *Infect Immun* **78**:2877-2889.
- Herwald H, Cramer H, Morgelin M, Russell W, Sollenberg U, Norrby-Teglund A, Flodgaard H, Lindbom L, Bjorck L.** 2004. M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell* **116**:367-379.
- Higgins J, Loughman A, van Kessel KP, van Strijp JA, Foster TJ.** 2006. Clumping factor A of *Staphylococcus aureus* inhibits phagocytosis by human polymorphonuclear leucocytes. *FEMS Microbiol Lett* **258**:290-296.
- Hill JA, Martin SA, Biller JE, Biemann K.** 1988. Use of a microchannel array detector in a four-sector tandem mass spectrometer. *Biomed Environ Mass Spectrom* **17**:147-151.
- Hillson JL, Karr NS, Oppliger IR, Mannik M, Sasso EH.** 1993. The structural basis of germline-encoded VH3 immunoglobulin binding to staphylococcal protein A. *J Exp Med* **178**:331-336.
- Holden MT, Lindsay JA, Corton C, Quail MA, Cockfield JD, Pathak S, Batra R, Parkhill J, Bentley SD, Edgeworth JD.** 2010. Genome sequence of a recently emerged, highly transmissible, multi-antibiotic- and antiseptic-resistant variant of methicillin-resistant *Staphylococcus aureus*, sequence type 239 (TW). *J Bacteriol* **192**:888-892.
- Hunt DF, Yates JR, 3rd, Shabanowitz J, Winston S, Hauer CR.** 1986. Protein sequencing by tandem mass spectrometry. *Proc Natl Acad Sci U S A* **83**:6233-6237.
- Huntzinger E, Boisset S, Saveanu C, Benito Y, Geissmann T, Namane A, Lina G, Etienne J, Ehresmann B, Ehresmann C, Jacquier A, Vandenesch F, Romby P.** 2005. *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J* **24**:824-835.
- Inoshima I, Inoshima N, Wilke GA, Powers ME, Frank KM, Wang Y, Bubeck Wardenburg J.** 2011. A *Staphylococcus aureus* pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. *Nat Med* **17**:1310-1314.
- Ito T, Katayama Y, Asada K, Mori N, Tsutsumimoto K, Tiensasitorn C, Hiramatsu K.** 2001. Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* **45**:1323-1336.
- Itoh S, Yokoyama R, Kamoshida G, Fujiwara T, Okada H, Takii T, Tsuji T, Fujii S, Hashizume H, Onozaki K.** 2013. Staphylococcal superantigen-like protein 10 (SSL10) inhibits blood coagulation by binding to prothrombin and factor Xa via their gamma-carboxyglutamic acid (Gla) domain. *J Biol Chem* **288**:21569-21580.
- Jansen KU, Girgenti DQ, Scully IL, Anderson AS.** 2013. Vaccine review: "*Staphylococcus aureus* vaccines: problems and prospects". *Vaccine* **31**:2723-2730.
- Janzon L, Löfdahl S, Arvidson S.** 1986. Evidence for a coordinate transcriptional control of alpha-toxin and protein A synthesis in *Staphylococcus aureus*. *FEMS Microbiology Letters* **33**:193-198.
- Jayasinghe L, Bayley H.** 2005. The leukocidin pore: evidence for an octamer with four LukF subunits and four LukS subunits alternating around a central axis. *Protein Sci* **14**:2550-2561.
- Jevons MP, Coe AW, Parker MT.** 1963. Methicillin resistance in staphylococci. *Lancet* **1**:904-907.
- Ji Y, Zhang B, Van SF, Horn, Warren P, Woodnutt G, Burnham MK, Rosenberg M.** 2001. Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science* **293**:2266-2269.

- Johnson AP, Aucken HM, Cavendish S, Ganner M, Wale MC, Warner M, Livermore DM, Cookson BD, participants UE.** 2001. Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). *J Antimicrob Chemother* **48**:143-144.
- Johnson JK, Khoie T, Shurland S, Kreisel K, Stine OC, Roghmann MC.** 2007. Skin and soft tissue infections caused by methicillin-resistant *Staphylococcus aureus* USA300 clone. *Emerg Infect Dis* **13**:1195-1200.
- Jones RC, Deck J, Edmondson RD, Hart ME.** 2008. Relative quantitative comparisons of the extracellular protein profiles of *Staphylococcus aureus* UAMS-1 and its *sarA*, *agr*, and *sarA agr* regulatory mutants using one-dimensional polyacrylamide gel electrophoresis and nanocapillary liquid chromatography coupled with tandem mass spectrometry. *J Bacteriol* **190**:5265-5278.
- Jongerijs I, Garcia BL, Geisbrecht BV, van Strijp JA, Rooijakkers SH.** 2010. Convertase inhibitory properties of Staphylococcal extracellular complement-binding protein. *J Biol Chem* **285**:14973-14979.
- Jongerijs I, Kohl J, Pandey MK, Ruyken M, van Kessel KP, van Strijp JA, Rooijakkers SH.** 2007. Staphylococcal complement evasion by various convertase-blocking molecules. *J Exp Med* **204**:2461-2471.
- Jongerijs I, Puister M, Wu J, Ruyken M, van Strijp JA, Rooijakkers SH.** 2010. Staphylococcal complement inhibitor modulates phagocyte responses by dimerization of convertases. *J Immunol* **184**:420-425.
- Jongerijs I, von Kockritz-Blickwede M, Horsburgh MJ, Ruyken M, Nizet V, Rooijakkers SH.** 2012. *Staphylococcus aureus* virulence is enhanced by secreted factors that block innate immune defenses. *J Innate Immun* **4**:301-311.
- Jonsson IM, Mazmanian SK, Schneewind O, Verdrengh M, Bremell T, Tarkowski A.** 2002. On the role of *Staphylococcus aureus* sortase and sortase-catalyzed surface protein anchoring in murine septic arthritis. *J Infect Dis* **185**:1417-1424.
- Josefsson E, McCrea KW, Ni Eidhin D, O'Connell D, Cox J, Hook M, Foster TJ.** 1998. Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. *Microbiology* **144** (Pt 12):3387-3395.
- Joshi GS, Spontak JS, Klapper DG, Richardson AR.** 2011. Arginine catabolic mobile element encoded *speG* abrogates the unique hypersensitivity of *Staphylococcus aureus* to exogenous polyamines. *Mol Microbiol* **82**:9-20.
- Kajimura J, Fujiwara T, Yamada S, Suzawa Y, Nishida T, Oyamada Y, Hayashi I, Yamagishi J, Komatsuzawa H, Sugai M.** 2005. Identification and molecular characterization of an N-acetylmuramyl-L-alanine amidase Sle1 involved in cell separation of *Staphylococcus aureus*. *Mol Microbiol* **58**:1087-1101.
- Kang M, Ko YP, Liang X, Ross CL, Liu Q, Murray BE, Hook M.** 2013. Collagen-binding microbial surface components recognizing adhesive matrix molecule (MSCRAMM) of Gram-positive bacteria inhibit complement activation via the classical pathway. *J Biol Chem* **288**:20520-20531.
- Karlsson A, Saravia-Otten P, Tegmark K, Morfeldt E, Arvidson S.** 2001. Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in *Staphylococcus aureus sarA* mutants due to up-regulation of extracellular proteases. *Infect Immun* **69**:4742-4748.
- Kastern W, Sjobring U, Bjorck L.** 1992. Structure of peptostreptococcal protein L and identification of a repeated immunoglobulin light chain-binding domain. *J Biol Chem* **267**:12820-12825.

- Keane FM, Loughman A, Valtulina V, Brennan M, Speziale P, Foster TJ.** 2007. Fibrinogen and elastin bind to the same region within the A domain of fibronectin binding protein A, an MSCRAMM of *Staphylococcus aureus*. *Mol Microbiol* **63**:711-723.
- Kempker RR, Farley MM, Ladson JL, Satola S, Ray SM.** 2010. Association of methicillin-resistant *Staphylococcus aureus* (MRSA) USA300 genotype with mortality in MRSA bacteremia. *J Infect* **61**:372-381.
- Kim H, Thammavongsa V, Schneewind O, Missiakas D.** 2012. Recurrent infections and immune evasion strategies of *Staphylococcus aureus*. *Current opinion in microbiology* **15**:92-99.
- Kim HK, Cheng AG, Kim HY, Missiakas DM, Schneewind O.** 2010. Nontoxic protein A vaccine for methicillin-resistant *Staphylococcus aureus* infections in mice. *J Exp Med* **207**:1863-1870.
- Kim HK, Emolo C, DeDent AC, Falugi F, Missiakas DM, Schneewind O.** 2012. Protein A-specific monoclonal antibodies and prevention of *Staphylococcus aureus* disease in mice. *Infect Immun* **80**:3460-3470.
- Kim HK, Falugi F, Thomer L, Missiakas DM, Schneewind O.** 2015. Protein A suppresses immune responses during *Staphylococcus aureus* bloodstream infection in guinea pigs. *MBio* **6**.
- Kim HK, Kim HY, Schneewind O, Missiakas D.** 2011. Identifying protective antigens of *Staphylococcus aureus*, a pathogen that suppresses host immune responses. *FASEB J* **25**:3605-3612.
- Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK, Active Bacterial Core surveillance MI.** 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* **298**:1763-1771.
- Ko YP, Kuipers A, Freitag CM, Jongerius I, Medina E, van Rooijen WJ, Spaan AN, van Kessel KP, Hook M, Rooijackers SH.** 2013. Phagocytosis escape by a *Staphylococcus aureus* protein that connects complement and coagulation proteins at the bacterial surface. *PLoS Pathog* **9**:e1003816.
- Kobayashi SD, Braughton KR, Palazzolo-Ballance AM, Kennedy AD, Sampaio E, Kristosturyan E, Whitney AR, Sturdevant DE, Dorward DW, Holland SM, Kreiswirth BN, Musser JM, DeLeo FR.** 2010. Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J Innate Immun* **2**:560-575.
- Kobayashi SD, DeLeo FR.** 2013. *Staphylococcus aureus* protein A promotes immune suppression. *MBio* **4**:e00764-00713.
- Kolaczowska E, Kubes P.** 2013. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* **13**:159-175.
- Kolar SL, Ibarra JA, Rivera FE, Mootz JM, Davenport JE, Stevens SM, Horswill AR, Shaw LN.** 2013. Extracellular proteases are key mediators of *Staphylococcus aureus* virulence via the global modulation of virulence-determinant stability. *Microbiologyopen* **2**:18-34.
- Korkmaz B, Horwitz MS, Jenne DE, Gauthier F.** 2010. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharmacol Rev* **62**:726-759.
- Koziel J, Maciag-Gudowska A, Mikolajczyk T, Bzowska M, Sturdevant DE, Whitney AR, Shaw LN, DeLeo FR, Potempa J.** 2009. Phagocytosis of *Staphylococcus aureus* by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors. *PLoS One* **4**:e5210.
- Kretschmer D, Gleske AK, Rautenberg M, Wang R, Koberle M, Bohn E, Schoneberg T, Rabiet MJ, Boulay F, Klebanoff SJ, van Kessel KA, van Strijp JA, Otto M, Peschel A.** 2010. Human formyl peptide receptor 2 senses highly pathogenic *Staphylococcus aureus*. *Cell Host Microbe* **7**:463-473.

- Kuklin NA, Clark DJ, Secore S, Cook J, Cope LD, McNeely T, Noble L, Brown MJ, Zorman JK, Wang XM, Pancari G, Fan H, Isett K, Burgess B, Bryan J, Brownlow M, George H, Meinz M, Liddell ME, Kelly R, Schultz L, Montgomery D, Onishi J, Losada M, Martin M, Ebert T, Tan CY, Schofield TL, Nagy E, Meineke A, Joyce JG, Kurtz MB, Caulfield MJ, Jansen KU, McClements W, Anderson AS. 2006. A novel *Staphylococcus aureus* vaccine: iron surface determinant B induces rapid antibody responses in rhesus macaques and specific increased survival in a murine *S. aureus* sepsis model. *Infect Immun* **74**:2215-2223.
- Kwiecinski J, Jin T, Josefsson E. 2014. Surface proteins of *Staphylococcus aureus* play an important role in experimental skin infection. *APMIS* **122**:1240-1250.
- Laarman AJ, Mijnheer G, Mootz JM, van Rooijen WJ, Ruyken M, Malone CL, Heezius EC, Ward R, Milligan G, van Strijp JA, de Haas CJ, Horswill AR, van Kessel KP, Rooijakkers SH. 2012. *Staphylococcus aureus* Staphopain A inhibits CXCR2-dependent neutrophil activation and chemotaxis. *EMBO J* **31**:3607-3619.
- Laarman AJ, Ruyken M, Malone CL, van Strijp JA, Horswill AR, Rooijakkers SH. 2011. *Staphylococcus aureus* metalloprotease aureolysin cleaves complement C3 to mediate immune evasion. *J Immunol* **186**:6445-6453.
- Lachenauer CS, Creti R, Michel JL, Madoff LC. 2000. Mosaicism in the alpha-like protein genes of group B streptococci. *Proc Natl Acad Sci U S A* **97**:9630-9635.
- Lambris JD, Ricklin D, Geisbrecht BV. 2008. Complement evasion by human pathogens. *Nat Rev Microbiol* **6**:132-142.
- Langley R, Patel D, Jackson N, Clow F, Fraser JD. 2010. Staphylococcal superantigen super-domains in immune evasion. *Crit Rev Immunol* **30**:149-165.
- Lauderdale KJ, Boles BR, Cheung AL, Horswill AR. 2009. Interconnections between Sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. *Infect Immun* **77**:1623-1635.
- Li J, Kasper DL, Ausubel FM, Rosner B, Michel JL. 1997. Inactivation of the alpha C protein antigen gene, *bca*, by a novel shuttle/suicide vector results in attenuation of virulence and immunity in group B Streptococcus. *Proc Natl Acad Sci U S A* **94**:13251-13256.
- Li M, Cheung GY, Hu J, Wang D, Joo HS, Deleo FR, Otto M. 2010. Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. *J Infect Dis* **202**:1866-1876.
- Li M, Diep BA, Villaruz AE, Braughton KR, Jiang X, DeLeo FR, Chambers HF, Lu Y, Otto M. 2009. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **106**:5883-5888.
- Li M, Du X, Villaruz AE, Diep BA, Wang D, Song Y, Tian Y, Hu J, Yu F, Lu Y, Otto M. 2012. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. *Nat Med* **18**:816-819.
- Li MZ, Elledge SJ. 2012. SLIC: a method for sequence- and ligation-independent cloning. *Methods Mol Biol* **852**:51-59.
- Lim H, Eng J, Yates JR, 3rd, Tollaksen SL, Giometti CS, Holden JF, Adams MW, Reich CI, Olsen GJ, Hays LG. 2003. Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to mu LC-ESI tandem mass spectrometry. *J Am Soc Mass Spectrom* **14**:957-970.
- Lindmark R, Movitz J, Sjoquist J. 1977. Extracellular protein A from a methicillin-resistant strain of *Staphylococcus aureus*. *Eur J Biochem* **74**:623-628.
- Lindsay JA, Foster SJ. 1999. Interactive regulatory pathways control virulence determinant production and stability in response to environmental conditions in *Staphylococcus aureus*. *Mol Gen Genet* **262**:323-331.

- Linge HM, Collin M, Nordenfelt P, Morgelin M, Malmsten M, Egesten A.** 2008. The human CXC chemokine granulocyte chemotactic protein 2 (GCP-2)/CXCL6 possesses membrane-disrupting properties and is antibacterial. *Antimicrob Agents Chemother* **52**:2599-2607.
- Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, Bastian JF, Fierer J, Nizet V.** 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med* **202**:209-215.
- Liu Q, Du X, Hong X, Li T, Zheng B, He L, Wang Y, Otto M, Li M.** 2015. Targeting surface protein SasX by active and passive vaccination to reduce *Staphylococcus aureus* colonization and infection. *Infect Immun* **83**:2168-2174.
- Liu Y, Wang H, Du N, Shen E, Chen H, Niu J, Ye H, Chen M.** 2009. Molecular evidence for spread of two major methicillin-resistant *Staphylococcus aureus* clones with a unique geographic distribution in Chinese hospitals. *Antimicrob Agents Chemother* **53**:512-518.
- Loffler B, Hussain M, Grundmeier M, Bruck M, Holzinger D, Varga G, Roth J, Kahl BC, Proctor RA, Peters G.** 2010. *Staphylococcus aureus* panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog* **6**:e1000715.
- Loughran AJ, Atwood DN, Anthony AC, Harik NS, Spencer HJ, Beenken KE, Smeltzer MS.** 2014. Impact of individual extracellular proteases on *Staphylococcus aureus* biofilm formation in diverse clinical isolates and their isogenic sarA mutants. *Microbiologyopen* **3**:897-909.
- Ludwig W, Seewaldt E, Kilpper-Balz R, Schleifer KH, Magrum L, Woese CR, Fox GE, Stackebrandt E.** 1985. The phylogenetic position of Streptococcus and Enterococcus. *J Gen Microbiol* **131**:543-551.
- Maguin E, Duwat P, Hege T, Ehrlich D, Gruss A.** 1992. New thermosensitive plasmid for gram-positive bacteria. *J Bacteriol* **174**:5633-5638.
- Malackowa N, DeLeo FR.** 2011. *Staphylococcus aureus* survival in human blood. *Virulence* **2**:567-569.
- Malackowa N, Kobayashi SD, Freedman B, Dorward DW, DeLeo FR.** 2013. *Staphylococcus aureus* leukotoxin GH promotes formation of neutrophil extracellular traps. *J Immunol* **191**:6022-6029.
- Malackowa N, Whitney AR, Kobayashi SD, Sturdevant DE, Kennedy AD, Broughton KR, Shabb DW, Diep BA, Chambers HF, Otto M, DeLeo FR.** 2011. Global changes in *Staphylococcus aureus* gene expression in human blood. *PLoS One* **6**:e18617.
- Manting EH, Driessen AJ.** 2000. *Escherichia coli* translocase: the unravelling of a molecular machine. *Mol Microbiol* **37**:226-238.
- Marrafini LA, Dedent AC, Schneewind O.** 2006. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. *Microbiol Mol Biol Rev* **70**:192-221.
- Marrafini LA, Schneewind O.** 2005. Anchor structure of staphylococcal surface proteins. V. Anchor structure of the Sortase B substrate IsdC. *J Biol Chem* **280**:16263-16271.
- Marrafini LA, Schneewind O.** 2006. Targeting proteins to the cell wall of sporulating *Bacillus anthracis*. *Mol Microbiol* **62**:1402-1417.
- Marti M, Trottonda MP, Tormo-Mas MA, Vergara-Irigaray M, Cheung AL, Lasa I, Penades JR.** 2010. Extracellular proteases inhibit protein-dependent biofilm formation in *Staphylococcus aureus*. *Microbes Infect* **12**:55-64.
- Martin FJ, Gomez MI, Wetzel DM, Memmi G, O'Seaghdha M, Soong G, Schindler C, Prince A.** 2009. *Staphylococcus aureus* activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A. *J Clin Invest* **119**:1931-1939.
- Mascari LM, Ross JM.** 2003. Quantification of staphylococcal-collagen binding interactions in whole blood by use of a confocal microscopy shear-adhesion assay. *J Infect Dis* **188**:98-107.

- Matsushita M, Endo Y, Fujita T.** 2000. Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease. *J Immunol* **164**:2281-2284.
- Matsushita M, Fujita T.** 2001. Ficolins and the lectin complement pathway. *Immunol Rev* **180**:78-85.
- Mazmanian SK, Liu G, Jensen ER, Lenoy E, Schneewind O.** 2000. *Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. *Proc Natl Acad Sci U S A* **97**:5510-5515.
- Mazmanian SK, Liu G, Ton-That H, Schneewind O.** 1999. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. *Science* **285**:760-763.
- Mazmanian SK, Skaar EP, Gaspar AH, Humayun M, Gornicki P, Jelenska J, Joachmiak A, Missiakas DM, Schneewind O.** 2003. Passage of heme-iron across the envelope of *Staphylococcus aureus*. *Science* **299**:906-909.
- Mazmanian SK, Ton-That H, Schneewind O.** 2001. Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. *Mol Microbiol* **40**:1049-1057.
- Mazmanian SK, Ton-That H, Su K, Schneewind O.** 2002. An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis. *Proc Natl Acad Sci U S A* **99**:2293-2298.
- McAdow M, Kim HK, Dedent AC, Hendrickx AP, Schneewind O, Missiakas DM.** 2011. Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood. *PLoS Pathog* **7**:e1002307.
- McAleese FM, Walsh EJ, Sieprawska M, Potempa J, Foster TJ.** 2001. Loss of clumping factor B fibrinogen binding activity by *Staphylococcus aureus* involves cessation of transcription, shedding and cleavage by metalloprotease. *J Biol Chem* **276**:29969-29978.
- McCarthy AJ, Lindsay JA.** 2010. Genetic variation in *Staphylococcus aureus* surface and immune evasion genes is lineage associated: implications for vaccine design and host-pathogen interactions. *BMC Microbiol* **10**:173.
- McCormack N, Foster TJ, Geoghegan JA.** 2014. A short sequence within subdomain N1 of region A of the *Staphylococcus aureus* MSCRAMM clumping factor A is required for export and surface display. *Microbiology* **160**:659-670.
- McCourt J, O'Halloran DP, McCarthy H, O'Gara JP, Geoghegan JA.** 2014. Fibronectin-binding proteins are required for biofilm formation by community-associated methicillin-resistant *Staphylococcus aureus* strain LAC. *FEMS Microbiol Lett* **353**:157-164.
- McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P.** 2012. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe* **12**:324-333.
- McGavin MJ, Zahradka C, Rice K, Scott JE.** 1997. Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease. *Infect Immun* **65**:2621-2628.
- McKenney D, Pouliot KL, Wang Y, Murthy V, Ulrich M, Doring G, Lee JC, Goldmann DA, Pier GB.** 1999. Broadly protective vaccine for *Staphylococcus aureus* based on an *in vivo*-expressed antigen. *Science* **284**:1523-1527.
- Mehlin C, Headley CM, Klebanoff SJ.** 1999. An inflammatory polypeptide complex from *Staphylococcus epidermidis*: isolation and characterization. *J Exp Med* **189**:907-918.
- Menzies BE, Kernodle DS.** 1996. Passive immunization with antiserum to a nontoxic alpha-toxin mutant from *Staphylococcus aureus* is protective in a murine model. *Infect Immun* **64**:1839-1841.
- Mishra NN, Liu GY, Yeaman MR, Nast CC, Proctor RA, McKinnell J, Bayer AS.** 2011. Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. *Antimicrob Agents Chemother* **55**:526-531.

- Moks T, Abrahmsen L, Nilsson B, Hellman U, Sjoquist J, Uhlen M.** 1986. Staphylococcal protein A consists of five IgG-binding domains. *Eur J Biochem* **156**:637-643.
- Monecke S, Coombs G, Shore AC, Coleman DC, Akpaka P, Borg M, Chow H, Ip M, Jatzwauk L, Jonas D, Kadlec K, Kearns A, Laurent F, O'Brien FG, Pearson J, Ruppelt A, Schwarz S, Scicluna E, Slickers P, Tan HL, Weber S, Ehricht R.** 2011. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. *PLoS One* **6**:e17936.
- Monk IR, Shah IM, Xu M, Tan MW, Foster TJ.** 2012. Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *MBio* **3** (2): e00277-11.
- Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ.** 2015. Complete Bypass of Restriction Systems for Major *Staphylococcus aureus* Lineages. *MBio* **6**:e00308-00315.
- Monteiro JM, Fernandes PB, Vaz F, Pereira AR, Tavares AC, Ferreira MT, Pereira PM, Veiga H, Kuru E, VanNieuwenhze MS, Brun YV, Filipe SR, Pinho MG.** 2015. Cell shape dynamics during the staphylococcal cell cycle. *Nat Commun* **6**:8055.
- Moore KL, Patel KD, Bruehl RE, Li F, Johnson DA, Lichenstein HS, Cummings RD, Bainton DF, McEver RP.** 1995. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J Cell Biol* **128**:661-671.
- Moran GJ, Krishnadasan A, Gorwitz RJ, Fosheim GE, McDougal LK, Carey RB, Talan DA, Group EMINS.** 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. *N Engl J Med* **355**:666-674.
- Morinaga N, Kaihou Y, Noda M.** 2003. Purification, cloning and characterization of variant LukE-LukD with strong leukocidal activity of staphylococcal bi-component leukotoxin family. *Microbiol Immunol* **47**:81-90.
- Movitz J.** 1976. Formation of extracellular protein A by *Staphylococcus aureus*. *Eur J Biochem* **68**:291-299.
- Mulcahy ME, Geoghegan JA, Monk IR, O'Keeffe KM, Walsh EJ, Foster TJ, McLoughlin RM.** 2012. Nasal colonisation by *Staphylococcus aureus* depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. *PLoS Pathog* **8**:e1003092.
- Munoz P, Hortal J, Giannella M, Barrio JM, Rodriguez-Creixems M, Perez MJ, Rincon C, Bouza E.** 2008. Nasal carriage of *S. aureus* increases the risk of surgical site infection after major heart surgery. *J Hosp Infect* **68**:25-31.
- Nallapareddy SR, Singh KV, Murray BE.** 2006. Construction of improved temperature-sensitive and mobilizable vectors and their use for constructing mutations in the adhesin-encoding *acm* gene of poorly transformable clinical *Enterococcus faecium* strains. *Appl Environ Microbiol* **72**:334-345.
- Navarre WW, Schneewind O.** 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63**:174-229.
- Neuhaus FC, Baddiley J.** 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiol Mol Biol Rev* **67**:686-723.
- Nickerson N, Ip J, Passos DT, McGavin MJ.** 2010. Comparison of Staphopain A (ScpA) and B (SspB) precursor activation mechanisms reveals unique secretion kinetics of proSspB (Staphopain B), and a different interaction with its cognate Staphostatin, SspC. *Mol Microbiol* **75**:161-177.
- Nickerson NN, Joag V, McGavin MJ.** 2008. Rapid autocatalytic activation of the M4 metalloprotease aureolysin is controlled by a conserved N-terminal fungalsin-thermolysin-propeptide domain. *Mol Microbiol* **69**:1530-1543.

- Nitsche-Schmitz DP, Johansson HM, Sastalla I, Reissmann S, Frick IM, Chhatwal GS. 2007. Group G streptococcal IgG binding molecules FOG and protein G have different impacts on opsonization by C1q. *J Biol Chem* **282**:17530-17536.
- Nordenfelt P, Tapper H. 2011. Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol* **90**:271-284.
- Nordenfelt P, Waldemarson S, Linder A, Morgelin M, Karlsson C, Malmstrom J, Bjorck L. 2012. Antibody orientation at bacterial surfaces is related to invasive infection. *J Exp Med* **209**:2367-2381.
- Nygaard TK, Pallister KB, DuMont AL, DeWald M, Watkins RL, Pallister EQ, Malone C, Griffith S, Horswill AR, Torres VJ, Voyich JM. 2012. Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. *PLoS One* **7**:e36532.
- O'Brien LM, Walsh EJ, Massey RC, Peacock SJ, Foster TJ. 2002. *Staphylococcus aureus* clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. *Cell Microbiol* **4**:759-770.
- O'Gara JP, Humphreys H. 2001. *Staphylococcus epidermidis* biofilms: importance and implications. *J Med Microbiol* **50**:582-587.
- O'Keeffe KM, Wilk MM, Leech JM, Murphy AG, Laabei M, Monk IR, Massey RC, Lindsay JA, Foster TJ, Geoghegan JA, McLoughlin RM. 2015. Manipulation of Autophagy in Phagocytes Facilitates *Staphylococcus aureus* Bloodstream Infection. *Infect Immun* **83**:3445-3457.
- O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson D, Loughman A, Foster T, O'Gara J. 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *Journal of bacteriology* **190**:3835-3850.
- O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, Foster TJ, O'Gara JP. 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *J Bacteriol* **190**:3835-3850.
- O'Neill E, Pozzi C, Houston P, Smyth D, Humphreys H, Robinson DA, O'Gara JP. 2007. Association between methicillin susceptibility and biofilm regulation in *Staphylococcus aureus* isolates from device-related infections. *J Clin Microbiol* **45**:1379-1388.
- O'Riordan K, Lee JC. 2004. *Staphylococcus aureus* capsular polysaccharides. *Clin Microbiol Rev* **17**:218-234.
- O'Seaghda M, van Schooten CJ, Kerrigan SW, Emsley J, Silverman GJ, Cox D, Lenting PJ, Foster TJ. 2006. *Staphylococcus aureus* protein A binding to von Willebrand factor A1 domain is mediated by conserved IgG binding regions. *FEBS J* **273**:4831-4841.
- Oliver DB, Beckwith J. 1981. *E. coli* mutant pleiotropically defective in the export of secreted proteins. *Cell* **25**:765-772.
- Olsen JV, Ong SE, Mann M. 2004. Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Mol Cell Proteomics* **3**:608-614.
- Otto M. 2010. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu Rev Microbiol* **64**:143-162.
- Otto R, de Vos WM, Gavrieli J. 1982. Plasmid DNA in *Streptococcus cremoris* Wg2: Influence of pH on Selection in Chemostats of a Variant Lacking a Protease Plasmid. *Appl Environ Microbiol* **43**:1272-1277.
- Padilla-Carlin DJ, McMurray DN, Hickey AJ. 2008. The guinea pig as a model of infectious diseases. *Comp Med* **58**:324-340.
- Palmqvist N, Foster T, Tarkowski A, Josefsson E. 2002. Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. *Microb Pathog* **33**:239-249.

- Pang YY, Schwartz J, Thoendel M, Ackermann LW, Horswill AR, Nauseef WM.** 2010. *agr*-Dependent interactions of *Staphylococcus aureus* USA300 with human polymorphonuclear neutrophils. *J Innate Immun* **2**:546-559.
- Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A.** 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol* **191**:677-691.
- Park JT, Shaw DR, Chatterjee AN, Mirelman D, Wu T.** 1974. Mutants of staphylococci with altered cell walls. *Ann N Y Acad Sci* **236**:54-62.
- Patel AH, Nowlan P, Weavers ED, Foster T.** 1987. Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. *Infect Immun* **55**:3103-3110.
- Patel D, Wines BD, Langley RJ, Fraser JD.** 2010. Specificity of staphylococcal superantigen-like protein 10 toward the human IgG1 Fc domain. *J Immunol* **184**:6283-6292.
- Patella V, Casolaro V, Bjorck L, Marone G.** 1990. Protein L. A bacterial Ig-binding protein that activates human basophils and mast cells. *J Immunol* **145**:3054-3061.
- Patti JM, Allen BL, McGavin MJ, Hook M.** 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol* **48**:585-617.
- Peacock SJ, de Silva I, Lowy FD.** 2001. What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiol* **9**:605-610.
- Peacock SJ, Foster TJ, Cameron BJ, Berendt AR.** 1999. Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. *Microbiology* **145** (Pt 12):3477-3486.
- Perry AM, Ton-That H, Mazmanian SK, Schneewind O.** 2002. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. III. Lipid II is an in vivo peptidoglycan substrate for sortase-catalyzed surface protein anchoring. *J Biol Chem* **277**:16241-16248.
- Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, van Kessel KP, van Strijp JA.** 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J Exp Med* **193**:1067-1076.
- Peschel A, Otto M.** 2013. Phenol-soluble modulins and staphylococcal infection. *Nat Rev Microbiol* **11**:667-673.
- Peterson PK, Verhoef J, Sabath LD, Quie PG.** 1977. Effect of protein A on staphylococcal opsonization. *Infect Immun* **15**:760-764.
- Plouin-Gaudon I, Clement S, Huggler E, Chaponnier C, Francois P, Lew D, Schrenzel J, Vaudaux P, Lacroix JS.** 2006. Intracellular residency is frequently associated with recurrent *Staphylococcus aureus* rhinosinusitis. *Rhinology* **44**:249-254.
- Popov L, Kovalski J, Grandi G, Bagnoli F, Amieva MR.** 2014. Three-Dimensional Human Skin Models to Understand *Staphylococcus aureus* Skin Colonization and Infection. *Front Immunol* **5**:41.
- Postma B, Poppelier MJ, van Galen JC, Prossnitz ER, van Strijp JA, de Haas CJ, van Kessel KP.** 2004. Chemotaxis inhibitory protein of *Staphylococcus aureus* binds specifically to the C5a and formylated peptide receptor. *J Immunol* **172**:6994-7001.
- Prat C, Bestebroer J, de Haas CJ, van Strijp JA, van Kessel KP.** 2006. A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. *J Immunol* **177**:8017-8026.
- Prax M, Lee CY, Bertram R.** 2013. An update on the molecular genetics toolbox for staphylococci. *Microbiology* **159**:421-435.

- Proctor RA.** 2012. Challenges for a universal *Staphylococcus aureus* vaccine. Clin Infect Dis **54**:1179-1186.
- Pugsley AP.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol Rev **57**:50-108.
- Que YA, Haeffliger JA, Piroth L, Francois P, Widmer E, Entenza JM, Sinha B, Herrmann M, Francioli P, Vaudaux P, Moreillon P.** 2005. Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis. J Exp Med **201**:1627-1635.
- Raeder R, Woischnik M, Podbielski A, Boyle MD.** 1998. A secreted streptococcal cysteine protease can cleave a surface-expressed M1 protein and alter the immunoglobulin binding properties. Res Microbiol **149**:539-548.
- Rajan V, Schoenfelder SM, Ziebuhr W, Gopal S.** 2015. Genotyping of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) in a tertiary care centre in Mysore, South India: ST2371-SCCmec IV emerges as the major clone. Infect Genet Evol **34**:230-235.
- Ramadurai L, Lockwood KJ, Nadakavukaren MJ, Jayaswal RK.** 1999. Characterization of a chromosomally encoded glycylglycine endopeptidase of *Staphylococcus aureus*. Microbiology **145** (Pt 4):801-808.
- Reed SB, Wesson CA, Liou LE, Trumble WR, Schlievert PM, Bohach GA, Bayles KW.** 2001. Molecular characterization of a novel *Staphylococcus aureus* serine protease operon. Infect Immun **69**:1521-1527.
- Reyes-Robles T, Alonzo F, 3rd, Kozhaya L, Lacy DB, Unutmaz D, Torres VJ.** 2013. *Staphylococcus aureus* leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection. Cell Host Microbe **14**:453-459.
- Rice K, Peralta R, Bast D, de Azavedo J, McGavin MJ.** 2001. Description of staphylococcus serine protease (*ssp*) operon in *Staphylococcus aureus* and nonpolar inactivation of *sspA*-encoded serine protease. Infect Immun **69**:159-169.
- Rigby KM, DeLeo FR.** 2012. Neutrophils in innate host defense against *Staphylococcus aureus* infections. Semin Immunopathol **34**:237-259.
- Robbins JB, Schneerson R, Horwith G, Naso R, Fattom A.** 2004. *Staphylococcus aureus* types 5 and 8 capsular polysaccharide-protein conjugate vaccines. Am Heart J **147**:593-598.
- Roberts GA, Houston PJ, White JH, Chen K, Stephanou AS, Cooper LP, Dryden DT, Lindsay JA.** 2013. Impact of target site distribution for Type I restriction enzymes on the evolution of methicillin-resistant *Staphylococcus aureus* (MRSA) populations. Nucleic Acids Res **41**:7472-7484.
- Rohde H, Burdelski C, Bartscht K, Hussain M, Buck F, Horstkotte MA, Knobloch JK, Heilmann C, Herrmann M, Mack D.** 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol Microbiol **55**:1883-1895.
- Rooijakkers SH, Ruyken M, Roos A, Daha MR, Presanis JS, Sim RB, van Wamel WJ, van Kessel KP, van Strijp JA.** 2005. Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol **6**:920-927.
- Rooijakkers SH, Ruyken M, van Roon J, van Kessel KP, van Strijp JA, van Wamel WJ.** 2006. Early expression of SCIN and CHIPS drives instant immune evasion by *Staphylococcus aureus*. Cell Microbiol **8**:1282-1293.
- Rooijakkers SH, van Strijp JA.** 2007. Bacterial complement evasion. Mol Immunol **44**:23-32.
- Rosenstein R, Gotz F.** 2000. Staphylococcal lipases: biochemical and molecular characterization. Biochimie **82**:1005-1014.

- Rudkin JK, Edwards AM, Bowden MG, Brown EL, Pozzi C, Waters EM, Chan WC, Williams P, O'Gara JP, Massey RC. 2012. Methicillin resistance reduces the virulence of healthcare-associated methicillin-resistant *Staphylococcus aureus* by interfering with the *agr* quorum sensing system. *J Infect Dis* **205**:798-806.
- Rudkin JK, Laabei M, Edwards AM, Joo HS, Otto M, Lennon KL, O'Gara JP, Waterfield NR, Massey RC. 2014. Oxacillin alters the toxin expression profile of community-associated methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* **58**:1100-1107.
- Rzychon M, Sabat A, Kosowska K, Potempa J, Dubin A. 2003. Staphostatins: an expanding new group of proteinase inhibitors with a unique specificity for the regulation of staphopains, *Staphylococcus spp.* cysteine proteinases. *Mol Microbiol* **49**:1051-1066.
- Sahu A, Lambris JD. 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunol Rev* **180**:35-48.
- Sanchez CJ, Shivshankar P, Stol K, Trakhtenbroit S, Sullam PM, Sauer K, Hermans PW, Orihuela CJ. 2010. The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation *in vivo* and in biofilms. *PLoS Pathog* **6**:e1001044.
- Sasano M, Burton DR, Silverman GJ. 1993. Molecular selection of human antibodies with an unconventional bacterial B cell antigen. *J Immunol* **151**:5822-5839.
- Sasso EH, Silverman GJ, Mannik M. 1989. Human IgM molecules that bind staphylococcal protein A contain VHIII H chains. *J Immunol* **142**:2778-2783.
- Savolainen K, Paulin L, Westerlund-Wikstrom B, Foster TJ, Korhonen TK, Kuusela P. 2001. Expression of *pls*, a gene closely associated with the *mecA* gene of methicillin-resistant *Staphylococcus aureus*, prevents bacterial adhesion *in vitro*. *Infect Immun* **69**:3013-3020.
- Schaffer AC, Solinga RM, Cocchiari J, Portoles M, Kiser KB, Risley A, Randall SM, Valtulina V, Speziale P, Walsh E, Foster T, Lee JC. 2006. Immunization with *Staphylococcus aureus* clumping factor B, a major determinant in nasal carriage, reduces nasal colonization in a murine model. *Infect Immun* **74**:2145-2153.
- Schlag M, Biswas R, Krismer B, Kohler T, Zoll S, Yu W, Schwarz H, Peschel A, Gotz F. 2010. Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. *Mol Microbiol* **75**:864-873.
- Schleifer KH, Kandler O. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**:407-477.
- Schneewind O, Fowler A, Faull KF. 1995. Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. *Science* **268**:103-106.
- Schneewind O, Mihaylova-Petkov D, Model P. 1993. Cell wall sorting signals in surface proteins of gram-positive bacteria. *EMBO J* **12**:4803-4811.
- Schneewind O, Model P, Fischetti VA. 1992. Sorting of protein A to the staphylococcal cell wall. *Cell* **70**:267-281.
- Schroeder K, Jularic M, Horsburgh SM, Hirschhausen N, Neumann C, Bertling A, Schulte A, Foster S, Kehrel BE, Peters G, Heilmann C. 2009. Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. *PLoS One* **4**:e7567.
- Schwarz-Linek U, Werner JM, Pickford AR, Gurusiddappa S, Kim JH, Pilka ES, Briggs JA, Gough TS, Hook M, Campbell ID, Potts JR. 2003. Pathogenic bacteria attach to human fibronectin through a tandem beta-zipper. *Nature* **423**:177-181.
- Sendi P, Proctor RA. 2009. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. *Trends Microbiol* **17**:54-58.

- Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L, Finnerty CC, Lopez CM, Honari S, Moore EE, Minei JP, Cuschieri J, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Jeschke MG, Klein MB, Gamelli RL, Gibran NS, Brownstein BH, Miller-Graziano C, Calvano SE, Mason PH, Cobb JP, Rahme LG, Lowry SF, Maier RV, Moldawer LL, Herndon DN, Davis RW, Xiao W, Tompkins RG, **Inflammation, Host Response to Injury LSCRP**. 2013. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* **110**:3507-3512.
- Seybold U, Kourbatova EV, Johnson JG, Halvosa SJ, Wang YF, King MD, Ray SM, **Blumberg HM**. 2006. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. *Clin Infect Dis* **42**:647-656.
- Shalaby MR, Aggarwal BB, Rinderknecht E, Svedersky LP, Finkle BS, Palladino MA, Jr. 1985. Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. *J Immunol* **135**:2069-2073.
- Shankar V, Baghdayan AS, Huycke MM, Lindahl G, Gilmore MS. 1999. Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. *Infect Immun* **67**:193-200.
- Sharp JA, Echague CG, Hair PS, Ward MD, Nyalwidhe JO, Geoghegan JA, Foster TJ, **Cunnion KM**. 2012. *Staphylococcus aureus* surface protein SdrE binds complement regulator factor H as an immune evasion tactic. *PLoS One* **7**:e38407.
- Sharp KH, Schneider S, Cockayne A, Paoli M. 2007. Crystal structure of the heme-IsdC complex, the central conduit of the Isd iron/heme uptake system in *Staphylococcus aureus*. *J Biol Chem* **282**:10625-10631.
- Shaw L, Golonka E, Potempa J, Foster SJ. 2004. The role and regulation of the extracellular proteases of *Staphylococcus aureus*. *Microbiology* **150**:217-228.
- Shinefield H, Black S, Fattom A, Horwith G, Rasgon S, Ordonez J, Yeoh H, Law D, Robbins JB, Schneerson R, Muenz L, Fuller S, Johnson J, Fireman B, Alcorn H, Naso R. 2002. Use of a *Staphylococcus aureus* conjugate vaccine in patients receiving hemodialysis. *N Engl J Med* **346**:491-496.
- Shockman GD, Barrett JF. 1983. Structure, function, and assembly of cell walls of gram-positive bacteria. *Annu Rev Microbiol* **37**:501-527.
- Sibbald MJ, Ziebandt AK, Engelmann S, Hecker M, de Jong A, Harmsen HJ, Raangs GC, Stokroos I, Arends JP, Dubois JY, van Dijk JM. 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol Mol Biol Rev* **70**:755-788.
- Siboo IR, Chaffin DO, Rubens CE, Sullam PM. 2008. Characterization of the accessory Sec system of *Staphylococcus aureus*. *J Bacteriol* **190**:6188-6196.
- Siboo IR, Chambers HF, Sullam PM. 2005. Role of SraP, a Serine-Rich Surface Protein of *Staphylococcus aureus*, in binding to human platelets. *Infect Immun* **73**:2273-2280.
- Sieprawska-Lupa M, Mydel P, Krawczyk K, Wojcik K, Puklo M, Lupa B, Suder P, Silberring J, Reed M, Pohl J, Shafer W, McAleese F, Foster T, Travis J, Potempa J. 2004. Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrob Agents Chemother* **48**:4673-4679.
- Sieradzki K, Tomasz A. 1999. Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of *Staphylococcus aureus*. *J Bacteriol* **181**:7566-7570.
- Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, Fridkin S, National Healthcare Safety Network T, Participating NF. 2013. Antimicrobial-

- resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infect Control Hosp Epidemiol* **34**:1-14.
- Sinha B, Francois PP, Nusse O, Foti M, Hartford OM, Vaudaux P, Foster TJ, Lew DP, Herrmann M, Krause KH.** 1999. Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin $\alpha 5\beta 1$. *Cell Microbiol* **1**:101-117.
- Smith EJ, Corrigan RM, van der Sluis T, Grundling A, Speziale P, Geoghegan JA, Foster TJ.** 2012. The immune evasion protein Sbi of *Staphylococcus aureus* occurs both extracellularly and anchored to the cell envelope by binding lipoteichoic acid. *Mol Microbiol* **83**:789-804.
- Smith EJ, Visai L, Kerrigan SW, Speziale P, Foster TJ.** 2011. The Sbi protein is a multifunctional immune evasion factor of *Staphylococcus aureus*. *Infect Immun* **79**:3801-3809.
- Song WC, Sarrias MR, Lambris JD.** 2000. Complement and innate immunity. *Immunopharmacology* **49**:187-198.
- Spaan AN, Henry T, van Rooijen WJ, Perret M, Badiou C, Aerts PC, Kemmink J, de Haas CJ, van Kessel KP, Vandenesch F, Lina G, van Strijp JA.** 2013. The staphylococcal toxin Panton-Valentine Leukocidin targets human C5a receptors. *Cell Host Microbe* **13**:584-594.
- Spaan AN, Reyes-Robles T, Badiou C, Cochet S, Boguslawski KM, Yoong P, Day CJ, de Haas CJ, van Kessel KP, Vandenesch F, Jennings MP, Le Van Kim C, Colin Y, van Strijp JA, Henry T, Torres VJ.** 2015. *Staphylococcus aureus* Targets the Duffy Antigen Receptor for Chemokines (DARC) to Lyse Erythrocytes. *Cell Host Microbe* **18**:363-370.
- Spaan AN, Surewaard BG, Nijland R, van Strijp JA.** 2013. Neutrophils versus *Staphylococcus aureus*: a biological tug of war. *Annu Rev Microbiol* **67**:629-650.
- Spaan AN, Vrieling M, Wallet P, Badiou C, Reyes-Robles T, Ohneck EA, Benito Y, de Haas CJ, Day CJ, Jennings MP, Lina G, Vandenesch F, van Kessel KP, Torres VJ, van Strijp JA, Henry T.** 2014. The staphylococcal toxins gamma-haemolysin AB and CB differentially target phagocytes by employing specific chemokine receptors. *Nat Commun* **5**:5438.
- Spaulding AR, Salgado-Pabon W, Kohler PL, Horswill AR, Leung DY, Schlievert PM.** 2013. Staphylococcal and streptococcal superantigen exotoxins. *Clin Microbiol Rev* **26**:422-447.
- Stackebrandt E, Teuber M.** 1988. Molecular taxonomy and phylogenetic position of lactic acid bacteria. *Biochimie* **70**:317-324.
- Stapels DA, Ramyar KX, Bischoff M, von Kockritz-Blickwede M, Milder FJ, Ruyken M, Eisenbeis J, McWhorter WJ, Herrmann M, van Kessel KP, Geisbrecht BV, Rooijackers SH.** 2014. *Staphylococcus aureus* secretes a unique class of neutrophil serine protease inhibitors. *Proc Natl Acad Sci U S A* **111**:13187-13192.
- Steven AC, Steinert PM.** 1994. Protein composition of cornified cell envelopes of epidermal keratinocytes. *J Cell Sci* **107** (Pt 2):693-700.
- Stranger-Jones YK, Bae T, Schneewind O.** 2006. Vaccine assembly from surface proteins of *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **103**:16942-16947.
- Sun F, Cho H, Jeong DW, Li C, He C, Bae T.** 2010. Aureusimines in *Staphylococcus aureus* are not involved in virulence. *PLoS One* **5**:e15703.
- Surewaard BG, de Haas CJ, Vervoort F, Rigby KM, DeLeo FR, Otto M, van Strijp JA, Nijland R.** 2013. Staphylococcal alpha-phenol soluble modulins contribute to neutrophil lysis after phagocytosis. *Cell Microbiol* **15**:1427-1437.
- Surewaard BG, Nijland R, Spaan AN, Kruijtz JA, de Haas CJ, van Strijp JA.** 2012. Inactivation of staphylococcal phenol soluble modulins by serum lipoprotein particles. *PLoS Pathog* **8**:e1002606.

- Talan DA, Krishnadasan A, Gorwitz RJ, Fosheim GE, Limbago B, Albrecht V, Moran GJ, Group EMINS.** 2011. Comparison of *Staphylococcus aureus* from skin and soft-tissue infections in US emergency department patients, 2004 and 2008. *Clin Infect Dis* **53**:144-149.
- Tegla CA, Cudrici C, Patel S, Trippe R, 3rd, Rus V, Niculescu F, Rus H.** 2011. Membrane attack by complement: the assembly and biology of terminal complement complexes. *Immunol Res* **51**:45-60.
- Tenover FC, Goering RV.** 2009. Methicillin-resistant *Staphylococcus aureus* strain USA300: origin and epidemiology. *J Antimicrob Chemother* **64**:441-446.
- Thakker M, Park JS, Carey V, Lee JC.** 1998. *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. *Infect Immun* **66**:5183-5189.
- Thammavongsa V, Kern JW, Missiakas DM, Schneewind O.** 2009. *Staphylococcus aureus* synthesizes adenosine to escape host immune responses. *J Exp Med* **206**:2417-2427.
- Thammavongsa V, Kim HK, Missiakas D, Schneewind O.** 2015. Staphylococcal manipulation of host immune responses. *Nat Rev Microbiol* **13**:529-543.
- Thammavongsa V, Missiakas DM, Schneewind O.** 2013. *Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death. *Science* **342**:863-866.
- Thiel S.** 2007. Complement activating soluble pattern recognition molecules with collagen-like regions, mannan-binding lectin, ficolins and associated proteins. *Mol Immunol* **44**:3875-3888.
- Thomer L, Becker S, Emolo C, Quach A, Kim HK, Rauch S, Anderson M, Leblanc JF, Schneewind O, Faull KF, Missiakas D.** 2014. N-acetylglucosaminylation of serine-aspartate repeat proteins promotes *Staphylococcus aureus* bloodstream infection. *J Biol Chem* **289**:3478-3486.
- Thwaites GE, Gant V.** 2011. Are bloodstream leukocytes Trojan Horses for the metastasis of *Staphylococcus aureus*? *Nat Rev Microbiol* **9**:215-222.
- Ton-That H, Liu G, Mazmanian SK, Faull KF, Schneewind O.** 1999. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. *Proc Natl Acad Sci U S A* **96**:12424-12429.
- Tzagoloff H, Novick R.** 1977. Geometry of cell division in *Staphylococcus aureus*. *J Bacteriol* **129**:343-350.
- Uhlen M, Guss B, Nilsson B, Gatenbeck S, Philipson L, Lindberg M.** 1984. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *J Biol Chem* **259**:1695-1702.
- Upadhyay A, Burman JD, Clark EA, Leung E, Isenman DE, van den Elsen JM, Bagby S.** 2008. Structure-function analysis of the C3 binding region of *Staphylococcus aureus* immune subversion protein Sbi. *J Biol Chem* **283**:22113-22120.
- Valeva A, Walev I, Pinkernell M, Walker B, Bayley H, Palmer M, Bhakdi S.** 1997. Transmembrane beta-barrel of staphylococcal alpha-toxin forms in sensitive but not in resistant cells. *Proc Natl Acad Sci U S A* **94**:11607-11611.
- van Belkum A, Verkaik NJ, de Vogel CP, Boelens HA, Verveer J, Nouwen JL, Verbrugh HA, Wertheim HF.** 2009. Reclassification of *Staphylococcus aureus* nasal carriage types. *J Infect Dis* **199**:1820-1826.
- van Wamel WJ, Rooijackers SH, Ruyken M, van Kessel KP, van Strijp JA.** 2006. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. *J Bacteriol* **188**:1310-1315.
- Vaudaux PE, Francois P, Proctor RA, McDevitt D, Foster TJ, Albrecht RM, Lew DP, Wabers H, Cooper SL.** 1995. Use of adhesion-defective mutants of *Staphylococcus aureus* to define

- the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. *Infect Immun* **63**:585-590.
- Veiga H, Pinho MG.** 2009. Inactivation of the *SauI* type I restriction-modification system is not sufficient to generate *Staphylococcus aureus* strains capable of efficiently accepting foreign DNA. *Appl Environ Microbiol* **75**:3034-3038.
- Ventura CL, Malachowa N, Hammer CH, Nardone GA, Robinson MA, Kobayashi SD, DeLeo FR.** 2010. Identification of a novel *Staphylococcus aureus* two-component leukotoxin using cell surface proteomics. *PLoS one* **5**:e11634.
- Votintseva AA, Fung R, Miller RR, Knox K, Godwin H, Wyllie DH, Bowden R, Crook DW, Walker AS.** 2014. Prevalence of *Staphylococcus aureus* protein A (*spa*) mutants in the community and hospitals in Oxfordshire. *BMC Microbiol* **14**:63.
- Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, Porcella SF, Long RD, Dorward DW, Gardner DJ, Kreiswirth BN, Musser JM, DeLeo FR.** 2005. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* **175**:3907-3919.
- Waldron DE, Lindsay JA.** 2006. *SauI*: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. *J Bacteriol* **188**:5578-5585.
- Walport MJ.** 2001. Complement. First of two parts. *N Engl J Med* **344**:1058-1066.
- Walport MJ.** 2001. Complement. Second of two parts. *N Engl J Med* **344**:1140-1144.
- Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, Kennedy AD, Dorward DW, Klebanoff SJ, Peschel A, DeLeo FR, Otto M.** 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* **13**:1510-1514.
- Ward JB.** 1981. Teichoic and teichuronic acids: biosynthesis, assembly, and location. *Microbiol Rev* **45**:211-243.
- Weidenmaier C, Kokai-Kun JF, Kristian SA, Chanturiya T, Kalbacher H, Gross M, Nicholson G, Neumeister B, Mond JJ, Peschel A.** 2004. Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nat Med* **10**:243-245.
- Weidenmaier C, Kokai-Kun JF, Kulauzovic E, Kohler T, Thumm G, Stoll H, Gotz F, Peschel A.** 2008. Differential roles of sortase-anchored surface proteins and wall teichoic acid in *Staphylococcus aureus* nasal colonization. *Int J Med Microbiol* **298**:505-513.
- Weidenmaier C, Peschel A, Kempf VA, Lucindo N, Yeaman MR, Bayer AS.** 2005. DltABCD- and MprF-mediated cell envelope modifications of *Staphylococcus aureus* confer resistance to platelet microbicidal proteins and contribute to virulence in a rabbit endocarditis model. *Infect Immun* **73**:8033-8038.
- Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC.** 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* **302**:1569-1571.
- Weinrauch Y, Drujan D, Shapiro SD, Weiss J, Zychlinsky A.** 2002. Neutrophil elastase targets virulence factors of enterobacteria. *Nature* **417**:91-94.
- Weiss WJ, Lenoy E, Murphy T, Tardio L, Burgio P, Projan SJ, Schneewind O, Alksne L.** 2004. Effect of *srtA* and *srtB* gene expression on the virulence of *Staphylococcus aureus* in animal models of infection. *J Antimicrob Chemother* **53**:480-486.
- Weissman IL, Anderson DJ, Gage F.** 2001. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annu Rev Cell Dev Biol* **17**:387-403.
- Werbick C, Becker K, Mellmann A, Juuti KM, von Eiff C, Peters G, Kuusela PI, Friedrich AW, Sinha B.** 2007. *Staphylococcal* chromosomal cassette *mec* type I, *spa* type, and expression of

Pls are determinants of reduced cellular invasiveness of methicillin-resistant *Staphylococcus aureus* isolates. J Infect Dis **195**:1678-1685.

- Wertheim HF, Verveer J, Boelens HA, van Belkum A, Verbrugh HA, Vos MC.** 2005. Effect of mupirocin treatment on nasal, pharyngeal, and perineal carriage of *Staphylococcus aureus* in healthy adults. Antimicrob Agents Chemother **49**:1465-1467.
- Wertheim HF, Vos MC, Ott A, van Belkum A, Voss A, Kluytmans JA, van Keulen PH, Vandenbroucke-Grauls CM, Meester MH, Verbrugh HA.** 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet **364**:703-705.
- Wertheim HF, Walsh E, Choudhury R, Melles DC, Boelens HA, Miajlovic H, Verbrugh HA, Foster T, van Belkum A.** 2008. Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. PLoS Med **5**:e17.
- Wilke GA, Bubeck Wardenburg J.** 2010. Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury. Proc Natl Acad Sci U S A **107**:13473-13478.
- Xia G, Kohler T, Peschel A.** 2010. The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. Int J Med Microbiol **300**:148-154.
- Xu SY, Corvaglia AR, Chan SH, Zheng Y, Linder P.** 2011. A type IV modification-dependent restriction enzyme SauUSI from *Staphylococcus aureus* subsp. aureus USA300. Nucleic Acids Res **39**:5597-5610.
- Xu Y, Narayana SV, Volanakis JE.** 2001. Structural biology of the alternative pathway convertase. Immunol Rev **180**:123-135.
- Yamashita K, Kawai Y, Tanaka Y, Hirano N, Kaneko J, Tomita N, Ohta M, Kamio Y, Yao M, Tanaka I.** 2011. Crystal structure of the octameric pore of staphylococcal gamma-hemolysin reveals the beta-barrel pore formation mechanism by two components. Proc Natl Acad Sci U S A **108**:17314-17319.
- Yang YH, Jiang YL, Zhang J, Wang L, Bai XH, Zhang SJ, Ren YM, Li N, Zhang YH, Zhang Z, Gong Q, Mei Y, Xue T, Zhang JR, Chen Y, Zhou CZ.** 2014. Structural insights into SraP-mediated *Staphylococcus aureus* adhesion to host cells. PLoS Pathog **10**:e1004169.
- Yates JR, 3rd, McCormack AL, Schieltz D, Carmack E, Link A.** 1997. Direct analysis of protein mixtures by tandem mass spectrometry. J Protein Chem **16**:495-497.
- Yoong P, Torres VJ.** 2015. Counter inhibition between leukotoxins attenuates *Staphylococcus aureus* virulence. Nat Commun **6**:8125.
- Yung SC, Murphy PM.** 2012. Antimicrobial chemokines. Front Immunol **3**:276.
- Yung SC, Parenti D, Murphy PM.** 2011. Host chemokines bind to *Staphylococcus aureus* and stimulate protein A release. J Biol Chem **286**:5069-5077.
- Zapotoczna M, Jevnikar Z, Miajlovic H, Kos J, Foster TJ.** 2013. Iron-regulated surface determinant B (IsdB) promotes *Staphylococcus aureus* adherence to and internalization by non-phagocytic human cells. Cell Microbiol **15**:1026-1041.
- Zhang L, Jacobsson K, Vasi J, Lindberg M, Frykberg L.** 1998. A second IgG-binding protein in *Staphylococcus aureus*. Microbiology **144** (Pt 4):985-991.
- Zielinska AK, Beenken KE, Mrak LN, Spencer HJ, Post GR, Skinner RA, Tackett AJ, Horswill AR, Smeltzer MS.** 2012. *sarA*-mediated repression of protease production plays a key role in the pathogenesis of *Staphylococcus aureus* USA300 isolates. Mol Microbiol **86**:1183-1196.
- Zimmerli W, Trampuz A, Ochsner PE.** 2004. Prosthetic-joint infections. N Engl J Med **351**:1645-1654.
- Zimmermann R, Eyrich S, Ahmad M, Helms V.** 2011. Protein translocation across the ER membrane. Biochim Biophys Acta **1808**:912-924.

- Zong Y, Bice TW, Ton-That H, Schneewind O, Narayana SV.** 2004. Crystal structures of *Staphylococcus aureus* sortase A and its substrate complex. *J Biol Chem* **279**:31383-31389.
- Zong Y, Mazmanian SK, Schneewind O, Narayana SV.** 2004. The structure of sortase B, a cysteine transpeptidase that tethers surface protein to the *Staphylococcus aureus* cell wall. *Structure* **12**:105-112.
- Zong Y, Xu Y, Liang X, Keene DR, Hook A, Gurusiddappa S, Hook M, Narayana SV.** 2005. A 'Collagen Hug' model for *Staphylococcus aureus* CNA binding to collagen. *EMBO J* **24**:4224-4236.