

Investigating factors that contribute to the ability of *Staphylococcus aureus* to colonise atopic dermatitis skin

A thesis submitted for the degree of

Doctor of Philosophy

2019

by

Aisling Towell



Moyne Institute of Preventive Medicine

Department of Microbiology

School of Genetics and Microbiology

Trinity College Dublin

Declaration of Authorship

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university. It is the result of my own work and includes work done in collaboration which is specifically indicated here and in the text;

Chapter 3: Figure 3.17, Figure 3.18, Figure 3.25, Figure 3.26 Atomic force microscopy measuring bacterial adherence to corneocytes was performed by Dr. Cécile Formosa-Dague at the Louvain Institute of Biomolecular Science and Technology, Université catholique de Louvain, Belgium.

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.

I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish.

Aisling Towell

Acknowledgements

Firstly, my sincerest thanks to my supervisor Joan Geoghegan. I can't express the level of gratitude I have for the endless patience, support and encouragement you've given me the past number of years. After my master's project (for some unknown reason) you presented me with this opportunity and I fully attribute the scientist I am today, to your amazing teaching. You've always been available to help and I genuinely could not have had a better supervisor.

To all the members of the Geoghegan lab past and present, Daz, Lee, Orla, Joana, Keenan, Marta, Marty and Thaina; thank you for making the lab such a welcoming and friendly environment to work in. A big thank you to my lab-mom Dee for support during meltdowns when you just need a listening ear and a hug. Special thanks to Prof. Tim Foster for providing me with knowledge and advice, and of course for all the recent reviews! To all the staff of the Moyne, I'll be hard pressed to find a workplace full of friendlier people in the future.

Michelle, my science rock, we started this together and you're central to the best memories of my PhD. Through all the laughs and tears, I really have gained a best friend.

Mick and Marina, you led me down a dark but necessary rabbit hole straight to the pav that I've so much gratitude for (about a golden planets worth!). Amz and Dan -Amyizms, West Spain and the hierarchy have kept me sane and usually in tears laughing. You guys are stuck with me as a friend for life! To all the Moynester postgrads, you've made this place like a big happy family and I just have one thing to say (to Eoin in particular); 'Do you hear the people sing?'. Shoutout to Maz, always the Steve to my Ryan. Oisín (hey cutie), meeting you was just the cherry on top and the **perfect** way to end my PhD experience, fingerguns!

My friends, particularly Kat, Patsy, Mary, Aoife, Ro and Fiona, you've always been there for me, encouraging me all the way. You gave me welcome distractions and more importantly, helped pick me up when I needed it most. Thank you Emer and Fay, you got the brunt of my stress and helped ease it with garden parties and far too much munch from centra.

Finally thank you to my family. My siblings Ailbhe and Kevin, and Ciaran (soon to be official!), thank you for always being so supportive, even when I was a demon! Movie weekends at home were the perfect therapy, especially with little Brunz. To my wonderful parents, Andrew and Eimear, you've always believed in me unconditionally, through this PhD and in everything else before. I couldn't have done this without you.

Summary

The skin of atopic dermatitis (AD) patients is frequently colonised by *Staphylococcus aureus*. Colonisation by *S. aureus* correlates with disease severity and exacerbated disease symptoms. The factors involved in *S. aureus* colonisation of AD skin have not been fully described. This thesis aimed to identify molecular determinants contributing to the colonisation of AD skin by *S. aureus*.

A skin protein corneodesmosin (CDSN) is abnormally displaced in AD skin and is potentially more accessible to bacteria. Here, CDSN was identified as a novel ligand for *S. aureus*. To characterise the interaction between *S. aureus* and CDSN at the molecular level, an expression system was generated in *Escherichia coli* to allow for the manipulation of the *CDSN* gene and purification of GST-tagged recombinant CDSN (rCDSN) from bacteria. Allelic exchange was used to construct mutants of a clinically relevant AD strain AD08, deficient in ClfB and/or FnBPA and/or FnBPB to examine the role of these proteins. AD08 did not adhere to CDSN in the absence of ClfB and FnBPB indicating that these two proteins promote adherence to CDSN. Surface plasmon resonance was employed to compare binding of the recombinant ligand binding domain of ClfB, FnBPB and FnBPA to CDSN. Recombinant FnBPB and ClfB bound CDSN at similar affinities while recombinant FnBPA had a 10-fold higher affinity for CDSN. The disconnection between bacterial and recombinant data suggests that AD08 expressed an insufficient amount of FnBPA to promote adherence to CDSN under the conditions tested. Measuring *S. aureus* adherence to corneocytes isolated from AD patients showed that ClfB promotes adherence of exponential phase bacteria while FnBPA and/or FnBPB mediate attachment of stationary phase bacteria. The *S. aureus* binding site in CDSN was mapped to the N-terminal omega loop region and blocking this region with anti-CDSN antibody raised against the N-terminus of CDSN reduced *S. aureus* adherence to AD corneocytes implying that *S. aureus* targets CDSN to adhere to AD corneocytes.

Having shown that FnBPA and FnBPB bind to CDSN in vitro, the ability of these proteins to support bacterial adhesion to loricrin and cytokeratin 10

(K10) was examined. Both proteins are established ligands for ClfB however this study showed that *S. aureus* mutants deficient in FnBPA and/or FnBPB had reduced adherence to both ligands. Furthermore, overexpressing either FnBPA or FnBPB from a plasmid promoted bacterial adherence, showing that both proteins are capable of binding to loricrin and K10 independently.

S. aureus strains of clonal complex 1 (CC1) are frequently isolated from Irish and British AD patients and the factors contributing to CC1 persistence in AD skin are unknown. Here, it was shown that a panel of CC1 strains from AD (AD_{CC1}) adhered more strongly to CDSN than commensals isolated from the nasal cavity of healthy children. CC30 strains are prevalent commensals of the nose of the healthy population. Three *S. aureus* AD_{CC1} strains, and two CC30 commensal strains were labelled with antibiotic resistance genes and competitive growth assays were carried out to determine whether AD_{CC1} strains have a competitive advantage over CC30 strains. Two of the three AD_{CC1} strains outcompeted growth of both CC30 isolates. Furthermore, a mutant of CC1 strain AD08 deficient in the Type VII Secretion System (T7SS) nuclease toxin EsaD and its repertoire of EsaG anti-toxins lost the ability to outcompete one CC30 isolate which implies that the T7SS of *S. aureus* may function in intraspecies interference competition among natural isolates. Likely, the ability of AD_{CC1} strains to adhere strongly to CDSN in AD skin gives them a competitive advantage where they can then proliferate and outcompete other *S. aureus* isolates, in some cases potentially driven by substrates of the T7SS.

Finally, the link between staphylococci and AD is not specific to humans. Here, CDSN was identified as a novel ligand for *S. pseudintermedius*, a canine commensal that is associated with canine pyoderma. *In vitro* adherence assays using mutants of *S. pseudintermedius* demonstrated that adherence is mediated by cell wall-anchored proteins SpsD and SpsL, which bound to the N-terminal omega loop region and to another unidentified site in CDSN.

Publications

Fleury, O.M., McAleer, M.A., Feuillie, C., Formosa-Dague, Sansevere, E., Bennett, D.E., Towell, A.M., McLean, W.H.I., Kezic, S., Robinson, D.A., Fallon, P.G., Foster T.J., Dufrêne, Y.F., Irvine, A.D., and Geoghegan, J.A. (2017) Clumping Factor B Promotes Adherence of *Staphylococcus aureus* to Corneocytes in Atopic Dermatitis. *Infect Immun* **85**: e00994-16.

Lacey, K.A., Mulcahy, M.E., Towell, A.M., Geoghegan, J.A., and McLoughlin, R.M. (2019) Clumping factor B is an important virulence factor during *Staphylococcus aureus* skin infection and a promising vaccine target. *PLoS Pathog* **15**: e1007713.

Towell, A.M., Feuillie, C., Vitry, P., Fleury, O.M., McAleer, A.M., Dufrêne, Y.F., Irvine, A.D., and Geoghegan, J.A. Corneodesmosin is a key ligand for *Staphylococcus aureus* adhesion to corneocytes in atopic dermatitis. Manuscript in preparation.

Table of contents

Declaration of Authorship	i
Acknowledgements	ii
Summary	iii
Publications	v
Table of contents	vi
List of Figures	xi
List of Tables.....	xiii

Chapter 1 Introduction

1.1 Biological characteristics of staphylococci	2
1.2 <i>Staphylococcus aureus</i> colonisation	2
1.3 Infection	4
1.4 Virulence factors	6
1.4.1 Cell envelope components	6
1.4.2 Cell wall-anchored proteins	7
1.4.2.1 Clf/Sdr protein subfamily	13
1.4.2.1.1 Clumping Factor A.....	13
1.4.2.1.2 Clumping Factor B.....	14
1.4.2.1.3 Fibronectin-binding protein subfamily.....	15
1.4.2.2 Contribution of CWAPs to infection.....	18
1.4.2.2.1 Adherence to Fibrinogen	18
1.4.2.2.2 Adhesion to and Invasion of host cells	18
1.4.2.2.3 Immune evasion	19
1.4.2.2.4 Biofilm formation	20
1.4.3 <i>S. aureus</i> Type VII secretion System.....	21
1.4.3.1 EsaD and EsaG	22

1.5 Genetic manipulation of <i>S. aureus</i>	25
1.5.1 Allelic exchange.....	25
1.5.1.1 Temperature sensitive plasmids	25
1.5.1.2 Counter selection in <i>S. aureus</i>	25
1.5.1.3 Bypass of <i>S. aureus</i> restriction barriers	26
1.6 The stratum corneum of human skin	28
1.6.1 Cornification of keratinocytes	28
1.6.2 Proteins of the cornified envelope	30
1.6.2.1 Corneodesmosin.....	30
1.6.2.2 Loricrin.....	32
1.6.2.3 Cytokeratin 10.....	32
1.6.2.4 Filaggrin and the natural moisturising factor (NMF)	33
1.6.3 Defence mechanisms of the skin.....	34
1.6.3.1 Host defence mechanisms.....	34
1.6.3.2 Natural skin commensal organisms	35
1.6.4 Mechanisms of <i>S. aureus</i> survival on skin.....	36
1.7 Atopic dermatitis	37
1.7.1 Host genetic susceptibility	37
1.7.2 Skin barrier defect in atopic dermatitis.....	38
1.7.2 Immune Dysregulation.....	40
1.7.3 Models to investigate atopic dermatitis.....	43
1.7.4 The role of <i>S. aureus</i> in the pathogenesis of atopic dermatitis	43
1.7.4.1 <i>S. aureus</i> colonisation of atopic dermatitis skin	45
1.7.4.2 <i>S. aureus</i> toxins in atopic dermatitis	46
1.7.5 Current therapies for atopic dermatitis.....	47
1.8 Aims and Objectives	49

Chapter 2 Materials and Methods

2.1 Bacterial strains and culture conditions.....	51
2.2 DNA sequencing.....	51
2.2.1 Isolation of plasmid and genomic DNA.....	51
2.2.2 Polymerase Chain Reaction	51
2.2.3 Agarose gel electrophoresis	52
2.2.4 Sequence and ligase independent cloning	52
2.3 Transformation.....	53
2.3.1 Preparation and transformation of <i>E. coli</i>	53
2.3.2 Preparation and transformation of <i>S. aureus</i>	53
2.3.3 Screening transformants.....	54
2.4.1 Plasmid construction.....	54
2.4.2 Strain construction by allelic exchange.....	55
2.5 Protein electrophoresis and western immunoblotting	58
2.5.1 SDS-PAGE	58
2.5.2 Western immunoblotting	58
2.6 Protein purification	58
2.6.1 Purification of GST-tagged recombinant protein.....	59
2.6.2 Purification of His-tagged recombinant protein	59
2.6.3 Purification of Strep-tagged recombinant protein.....	60
2.6.4 Protein Concentration Determination.....	60
2.7 Solid phase bacterial adherence assays.....	60
2.8 Growth curves.....	61
2.9 Surface plasmon resonance	61
2.10 Generation of F(ab) ₂ fragments	61
2.11 Intraspecies bacterial competition assays.....	61

2.12 Atomic force microscopy.....	62
2.13 Statistical analysis	62

Chapter 3 Characterisation of the interaction between *Staphylococcus aureus* and corneodesmosin

3.1 Introduction.....	76
3.2 Results.....	80
3.2.1 <i>S. aureus</i> adheres to immobilized corneodesmosin	80
3.2.2 Construction, expression and purification of recombinant GST-CDSN	82
3.2.3 <i>S. aureus</i> AD _{CC1} isolates adhere more strongly to CDSN than commensal CC30 isolates.....	86
3.2.4 Construction and validation of a <i>clfB</i> null mutation in <i>S. aureus</i> AD08	88
3.2.5 Adherence of AD08 to CDSN is not solely dependent by ClfB	89
3.2.6 Construction and validation of FnBPA, FnBPB and ClfB deficient mutants of <i>S. aureus</i> AD08	92
3.2.7 ClfB and FnBPB mediate <i>S. aureus</i> adherence to CDSN	98
3.2.8 Investigating <i>S. aureus</i> adherence to rCDSN under different conditions	98
3.2.9 Carriage of <i>fnbB</i> in AD _{CC1} strains and nasal CC30 isolates.....	102
3.2.10 Purification of CC1-type recombinant His-tagged ClfB N2N3, FnBPA N2N3 and FnBPB N2N3	104
3.2.11 Recombinant ClfB N2N3, FnBPA N2N3 and FnBPB N2N3 bind to human CDSN	104
3.2.12 ClfB, FnBPA and FnBPB mediate <i>S. aureus</i> adherence to AD corneocytes	112
3.2.13 Localising the <i>S. aureus</i> binding site in CDSN	116
3.2.14 ClfB and FnBPB facilitate AD08 adherence to the N-terminal Ω loop of CDSN	117
3.2.15 Generation of anti-CDSN F(ab) ₂ fragments.....	123

3.2.16 More CDSN is detected on the surface of low NMF corneocytes ..	125
3.2.17 <i>S. aureus</i> targets CDSN to adhere to AD corneocytes	125
3.2.18 CDSN is not a ligand for coagulase-negative staphylococci	129
3.3 Discussion	131

Chapter 4 Molecular analysis of the interaction between staphylococci and corneocyte proteins

4.1 Introduction	138
4.2 Results.....	141
4.2.1 Late cornified envelope proteins are not ligands for <i>S. aureus</i>	141
4.2.2 Adherence of <i>S. aureus</i> to recombinant GST-tagged L2v	143
4.2.3 FnBPA and FnBPB mediate adherence to loricrin	145
4.2.4 Adherence of <i>S. aureus</i> to recombinant GST-HK10	149
4.2.5 Adherence of <i>S. aureus</i> to fibrinogen	153
4.2.6 Identification of CDSN as a novel ligand for <i>S. pseudintermedius</i> ...	155
4.2.7 Localising the <i>S. pseudintermedius</i> binding site in CDSN	157
4.2.8 SpsD and SpsL mediate <i>S. pseudintermedius</i> adherence to CDSN	160
4.3 Discussion	162

Chapter 5 Investigating the importance of *Staphylococcus aureus* intraspecies competition in colonisation of atopic dermatitis skin

5.1 Introduction	168
5.2 Results.....	171
5.2.1 Construction of pIMAY::Int. <i>S.aphA3</i> and pIMAY::Int. <i>S.ermAM</i>	171
5.2.2 Generation and validation of Kan ^r and Ery ^r labelled strains.....	173
5.2.3 Analysis of the effect of <i>aphA3</i> and <i>ermAM</i> carriage	176
5.2.4 Competitive growth of AD08 _{CC1} against TS1064 _{CC30} and TS1157 _{CC30}	180

5.2.5 Construction and Validation of AD08 Δ esaDG	184
5.3 Discussion	190
Chapter 6 Discussion	
6.1 Discussion	196
Chapter 7 References	
References	200

List of Figures

Figure 1.1 Structural organisation of staphylococcal MSCRAMM proteins.	17
Figure 1.2. <i>S. aureus</i> Type VII Secretion System.	24
Figure 1.3. Allelic exchange using pIMAY	27
Figure 1.4. Cornification in the epidermis.....	29
Figure 1.5. Physiological differences between the epidermis of healthy and atopic dermatitis skin.....	42
Figure 3.1. <i>S. aureus</i> Newman adheres to corneodesmosin.....	81
Figure 3.3. Purification of rCDSN	85
Figure 3.4. AD _{CC1} isolates adhere more strongly to CDSN than commensal CC30 isolates.....	87
Figure 3.5. Schematic representation of the <i>clfB</i> mutation.....	90
Figure 3.6. <i>S. aureus</i> adherence to immobilized rCDSN is not dependent on ClfB.	91
Figure 3.7. Schematic representation of <i>fnbA</i> and <i>fnbB</i> mutations.	95
Figure 3.8. Schematic representation of the <i>fnbA</i> restoration.	96
Figure 3.9. Validation of AD08 mutants.....	97
Figure 3.10. ClfB, FnBPA and FnBPB mediate <i>S. aureus</i> adherence to CDSN...100	

Figure 3.11. Adherence of <i>S. aureus</i> AD08 to rCDSN under variable bacterial growth conditions.	101
Figure 3.12. Carriage of <i>fnbB</i> in AD _{CC1} and CC30 nasal isolates. A	103
Figure 3.13. SDS-PAGE of rClfBN2N3 _{CC1} , rFnBPAN2N3 _{CC1} and rFnBPBN2N3 _{CC1}	107
Figure 3.14. rFnBPAN2N3 _{CC1} binds directly to His-CDSN.	108
Figure 3.15. rFnBPBN2N3 _{CC1} binds directly to His-CDSN.	109
Figure 3.16. rClfBN2N3 _{CC1} binds directly to His-CDSN.....	110
Figure 3.17. ClfB, FnBPA and/or FnBPB mediate adherence of exponential phase <i>S. aureus</i> to AD corneocytes.....	114
Figure 3.18. ClfB, FnBPA and/or FnBPB mediate adherence of stationary phase <i>S. aureus</i> toAD corneocytes.....	115
Figure 3.19. CDSN loop domain structure.....	118
Figure 3.21 Construction of CDSN N-terminal Ω loop.....	120
Figure 3.22. The N-terminal Ω loop of CDSN comprises the <i>S. aureus</i> binding site.....	121
Figure 3.23. ClfB and FnBPB facilitate AD08 adherence to the N-terminal Ω loop of CDSN.	122
Figure 3.24. Generation of anti-CDSN F(ab) ₂ fragments.....	124
Figure 3.25. F(ab) ₂ bind more strongly to low NMF corneocytes than to high NMF corneocytes.	127
Figure 3.26. Blocking the bacterial binding site in CDSN reduces <i>S. aureus</i> adherence to AD corneocytes.	128
Figure 3.27. CDSN is not a ligand for coagulase-negative staphylococci.	130
Figure 4.1. <i>S. aureus</i> AD08 does not adhere to recombinant LCE proteins.....	142
Figure 4.2. <i>S. aureus</i> adherence to loricrin is not solely mediated by ClfB.....	144
Figure 4.3. ClfB, FnBPA and FnBPB are capable of mediating <i>S. aureus</i> adherence to loricrin.....	147

Figure 4.4. ClfB, FnBPA and FnBPB mediate AD08 adherence to loricrin.....	148
Figure 4.5. ClfB, FnBPA and FnBPB mediate adherence of <i>S. aureus</i> to cytokeratin 10.....	151
Figure 4.6. ClfB and FnBPB mediate AD08 adherence to cytokeratin 10.	152
Figure 4.7. <i>S. aureus</i> adheres to fibrinogen in the absence of ClfB, FnBPA and FnBPB.....	154
Figure 4.8. <i>S. pseudintermedius</i> ED99 adheres to rCDSN	156
Figure 4.9. Clustal alignment of Human CDSN and Canine CDSN.....	158
Figure 4.10. <i>S. pseudintermedius</i> ED99 adheres to the N-terminal Ω loop of CDSN and to another unidentified region.....	159
Figure 5.1. Schematic representation of pIMAY::Int. <i>S.aphA3</i> _{CC30} construction....	172
Figure 5.2. Generation of Kan ^r and Ery ^r <i>S. aureus</i> strains.	174
Figure 5.4. <i>ermAM</i> and <i>aphA3</i> carriage do not effect AD08 fitness	178
Figure 5.5. <i>ermAM</i> and <i>aphA3</i> carriage do not effect bacterial fitness of TS1064 and TS1157.....	179
Figure 5.6. AD08 has increased growth compared to TS1064 and TS1157.	181
Figure 5.7. AD24 has increased growth compared to TS1064 and TS1157.	182
Figure 5.8. Growth of AD17 is similar to TS1064 and TS1157.....	183
Figure 5.9. Schematic representation of pIMAY:: Δ <i>esaDG</i> construction.	186
Figure 5.10. Validation of AD08 Δ <i>esaDG</i>	187
Figure 5.11. <i>esaD</i> and/or <i>esaG</i> and/or <i>esaG</i> homologues contribute to the competitive growth of AD08.	189
Figure 6.1. Proposed model of the molecular factors contributing to <i>S. aureus</i> colonisation of atopic dermatitis skin.....	197

List of Tables

Table 1.1. <i>S. aureus</i> cell wall-anchored proteins.....	10
--	----

Table 2.1. Bacterial strains.....	64
Table 2.2 Plasmids.....	69
Table 2.3 Primers	73
Table 3.1. Affinities of rFnBPAN2N3 _{CC1} , rFnBPBN2N3 _{CC1} , rClfBN2N3 _{CC1} for His-CDSN determined using surface plasmon resonance.	111

Key to Abbreviations

AD	Atopic dermatitis
AFM	Atomic force microscopy
Agr	Accessory gene regulator
AMP	Antimicrobial peptide
Am ^r	Ampicillin resistance
Bp	Base pair
CC	Clonal complex
CDSN	Corneodesmosin
CE	Cornified envelope
CFU	Colony forming unit
Clf	Clumping factor
Cm ^r	Chloramphenicol
CoNS	Coagulase-negative staphylococci
CWAP	Cell wall-anchored protein
DLL	Dock, lock and latch
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDC	Epidermal differentiation complex
Ery ^r	Erythromycin resistance
Fg	Fibrinogen
Fn	Fibronectin
FnBP	Fibronectin-binding protein
GST	Glutathione S-transferase
HBD	Human β -defensin
His	Hexahistidine
Ig	Immunoglobulin
IL	Interleukin
Int.S.	Integration site
Isd	Iron-regulated surface determinant

K	Keratin
Kan ^r	Kanamycin resistance
kb	Kilobase
kDa	kilodalton
KIF	Keratin intermediate filament
L2v	Loricrin loop 2 variant
LCE	Late cornified envelope protein
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
NMF	Natural moisturising factor
OD ₆₀₀	Optical density measured at 600 nm
PBS	Phosphate buffered saline
PCA	Pyrrolidone carboxylic acid
PCR	Polymerase chain reaction
PSM	Phenol soluble modulin
PVDF	Polyvinylidene fluoride
SAg	Superantigen
SC	Stratum corneum
SCFS	Single cell force spectroscopy
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SLIC	Sequence and ligase independent cloning
SPR	Surface plasmon resonance
Sps	<i>S. pseudintermedius</i> surface protein
T7SS	Type VII secretion system
UCA	Urocanic acid
VP	‘Villus-like’ projection
WT	Wild-type

Chapter 1

Introduction

1.1 Biological characteristics of staphylococci

Staphylococci are spherical, non-motile, Gram-positive organisms that are typically white or golden in colour and divide in three planes to form grape-like clusters when viewed under a microscope (Tzagoloff & Novick, 1977). Staphylococcal species in this genus are part of the phylum Firmicutes, class Bacilli and order Bacillales (Becker *et al.*, 2011). Closely related organisms include *Enterococcus*, *Bacillus* and *Listeria*. Typical characteristics of staphylococci include a genome consisting of a low guanine and cytosine content (30–39%), a high level of halotolerance and resistance to desiccation (Ludwig *et al.*, 1985, Stackebrandt & Teuber, 1988, Parfentjev & Catelli, 1964).

Staphylococci are divided into two groups based on their ability to produce coagulases; coagulase-positive staphylococci and coagulase-negative staphylococci. Coagulase-positive staphylococci include *Staphylococcus aureus* (Fahlberg & Marston, 1960), *S. argenteus*, *S. schweitzeri* (Tong *et al.*, 2015b), *S. intermedius*, *S. delphini* and *S. pseudintermedius* (Bannoehr *et al.*, 2007). The coagulase-negative staphylococci (CoNS) comprise >35 species including the human skin commensal, *S. epidermidis* as well as other human commensals such as *S. hominis*, *S. lugdunensis* and *S. haemolyticus*. Historically, CoNS were considered less pathogenic than *S. aureus* however recent evidence demonstrates that CoNS cause serious human infections, particularly in a nosocomial setting. *S. epidermidis* for example, is one of the leading causes of indwelling medical device related infections (Becker *et al.*, 2014, Sievert *et al.*, 2013). *S. aureus* is visibly distinguishable from other staphylococci through the production of the orange carotenoid pigment staphyloxanthin, which gives *S. aureus* a distinctive golden colour (Marshall & Wilmoth, 1981).

1.2 *Staphylococcus aureus* colonisation

S. aureus is a commensal organism that colonises the nasal cavity of the healthy human population. *S. aureus* nasal carriage is divided into two classes; 20% of the population are persistently colonised and the remaining 80% are intermittent carriers (van Belkum *et al.*, 2009). The difference in persistent and intermittent carriage is likely influenced by host genetic factors. Risk factors for

persistent carriage include single nucleotide polymorphisms (SNPs) in genes associated with immunity such as genes encoding the glucocorticoid receptor NR3C1, complement factor H, C-reactive proteins, interleukin-4 and β -Defensin-1 (van den Akker *et al.*, 2006, Emonts *et al.*, 2008, Ruimy *et al.*, 2010, Zanger *et al.*, 2011). It's possible that these SNPs allow bacteria to overcome the immune response in the nasal cavity.

Within *S. aureus*, strains are divided into groups comprising genetically related strains. This is achieved by molecular typing methods such as multilocus sequence typing (MLST). MLST involves sequencing seven housekeeping genes (*araC*, *aroE*, *glpF*, *gmK*, *pta*, *tpiA* and *ypjL*) and isolates are assigned sequence types based on their allelic profile (Enright *et al.*, 2000). Sequence types comprising isolates where at least five of the seven gene sequences are closely related are grouped into clonal complexes (CCs) (Feil *et al.*, 2003). Specific CCs are uniquely adapted to colonise different niches. Several studies have shown that strains of CC30 are associated with colonisation of the nares of healthy humans (Fleury *et al.*, 2017, Rasmussen *et al.*, 2013, Holtfreter *et al.*, 2007, Melles *et al.*, 2004, Feil *et al.*, 2003, Argudin *et al.*, 2013). The molecular factors that give CC30 strains an advantage in the nasal cavity have not been fully described however there is an association between persistent colonisation and attenuated virulence. CC30 commensal isolates have reduced activity of the global regulator Agr and a reduction in toxic activity by α -haemolysin due to conserved polymorphisms in the *agrC* and *hla* genes respectively (DeLeo *et al.*, 2011). *hla* mutants were associated with reduced toxicity to and prolonged survival on airway epithelial cells *in vitro* suggesting that α -haemolysin deficiency may be beneficial for long-term colonisation (Kiedrowski *et al.*, 2016). Despite this apparent attenuated virulence, persistent colonisation in itself is a risk factor for infection as strains involved in infection frequently result from an individual's autologous strain (von Eiff *et al.*, 2001, Wertheim *et al.*, 2004, Munoz *et al.*, 2008). Furthermore, genomic analysis of *S. aureus* core genomes suggests that strains involved in infection are also frequently associated with colonisation (Lindsay *et al.*, 2006). Conversely, individuals may develop immunity to their autologous strain as non-

carriers have reduced survival rates from *S. aureus* mediated bacteraemia-related deaths compared to persistent carriers (Wertheim *et al.*, 2004).

The predominant site of colonisation by *S. aureus* is the moist squamous epithelium of the anterior nares which is made up of layers of dead desquamated epithelial cells (Mulcahy *et al.*, 2012, Cole *et al.*, 2001, Peacock *et al.*, 2001). Attachment to the anterior nares relies on interactions between *S. aureus* cell wall-anchored proteins (CWAPs) and host ligands, specifically clumping factor B (ClfB), which mediates *S. aureus* adherence to the skin protein loricrin *in vivo* (Mulcahy *et al.*, 2012). Other *S. aureus* CWAPs have been implicated in colonisation including the iron-regulated surface determinant protein A (IsdA) which mediates bacterial adherence to desquamated epithelial cells *in vitro* (Corrigan *et al.*, 2009, Clarke & Foster, 2008) and to the nasal cavity of cotton rats (Clarke *et al.*, 2006). Surface protein G (SasG) (Corrigan *et al.*, 2007, Roche *et al.*, 2003), and serine aspartate repeat proteins C and D (SdrC and SdrD) (Corrigan & Foster, 2009) also mediate bacterial adherence to desquamated epithelial cells *in vitro* however the host ligand(s) for the majority of these interactions have not been proven *in vivo*. It is likely that SdrD facilitates adherence by binding to the desmosome protein desmoglein-1 (Askarian *et al.*, 2016). *S. aureus* can also colonise deeper in the nasal cavity where adherence is dependent on wall teichoic acid (WTA) which binds to a type F-scavenger receptor SREC-1 expressed by the ciliated epithelial cells in the posterior nares. Blocking the interaction of WTA and SREC-1 in a rat model reduced colonisation of *S. aureus* (Baur *et al.*, 2014).

1.3 Infection

S. aureus is an opportunistic pathogen in humans that is capable of causing a wide range of diseases. The most common infections caused by *S. aureus* include superficial soft skin and tissue infections such as styes, boils and impetigo (Foster, 1996). *S. aureus* can also cause more severe and potentially life threatening infections if it breaches the skin or mucosal barrier including deep-seated abscesses. Upon entry to the bloodstream, *S. aureus* can disseminate through the body and cause serious infections including sepsis, endocarditis, osteomyelitis and toxic shock syndrome. This is

particularly prevalent in a nosocomial setting where *S. aureus* gains entry to the bloodstream through indwelling medical devices such as catheters and intravenous lines (Tong *et al.*, 2015a, Lowy, 1998).

The success of *S. aureus* as a disease-causing pathogen can be attributed to its vast array of virulence factors including CWAPs and toxin activity. The role of CWAPs in *S. aureus* infection is discussed in 1.4.2.2. *S. aureus* secretes a number of cytolytic toxins that contribute to infection by directly damaging host cells and tissues and evading immune responses. Pore-forming toxins α -toxin, β -toxin, LukAB, LukED, HlgAB, HlgCB and PVL cause cell lysis by forming pores in the membrane of host cells including erythrocytes, leukocytes, platelets, endothelial cells and epithelial cells (Valeva *et al.*, 1997, Alonzo & Torres, 2014, Berube & Bubeck Wardenburg, 2013), which can promote bacterial escape from within a cell or help protect the bacteria from phagocytosis. *S. aureus* superantigens (SAGs) including enterotoxins and toxic shock syndrome toxin (TSST) bind to major histocompatibility complex II (MHCII) molecules on the surface of immune cells triggering hyper T-cell activation and a cytokine storm that can lead to the potentially fatal disease toxic shock syndrome (Krakauer *et al.*, 2016, Holtfreter & Broker, 2005). Phenol soluble modulins (PSMs), PSM α peptides, PSM β peptides and δ -toxin, have multiple functions that contribute to *S. aureus* infection including lysing erythrocytes and leukocytes, stimulating release of proinflammatory cytokines and mediating biofilm dispersal (Peschel & Otto, 2013, Syed *et al.*, 2015, Periasamy *et al.*, 2012).

Treatment of invasive *S. aureus* infection is complicated by antibiotic resistance. Resistance to β -lactam antibiotics dates back to the introduction of penicillin as an antibiotic. β -lactam antibiotics work by inhibiting the transpeptidation step during peptidoglycan biosynthesis (Bycroft, 1985). Resistance to penicillin occurs by expression of β -lactamase, encoded by the *blaZ* gene. This enzyme hydrolyses the β -lactam ring of β -lactam antibiotics (Lowy, 2003). This led to the synthesis of methicillin which is a penicillin derivative that is not sensitive to β -lactamase. However, methicillin resistant *S. aureus* (MRSA) isolates were in circulation as they had been selected for by widespread use of penicillin (Harkins *et al.*, 2017). Acquisition of a mobile

genetic element termed staphylococcal cassette chromosome *mec* (SCC*mec*) by MRSA strains confers resistance to all β -lactam antibiotics most commonly via the *mecA* gene that encodes the transpeptidase penicillin-binding-protein 2a (Chambers, 1997, Liu *et al.*, 2016).

Currently, MRSA strains are grouped into two groups; healthcare associated MRSA (HA-MRSA) and the more virulent community acquired MRSA (CA-MRSA) (Rudkin *et al.*, 2012). CA-MRSA strains are particularly successful pathogens due to an increased repertoire of virulence factors often expressed by genes located on mobile genetic elements including the Panton-Valentine leucocidin toxin (PVL) and arginine catabolic mobile element (ACME) (Diep *et al.*, 2008, Shukla *et al.*, 2012). ACME aids bacterial survival in the presence of spermidine and spermine polyamines present on human skin via expression of the *speG* gene encoding spermidine acetyltransferase (SpeG) (Planet *et al.*, 2013). It is important for virulence as a mutant of a CA-MRSA isolate lacking this element had attenuated pathogenicity in a rabbit model of bacteraemia (Diep & Otto, 2008). Furthermore, ACME carries the *copX* and *copL* genes which confer hyper resistance to copper, thus contributing to the virulence of CA-MRSA by protecting the bacteria from the antibacterial toxic effects of copper within host immune cells (Purves *et al.*, 2018). PVL contributes to *S. aureus* infection by secreting pore-forming components resulting in neutrophil lysis (Loffler *et al.*, 2010).

1.4 Virulence factors

1.4.1 Cell envelope components

Peptidoglycan accounts for approximately 60% of the *S. aureus* cell wall and consists of alternating disaccharide subunits comprising *N*-acetylmuramic acid-(β 1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc) (Ghuysen & Strominger, 1963). Tetrapeptides (L-Ala-D-Glu-L-Lys-D-Ala) act to cross link the MurNAc moieties in the glycan chains generating a rigid three dimensional structure (Ghuysen *et al.*, 1965). Penicillin-binding proteins catalyse a reaction called transpeptidation where the tetrapeptides on adjacent glycan strands are crosslinked. *S. aureus* peptidoglycan can be cleaved by the endopeptidase

lysostaphin due to the presence of pentaglycine bridges (Schleifer & Kandler, 1972).

Teichoic acids include wall teichoic acid (WTA) and lipoteichoic acid (LTA). WTA is composed of ribitol-phosphate polymers substituted with N-acetylglucosamine and D-alanine residues. It is covalently anchored to the N-acetylmuramic acid in peptidoglycan via a disaccharide unit (Brown *et al.*, 2013). LTA consists of a poly(glycerol-phosphate) backbone and glycolipid anchor to attach to the cytoplasmic membrane (Xia *et al.*, 2010). WTA and LTA are required for structural integrity of the cell envelope (Schlag *et al.*, 2010, Corrigan *et al.*, 2011).

Several *S. aureus* strains produce a capsule polysaccharide (CP) which is covalently linked to the cell wall. Serotypes 5 and 8 are predominant in clinical isolates. Expression of CP provides *S. aureus* with protection from phagocytosis (O'Riordan & Lee, 2004) and strains expressing CP have increased virulence in animal infection models (Thakker *et al.*, 1998, Luong *et al.*, 2002).

1.4.2 Cell wall-anchored proteins

The surface of staphylococci is decorated by a number of cell wall-anchored proteins (CWAPs). *S. aureus* can express up to 25 different CWAPs. Following translation, CWAPs are directed to the secretory (Sec) apparatus due to the presence of a signal sequence in the N-terminus of the protein where they are translocated across the bacterial membrane and covalently linked to the peptidoglycan in the cell wall. The signal sequence is cleaved by membrane-anchored signal peptidase enzymes upon translocation across the membrane (Mazmanian *et al.*, 2001). CWAPs contain a sorting signal (SS) in a carboxyl terminal domain including an LPXTG motif for recognition by a membrane bound sortase enzyme. This is followed by a hydrophobic membrane spanning domain and several positively charged residues that hold the protein within the cell membrane before it is finally covalently attached the peptidoglycan precursor lipid II by sortase (Schneewind *et al.*, 1993, Clancy *et al.*, 2010, Marraffini *et al.*, 2006). The majority of *S. aureus* CWAPs are covalently anchored by sortase A (Ton-That *et al.*, 1999) which cleaves the

threonine and glycine residues of the LPXTG motif. Sortase A attaches the cleaved protein via the threonine to the uncrosslinked pentaglycine bridge of the peptidoglycan precursor lipid II (Mazmanian *et al.*, 2001). *S. aureus* sortase B is responsible for anchoring the iron-regulated surface determinant C (IsdC) protein by recognition of a C-terminal NPQTN containing sorting signal (Mazmanian *et al.*, 2002). Sortase A is critical for virulence as *srtA* mutants of *S. aureus* are attenuated in infection models such as sepsis and endocarditis (Jonsson *et al.*, 2002, Weiss *et al.*, 2004) and in murine and rat models of colonisation (Schaffer *et al.*, 2006, Weidenmaier & Peschel, 2008).

S. aureus CWAPs have been classified into five families based on conserved structural domains (Speziale *et al.*, 2014). The microbial surface components recognising adhesive matrix molecules (MSCRAMM) proteins share a common domain structure with two tandem IgG-like folded subdomains followed by a long unstructured repeat region (section 1.4.2.1) (Foster *et al.*, 2014). MSCRAMM CWAPs are expressed by multiple staphylococcal strains and several MSCRAMMs bind to common ligands. Two *S. pseudintermedius* wall-anchored proteins SpsD and SpsL have a typical MSCRAMM domain structure (Figure 1.1 C, D) (Pietrocola *et al.*, 2015) and adhere to *S. aureus* ligands fibrinogen, fibronectin and for SpsD, cytokeratin 10 (Geoghegan *et al.*, 2009, Bannoehr *et al.*, 2011). The near iron transporter (NEAT) motif family consists of iron regulated surface determinant (Isd) proteins IsdA, IsdB, IsdC and IsdH which are involved in iron acquisition from haem and haemoglobin. This is facilitated by the presence of one or more NEAT motifs (Foster *et al.*, 2014, Grigg *et al.*, 2007, Andrade *et al.*, 2002). IsdA, IsdB and IsdH are anchored to the cell wall by sortase A (Ton-That *et al.*, 1999) while IsdC is covalently linked by sortase B (Mazmanian *et al.*, 2002). *S. aureus* surface protein G (SasG) is the only member of the G5-E repeat family found in *S. aureus*. It harbours an N-terminal A domain containing a legume-lectin like domain (Speziale *et al.*, 2014) followed by a repeat region of G5 domains separated by independently folded domains known as E regions (Foster *et al.*, 2014). The legume-lectin fold is found in the serine-rich adhesin for platelets (SraP) and SasG, and the three-helical bundle domains occur in 4-5 copies in

staphylococcal protein A (SpA). Functions of *S. aureus* CWAPs are listed in Table 1.1.

Table 1.1. *S. aureus* cell wall-anchored proteins

Protein group	Ligand	Function	Reference
MSCRAMMs			
Clumping factor (Clf) A	Fibrinogen γ -chain	Adherence to fibrinogen; Immune evasion	(Ganesh <i>et al.</i> , 2008)
	Complement factor I	Cleavage of C3b	(Hair <i>et al.</i> , 2010)
ClfB	Fibrinogen α -chain, Keratin 10	Adherence to fibrinogen and cytokeratin 10	(Ni Eidhin <i>et al.</i> , 1998, Walsh <i>et al.</i> , 2004)
	Loricrin	Nasal colonisation	(Mulcahy <i>et al.</i> , 2012)
Serine aspartate repeat (Sdr) protein C	Unknown	Adherence to desquamated epithelial cells	(Corrigan <i>et al.</i> , 2009)
	β -neurexin	Unknown	(Barbu <i>et al.</i> , 2010)
	SdrC	Biofilm formation	(Barbu <i>et al.</i> , 2014, Feuillie <i>et al.</i> , 2017)
SdrD	Desmoglein 1	Adherence to desquamated epithelial cells	(Askarian <i>et al.</i> , 2016, Corrigan <i>et al.</i> , 2009)
SdrE/Bbp	Complement factor H	Immune evasion; Cleavage of C3b	(Sharp <i>et al.</i> , 2012)
	C4b binding protein	Immune evasion; Cleavage of C4b	(Hair <i>et al.</i> , 2013)
	Fibrinogen α -chain	Adherence to the extracellular matrix (ECM)	(Vazquez <i>et al.</i> , 2011)
Collagen adhesin (Cna)	Collagen triple helix	Adherence to collagen-rich tissue	(Zong <i>et al.</i> , 2005)
	Complement protein C1q	Immune evasion; Inhibition of classical complement pathway	(Kang <i>et al.</i> , 2013)
Fibronectin binding protein (FnBP) A	Fibronectin	Adherence to fibronectin; Invasion of host cells	(Greene <i>et al.</i> , 1995, Liang <i>et al.</i> , 2016)
	Fibrinogen γ -chain, elastin	Adherence to the ECM	(Keane <i>et al.</i> , 2007, Roche <i>et al.</i> , 2004)
	Plasminogen	Immune evasion; Cleavage of IgG and C3b	(Pietrocola <i>et al.</i> , 2016)
	FnBPA	Biofilm formation	(Herman-Bausier <i>et al.</i> , 2015)

FnBPB	Fibronectin	Adherence to fibronectin; Invasion of host cells	(Greene <i>et al.</i> , 1995, Liang <i>et al.</i> , 2016)
	Fibrinogen γ -chain, elastin	Adherence to the ECM	(Burke <i>et al.</i> , 2011, Roche <i>et al.</i> , 2004)
	Plasminogen	Immune evasion; Cleavage of IgG and C3b	(Pietrocola <i>et al.</i> , 2016)
	FnBPB	Biofilm formation	(O'Neill <i>et al.</i> , 2008)
	Histones	Immune evasion; Prevents membrane damage	(Pietrocola <i>et al.</i> , 2019)
NEAT motif family			
Iron-regulated surface determinant (Isd) protein A	Haem	Iron acquisition	(Grigg <i>et al.</i> , 2007)
	Unknown ligand	Adherence to desquamated epithelial cells	(Corrigan <i>et al.</i> , 2009)
	-	Resistance to lipids and antimicrobial peptides	(Clarke <i>et al.</i> , 2007)
	-	Resistance to H ₂ O ₂ in neutrophils	(Palazzolo-Ballance <i>et al.</i> , 2008)
IsdB	Haem, Haemoglobin	Iron acquisition	(Grigg <i>et al.</i> , 2010)
	β 3 integrins	Invasion of host cells	(Zapotoczna <i>et al.</i> , 2013)
IsdC	Haem	Iron acquisition	(Villareal <i>et al.</i> , 2008)
IsdH	Haem, Haemoglobin	Iron acquisition	(Grigg <i>et al.</i> , 2010)
	Complement factor I	Immune evasion; Cleavage of C3b	(Visai <i>et al.</i> , 2009)
G5-repeat family			
<i>S. aureus</i> surface protein G (SasG)	Unknown ligand	Adherence to desquamated nasal epithelial cells	(Corrigan <i>et al.</i> , 2007)
	SasG	Biofilm formation	(Geoghegan <i>et al.</i> , 2010)

<i>Three helical bundle family</i>			
Protein A	IgG	Immune evasion; Inhibition of opsonophagocytosis	(Atkins et al., 2008)
	V _H 3 IgM	Immune modulation; B-cell expansion	(Goodyear & Silverman, 2003)
	TNFR1	Immune modulation; Proinflammatory	(Gomez et al., 2004)
	Von Willebrand factor	Adhesion to endothelial cells; Infective endocarditis	(Gomez et al., 2006)
<i>Legume-lectin domain family</i>			
Serine-rich adhesin for platelets (SraP)	N-acetylneuraminic acid	Invasion of epithelial cells	(Yang et al., 2014)
	Unknown ligand on platelets	Infective endocarditis	(Siboo et al., 2005)

1.4.2.1 Clf/Sdr protein subfamily

The Clf/Sdr subfamily of the MSCRAMMs in *S. aureus* consists of clumping factors A (ClfA) and B (ClfB) and the serine aspartate repeat (Sdr) proteins (SdrC, SdrD and SdrE) (Foster *et al.*, 2014). These proteins have similar domain structure with an N-terminal signal sequence, followed by a ligand binding A domain comprising N1, N2 and N3 subdomains where N2 and N3 consist of tandem IgG-like folds (Foster & Hook, 1998, Foster *et al.*, 2014). Between the N1 and N2 subdomain of ClfA lies a secretion motif that is necessary for export of the protein (McCormack *et al.*, 2014). No function of subdomain N1 has been identified. The N2 N3 subdomains function in ligand binding (Ganesh *et al.*, 2011). The A domain is followed by a flexible unstructured region (R) composed of serine-aspartate repeats. This region acts as a stalk to project the A domain from the surface of the cell. The R region is followed by a wall spanning region and sorting signal (Figure 1.1A) (Foster *et al.*, 2014).

1.4.2.1.1 Clumping Factor A

The archetypal MSCRAMM protein clumping factor A (ClfA) was the first fibrinogen-binding protein described in *S. aureus* and it binds to the C-terminus of the γ -chain of fibrinogen (Fg) (McDevitt *et al.*, 1997). The crystal structure of ClfA in complex with a Fg peptide has been solved in the apo form with the ligand peptide bound and shows that ClfA binding to Fg occurs by a mechanism known as 'dock, lock and latch' (DLL) (Deivanayagam *et al.*, 2002). This is described in detail in section 1.4.2.1.4 ClfA binding Fg shields bacteria from immune cells and protects *S. aureus* from phagocytosis by macrophages and neutrophils (Higgins *et al.*, 2006). ClfA also binds to complement factor I, to increase cleavage of the immune complement component C3b into inactive C3b resulting in reduced phagocytosis of *S. aureus* (Hair *et al.*, 2008). The role of ClfA as a potent virulence factor contributing to *S. aureus* infection has been demonstrated in several animal models. ClfA deficient *S. aureus* mutants were less pathogenic compared to wild-type strains in a rat model of endocarditis (Moreillon *et al.*, 1995), a murine model of arthritis (Josefsson *et al.*, 2001) and a murine model of sepsis (McAdow *et al.*, 2011) indicating that ClfA contributes to a range of *S. aureus* infections.

1.4.2.1.2 Clumping Factor B

Clumping factor B (ClfB) is encoded by the *clfB* gene carried by all sequenced *S. aureus* strains (Lindsay *et al.*, 2006). The amino acid sequence of the ClfB protein is identical within strains of the same clonal complex however variation exists between clonal complexes (Murphy *et al.*, 2011). While the SD repeat region is highly conserved between ClfA and ClfB, the ligand-binding A domain shares only 26% amino acid identity. Fg was the first ligand identified for ClfB (Ni Eidhin *et al.*, 1998). Unlike ClfA, which binds to the γ -chain of Fg, the ClfB binding site has been mapped to a repeat region in the α C domain at the C-terminus of the A α chain (Walsh *et al.*, 2008). ClfB also binds to the stratum corneum protein cytokeratin 10 (K10). The binding site was mapped to a region present in the V2 subdomain of the C-terminal tail region of K10. This region is known as the 'YY' loop and consists of quasi-repeats of Y(glycine/serine)_nY which form an omega loop (Ω) (Walsh *et al.*, 2004, Zhou *et al.*, 1988). Another ligand for ClfB is the cornified envelope protein loricrin which, similar to K10, is composed of glycine/serine rich Ω loops. Analysis of ClfB binding to different loop regions of loricrin showed that ClfB has binding sites throughout the protein and binds with the highest affinity to loop region 2v (L2v) (Mulcahy *et al.*, 2012). ClfB binds to Fg, K10 and loricrin by the DLL mechanism (Ni Eidhin *et al.*, 1998, Walsh *et al.*, 2004, Mulcahy *et al.*, 2012, Ganesh *et al.*, 2011).

ClfB has a fundamental role in nasal colonisation by *S. aureus*. ClfB facilitates bacterial adherence to desquamated nasal epithelial cells *in vitro* (Corrigan *et al.*, 2009) and ClfB deficient mutants of *S. aureus* have reduced colonisation in both murine models and human volunteers (Mulcahy *et al.*, 2012, Schaffer *et al.*, 2006, Wertheim *et al.*, 2008). In a murine model of *S. aureus* colonisation, the interaction between ClfB and loricrin was shown to be necessary for colonisation as loricrin^{-/-} mice were colonised poorly (Mulcahy *et al.*, 2012). ClfB has also been implicated in skin colonisation, specifically colonisation of atopic dermatitis skin as it has been shown that ClfB promotes *S. aureus* adherence to corneocytes isolated from AD patients (Fleury *et al.*, 2017, Feuillie *et al.*, 2018).

1.4.2.1.3 Fibronectin-binding protein subfamily

Fibronectin binding protein A (FnBPA) and B (FnBPB) are closely related CWAPs of *S. aureus* which were initially characterised based on their ability to bind to the extracellular matrix (ECM) and plasma glycoprotein fibronectin (Fn). The genes encoding FnBPA and FnBPB, *fnbA* and *fnbB* respectively, lie adjacent on the chromosome but are independently transcribed (Signas *et al.*, 1989, Jonsson *et al.*, 1991, Greene *et al.*, 1995). While the majority of *S. aureus* strains carry both *fnbA* and *fnbB*, some strains carry only *fnbA* (Peacock *et al.*, 2000). FnBPA and FnBPB (FnBPs) have a similar domain structure to other MSCRAMM proteins with an N-terminal A domain divided into N1, N2 and N3 subdomains related to ClfA (Deivanayagam *et al.*, 2002), followed by a disordered repeat region comprising 11 (FnBPA) or 10 (FnBPB) tandemly repeated Fn-binding motifs (Figure 1.1B) (Schwarz-Linek *et al.*, 2003). The Fn-repeat region is highly conserved between FnBPA and FnBPB with 95% shared amino acid identity (Burke *et al.*, 2010). There is greater variation in the A domain N2 N3 subdomains and seven allelic variants of both FnBPA and FnBPB have been identified. Despite this sequence variation, the ligand-binding activity by the A domain is conserved across all isotypes (Loughman *et al.*, 2008b, Burke *et al.*, 2010).

FnBP ligand binding contributes to several aspects of *S. aureus* virulence including intracellular invasion of host cells, adherence to host cells and tissues, immune evasion and biofilm formation (Foster, 2016). FnBP binding to Fn is primarily facilitated by the Fn-binding repeat region. The binding site in Fn has been mapped to a region in the N-terminus comprising five type-1 modules. Binding occurs by a tandem β -zipper mechanism (Schwarz-Linek *et al.*, 2003) where the repeated Fn-binding motifs form anti-parallel strands along adjacent Fn modules. An individual FnBP can bind up to nine Fn modules (Bingham *et al.*, 2008) and the Fn-binding repeats bind to the type-1 modules with different affinities (Meenan *et al.*, 2007). FnBP mediated adherence to Fn can facilitate intracellular invasion of non-phagocytes through the formation of a Fn-bridge between *S. aureus* and $\alpha 5\beta 1$ integrins expressed on the surface of host cells (Sinha *et al.*, 1999, Fowler *et al.*, 2000).

Similar to ClfA, the N2 N3 subdomains of FnBPs facilitate binding to the C-terminal γ -chain of Fg. However unlike ClfA, the N2 N3 subdomains of FnBPs also bind to the ECM protein elastin. Binding to both ligands likely occurs by the DLL mechanism (Stemberk *et al.*, 2014, Wann *et al.*, 2000, Roche *et al.*, 2004). A further ligand of FnBPs is the plasmin precursor plasminogen, an interaction that presumably contributes to *S. aureus* immune evasion. Binding occurs by an unknown mechanism that for FnBPB is dependent on the N3 subdomain (Pietrocola *et al.*, 2016). FnBPB also contributes to immune evasion by binding antibacterial histones secreted from neutrophils (Pietrocola *et al.*, 2019).

1.4.2.1.4 'Dock, lock and latch'

The first report of the 'dock, lock and latch' mechanism described binding of the *S. epidermidis* protein SdrG binding to Fg (Ponnuraj *et al.*, 2003). Binding of SdrG to Fg by DLL forms an extremely stable bond that rivals that of a covalent bond in terms of mechanical strength (Milles *et al.*, 2018). Comparison of crystal structures of SdrG both in apo-form and in complex with a synthetic peptide mimicking the Fg binding site allowed in-depth analysis into the molecular dynamics of the interaction. The SdrG apo-protein comprises a ligand-binding trench located between the N2 and N3 subdomains of the A domain. Docking of a ligand into the exposed trench results in a conformational change and a C-terminal extension in the N3 subdomain, known as the latching peptide, is redirected to cover the bound ligand in the trench thus locking it in place. Hydrophobic interactions between the N3 extension and the bound peptide stabilise the structure (locking) and residues on the N3 extension interact with a trench on the surface of the N2 domain by β -strand complementation (latching) (Ponnuraj *et al.*, 2003, Bowden *et al.*, 2008). *S. aureus* MSCRAMMs have a similar domain structure to SdrG, particularly in the A domain and it is now established that many MSCRAMM-ligand interactions occur by DLL. The crystal structure of the N2 N3 subdomains of ClfB was solved in apo-form and in complex with Fg and K10 peptides which confirmed that ClfB ligand-binding occurs by DLL (Ganesh *et al.*, 2011, Xiang *et al.*, 2012).

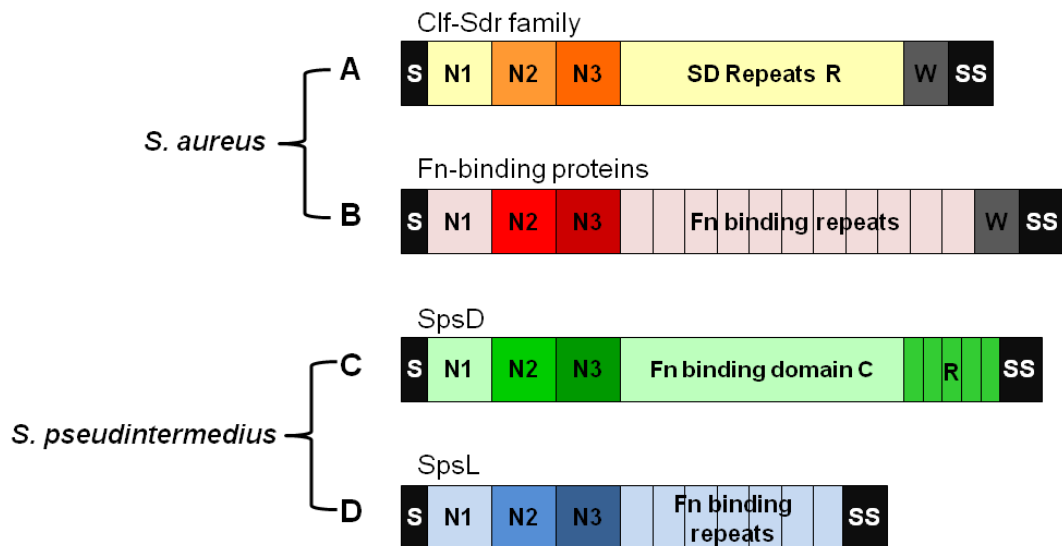


Figure 1.1 Structural organisation of staphylococcal MSCRAMM proteins.

Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) have an N-terminal signal sequence (S) for secretion via the Sec machinery. This is followed by an A domain comprising three separately folded subdomains N1, N2 and N3 where N2 and N3 consist of IgG-like folds. Each protein possesses a C-terminal sorting signal (SS) for anchoring to the cell wall. The region lying between the A domain and SS differs between the four protein subfamilies shown here and is comprised of **A)** a repeat region (R) of serine-aspartate (SD) dipeptide repeats, **B)** Fibronectin-binding (Fn) repeats or **D)** putative Fn-binding repeats. **C)** A connecting domain that facilitates binding to fibronectin (C) links the A domain to a repeat region (R). Adapted from Foster *et al.*, (2014) and Pietrocola *et al.*, (2015).

1.4.2.2 Contribution of CWAPs to infection

The role of *S. aureus* CWAPs in colonisation has been discussed in section 1.2. However CWAPs also function as important virulence factors that contribute to *S. aureus* infection (Foster *et al.*, 2014).

1.4.2.2.1 Adherence to Fibrinogen

Several *S. aureus* surface proteins bind to the plasma protein Fg including ClfA, ClfB, FnBPA, FnBPB and Bbp (McDevitt *et al.*, 1997, Ni Eidhin *et al.*, 1998, Wann *et al.*, 2000, Vazquez *et al.*, 2011). Binding to soluble Fg allows *S. aureus* to agglutinate so that large clumps of bacteria are formed (McAdow *et al.*, 2011, Walker *et al.*, 2013). Agglutination promotes the formation of thromboembolic lesions during infection. CWAP binding to Fg is involved in establishing several *S. aureus* infections including bacteraemia (Josefsson *et al.*, 2008), septicaemia (Flick *et al.*, 2013), endocarditis (Moreillon *et al.*, 1995, Que *et al.*, 2005), kidney abscesses (Cheng *et al.*, 2009) and septic arthritis (Josefsson 2001). Binding to Fg promotes platelet aggregation which is important for the pathogenesis of infective endocarditis (Bayer *et al.*, 1995). Flick *et al.*, (2013) demonstrated that Fg deficient mice were less susceptible to *S. aureus* infection indicating that binding to Fg is important for *S. aureus* to establish blood stream infection (Flick *et al.*, 2013). The recruitment of fibrinogen to the bacterial surface by ClfA is an immune evasion tactic and protects the bacteria from neutrophil and macrophage phagocytosis (Higgins *et al.*, 2006).

1.4.2.2.2 Adhesion to and Invasion of host cells

S. aureus employs several mechanisms that allow the bacteria to survive within professional phagocytes including neutrophils (McGuinness *et al.*, 2016), macrophages (Horn *et al.*, 2018, Zapotoczna *et al.*, 2018) and dendritic cells (O'Keeffe *et al.*, 2015) which can aid bacterial dissemination through the host. *S. aureus* can also invade various non-professional phagocytes such as endothelial cells, keratinocytes and epithelial cells to evade the immune response and avoid antibiotics. Internalisation into mammalian cells is largely mediated by FnBPA and FnBPB (Dziewanowska *et al.*, 1999, Peacock *et al.*, 1999, Massey *et al.*, 2001, Edwards *et al.*, 2011). FnBP binding

to the N-terminal of Fn engenders a conformational change in Fn exposing an integrin binding site that is recognised by the surface expressed $\alpha 5 \beta 1$ integrin on mammalian cells, resulting in a Fn-bridge between the bacterium and the host cell (Liang *et al.*, 2016). Binding of an FnBP protein to multiple Fn molecules causes integrin clustering and triggers intracellular signalling in the host cell resulting in endocytosis of the bacterium (Agerer *et al.*, 2005). FnBP Fn-binding motifs have differing affinities for Fn and internalisation into host cells depends on the presence of at least one high affinity motif (Edwards *et al.*, 2010, Edwards *et al.*, 2011). The N2 N3 subdomains of FnBPB can also bind Fn by an unknown mechanism (Burke *et al.*, 2011) Other CWAPs facilitate *S. aureus* internalisation into host cells. IsdB promotes *S. aureus* invasion by direct binding to $\beta 3$ integrins expressed on the surface of host cells (Zapotoczna *et al.*, 2013). The legume-lectin like subdomain of the BR domain of SraP can bind to N-acetyl neuraminic acid present on mammalian cells, promoting *S. aureus* uptake into epithelial cells (Yang *et al.*, 2014).

1.4.2.2.3 Immune evasion

Several *S. aureus* CWAPs aid in bacterial immune evasion. Intracellular invasion of host cells, as described in section 1.4.2.2.2, provides protection from immune attack. Expression of protein A is one of the most important mechanisms of *S. aureus* immune evasion. Protein A binds to the Fc region of IgG and the bacteria become coated in IgG molecules in the incorrect orientation. This prevents bacterial recognition by neutrophils thus protecting the bacteria from phagocytosis and inhibiting complement activation (Foster, 2009, Atkins *et al.*, 2008). Protein A is also a B-cell superantigen capable of binding to the $V_H 3$ -class IgM molecules on the surface of B-cells, stimulating clonal expansion and subsequent apoptosis which reduces antibody production and limits adaptive immune responses (Goodyear & Silverman, 2003). This reduction in adaptive immune function limits host protection against future *S. aureus* infection (Falugi *et al.*, 2013) which hampers the successful development of a vaccine against *S. aureus*. Protein A is also a potent activator of tumour necrosis factor receptor 1 (TNFR1) resulting in pro-inflammatory signalling (Gomez *et al.*, 2004). TNFR1 is the usual receptor for the cytokine tumour necrosis factor- α (TNF α), which is critical for the host response to

infection. Protein A binding TNFR1 mimics TNF α and stimulates TNFR1 shedding from the surface of epithelial cells and macrophages, thus limiting TNF α signalling activity (Gomez *et al.*, 2007, Gomez *et al.*, 2006).

CWAPs contribute to *S. aureus* immune evasion by interfering with the host complement system to protect against complement mediated phagocytosis. ClfA and IsdH promote cleavage of the complement opsonin C3b possibly through binding to complement regulator factor I (Hair *et al.*, 2010, Visai *et al.*, 2009). SdrE binds complement factor H resulting in increased C3b cleavage (Sharp *et al.*, 2012). SdrE and Bbp bind to complement regulator C4BP promoting cleavage of C4b and decreasing C3b opsonisation (Hair *et al.*, 2013). FnBPA and FnBPB bind to the host serum protein plasminogen (Pietrocola *et al.*, 2016). Host or bacterial plasminogen activators stimulate the generation of plasmin (Collen, 1998). Plasmin is a potent serine protease that cleaves human IgG and C3b from the bacterial cell surface to protect the bacteria from neutrophil recognition (Roosjakkers *et al.*, 2005). Histones are prominent components in neutrophil extracellular traps (NETs) that disrupt the cell membrane of bacteria (Sollberger *et al.*, 2018). FnBPB captures and binds to histones released from neutrophils and simultaneous binding to activated plasminogen by FnBPB and/or another unidentified *S. aureus* CWAPs results in histone cleavage. *S. aureus* is thereby protected from histone-mediated membrane damage (Pietrocola *et al.*, 2019). Adenosine synthase (AdsA) comprises a nucleotidase motif that contributes to immune evasion by converting intracellular ATP to adenosine, inhibiting oxidative burst in neutrophils (Thammavongsa *et al.*, 2009).

1.4.2.2.4 Biofilm formation

S. aureus is a leading cause in indwelling medical device related infections driven by the ability of *S. aureus* to form biofilms (Otto, 2008). The first phase of biofilm formation involves CWAP-mediated attachment to a conditioned or unconditioned surface (Speziale *et al.*, 2014, Feuillie *et al.*, 2017, Vaudaux *et al.*, 1995). The accumulation and maturation phase involves bacterial aggregation which can be mediated by the *icaADBC*-encoded polysaccharide intercellular adhesin (Cramton *et al.*, 1999) or by CWAPs in an *ica*-independent manner (O'Neill *et al.*, 2007b). *S. aureus* proteins display

functional redundancy and several CWAPs are capable of mediating biofilm accumulation. The specific CWAP responsible for mediating biofilm differs between strains and in some cases multiple CWAPs are involved (O'Neill *et al.*, 2008, Barbu *et al.*, 2014). FnBPA and FnBPB mediated biofilm has been demonstrated with HA- and CA-MRSA strains (O'Neill *et al.*, 2008, McCourt *et al.*, 2014, Geoghegan *et al.*, 2013) and occurs by low affinity homophillic interactions between the N2 N3 subdomains of adjacent bacteria (Herman-Bausier *et al.*, 2015, Geoghegan *et al.*, 2013). SasG mediated biofilm occurs by homophillic interactions between the G5-E repeats in the B region of adjacent bacteria. This requires removal of the A domain of the protein, hypothesised to occur by limited cleavage within the B region (Geoghegan *et al.*, 2010). SdrC mediates attachment to both biotic and abiotic surfaces and promotes biofilm accumulation by low affinity homophillic interactions between the N2 subdomains of neighbouring cells (Barbu *et al.*, 2014, Feuillie *et al.*, 2017). During *in vivo* biofilm formation in the presence of plasma, Fg-binding MSCRAMMs including ClfA, promote bacterial aggregation with fibrinogen in the biofilm, enhancing biofilm resistance to antimicrobials (Zapotoczna *et al.*, 2015). Similarly during bone joint infections, free-floating biofilm-like clumps of bacteria are formed in synovial fluid through interactions between MSCRAMMs and fibrinogen (Dastgheyb *et al.*, 2015).

1.4.3 *S. aureus* Type VII secretion System

The type VII secretion system (T7SS) was first described for Gram-positive bacteria in members of the genus mycobacterium following detection of two secreted substrates EsxA (formally ESAT-6) and EsxB (formally CFP-10). Potent T-cell antigens EsxA and EsxB lack a Sec-signal sequence which indicated that a novel Sec-independent secretion system was responsible (Abdallah *et al.*, 2007). T7SS has since been described in several members of the Firmicutes phyla where homologs of the gene encoding EsxA were identified in the genomes of *Bacillus subtilis*, *Listeria monocytogenes* and *S. aureus* (Pallen, 2002). *S. aureus* T7SS is encoded by genes on the *ess* locus comprising six core components *essC*, *esaA*, *essA*, *essB*, *esxA* and *esaB* (Burts *et al.*, 2005, Jager *et al.*, 2016, Kneuper *et al.*, 2014) which encode four membrane proteins, one extracellular protein and one cytoplasmic protein

respectively (Unnikrishnan *et al.*, 2017). *essH* encodes for a peptidoglycan hydrolase EssH which cleaves pentaglycine cross bridges in the cell wall, likely facilitating the secretion of *ess* substrates into the extracellular milieu (Bobrovskyy *et al.*, 2018). The *ess* locus is under complex transcriptional control by alternative sigma factor σ^B and is negatively regulated by the two-component system SaeRS (Schulthess *et al.*, 2012, Anderson *et al.*, 2013).

Proteins encoded by the *ess* locus (ESS proteins) are important *S. aureus* virulence factors. Four of the core component genes, *esxA*, *esaA*, *essA* and *essC*, are upregulated in the lung during persistent colonisation of cystic fibrosis patients (Windmuller *et al.*, 2015). Additionally ESS activity was shown to promote persistent abscesses in murine models of *S. aureus* infection (Burts *et al.*, 2005, Burts *et al.*, 2008) and contribute to virulence in murine pneumonia and skin infection models (Kneuper *et al.*, 2014, Wang *et al.*, 2016). T7SS substrates EsxA and EsxB have been implicated in the regulation of host cell apoptotic pathways and the release of intracellular *S. aureus* from epithelial cells (Korea *et al.*, 2014). ESS activity may also function to protect *S. aureus* against neutrophils and support neutrophil lysis (Wang *et al.*, 2016). The majority of studies characterising the functional role of the *ess* locus however have focused on a select few *S. aureus* strains and one study showed that the contribution of ESS proteins to virulence was strain specific (Kneuper *et al.*, 2014). There is some indication that ESS proteins are involved in iron homeostasis as genes involved in iron acquisition are upregulated in the absence of EsaB and EssC activity (Casabona *et al.*, 2017a, Casabona *et al.*, 2017b). However, the biological function of several ESS substrates has not been elucidated and the molecular determinants by which T7SS contributes to *S. aureus* pathogenesis has have not been fully defined.

1.4.3.1 EsaD and EsaG

The *essC* gene, a homolog of mycobacterial *eccC*, is conserved in T7SS between organisms. In *S. aureus* four *essC* allelic variants have been identified based on extensive sequence variation in the 3' end of the gene. A specific subset of genes directly downstream of *essC* is associated with each distinct cluster. Among the subset of genes following *essC* variant 1 (*essC1*) are genes encoding the putative chaperone protein *esaE* (Warne *et al.*, 2016, Cao *et al.*,

2016) and secreted substrates EsxB, EsxC, EsxD and EsaD where only EsaD has been assigned a function. Carriage of *essC1* displays a degree of lineage specificity with association to important clonal complexes including CC1, CC5, CC8 and CC45. Of the four distinct *essC* gene clusters, only those with *essC1* carry the *esxB/esxC/esxD/esaE/esaD* subset of genes (Warne *et al.*, 2016, Jager *et al.*, 2018). Sequence variation within *esaD* exists between strains and immediately downstream of every *esaD* variant is a specific variant of a gene of the DUF600 (domain of unknown function 600) family termed *esaG* (Warne *et al.*, 2016, Cao *et al.*, 2016).

Initial identification of EsaD reported a role in virulence in contributing to kidney abscesses in a murine infection model (Anderson *et al.*, 2013). EsaD has since been characterised as a secreted nuclease toxin with a C-terminal nuclease domain that cleaves DNA of a sensitive *S. aureus* strain. The cognate EsaG protein acts as an anti-toxin and interacts with the C-terminal nuclease domain of EsaD in the cytoplasm, neutralising its nuclease ability to protect the host cell from self-intoxication. EsaG is only detected in the cytoplasm and is likely cleaved from EsaD during secretion thus releasing an active form of the toxin. A second protein essential for EsaD secretion is the putative chaperone EsaE, which binds to the N-terminal domain of EsaD and has a potential role in secretion of EsaD (Cao *et al.*, 2016, Aly *et al.*, 2017). A proposed model of the *S. aureus* T7SS machinery is depicted in Figure 1.2. While only strains carrying the *essC1* variant are associated with *esaE/esaD/esaG*, every *S. aureus* strain carries at least two copies of DUF600 genes, downstream of *essC* (Warne *et al.*, 2016). These genes have since been described as *esaG* homologs and they encode for variants of EsaG which are predicted to neutralise the nuclease ability of variant EsaD toxins from other *S. aureus* strains (Cao *et al.*, 2016). Every *S. aureus* strain analysed has at least two *esaG*-like genes and as many as 13 within one strain have been reported (Warne *et al.*, 2016).

A putative role of *S. aureus* T7SS in intraspecies competition was reported using lab strains of *S. aureus* where one strain inhibited growth of a second sensitive strain in an EsaD dependent manner thus confirming that EsaD is capable of competitively targeting other bacteria (Cao *et al.*, 2016).

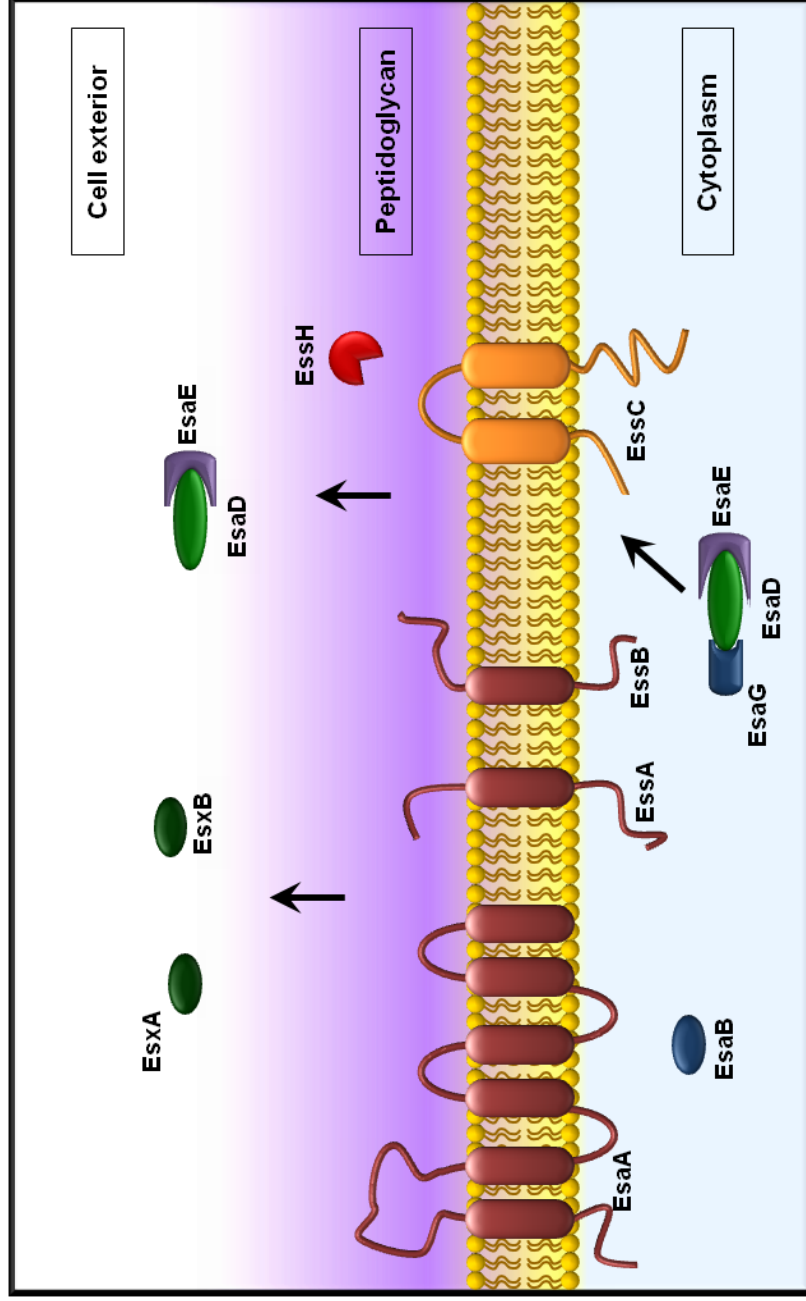


Figure 1.2. *S. aureus* Type VII Secretion System. A proposed model depicting the ESS secretion system. Transmembrane proteins (Dark red), secreted proteins (Green), dual location proteins (Purple), peptidoglycan hydrolase (Red), cytoplasmic proteins (Blue) and transmembrane ATPase (Orange) are indicated. Adapted from Unnikrishnan et al., (2017).

1.5 Genetic manipulation of *S. aureus*

Studying the molecular determinants involved in *S. aureus* pathogenesis and virulence has been greatly aided by advancements in genetic manipulation techniques which allow the manipulation of, or indeed, the elimination or expression of specific genes.

1.5.1 Allelic exchange

Allelic exchange is an extensively used technique for the generation of site-specific chromosomal mutations (Monk *et al.*, 2012). Using this approach, point mutations can be introduced into specific genes or a gene can be inserted into or deleted from the chromosome at a specific location. This is achieved by first cloning the immediate 5' and 3' flanking regions of the target DNA into a temperature-sensitive (TS) plasmid in *E. coli*.

1.5.1.1 Temperature sensitive plasmids

Temperature sensitive plasmids contain a temperature-dependent replication system. In *S. aureus* the plasmid pKOR1 containing the pE194ts replicon is a commonly used for mutagenesis in *S. aureus* (Bae & Schneewind, 2006). pKOR1 is restricted for replication at 43 °C. However growth of *S. aureus* at elevated temperatures can give rise to secondary mutations in the *saeRS* genes (Sun *et al.*, 2010). To overcome this, Monk *et al.*, (2012) generated the TS plasmid pIMAY containing the pWV01ts replicon which is permissive for replication at 30 °C and allows for selection of integrants at 37 °C, thus reducing the likelihood of secondary mutations (Monk *et al.*, 2012).

1.5.1.2 Counter selection in *S. aureus*

Plasmid pIMAY carrying the mutation cassette is introduced into *S. aureus* and the cells are propagated at 28 °C which is permissive for plasmid replication. A temperature upshift to 37 °C, which is restrictive for replication, selects for a single cross-over event by which the plasmid integrates into the chromosome at a site of homology. Propagating integrants at the permissive temperature then induces a second single cross-over event by homologous recombination. The plasmid is eliminated from the cell by growth in the absence of selective pressure. The resulting cells are wild-type or have the mutant

genotype depending on where the second single cross-over event occurred (Monk *et al.*, 2012). The pIMAY plasmid employs inducible *secY* antisense-RNA counter-selection to identify cells that have lost the plasmid. Expression of the *secY* antisense-RNA is controlled by the *tet* promoter/repressor and is induced by the gratuitous inducer anhydrotetracycline (ATc). The Sec system, of which SecY is a component, is essential for survival of prokaryotes (Manting & Driessen, 2000) and inhibiting *secY* expression by antisense RNA impedes bacterial growth (Ji *et al.*, 2001). A schematic representation of the allelic exchange process in *S. aureus* using pIMAY is depicted in figure 1.3.

1.5.1.3 Bypass of *S. aureus* restriction barriers

Staphylococcal strains contain strong restriction barriers which prevent uptake of foreign DNA. As such, transformation of foreign DNA into many *S. aureus* has previously been impossible (Corvaglia *et al.*, 2010, Waldron & Lindsay, 2006). Two restriction modification (RM) systems in *S. aureus*, type I (Roberts *et al.*, 2013, Waldron & Lindsay, 2006) and type IV (Xu *et al.*, 2011) recognise and degrade DNA that displays a different methylation profile to the host strain. The type I Sau1 RM system is encoded by three host specificity determinant (*hsd*) genes *hsdR*, *hsdM* and *hsdS* and recognises distinct adenine methylation profiles that are characteristic of a particular host clonal complex (Monk *et al.*, 2015, Waldron & Lindsay, 2006). The type IV RM system relies on the restriction endonuclease SauUSI which recognises cytosine-methylated DNA (Corvaglia *et al.*, 2010, Xu *et al.*, 2011). The DCM system in *E. coli* generates a species specific cytosine methylation profile which is recognised as foreign by *S. aureus* SauUSI (Xu *et al.*, 2011). To bypass the type IV RM system in *S. aureus*, Monk *et al.* (2012) engineered a mutant of *E. coli* deficient in *dcm* (DC10B). This allowed transfer of plasmids isolated from DC10B into *S. aureus* CC8 strains at an acceptable frequency (Monk *et al.*, 2012, Waldron & Lindsay, 2006). Further derivatives of DC10B possessing type I modification genes to confer lineage-specific adenine methylation profiles were generated including IM01B, IM08B and IM30B which allow for efficient introduction of plasmid DNA into *S. aureus* clonal complexes 1, 8 and 30 respectively (Monk *et al.*, 2015) by simultaneously by-passing both RM systems.

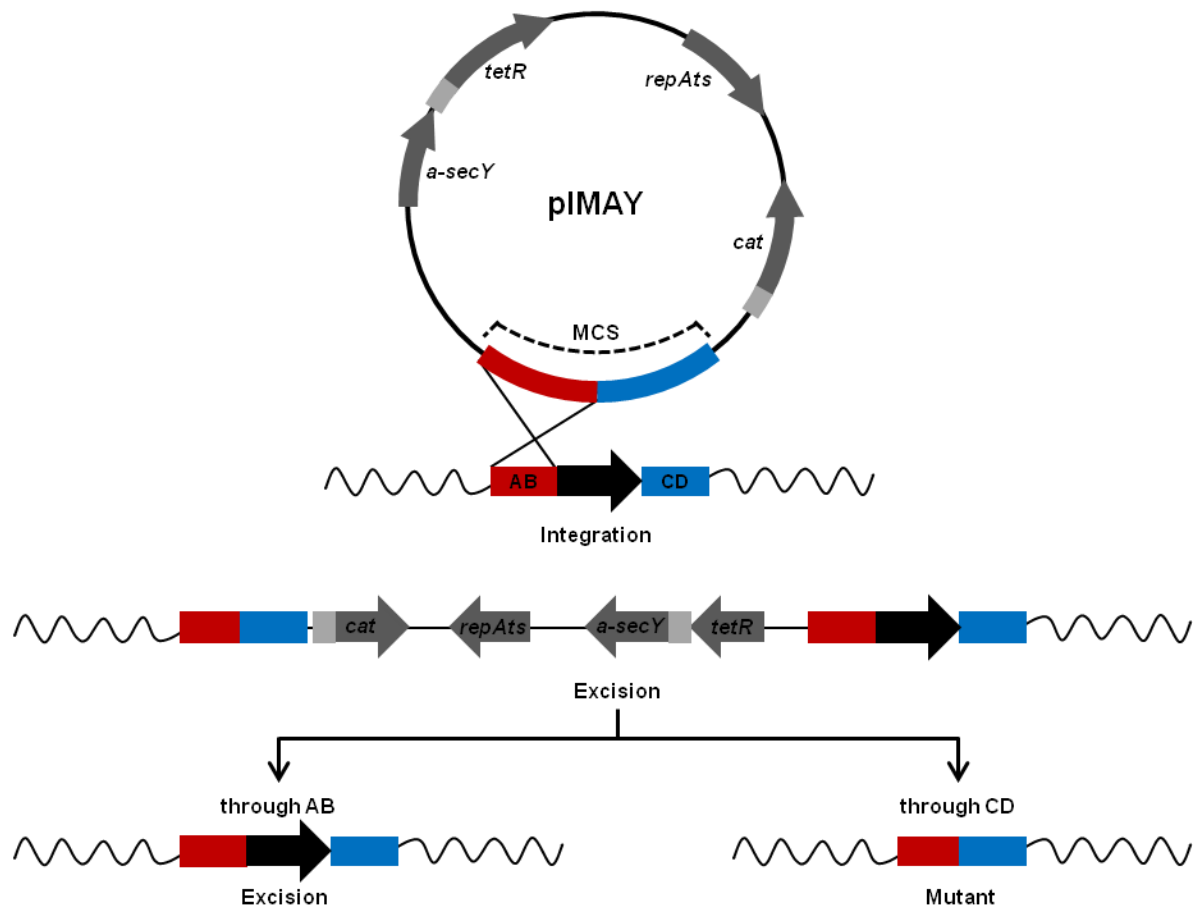


Figure 1.3. Allelic exchange using pIMAY. Following transformation, bacteria are grown at 28 °C which is permissive for plasmid replication. pIMAY has a temperature sensitive replicon (*repAts*) and integration into the chromosome is selected for growing the bacteria 37 °C. This diagram depicts an integration event occurring upstream of the gene of interest (AB). Replicating plasmid is eliminated by growth at 28 °C in the absence of selective pressure. Bacteria are grown in the presence of anhydrotetracycline to induce expression of *secY* anti-sense RNA (α -*secY*) to select for cells that have lost the plasmid. Plasmid excision through the AB side recreates the wild-type locus, while CD yields a mutated gene. *cat*; chloramphenicol resistance, *tetR*; tet repressor. Adapted from (Monk *et al.*, 2012).

1.6 The stratum corneum of human skin

Human skin is divided into two main layers. The uppermost layer, the epidermis, acts as a physical barrier against invading microbes and allergens, while the dermis below consists of fibrous and connective tissues to provide a structural framework. A further layer below the dermis, the subcutaneous layer, consisting mainly of fat stores, provides insulation and additional protection to deeper bones and tissues (SanMiguel & Grice, 2015).

1.6.1 Cornification of keratinocytes

The epidermis provides the first line of antimicrobial defence and is subdivided into stratified layers, each with a distinct composition of proteins and in some cases, cell types. To maintain continuous rejuvenation of the skin, keratinocytes, the primary cell type of the epidermis, undergo a programmed cell death pathway known as cornification (Figure 1.4). Terminally differentiating keratinocytes migrate from the lower basal layer to the uppermost layer, known as the stratum corneum (SC), where they are eventually shed off the surface in a process known as desquamation (Simpson *et al.*, 2011).

Keratins (K) are abundant in the epidermis and function to support epithelial cells by assembling into keratin intermediate filaments (KIFs) to form a structural cytoskeleton. Keratinocytes in the basal layer are supported by a network of keratin K5 and K14 proteins. Once epidermal differentiation has been initiated, basal keratinocytes begin to migrate into the spinous layer and express K1 and K10 to replace the pre-existing KIFs in the cytoskeleton. In the granular layer, the cell loses all mitotic activity and a gene cluster named the 'epidermal differentiation complex' (EDC) is expressed which encodes for proteins characteristic of the cornification process. Among these is the protein filaggrin which acts to aggregate the KIFs into tight bundles forcing the cells to collapse into the flattened shape typical of terminally differentiated keratinocytes, also known as corneocytes (Candi *et al.*, 2005, Eckhart *et al.*, 2013).

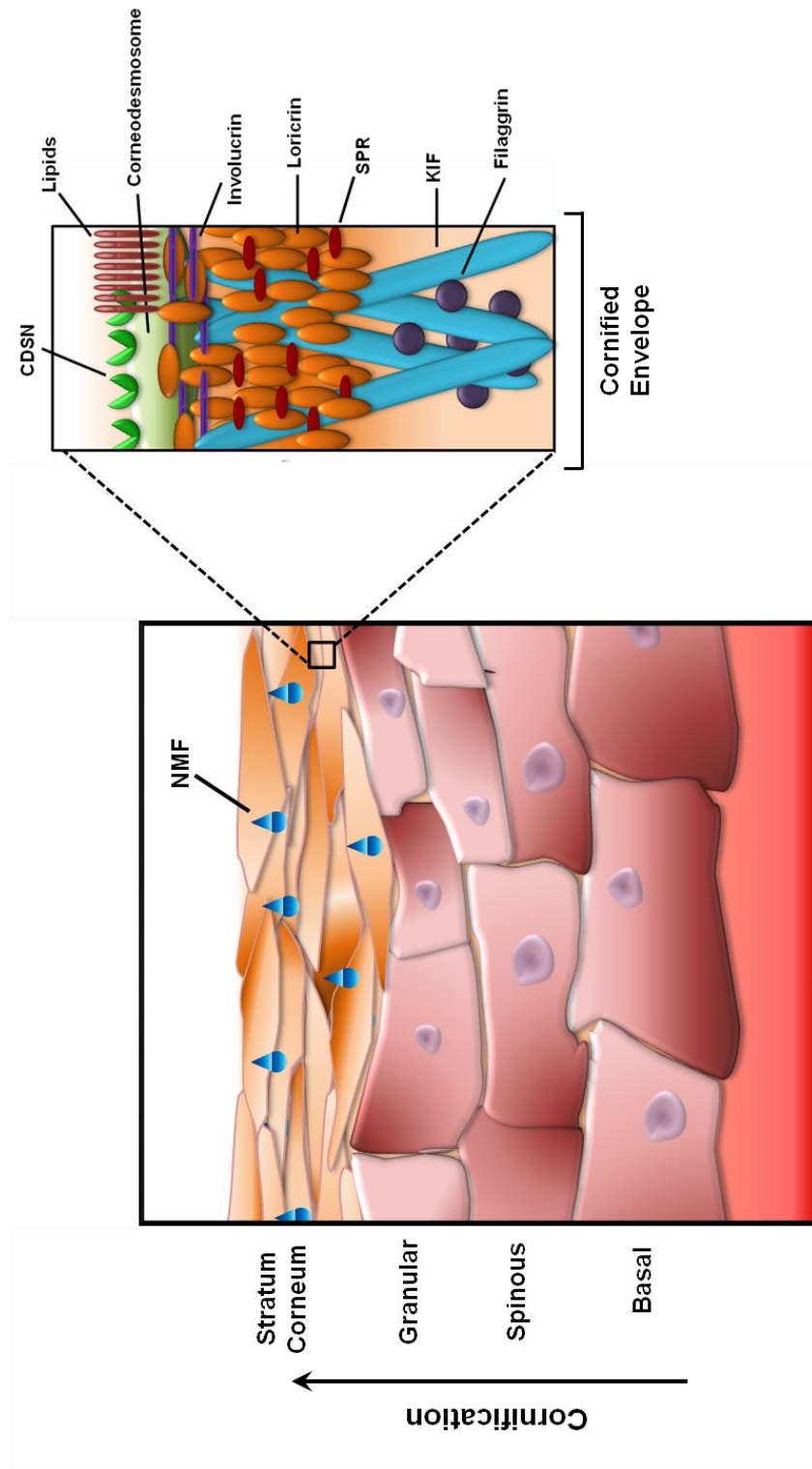


Figure 1.4. Cornification in the epidermis. Terminally differentiating keratinocytes migrate up from the basal layer to the stratum corneum and are forced into a flattened shape by a complex of filaggrin embedded in a KIF matrix. Structural proteins of the cornified envelope assemble and replace the plasma membrane to form corneocytes. Lipids are covalently linked to the cornified envelope and corneocyte adherence is facilitated by CDSN. NMF; Natural Moisturising Factor, CDSN; Corneodesmosin, KIF; Keratin intermediate filament, SPR; Small proline rich protein. Adapted from (Candi *et al.*, 2005).

1.6.2 Proteins of the cornified envelope

The KIF/filaggrin complex provides a scaffold for the formation of the cornified envelope (CE), which is an insoluble structure that replaces the plasma membrane of the anucleated corneocytes in the SC. The CE comprises a number of structural proteins including loricrin, late cornified envelope proteins, involucrin and small proline-rich proteins which become extensively cross-linked by enzymatic activity of several transglutaminases (Candi *et al.*, 2005). Concurrently, a series of lipids are synthesised and some of these, predominantly long-chain ceramides, are covalently crosslinked to the scaffold of keratins and CE structural proteins. Other lipids, mainly long chain fatty acids, cholesterol and ceramides, form the intercellular lamellae which surrounds the cell to complete the physical barrier of the SC (Rawlings & Harding, 2004). In the lower layers of the epidermis, structural integrity is maintained partly by intercellular adhesion structures known as desmosomes. The extracellular part of desmosomes, termed desmoglea, comprises desmoglein and desmocollin proteins which facilitate cell-cell adherence and anchor the plasma membrane of two adjacent cells to the KIF cytoskeleton. As the plasma membrane is altered to a lipid envelope during cornification, modified desmosomes termed corneodesmosomes, are incorporated into the CE (Ishida-Yamamoto *et al.*, 2018). In the uppermost layer of the SC where keratinocytes have undergone complete cornification, corneodesmosomes are located at the peripheral edges of corneocytes. Degradation of corneodesmosomes components by progressive proteolytic activity of serine proteases, primarily kallikreins (KLKs), results in desquamation, a process by which the corneocytes are shedded from the skin allowing for renewal of the skin (Ishida-Yamamoto & Igawa, 2015, Jonca *et al.*, 2011).

1.6.2.1 Corneodesmosin

A unique component of corneodesmosomes is the protein corneodesmosin (CDSN). This 54 kDa N-glycosylated protein has a high glycine and serine content (16% and 27.5% respectively). Two domains of the protein (amino acid residues 60-171 and 375-476) display tandem repetition of serine and/or glycine residues which are separated by aromatic or aliphatic residues (Guerrin *et al.*, 1998). These glycine/serine-rich regions are predicted

to form exposed structural motifs called omega (Ω) loops. Glycine/serine rich Ω loop regions are characteristic of several other SC proteins including loricrin and cytokeratin 10.

CDSN is expressed by differentiating keratinocytes and stored within lamellar granules before relocating to the desmosome desmoglea of granular keratinocytes before it is incorporated into the extracellular core of corneodesmosomes in the SC (Serre *et al.*, 1991, Simon *et al.*, 1997, Jonca *et al.*, 2011). In the SC, CDSN is responsible for cell-cell cohesion by homophillic interactions involving the N-terminal domain of two adjacent cells (Jonca *et al.*, 2002, Caubet *et al.*, 2004b). During cornification, CDSN is subject to recurrent proteolytic cleavage by two serine proteases KLK5 and KLK7 (Caubet *et al.*, 2004a). The initial proteolysis step occurs prior to the incorporation of CDSN into the desmoglea of granular keratinocytes and involves the removal of the peripheral N- and C- terminus of the protein giving rise to a 46-48 kDa protein that retains the Ω loop domains, presumably for corneocyte adhesion. As the corneocyte continues to migrate upwards, the N-terminal Ω loop domain is cleaved from the protein leaving a 30-36 kDa fragment. This correlates with a decrease in structural integrity of the SC. Final cleavage results in a 15 kDa fragment comprising the central part of CDSN that is likely devoid of adhesive properties and is detected on non-cohesive corneocytes that are shed from the outermost layer of the SC. Thus, proteolytic cleavage of CDSN is a fundamental component of desquamation (Simon *et al.*, 2001).

Matsumoto *et al.*, (2008) demonstrated the importance of CDSN using a murine model with a targeted deletion of the *CDSN* gene which produced a lethal phenotype in neonatal mice (Matsumoto *et al.*, 2008). Grafting the knockout skin onto adult mice showed hyperproliferation of epidermal cells, detachment of individual strata in the epidermis, increased skin permeability and rapid degeneration of the epidermis and hair follicles (Matsumoto *et al.*, 2008, Leclerc *et al.*, 2009). In humans, nonsense mutations in the *CDSN* gene are associated with rare genetic diseases. Peeling skin disease is characterised by continuous separation of the epidermal strata and is associated with atopy and pruritus. It is caused by mutations in *CDSN* that result in a premature stop codon (Ishida-Yamamoto *et al.*, 2014). Hypotrichosis simplex of the scalp leads

to complete baldness in young adults and is associated with nonsense mutations in *CDSN* resulting in the expression of a truncated form of *CDSN* that accumulates in the dermis and amyloid deposits (Levy-Nissenbaum *et al.*, 2003). Abnormality in the distribution of *CDSN* has recently been implicated in atopic dermatitis (Riethmuller *et al.*, 2015) which is discussed in section 1.7.2.

1.6.2.2 Loricrin

Loricrin, a 26 kDa protein, is a major component of the CE comprising 75% of the total protein mass. Loricrin is expressed initially in the granular layer before it is integrated into the CE and crosslinked by disulphide and N-(γ -glutamy1) lysine isopeptide bonds through the activity of transglutaminase-3. Further crosslinking to the CE-lipid protein scaffold then occurs by transglutaminase-1 (Candi *et al.*, 2005, Hohl *et al.*, 1991). The structure of loricrin comprises 3-4 unstructured domains rich in glycine/serine residues predicted to form exposed Ω loops (Hohl *et al.*, 1991). Ω loops consist of at least six consecutive amino acid residues forced into a loop structure when hydrophobic residues in the sequence associate, forming a rosette-like conformation that mediate adhesion in a Velcro-like manner (Steinert *et al.*, 1991). The loop domains are separated by regions containing glutamine and lysine residues for transglutaminase-recognition. The loricrin gene is part of the EDC and harbours natural polymorphisms. One such polymorphism in the *LOR* gene causes a 12 bp deletion resulting in a 4-amino acid deletion in the third loop domain, (hereafter referred to as L2v) (Yoneda *et al.*, 1992). Loricrin has been identified as a ligand for *S. aureus*, mediated by CWAP ClfB. ClfB has multiple binding sites in the glycine/serine rich loop regions of loricrin, with the highest affinity for L2v. It is postulated that the interaction between ClfB and loricrin is required for nasal colonisation by *S. aureus* (Mulcahy *et al.*, 2012).

1.6.2.3 Cytokeratin 10

Each strata of the epidermis is associated with a distinct group of keratins which assemble into KIFs to provide structural support for differentiating keratinocytes. Type I (acidic) and Type II (basic/neutral) keratins are coexpressed from most epithelial cells. The assembly of KIFs requires a heteropolymeric pair formation of one type I and one type II keratin (Moll *et al.*,

2008). Keratins share a similar structure with a central α -helical rod flanked by non-helical head and tail domains. Each domain is further divided into subdomains. The head domain comprises two subdomains V1 and E1, the central α -helical rod domain consists of four subdomains 1A, 1B, 2A and 2B connected by linkers L1, L12 and L2 and the tail domain comprises subdomains E2 and V2 (Steinert, 1993). Cytokeratin 10 (K10) is a type I keratin that associates with K1 during cornification. The V2 subdomain in the C-terminal tail region of K10 is composed of quasi-repeats of Y[glycine/serine]_NY which adopt an Ω loop structure, similar to those found in loricrin (Steinert *et al.*, 1991). The glycine/serine rich Ω loop region of K10 contains a binding site for *S. aureus* CWAP ClfB (Walsh *et al.*, 2004). However the relevance of K10 as a ligand for *S. aureus in vivo* remains to be established.

1.6.2.4 Filaggrin and the natural moisturising factor (NMF)

Filaggrin is a key component in the cornification process and a major structural protein in the SC. The *FLG* gene encodes a large precursor protein, profilaggrin, composed of multiple filaggrin repeats joined by hydrophobic linker peptides. Profilaggrin is dephosphorylated and proteolysed to release multiple filaggrin monomers (Sandilands *et al.*, 2007). Following their release into the cytoplasm, filaggrin interacts with the keratin cytoskeleton forcing the collapse of KIFs and initiating cell death (Dale *et al.*, 1997). In the final stages of keratinocyte terminal differentiation filaggrin is subject to deamination, altering the net charge of the protein to neutral and disrupting the ionic interactions of the filaggrin/keratin complex (Ishida-Yamamoto *et al.*, 2002). Filaggrin monomers are then susceptible to further degradation into free amino acids by proteases caspase-14 (Denecker *et al.*, 2007) and calpain-1 (Kamata *et al.*, 2009) in response to the water gradient in the SC (Scott & Harding, 1986).

Restricted water loss from the body is carefully regulated by the SC which acts as a selectively permeable barrier to protect against desiccation. A controlled amount of water loss is necessary to hydrate the uppermost layers of the SC and to allow for enzymatic reactions involved in cornification and desquamation. Hydration in the skin is regulated by the natural moisturising factor (NMF). The composition of NMF is fundamentally reliant on controlled proteolytic cleavage of filaggrin into breakdown products urocanic acid (UCA)

and pyrrolidone carboxylic acid (PCA). Together with urea, citrate, lactate acid and sugars, these hygroscopic amino acids constitute the NMF in the SC. These metabolites function as osmolytes to draw water into the SC thus providing hydration (Rawlings & Harding, 2004).

1.6.3 Defence mechanisms of the skin

The skin has a unique microbiome that harbours a remarkable degree of microbial diversity. Both symbiotic commensals and opportunistic pathogens colonise physiologically and topographically distinct niche environments in the skin. Members of the skin microbiota are influenced by several host factors including local skin architecture, pH, moisture content and lipid content (SanMiguel & Grice, 2015, Grice *et al.*, 2009). To protect from these microbes, the skin possesses a number of defence mechanisms including surface pH, production of antimicrobial peptides and fatty acids, and synergy with some commensal species.

1.6.3.1 Host defence mechanisms

The skin has an acidic pH ranging from 4-6 which inhibits growth of pathogenic bacteria, including *S. aureus* which thrives at neutral pH. Furthermore, epidermal peptides such as dermcidin have increased antimicrobial properties at a lower pH (Ali & Yosipovitch, 2013, Schitteck *et al.*, 2001). The acidic pH is maintained primarily by endogenous mechanisms including the sodium-hydrogen antiporter (NHE1) expressed by keratinocytes and the hydrolysis of phospholipids to free fatty acids by secretory phospholipase A₂ enzymes (Chan & Mauro, 2011). Filaggrin breakdown products UCA and PCA are acidic components of the NMF, which contribute towards the acidity of the skin (O'Regan *et al.*, 2008).

Keratinocytes and secretory cells including sweat and sebaceous glands express antimicrobial peptides (AMPs) and fatty acids to kill or inhibit growth of pathogenic bacteria. AMPs produced in the skin are members of several protein families including β -defensins, cathelicidins, ribonucleases and S100 proteins. Cathelicidins, β -defensins and dermcidin of the skin mainly act by disrupting the bacterial cell membrane and the viral envelope (Gallo & Hooper, 2012). Cathelicidins are encoded by a single gene *CAMP* which encodes a precursor

protein that is cleaved to generate active AMPs including the 37-amino-acid peptide LL37. Expression of LL37 by keratinocytes is generally limited to infection or injury (Reinholz *et al.*, 2012). In addition to direct antimicrobial activity, LL37 also functions as a chemoattractant for neutrophils, mast cells, T-cells and monocytes (Niyonsaba *et al.*, 2002, De *et al.*, 2000). β -defensins are small cationic peptides that have antimicrobial activity. Four β -defensins expressed by keratinocytes have been characterised to date (Chieosilapatham *et al.*, 2017). Similar to LL37, expression of human β -defensin 2 (HBD-2) is upregulated in response to injury or inflammation while human β -defensin 1 (HBD-1) is expressed constitutively (Yamasaki & Gallo, 2008). β -defensins also have chemoattractive properties for the recruitment of immature-dendritic cells and memory T-cells (Yang *et al.*, 1999). Both cathelicidins and β -defensin induce expression of the pro-inflammatory IL-18 by keratinocytes (Niyonsaba *et al.*, 2005). Another AMP, dermcidin, is present continuously in human sweat and is not upregulated in response to inflammation (Schitteck *et al.*, 2001, Song *et al.*, 2013).

1.6.3.2 Natural skin commensal organisms

Skin colonisation by pathogenic bacteria, including *S. aureus*, is inhibited by both host factors and by commensal organisms present in the skin microbiome. Specifically, several coagulase-negative staphylococci (CoNS) species have potent anti-*S. aureus* activity. *S. epidermidis*, *S. lugdunensis* and *S. hominis* express antimicrobial peptides that can inhibit the growth of *S. aureus* (Cogen *et al.*, 2010, Zipperer *et al.*, 2016, Nakatsuji *et al.*, 2017). *S. epidermidis* can induce clearance of *S. aureus* from the nasal cavity by releasing a serine protease Esp (Iwase *et al.*, 2010). Esp degrades several *S. aureus* proteins including protein A and those involved in biofilm formation such as FnBPA. Furthermore, Esp may degrade host ligands for *S. aureus* including Fn and Fg (Sugimoto *et al.*, 2013). Numerous bacterial species express peptides known as bacteriocins which have antimicrobial properties to inhibit growth of other organisms competing for nutrients (Gallo & Hooper, 2012). Some commensals act to modulate host defences and synergise with host AMPs to inhibit pathogenic bacteria. *Propionibacterium* spp. induce the expression of AMPs and proinflammatory cytokines in human sebocytes (Nagy

et al., 2006). *S. hominis* expresses a lantibiotic that acts in synergy with host AMP LL37 leading to increased bactericidal activity against *S. aureus* (Nakatsuji et al., 2017). PSM- γ and δ -toxin expressed by *S. epidermidis* have a similar mode of action to LL37 and synergy between them increases antimicrobial activity (Cogen et al., 2010).

However not all commensal antimicrobial defence mechanisms are bactericidal. *S. aureus* Agr activity is reduced by an unknown mechanism facilitated by *Corynebacterium striatum* resulting in decreased *S. aureus* toxin expression and increased adherence to epithelial cells such that *S. aureus* is converted from a pathogen to a commensal (Ramsey et al., 2016). Similarly CoNS *S. caprae* expressing type I agr secretes an autoinducing peptide (AIP) that inhibits *S. aureus* quorum sensing by all four *S. aureus* agr types. *S. epidermidis* secretes a pheromone capable of inhibiting *S. aureus* quorum sensing by type I, II and III agr types. Furthermore, a murine model of infection had increased *S. aureus* clearance following co-inoculation with *S. caprae* demonstrating the host protection effect of CoNS (Paharik et al., 2017, Otto et al., 2001).

1.6.4 Mechanisms of *S. aureus* survival on skin

S. aureus employs a number of factors to evade host defence mechanisms on the skin, mainly by conferring resistance to AMPs. Expression of CWAP IsdA alters the *S. aureus* cell surface hydrophobicity which confers resistance to sebum fatty acids and lipids as they can no longer bind to the hydrophilic surface (Clarke et al., 2007). Reducing the negative charge at the cell surface renders the bacteria less susceptible to cationic defensins. This is achieved by modifications to teichoic acids and to the phospholipid phosphatidylglycerol where the addition of D-alanine and L-lysine residues respectively result in a positively charged surface to repulse cationic AMPs (Peschel et al., 1999, Staubitz et al., 2004). Furthermore, host AMPs can be directly targeted by secreted proteins including the metalloprotease aureolysin and serine protease V8 which degrade the cathelicidin LL37 (Sieprawska-Lupa et al., 2004).

1.7 Atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disorder that causes periodic flares of eczematous lesions accompanied by intense pruritus in the skin. AD affects 20% of children and 2-10% of adults in industrialised countries. The disease is characterised by a skin barrier defect and an abnormal IgE allergic immune response (Figure 1.5) (Deckers *et al.*, 2012, Bieber, 2008, Silverberg & Hanifin, 2013). The disease resolves in approximately 70% of childhood cases, however family history of AD and early and severe onset are risk factors for AD persistence into adulthood (Peters *et al.*, 2010, Illi *et al.*, 2004). Approximately 20% of children with AD subsequently develop other allergy-based diseases including asthma and allergic rhinitis (Illi *et al.*, 2004, Belgrave *et al.*, 2014).

1.7.1 Host genetic susceptibility

The genetic determinants that predispose humans to AD have not been fully defined. Mutations in the *FLG* gene encoding the filaggrin precursor profilaggrin have the highest association to AD. The majority of proteins involved in cornification, including filaggrin, are encoded by the EDC present at human chromosome band 1q21 (Volz *et al.*, 1993, Mischke *et al.*, 1996). Loss-of-function mutations in *FLG* resulting in filaggrin deficiency have been extensively reported in AD cohorts. In European cohorts specifically, null mutations R501X and 2282derl4 in *FLG* are an established risk factor for AD (Weidinger *et al.*, 2006, Baurecht *et al.*, 2007, Barker *et al.*, 2007, Marenholz *et al.*, 2006, Palmer *et al.*, 2006). Furthermore, carriage of *FLG* null mutations is associated with more severe AD disease (Rodriguez *et al.*, 2009). *FLG* is polymorphic with alleles encoding 10, 11 or 12 copies of the filaggrin monomer. It has been reported that copy number variation between individuals is associated with AD where patients have a reduced number of *FLG* repeats (Brown *et al.*, 2012). Several other studies have implicated genetic mutations in the development of AD, primarily mutations in genes of the EDC. However of these, *FLG* is the most significant. In addition, abnormal regulation of genes in the EDC is influenced by components of the Th2 immune response, discussed in section 1.7.2 (Boguniewicz & Leung, 2011).

1.7.2 Skin barrier defect in atopic dermatitis

A distinguishing feature of AD is an impaired skin barrier which has decreased structural integrity and hydration, increased permeability, water loss and pH, abnormal lipid composition and reduced expression of AMPs. Several factors give rise to the defective barrier, with filaggrin deficiency highlighted as the most important contributing factor (Weidinger & Novak, 2016). While null mutations in *FLG* are associated with a higher risk of AD development, a deficiency in filaggrin is characteristic in AD regardless of *FLG* genotype (Pellerin *et al.*, 2013), likely mediated by Th2 cytokines interleukin-4 (IL-4) and interleukin-13 (IL-13), discussed in section 1.7.2. Filaggrin is the major structural protein in the formation of the SC as it interacts with KIFs to form a scaffold for the formation of the CE during cornification (Dale *et al.*, 1997) and filaggrin breakdown products UCA and PCA are fundamental components of the NMF responsible for hydration of the skin (Rawlings & Harding, 2004). Filaggrin deficiency is associated with irregular keratinocyte differentiation, disorganised KIFs, impaired corneocyte cohesion, increased SC permeability, decreased water retention and altered lipid formation (Weidinger & Novak, 2016, Pendaries *et al.*, 2014, Gruber *et al.*, 2011, Mildner *et al.*, 2010).

The absence of filaggrin and subsequently UCA and PCA, results in reduced NMF levels in AD skin which correlates with disease severity (Kezic *et al.*, 2008) and abnormal corneocyte morphology. The surface area of AD corneocytes both in lesional and non-lesional skin are smaller than healthy corneocytes presumably due to inefficient corneocyte flattening by the structurally defective CE (Igawa *et al.*, 2013). Furthermore, in association with reduced NMF levels, AD corneocytes exhibit 'villus-like' projections (VPs) which protrude from the surface of the corneocyte. The corneodesmosome component corneodesmosin (CDSN) is normally confined to the peripheral edges of corneocytes in healthy skin. However CDSN is present on the tips of VPs in AD skin, densely covering the entire corneocyte surface (Riethmuller *et al.*, 2015, Igawa *et al.*, 2013). Correspondingly, knocking down filaggrin with siRNA in a 3D human epidermis equivalent showed an increase in both transcription and expression of CDSN. Corneodesmosomes were not detected in this model and localisation of CDSN was abnormal (Pendaries *et al.*, 2014).

CDSN undergoes proteolytic cleavage by KLK7 to facilitate desquamation. In AD skin expression of KLK7 is upregulated. However secretion of KLK7 from lamellar granules is impaired. Furthermore, a cognate protease inhibitor LEKTI is also upregulated, thus cleavage of CDSN is reduced in AD skin (Igawa *et al.*, 2017). Other corneodesmosome components desmoglein-1 and desmocollin-1 also display altered cellular distribution in AD skin (Igawa *et al.*, 2013). However these two proteins are downregulated in AD skin (Totsuka *et al.*, 2017). Other structural CE proteins including loricrin, K10, K1 and involucrin are downregulated in AD, which may contribute to the defective structural architecture of AD skin (Bao *et al.*, 2017, Kim *et al.*, 2008, Totsuka *et al.*, 2017).

Lipids including ceramides and fatty acids are essential for intact skin barrier function. However abnormalities in the lipid profile of the SC is distinctive in AD skin. In normal skin, a lipid monolayer of ceramides is covalently attached to the CE, thus the absence of filaggrin and altered CE assembly likely contributes to the disorganised lipid formation in AD skin. The structure of both free fatty acids and ceramides is atypical with an extreme reduction in chain length leading to decrease in lipid density. These aberrations in the lipid lamellae correlate with reduced NMF levels and AD disease severity (van Smeden *et al.*, 2014, Janssens *et al.*, 2012).

The pH of AD skin is elevated with a shift towards neutral which contributes to the skin barrier defect (Eberlein-Konig *et al.*, 2000). Many enzymes in the skin, including those involved in the formation of the lipid bilayers, are optimal at a lower pH thus maintaining the acidity of the skin is important for barrier formation and antimicrobial defences. Elevated pH in AD skin is due to the absence of acidic metabolites UCA and PCA, reduced sweat secretions and decreased levels of free fatty acids (Ali & Yosipovitch, 2013).

A further characteristic of AD skin is a decrease in AMPs, required for host defence against pathogenic microbes. The main AMPs in the skin include human β -defensins (HBD -1, -2, -3) and cathelicidins (LL37). Normal expression of AMPs in healthy skin is low and expression is then upregulated during inflammation (Chieosilapatham *et al.*, 2017). Presumably expression of AMPs in inflamed lesional skin typical of AD would be upregulated however several studies have reported a reduction in expression of AMPs LL37, HBD-2

and HBD-3 in inflamed lesions, a phenotype mediated by Th2 cytokines IL-4 and IL-13 (Ong *et al.*, 2002, Mallbris *et al.*, 2010, Hata *et al.*, 2010).

1.7.2 Immune Dysregulation

In concurrence with the disturbed skin barrier, a dysregulated immune response is believed to contribute to pathogenesis of AD. AD is a biphasic disease associated with increased serum IgE levels and a Th2-mediated response predominating the acute phase followed by switch to a Th1-mediated response as the disease shifts towards the chronic phase (Leung *et al.*, 2004). The acute phase in particular is characterised by overexpression of Th2 proinflammatory cytokines IL-4, IL-13 and IL-31 (Howell *et al.*, 2007b, Raap *et al.*, 2008). It is hypothesised that the barrier defect in AD skin allows invading microbes or allergens to penetrate deeper into the epidermis where they are detected by skin antigen-presenting cells such as Langerhans cells which stimulate differentiation of naïve T-cells into Th2 cells (Callard & Harper, 2007). Microbial factors including bacterial peptidoglycan and lipoteichoic acid, and both IL-4 and IL-13, stimulate overexpression of thymic stromal lymphoprotein (TSLP) from keratinocytes which drives proliferation of naïve T-cells into Th2 cells and Langerhan-mediated IL-4 and IL-13 production from Th2 cells (Cianferoni & Spengel, 2014).

IL-4, IL-13 and IL-31 have an established role in the pathogenesis of AD by indirectly downregulating expression of several genes in a STAT-6 dependent manner, including *FLG* (filaggrin), *LOR* (loricrin), *IVL* (involucrin), *DSG1* (desmoglein-1), *DSC1* (desmocollin-1), *KRT10* (K10), *KRT1* (K1) (Bao *et al.*, 2017, Kim *et al.*, 2008, Totsuka *et al.*, 2017, Hatano *et al.*, 2013, Howell *et al.*, 2009, Cornelissen *et al.*, 2012). Caspase-14, normally involved in cleavage of filaggrin to NMF metabolites, is also downregulated in AD skin due to IL-4/IL-13 (Hvid *et al.*, 2011). Given the role of these proteins in the formation of the cornified envelope, it is conceivable that this Th2-mediated dampening of gene expression largely contributes to the barrier defect in AD skin. Furthermore, downregulation of elongases 3 and 6 (*ELOVL3* and *ELOVL6*) by IL-4/IL-13 results in the formation of aberrant short-chain fatty acids associated with AD skin (Berdyshev *et al.*, 2018, Sawada *et al.*, 2012). IL-4 and IL-13 are also involved in indirectly regulating expression of AMPs in AD skin. During

inflammation, AMPs in healthy skin are typically upregulated by TNF- α and IFN- γ activity. However in AD skin, STAT-6 activation by IL-4 and IL-13 induces expression of the suppressors of cytokine signalling (SOCS) -1 and -3 which inhibit TNF α and IFN- γ mediated induction of HBD2 and HBD3 (Albanesi *et al.*, 2007, Nomura *et al.*, 2003). Additionally the cathelicidin LL37 is downregulated by IL-4 and IL-13 via STAT-6 (Howell *et al.*, 2006). IL-31 is highly pruritogenic as it stimulates the release of a neurotransmitter brain-derived natriuretic peptide (BNP) which transmits itch signals to the spinal cord (Meng *et al.*, 2018).

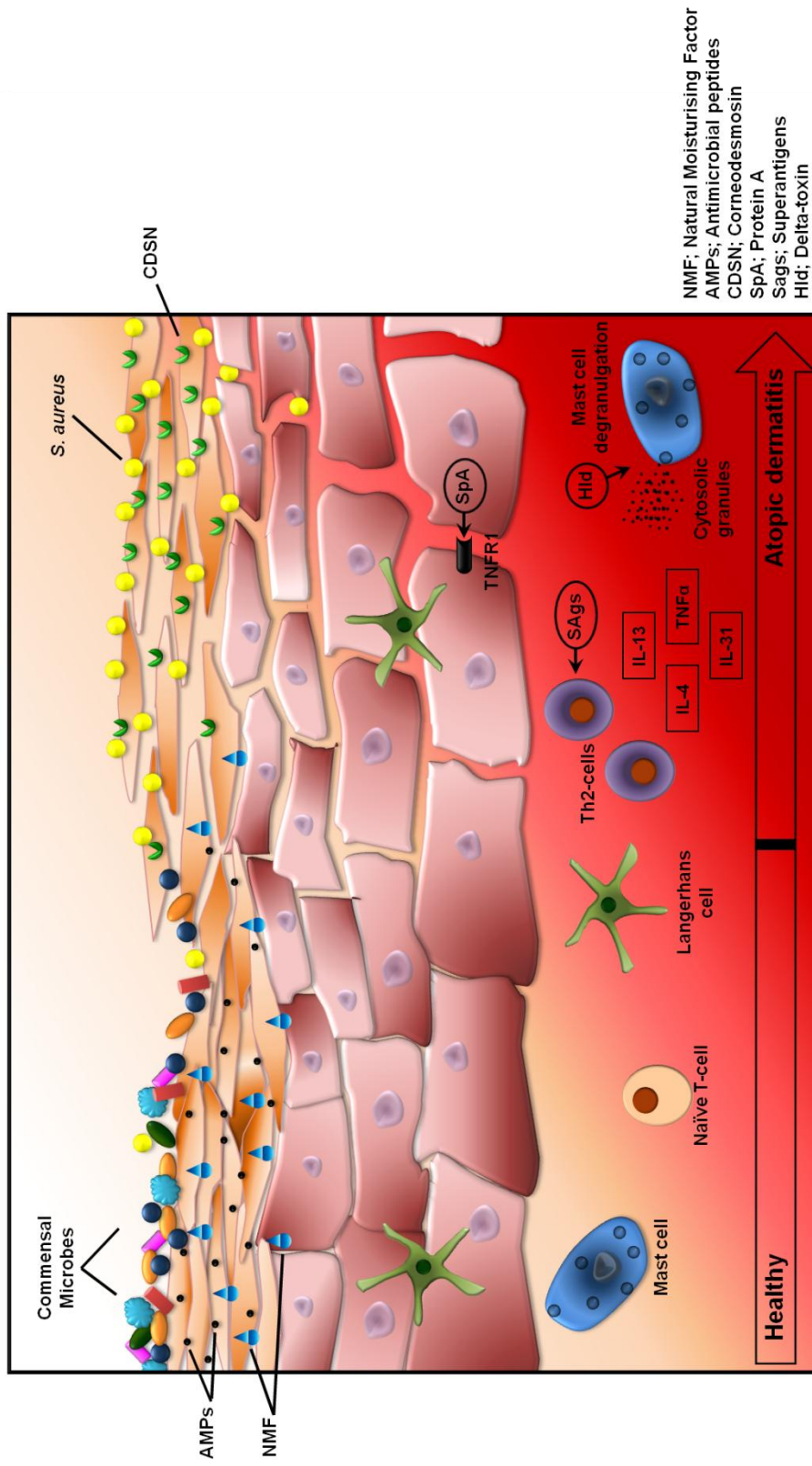


Figure 1.5. Physiological differences between the epidermis of healthy and atopic dermatitis skin. AD skin exhibits a heightened Th2 immune response with decreased NMF and AMPs. Microbial diversity on atopic dermatitis (AD) skin is reduced. Reduced structural integrity of AD corneocytes leads to an impaired barrier that permits entry of *S. aureus*. *S. aureus* secreted factors stimulate the Th2 mediated immune response further leading to inflamed skin lesions.

1.7.3 Models to investigate atopic dermatitis

Investigating AD has been hindered by the lack of a suitable *in vivo* animal model. Typically murine models are considered efficient model organisms due to their physiological similarity to humans, low maintenance, rapid breeding and relative ease of genomic manipulation (Perlman, 2016). AD is characterised by interplay between immune and barrier abnormalities and no AD murine model accurately displays both aspects phenotypically (Ewald *et al.*, 2017). The transcriptomes of multiple AD murine models were compared to the human AD transcriptome. Common AD murine models include NC/Nga, *FLG*^{-/-}, flaky tail, oxazolone-challenged, ovalbumin-challenged and a IL-23-injected psoriasis model. Homology to the human transcriptome was below 20% for each model except the IL-23 injected psoriasis model which had 37% homology. Thus murine models are not sufficient to accurately capture all aspects of AD (Ewald *et al.*, 2017).

Organotypic 3D models offer an attractive alternative to study AD as they replicate the human stratified epidermis. There is a degree of flexibility with 3D skin equivalents as they allow for gene knockdown and the addition of disease-associated cytokines. Knockdown of specific genes such as *FLG* and challenge with Th2 cytokines IL-4 and IL-13 has been shown to induce an AD phenotype in 3D skin equivalent models (Smits *et al.*, 2017, De Vuyst *et al.*, 2017, Popov *et al.*, 2014). A major limitation is the omission of immune cells from 3D skin equivalents. More recently atomic force microscopy (AFM) has been utilised for sensitive and in-depth analysis of protein-binding dynamics, microbe-host cell interactions and multiparametric imaging of host cells (Herman-Bausier *et al.*, 2018, Herman *et al.*, 2014, Fleury *et al.*, 2017). Specifically, AFM has been employed to investigate interactions between *S. aureus* and corneocytes isolated from AD patients (Feuillie *et al.*, 2018, Fleury *et al.*, 2017).

1.7.4 The role of *S. aureus* in the pathogenesis of atopic dermatitis

It is well established that the skin of AD patients exhibits a microbiome dysbiosis with a reduction in general microbial diversity. Typical commensal genera including *Corynebacterium spp.*, *Streptococcus spp.* and *Propionibacterium spp.* are reduced in AD skin, while Firmicutes such as

staphylococci are increased (Leyden *et al.*, 1974, Kong *et al.*, 2012, Nakatsuji *et al.*, 2017). *S. aureus* is not a natural member of the skin microbiota and carriage is transient (Oh *et al.*, 2012) however the skin of over 70% of AD patients is persistently colonised by *S. aureus* (Totte *et al.*, 2016) and the bacterial burden by *S. aureus* correlates with disease severity (Tauber *et al.*, 2016, Byrd *et al.*, 2017). *S. aureus* is ubiquitous in AD skin particularly during disease flares where it proliferates and dominates the microbiome in lesional skin (Kong *et al.*, 2012). Multiple factors associated with AD skin make it a more favourable environment for *S. aureus* colonisation. AD skin has reduced defence mechanisms, including a neutral pH which is permissive for *S. aureus* growth and decreased expression of bactericidal AMPs. In healthy skin, natural skin commensals such as CoNS outcompete and/or inhibit growth of *S. aureus* (described in section 1.6.3.2). However, CoNS with anti-*S. aureus* activity are less frequent in AD skin (Nakatsuji *et al.*, 2017). Furthermore, it was reported that *S. aureus* can penetrate past the epidermis in healthy human skin equivalents deficient in LL37 (Nakatsuji *et al.*, 2016). Given that LL37 is downregulated in AD skin, it is conceivable that *S. aureus* may penetrate further due to the defective structural integrity of the SC in AD skin.

S. aureus strains of clonal complex (CC) 30 are predominant in nasal carriage of the healthy population (Fleury *et al.*, 2017, Rasmussen *et al.*, 2013, Feil *et al.*, 2003). However, within the *S. aureus* spp., AD skin is associated with a distinct population structure. During disease flares, patients are usually colonised by a single strain of *S. aureus* (Byrd *et al.*, 2017, Harkins *et al.*, 2018). Studies based in Ireland, Britain, Denmark and Canada have reported frequent isolation of CC1 strains from the skin of AD patients (Fleury *et al.*, 2017, Harkins *et al.*, 2018, Clausen *et al.*, 2017, Yeung *et al.*, 2011). Fleury *et al.*, (2017) isolated forty-four *S. aureus* strains from the skin of Irish AD paediatric patients and forty-nine strains from the nasal cavity of age-matched healthy controls. CC1 strains were most frequently isolated from the skin of AD patients and represented 20% of AD strains isolated. Conversely, CC1 carriage in the healthy population was rare. CC30 was ubiquitous in nasal colonisation of the healthy population however these strains were underrepresented in AD patients (Fleury *et al.*, 2017). Harkins *et al.*, (2018) reported comparable

findings, where CC1 strains were associated with skin colonisation of paediatric AD patients and CC30 strains were dominant in the nasal cavity of healthy children (Harkins *et al.*, 2018). The sample cohort was relatively small in this study however, with only 9 AD skin isolates and 18 non-AD nasal isolates. CC1 was associated with AD skin colonisation in a cohort comprising seventy-four Danish AD adult patients, however there were no healthy controls included for comparison (Clausen *et al.*, 2017). A collection of *S. aureus* isolates from a large scale study based in Canada involving 119 paediatric and 41 adult AD patients reported frequent detection of CC1 strains and showed that carriage of a CC1 strain was associated with disease severity. Again, there were no healthy controls included in this study (Yeung *et al.*, 2011). Strains of CC8, CC15, CC45 and CC5 were also regularly detected in the skin of AD patients, albeit usually at a reduced frequency than CC1. CC30 strains were consistently underrepresented in AD skin. A large scale multicentre international study to examine CC carriage in the AD population is warranted to establish whether there is a geographical distribution of AD-associated *S. aureus* lineages, particularly given the prevalence of CC1 strains in both an Irish and a British AD population (Fleury *et al.*, 2017, Harkins *et al.*, 2018).

1.7.4.1 *S. aureus* colonisation of atopic dermatitis skin

The molecular determinants responsible for *S. aureus* colonisation of AD skin are ambiguous. *S. aureus* has a higher level of adherence to skin biopsies taken from AD patients compared to those taken from patients with psoriasis or healthy volunteers (Cho *et al.*, 2001). Recent evidence suggests that *S. aureus* adherence to AD skin is influenced by NMF levels. *S. aureus* adheres stronger to corneocytes isolated from AD patients with low NMF levels compared to high NMF levels (Feuillie *et al.*, 2018). Taking into consideration that both reduced NMF levels and *S. aureus* burden on AD skin correlate with more severe disease symptoms, this is indicative that adherence of *S. aureus* to AD skin directly contributes to disease pathogenesis. The first step in establishing successful colonisation is adherence to host cells and tissues which is typically dependent on protein-ligand interactions and several *S. aureus* CWAPs have been implicated in colonisation of AD skin. *S. aureus* adherence to AD

corneocytes is reduced in the absence of ClfB and strains isolated from AD patients have a higher level of ClfB-ligand binding activity than control strains from the nares of healthy controls (Fleury *et al.*, 2017, Feuillie *et al.*, 2018). FnBPA and/or FnBPB are capable of promoting stronger adherence to skin biopsies taken from AD patients compared to healthy controls and immunohistochemical staining for the FnBP ligand Fn in AD skin biopsies showed increased deposition of Fn on the surface of AD skin (Cho *et al.*, 2001). Furthermore, the expression of ClfB and FnBPs is increased at an elevated pH (Miajlovic *et al.*, 2010), characteristic of AD skin. The structural differences in AD skin including abnormal corneocyte morphology present the possibility of additional and/or more accessible ligands for *S. aureus* adherence in AD skin.

1.7.4.2 *S. aureus* toxins in atopic dermatitis

Carriage of *S. aureus* results in exacerbated AD symptoms likely due to the toxic activity of several secreted and wall-anchored factors (Figure 1.5). *S. aureus* protein A stimulates inflammation by signalling through TNFR1 expressed on keratinocytes which triggers the release of pro-inflammatory cytokines (Gomez *et al.*, 2004, Classen *et al.*, 2011). *S. aureus* expresses numerous superantigens (SAGs) including toxic shock syndrome toxin-1 (TSST-1) and enterotoxins SEA, SEB, SEC, SED, SEE or SEG. SAGs have proinflammatory activity by binding to MHCII molecules and activating T-cells which subsequently increases the level of circulating cytokines secreted by T-cells (Spaulding *et al.*, 2013). SAGs have been shown to trigger an IgE-mediated response in AD. IgE antibodies against SEA, SEB and TSST were detected in serum from AD patients that were colonised by SAg-expressing *S. aureus* but not in serum from psoriasis patients colonised by SAg-expressing strains (Leung *et al.*, 1993). During a normal IgE-mediated immune response, activated mast cells (MCs) undergo degranulation which releases proinflammatory cytosolic granules. In a murine model, MC degranulation was triggered by *S. aureus* PSM δ -toxin and this was increased in the presence of IgE. Additionally, δ -toxin has been detected in the culture supernatant of *S. aureus* strains isolated from AD skin lesions (Nakamura *et al.*, 2013). Collectively this suggests a role for δ -toxin in promoting inflammation in AD. The pore-forming α -toxin causes host cell death via binding to the membrane

lipid sphingomyelin. In healthy skin, sphingomyelin is reduced by the enzymatic activity of sphingomyelinase, thereby protecting keratinocytes from α -toxin-mediated cell death. In AD skin however sphingomyelinase is downregulated by IL-4 and IL-13, thus keratinocytes from AD skin are more susceptible to α -toxin-mediated lysis compared to keratinocytes from healthy individuals (Brauweiler *et al.*, 2014). As mentioned in section 1.7.4, *S. aureus* can penetrate further into a human skin equivalent model in the absence of host AMP LL37. This was dependent on *S. aureus* proteases by an undefined mechanism, as a protease deficient mutant of *S. aureus* was unable to penetrate (Nakatsuji *et al.*, 2016). *S. aureus* proteases aureolysin and the serine protease V8 degrade LL37 (Sieprawska-Lupa *et al.*, 2004) and may therefore contribute to the entry of *S. aureus* into the lower strata of the epidermis.

1.7.5 Current therapies for atopic dermatitis

Treatment of AD centres on reducing inflammation, itching and cosmetic symptoms such as characteristic red and dry skin lesions. Conventional AD therapies include moisturizers, topical corticosteroids and calcineurin inhibitors to target inflammation (Eichenfield *et al.*, 2014). Fusidic acid has been widely used in topical treatment of atopic dermatitis in combination with corticosteroids however this has been complicated by the emergence of fusidic acid resistant isolates (Harkins *et al.*, 2018, Edslev *et al.*, 2018). Fusidic acid is a narrow spectrum antibiotic that acts by targeting elongation factor G (EF-G) which is essential for translation in *S. aureus*. Resistance occurs by mutations in *fusA*, which encodes for EF-G (Besier *et al.*, 2003, O'Neill *et al.*, 2007a) and by the acquisition of genes *fusB* and *fusC* which encode proteins that confer protection by directly binding to EF-G in the presence of fusidic acid.

Due to treatment inefficiency and concerns over antibiotic resistance and long term steroid use, targeted therapies are being developed. Several modern therapies feature molecules to dampen the host immune response, thus limiting inflammation. Dupilumab is a monoclonal antibody that is now approved for treatment of moderate–severe AD in the US. It reduces the Th2 immune response by binding to the α -subunit of the IL-4 receptor and blocking IL-4 and IL-13 signal transduction (Gooderham *et al.*, 2018). Monoclonal antibodies Lebrikizumab and Tralokinumab were originally synthesised as possible asthma

therapies and block IL-13 signalling. Independent phase II clinical trials using Lebrikizumab and Tralokinumab to treat AD showed improved disease symptoms (Simpson *et al.*, 2018, Wollenberg *et al.*, 2019).

Dampening the immune response reduces AD disease symptoms. However it is unknown whether this type of treatment will result in decreased colonisation by *S. aureus*. *S. aureus* exacerbates AD symptoms and colonisation is a risk factor for infection, therefore eliminating *S. aureus* from the skin of AD patients is valuable. Antibiotics are not recommended for typical treatment of AD due to concerns over the growing rates of antibiotic resistance (Shah & Mohanraj, 2003, Jung *et al.*, 2015). Furthermore, following treatment with antibiotics, AD patients are rapidly re-colonised by *S. aureus* (Gilani *et al.*, 2005, Ewing *et al.*, 1998).

In healthy skin, natural commensals outcompete and/or inhibit colonisation by *S. aureus*. However such typical commensals are reduced in AD skin (Kong *et al.*, 2012). Manipulating the microbiome via microbiome transplants may provide a biotherapeutic approach to control AD disease symptoms. Probiotics contain viable cultures of specific commensal bacteria that are used to protect the host from pathogenic bacteria and restore a healthy microbiome. Several studies have examined the effect of probiotics containing *Lactobacillus* spp. and/or *Bifidobacterium* spp. for the treatment of AD. Meta-analysis show conflicting results between studies and suggest that current research on probiotics and AD is not significantly robust to show a benefit (Huang *et al.*, 2017, Rather *et al.*, 2016). Commensal Gram-negative bacteria (GNB) are ubiquitous in the microbiome of healthy skin but underrepresented in AD skin. Myles *et al.*, (2017) reported that *Roseomonas mucosa* from healthy volunteers inhibited *S. aureus* growth in murine and cell culture models of AD. Topical application of *Roseomonas mucosa* on AD patients resulted in improved disease symptoms and decreased *S. aureus* burden with no additional adverse effects (Myles *et al.*, 2016, Myles *et al.*, 2018). Similarly topical treatment containing CoNS spp. with anti-*S. aureus* activity reduced colonisation by *S. aureus* in AD patients. This study identified a lantibiotic expressed by *S. hominis* strain A9 with potent antimicrobial activity against *S. aureus* (Nakatsuji *et al.*, 2017). Topical treatment with *S. hominis* A9 is currently in phase II

clinical trials for treatment of AD. A bacteriophage endolysin known as Staphefekt is bactericidal to *S. aureus* specifically. Staphefekt is a component of several commercially available skincare products. A phase III clinical trial investigating the potential of Staphefekt as a topical agent to treat AD specifically is ongoing (Totte *et al.*, 2017).

Several studies have identified a link between low serum vitamin D levels and more severe AD disease. Furthermore, it has been reported that vitamin D stimulates expression of AMPs including LL37 and decreases expression of proinflammatory cytokines. Large scale studies are needed to confirm whether vitamin D may offer a therapeutic option for the treatment of AD (Kim & Bae, 2016, Roider *et al.*, 2013, Huang *et al.*, 2018).

1.8 Aims and Objectives

S. aureus colonises the skin of AD patients and exacerbates disease symptoms however there is very little known about how *S. aureus* adheres to AD skin and maintains its niche. Given that *S. aureus* is not usually a dominant member of the skin's microflora, and that a specific subset of *S. aureus* strains (clonal complex 1 isolates) are associated with AD, implies that unique host and bacterial factors contribute to allow *S. aureus* to persist in the AD skin environment. Thus the main aims and objectives of this project were to;

- Study corneodesmosin (CDSN) as a novel ligand for *S. aureus* and determine the relevance of this interaction to colonisation of AD skin.
- Identify the *S. aureus* proteins involved in binding to CDSN and characterise the interactions at the molecular level.
- Investigate the ability of cell wall-anchored proteins of *S. aureus* to bind to other corneocyte proteins.
- Determine the factors that contribute to the enhanced ability of *S. aureus* CC1 strains to dominate the microbiota of AD skin.
- Investigate CDSN as a potential ligand for other staphylococcal species.

Chapter 2

Materials and Methods

2.1 Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 2.1. Staphylococcal strains were grown in tryptic soy broth (TSB, Difco) or brain heart infusion (BHI) broth (Difco) at 37 °C with shaking (200 rpm) or on tryptic soy agar (TSA, Sigma) at 37 °C. Alternatively *S. aureus* was grown in nutrient limited medium RPMI 1640 (Sigma) in sterile plastic flasks. Initial RPMI cultures were grown overnight at 37 °C with shaking then diluted to an OD₆₀₀ = 0.05 in fresh RPMI for growth to stationary phase at 37 °C with shaking. *Escherichia coli* strains were grown in Luria (L) broth (Difco) at 37 °C with shaking or on Luria agar (Difco) at 37 °C. Broth was supplemented with antibiotics ampicillin (Amp, 100 µg/ml), chloramphenicol (Cm, 10 µg/ml), kanamycin (Kan, 50 µg/ml), erythromycin (Ery, 10 µg/ml) and anhydrotetracycline (Atc, 1 µg/ml) where appropriate.

2.2 DNA sequencing

Confirmatory DNA sequencing was carried out by GATC-Biotech or Eurofins Scientific.

2.2.1 Isolation of plasmid and genomic DNA

Plasmid DNA was isolated using Wizard® Plus SMiniprep kit (Promega) or Qiagen® Plasmid Plus MidiKit according to the manufacturers' instructions. DNA purification was carried out using the High Pure PCR Product Purification Kit (Roche) or Wizard® SV Gel and PCR Clean-up System (Promega). Genomic DNA was isolated using the Bacterial Genomic DNA purification kit (Edge BioSystems). Extraction of plasmid and genomic DNA from *S. aureus* required the addition of lysostaphin (200 µg/ml) to digest cell wall peptidoglycan.

2.2.2 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) amplification was carried out in a DNA thermal cycler (PIKO, Techne or Qcycler). Reaction volumes were typically carried out in 50 µl volumes using Velocity DNA polymerase (Bioline) according to the manufacturers' instructions. Plasmid DNA (1-10 ng), genomic DNA (150 ng) or synthetic DNA (1-10 ng) were used as template for PCR

amplification. Primers and dNTPs (Bioline) were used at final concentrations of 0.4 nM and 1 μ M, respectively. Primers are listed in Table 2.3 and were purchased from Integrated DNA Technologies. Synthetic DNA (gBlocks®) was synthesised by Integrated DNA Technologies. Initial denaturation was carried out at 98 °C (2 min) followed by 30-35 cycles of denaturation (30 s) at 98 °C, annealing (30 s, temperature dependent on primers) and extension at 72 °C. For plasmid DNA, an extension time of 15 s/kb of DNA was used and for genomic DNA an extension time of 30 s/kb was used. A final extension step was carried out at 72 °C for 10 min. PCR products amplified using plasmid DNA as template were incubated with 1 U DpnI (NEB) at 37 °C in a waterbath for 1-2 h to digest residual template DNA. PCR products were purified using High Pure PCR Product Purification Kit (Roche) or Wizard® SV gel and PCR clean-up system (Promega).

2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA molecules based on size. Gels containing 0.8-1% (w/v) agarose (Sigma) were dissolved by boiling in Tris-acetate-EDTA (TAE) buffer (Invitrogen) and cooled to 65 °C. SybrSafe Dye stain (ThermoFisher Scientific) was added and gels were cast in mini trays (Life Technologies). DNA samples were mixed with loading dye (NEB) and loaded into wells alongside the appropriate DNA ladder (NEB). Electrophoresis of samples was performed at 100 V. Gel images were visualised under UV lights using Alpha Imager™ software.

2.2.4 Sequence and ligase independent cloning

Sequence and ligase-independent cloning (SLIC) was carried out by a method described previously (Li & Elledge, 2012). The vector was amplified by inverse PCR. The insert was amplified by PCR using primers to incorporate 5' extensions with homology to the end regions of the amplified vector. Vector and insert DNA (1 μ g) were incubated independently with T4 polymerase (3 U, NEB) to generate products with single stranded ends. The reaction consisted of a final volume of 40 μ l containing NEB buffer 2, 1 X molecular grade BSA (NEB), dithiothreitol (DTT, 5 mM) and urea (200 mM). The reaction was incubated at 23 °C for 20 minutes and stopped with EDTA (25 mM), followed by

incubation at 75 °C for 20 min. Equal volume vector and insert (5 µl) were combined and allowed to anneal by a reaction involving initial incubation at 65 °C for 10 min, followed by a decreasing temperature gradient (65-25 °C) with 1 min hold for each degree. The entire reaction was used for transformation into chemically competent *E. coli* cells.

2.3 Transformation

2.3.1 Preparation and transformation of *E. coli*

Chemically competent *E. coli* were prepared using CaCl₂ treatment. An overnight culture of *E. coli* was used to inoculate 400 ml fresh L-broth in a 2 L flask which was grown until an OD₆₀₀ = 0.5 was reached. Cells were chilled on ice for 1 h and harvested by centrifugation. Cells were resuspended in ice cold MgCl₂ (100 mM) and harvested again by centrifugation. Cells were washed twice in ice cold buffer (60 mM CaCl₂, 10 mM PIPES, 15% (v/v) glycerol, pH 8) before being aliquoted. Cells were snap frozen in liquid nitrogen and stored at -70 °C.

Thawed aliquots (150 µl) were incubated with plasmid DNA (150 ng), SLIC reactions (10 µl) or ligation reactions (10 µl) for 30 min on ice. Samples were heated to 42 °C for 2 min and then chilled on ice for 2 min. 900 µl L-broth was added and the mixture was incubated for 1 h at 37 °C with shaking before plating onto L-agar containing the appropriate antibiotic. Plates were incubated for 16-24 h at 37 °C. Transformants were screened by colony PCR.

2.3.2 Preparation and transformation of *S. aureus*

Electrocompetent *S. aureus* cells were prepared by diluting an overnight culture of *S. aureus* to an OD₆₀₀ = 0.5 in 50 ml prewarmed TSB and incubated at 37 °C with shaking for 30 min. Cells were cooled in an ice-water slurry for 10 min. Cells were harvested by centrifugation at 3,900 x g for 10 min at 4 °C. Cells were resuspended in 50 ml ice-cold sterile water. Cells were harvested again by centrifugation and washed in decreasing volumes of ice-cold 10% (v/v) glycerol (5 ml, 1 ml, 210 µl). Aliquots were snap-frozen in liquid nitrogen and stored at -70 °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 resuspended in sterile 10% (v/v) glycerol/500 mM sucrose solution (50 µl). 1-5 µg of plasmid DNA in a final volume of 5 µl was added to the cells. The cells were added to a 0.1 cm electroporation cuvette (Biorad). The cells were pulsed at 21 kV/cm, 100 Ω and 25 uF. Following electroporation, cells were resuspended in 1 ml BHI incorporating 500 mM sucrose and incubated for 2 h at 37 °C with shaking. Cells were plated onto TSA containing appropriate antibiotic. Transformants were screen by PCR.

2.3.3 Screening transformants

Colonies intended for screening were toothpicked onto the appropriate agar. Half a toothpicked colony was resuspended in TE buffer (1 mM EDTA/10 mM Tris-HCL, pH 7.8) for *S. aureus* and sterile water for *E. coli*, until a milky suspension was achieved. The suspension was boiled at 100 °C for 10 min and centrifuged at 6000 rpm for 10 min. Supernatant (2 µl) was used as template for PCR using Phire Hot Start DNA polymerase (Finnzymes) as per the manufacturers' instructions.

2.4.1 Plasmid construction

Plasmids used in this study are listed in Table 2.2 and primers are listed in Table 2.3.

DNA encoding human CDSN (residues K33-P529) codon optimised (CO) for *E. coli* was synthesised commercially and included 5' and 3' restriction sites BglII and Sall respectively. The synthetic DNA fragment was amplified by PCR with primers CDSN F BglII and CDSN R Sall and subsequently cloned into vector pGEX-4T2 by conventional cloning methods with restriction enzymes BamHI and Sall, to generate plasmid pGEX-4T2::*CDSN*(CO).

Plasmid pGEX-4T2::*CDSN*(CO) was used as template for inverse PCR where primers CDSN F ST and CDSN R ST were used to add DNA encoding a Strep-tag II affinity tag to the 3' end of the *CDSN* gene. The PCR product was treated with DpnI and joined by blunt end ligation using the LigaFast® Rapid DNA Ligation System (Promega) to generate plasmid pGEX-4T2::*CDSN*.

Plasmids pGEX-4T2::*CDSN* Δ NT and pGEX-4T2::*CDSN* Δ CT were generated by inverse PCR using plasmid pGEX-4T2::*CDSN* as template with primers CDSN Δ NT F and CDSN Δ NT R to generate plasmid pGEX-4T2::*CDSN* Δ NT and primers CDSN Δ CT F and CDSN Δ CT R to generate plasmid pGEX-4T2::*CDSN* Δ CT. Inverse PCR products were treated with DpnI and blunt-end ligation was carried out.

Plasmid pGEX-4T2::*CDSN*NT Ω was constructed by two consecutive rounds of inverse PCR using pGEX-4T2::*CDSN* as template and primers CDSN Δ ₃₃₋₆₁ F, CDSN Δ ₃₃₋₆₁ R and CDSN Δ ₁₇₂₋₅₂₉ F, CDSN Δ ₁₇₂₋₅₂₉ R respectively. The inverse PCR products were treated with DpnI and joined by blunt-end ligation.

2.4.2 Strain construction by allelic exchange

S. aureus AD08 Δ *clfB*, AD08 Δ *fnbA*, AD08 Δ *fnbB*, AD08 Δ *fnbA* Δ *fnbB*, AD08 Δ *clfB* Δ *fnbA*, AD08 Δ *clfB* Δ *fnbB*, AD08 Δ *clfB* Δ *fnbA* Δ *fnbB*, AD08 Δ *esaDG*, AD08 Δ *ermAM*, AD08 Δ *aphA3*, TS1064 Δ *ermAM*, TS1064 Δ *aphA3*, TS1157 Δ *aphA3* were generated using plasmid pIMAY (Monk *et al.*, 2012). Cloning was carried out using the SLIC procedure as described in 2.2.4. Primers for amplifying insert sequences contained 5' extensions with homology to the target vector. Mutagenesis was carried out by allelic exchange as described previously in section 1.5.1. Each mutant strain was confirmed by DNA sequencing of a PCR amplicon generated using genomic DNA as template with primers OUT F and OUT R. Mutants were validated by cross streaking against *S. aureus* RN4220 on sheep blood agar to test for δ -toxin production and the OD₆₀₀ of cultures grown in TSB was monitored over 16 h to compare the growth profile to the parental wild-type strain.

Deletion of the *clfB* gene was achieved using plasmid pIMAY:: Δ *clfB* (Mulcahy *et al.*, 2012). Plasmid pIMAY:: Δ *clfB* was passaged through *E. coli* IM01B and transformed into electrocompetent AD08, AD08 Δ *fnbA* and AD08 Δ *fnbA* Δ *fnbB* cells by electroporation. *clfB* was deleted by allelic exchange.

Deletion of the *fnbA* gene was achieved using pIMAY:: Δ *fnbA*. Plasmid pIMAY:: Δ *fnbA* was constructed using AD08 genomic DNA as template DNA. The regions 500 bp upstream and 504 bp downstream of the *fnbA* gene were

amplified by PCR, where primers *fnbA* A and *fnbA* B annealed to the 5' and 3' upstream region and primers *fnbA* C and *fnbA* D annealed to the 5' and 3' region downstream of *fnbA*. The upstream and downstream PCR products were denatured and allowed to re-anneal via complementary sequences in primers *fnbA* B and *fnbA* C and this was used as template for subsequent PCR with primers *fnbA* A and *fnbA* D. The product was cloned into pIMAY between KpnI and SacI restriction sites by SLIC. The resulting plasmid pIMAY:: Δ *fnbA* was transformed into chemically competent *E. coli* IM01B by heat shock and verified by DNA sequencing. Following isolation from *E. coli*, the plasmid was transformed into electrocompetent *S. aureus* AD08 by electroporation and the *fnbA* gene was deleted by allelic exchange.

Deletion of the *fnbA* and *fnbB* genes was achieved using plasmid pIMAY:: Δ *fnbA* Δ *fnbB*. Plasmid pIMAY:: Δ *fnbA* Δ *fnbB* was passaged through *E. coli* IM01B and transformed into electrocompetent AD08 cells by electroporation. *fnbA* and *fnbB* were deleted by allelic exchange.

The *fnbA* gene was restored onto its original location on the chromosome of AD08 Δ *fnbA* Δ *fnbB* and AD08 Δ *clfB* Δ *fnbA* Δ *fnbB* to isolate AD08 Δ *fnbB* and AD08 Δ *clfB* Δ *fnbB* respectively using plasmid pIMAY::*fnbA*_restore. Primers *fnbA* A and *fnbA*_Res D were used to amplify the region 500 bp upstream of the *fnbA* gene and 527 bp downstream of the *fnbB* gene (Chapter 3, Figure 3.7). The product was cloned into pIMAY between KpnI and SacI restriction sites by SLIC. The resulting plasmid pIMAY::*fnbA**fnbB* was transformed into chemically competent *E. coli* XL1-Blue by heat shock and verified by DNA sequencing. An inverse PCR with plasmid pIMAY::*fnbA**fnbB* as template and primers *fnbB*_Del F and *fnbB*_Del R were used to delete the *fnbB* gene from plasmid pIMAY::*fnbA**fnbB*. The product was joined by blunt end ligation using the LigaFast® Rapid DNA Ligation System (Promega) to generate plasmid pIMAY::*fnbA*_restore. Plasmid pIMAY::*fnbA*_restore was passaged through *E. coli* IM01B and transformed into electrocompetent AD08 Δ *fnbA* Δ *fnbB* and AD08 Δ *clfB* Δ *fnbA* Δ *fnbB* by electroporation. *fnbA* was restored onto its original location on the chromosome by allelic exchange.

Deletion of the region encoding *esaD*, *esaG* and 10 *esaG*-like genes was achieved using plasmid pIMAY:: Δ *esaDG*. DNA with homology to 654 bp

upstream of *esaD* and 514 bp downstream of the final *esaG*-like gene was synthesised commercially and cloned into plasmid pIMAY between KpnI and SacI restriction sites by SLIC. Plasmid pIMAY:: Δ *esaDG* was passaged through *E. coli* IM01B and transformed into electrocompetent AD08 and the region was deleted by allelic exchange.

AD08*ermAM* and AD08::*aphA3* were generated using plasmids pIMAY::Int.S.*ermAM*_{CC1} and pIMAY::Int.S.*aphA3*_{CC1} respectively. Primers Int.S. A and Int.S._{CC1} B were designed to amplify 553 bp upstream and primers Int.S. C and Int.S. D amplified 586 bp downstream of the specific integration site in AD08. Primers Promoter_{CC1} F and Erm R were used with pIMC::*ery* as template to amplify *ermAM*. Primers Promoter_{CC1} F and Kan R were used with pIMC::*kan* as template to amplify *aphA3*. Complementary overhangs in primers Promoter_{CC1} F and Erm R and Kan R allowed the resistance gene products to be independently joined to the AB and CD products in a single overlap extension PCR using primers Int.S. A and Int.S. D. The resulting products were cloned into pIMAY between the KpnI and SacI restriction sites by SLIC to generate plasmids pIMAY::Int.S.*ermAM*_{CC1} and pIMAY::Int.S.*aphA3*_{CC1}. Both plasmids were passaged through *E. coli* IM01B and transformed into electrocompetent AD08. Insertion of *ermAM* and *aphA3* onto the AD08 chromosome was achieved by allelic exchange.

TS1064*ermAM*, TS1064*aphA3*, TS1157*ermAM* and TS1157*aphA3* were generated using plasmids pIMAY::Int.S.*ermAM*_{CC30} and pIMAY::Int.S.*aphA3*_{CC30}. The regions 553 bp upstream and 578 bp downstream of the integration site in MRSA252 were amplified by PCR where primers Int.S. A and Int.S._{CC30} B annealed to the 5' and 3' upstream region and primers Int.S. C and Int.S. D annealed to the 5' and 3' region downstream of the integration site. Primers Promoter_{CC30} F and Erm R were used with pIMC::*ery* as template to amplify *ermAM*. Primers Promoter_{CC30} F and Kan R were used with pIMC::*kan* as template to amplify *aphA3*. Complementary overhangs in primers Promoter_{CC30} F and Erm R and Kan R allowed each resistance gene product to be independently joined to the AB and CD products in single overlap extension PCRs using primers Int.S. A and Int.S. D (Chapter 5, Figure 3.1). The resulting products were cloned into pIMAY between the KpnI and SacI restriction sites by

SLIC to generate plasmids pIMAY::Int.S.*ermAM*_{CC30} and pIMAY::Int.S.*aphA3*_{CC30}. Both plasmids were passaged through *E. coli* IM30B and transformed into electrocompetent TS1064 and TS1157. Insertion of *ermAM* and *aphA3* onto the TS1064 and TS1157 chromosomes was achieved by allelic exchange.

2.5 Protein electrophoresis and western immunoblotting

2.5.1 SDS-PAGE

Protein samples were diluted 1:1 with 2 X final sample buffer (Laemmli, Sigma) and boiled for 5 min. 20 µl volume of sample was loaded onto acrylamide gels (4.5% stacking and 10-12.5% separating gel) and separated by SDS-PAGE. Electrophoresis was carried out at 120 V and proteins were visualised by InstantBlue protein stain (Expedeon) or transferred onto PVDF membranes (Roche) for Western Immunoblotting.

2.5.2 Western immunoblotting

Proteins were transferred onto a PVDF membrane (Roche) for 1 h at 100 V using a wet transfer cell (Bio Rad). Non-specific binding to membranes was blocked by incubation for 1-2 h in TS buffer (10 mM Tris-HCL, pH 7.4, 150 mM NaCl) containing 10% (w/v) skimmed milk (Marvel). Blots were probed with the appropriate primary antibody diluted in 10 % (w/v) Marvel/TS buffer for 1 h at room temperature with shaking. Blots were washed three times for 10 min with shaking in TS buffer. Secondary antibodies were diluted in 10% (w/v) Marvel/TS buffer and incubated with the membranes for 1 h at room temperature with shaking. Again, blots were washed three times for 10 min with shaking in TS buffer. Reactive bands were detected using the LumiGLO reagent and peroxidase detection system (Cell Signalling Technology) and visualised using ImageQuant TL software (GE).

2.6 Protein purification

Recombinant proteins were expressed from IPTG-inducible pQE30 vector with an N-terminal hexa histidine (His) affinity tag for purification by nickel affinity chromatography or from IPTG-inducible pGEX-4T2 vector with an N-terminal glutathione-S-transferase (GST) affinity tag for purification by

glutathione affinity chromatography or with a C-terminal Streptag II (Strep) affinity tag for purification by Strep-Tactin affinity chromatography. *E. coli* Topp3 or XL1-Blue cells carrying pQE30 or pGEX-4T2 plasmids were grown to OD₆₀₀ = 0.4–1 and induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 3 h at 37 °C with shaking. Cells were harvested by centrifugation and resuspended in 30 ml PBS containing EDTA-free protease inhibitors (Roche). Cells were mechanically lysed using a French pressure cell (1000–1500 psi). The lysate was centrifuged at 15,000 *g* for 20 min. 20–200 μ l DNase (1 mg/ml) was added to the supernatant followed by filtration through a 0.45 μ m filter (Whatman).

2.6.1 Purification of GST-tagged recombinant protein

GST-tagged proteins were purified using a 5 ml GSTTM FF column (GE Healthcare) according to the manufactures' instructions with the flow rate controlled using a peristaltic pump. The column was equilibrated with 5 x column volume PBS. The cleared lysate was loaded onto the column and the column was washed with 10 x column volumes of PBS. Protein was eluted from the column with glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris-HCL, pH 8.0) and analysed by SDS-PAGE. Eluted protein was dialysed against PBS using Spectra/PorTM Dialysis Membrane Tubing (Fisher Scientific) for 16 h at 4 °C.

2.6.2 Purification of His-tagged recombinant protein

His-tagged proteins were purified using a 5 ml HiTrapTM Chelating HP column (GE Healthcare) according to the manufacturers' instructions with the flow rate controlled using a peristaltic pump. The column was precharged with Ni²⁺ and equilibrated with 5 x column volume binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCL, pH 7.9). The cleared lysate was loaded onto the column and the column was washed with 10 x column volumes of binding buffer. Protein was eluted from the column with a continuous linear gradient of imidazole (5 – 100 mM) in binding buffer and analysed by SDS-PAGE. Eluted protein was dialysed against PBS using Spectra/PorTM Dialysis Membrane Tubing (FisherScientific) for 16 h at 4 °C.

2.6.3 Purification of Strep-tagged recombinant protein

Strep-tagged proteins were purified using a 5 ml StrepTrapTM column (GE Healthcare) according to the manufacturers' instructions with the flow rate controlled using a peristaltic pump. The column was equilibrated using 5 x column volumes binding buffer (100 mM Tris-HCL, 150 mM NaCl, 1mM EDTA, pH 8). The cleared lysate was loaded onto the column and the column was washed with 10 x column volumes of binding buffer. Protein was eluted from the column with desthiobiotin (2.5 mM) in binding buffer and analysed by SDS-PAGE. Eluted protein was dialysed against PBS using Spectra/PorTM Dialysis Membrane Tubing (FisherScientific) for 16 h at 4 °C.

2.6.4 Protein Concentration Determination

The concentration of purified recombinant protein was determined by measuring absorbance at 280 nm using a NanodropTM 1000 spectrophotometer according to Beer-Lambert Law ($A_{280} = E.c.l.$) or by BCA protein assay kit (Pierce) as per the manufacturers' instructions (ThermoFisher Scientific).

2.7 Solid phase bacterial adherence assays

Nunc MaxiSorpTM Microtiter flat-bottom plates (ThermoFisher Scientific) were coated with a single concentration or serial dilutions of recombinant protein diluted in sodium carbonate buffer (0.1 M NaHCO₃, pH 9.6) and incubated overnight at 4 °C. Wells were washed 3 times with PBS. To block non-specific binding, 5% (w/v) bovine serum albumin (BSA) in PBS was added to the wells and incubated for 2 h at 37 °C. Wells were washed 3 times with PBS. Bacterial cultures grown to exponential phase or stationary phase were adjusted to an OD₆₀₀ = 1 in PBS and added to the plate for 2 h incubation at 37 °C. Wells were washed 3 times in PBS and bound cells were fixed with formaldehyde (25% v/v) for 20 min and stained with crystal violet (0.5% v/v) for 1 min. Following washing with PBS, acetic acid (5% v/v) was added and incubated for 5 min at room temperature with gentle agitation. The absorbance was measured at 570 nm in an ELISA plate reader (Labsystems). Fibrinogen and fibronectin were diluted in PBS instead of sodium carbonate buffer and immobilised onto flat-bottomed plates (Sarstedt).

2.8 Growth curves

Stationary phase bacterial cultures were diluted to $OD_{600} = 0.025$ in TSB and added (200 μ l) to sterile round-bottomed microtitre plates (Sarstedt). Plates were incubated at 37 °C with continuous shaking in a Synergy H1 plate reader (Biotek) for 16 h with OD_{600} values measured at 30 min intervals.

2.9 Surface plasmon resonance

Surface plasmon resonance (SPR) was performed using the BIAcore X100 system (GE Healthcare). His-CDSN was diluted in 10 mM sodium acetate buffer (pH 4) and immobilized onto a CM5 sensor chip by amine coupling as per the manufacturers' instructions. A second flow cell remained uncoated and served as a reference surface. Increasing concentrations of recombinant rClfBN2N3_{CC1} (1.11 μ M–90 μ M), rFnBPAN2N3_{CC1} (0.48 μ M–39 μ M) and rFnBPBN2N3_{CC1} (1.11 μ M–80 μ M) in PBS were passed over the surface of the chip. The surface of the chip was regenerated between individual cycles. All sensorgram data was subtracted from the corresponding data of the reference flow cell and the response generated from passing buffer over the chip was also subtracted from each sensorgram. Data was analysed using the BIAevaluation software version 3.0. The dissociation constant (K_D) of each interaction was calculated by plotting the level of binding in response units (RU) against the concentration of rClfBN2N3_{CC1}, rFnBPAN2N3_{CC1} and rFnBPBN2N3_{CC1}.

2.10 Generation of F(ab)₂ fragments

Anti-CDSN F(ab)₂ fragments were generated using the PierceTM F(ab')₂ Micro Preparation Kit (ThermoFisher Scientific) as per the manufacturers' instructions. Undigested anti-CDSN and anti-CDSN F(ab)₂ fragments were analysed by SDS-PAGE and visualised using InstantBlue protein stain.

2.11 Intraspecies bacterial competition assays

S. aureus cultures were grown to stationary phase in RPMI as described in section 2.1. Cultures were washed and adjusted to $OD_{600} = 0.00056$ ($\sim 3 \times 10^5$ CFU/ml) in RPMI. 12.5 μ l of each adjusted culture was added to RPMI (475 μ l)

in a 1.5 ml plastic tube at a 1:1 ratio to give $\sim 1.5 \times 10^4$ CFU/ml of bacteria. Immediately 100 μ l of sample (T0) was taken and added to 900 μ l of RPMI and 10-fold serial dilutions were prepared. 100 μ l of the dilutions were plated out on TSA in triplicate to calculate input CFU. Co-cultures were incubated for 7 h at 37 °C with shaking (200 rpm). Samples (100 μ l) were taken after 3 h incubation (T3), after 5 h incubation (T5) and 7 h incubation (T7), diluted in RPMI and plated out onto TSA (100 μ l) in triplicate. Plates were incubated for 12–16 h at 37 °C. Colonies were replica plated onto agar containing Ery (10 μ g/ml) and/or Kan (50 μ g/ml) and TSA to obtain CFU counts of resistant bacteria. The CFU on antibiotic-containing agar was subtracted from the total CFU on TSA without antibiotic to obtain the CFU of a non-resistant strain. To determine differences in growth between two competing strains, the CFU/ml value of each individual strain at T0 was set to an arbitrary value of 100%. Viable counts at each of the timepoints were expressed relative to the starting inoculum of 100%.

2.12 Atomic force microscopy

All atomic force microscopy experiments were conducted by Dr. Cécile Formosa-Dague at the Louvain Institute of Biomolecular Science and Technology, Université catholique de Louvain, Belgium.

2.13 Statistical analysis

- Statistical analyses were performed using Prism Graphpad software version 5.01. *, ** and *** represent p-values of < 0.05, < 0.01 and < 0.001 respectively. ns represents non-significant p-values > 0.05. Unpaired two-tailed Student's t-test was used to compare the mean values between two independent samples where there was no other variable factor in the experiment.
- One-way ANOVA was used to compare the mean values of at least three samples, where there was one independent variable in the experimental procedure. This test was used for single-point bacterial adherence assays in which the adherence of one bacterial strain to a single concentration of multiple different recombinant proteins was measured, or where the adherence of multiple bacterial strains to a single concentration of one recombinant protein was measured. Similarly it was used to compare the binding affinity of one protein for multiple

concentrations of three independent recombinant proteins. Post-tests used with a one-way ANOVA were either Tukey post-test to allow for comparisons between every sample or Dunnett post-test to compare each sample to one specified sample.

- Two-way ANOVA was used to compare the mean values of at least two or more samples where there were also two or more independent variables in the experimental procedure. This included solid-phase bacterial adherence assays measuring the adherence of multiple bacterial strains to multiple concentrations of a recombinant protein. It was also used to analyse intraspecies competition involving the competitive growth of multiple strains at several different timepoints. A Bonferroni post-test was used with the two-way ANOVA to pinpoint where significant differences were.

Table 2.1. Bacterial strains

Strain	Description	Reference
<i>Escherichia coli</i>		
XL1-Blue	Host for cloning. <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i>	Stratagene
Topp3	Strain deficient in proteases for expression of recombinant proteins. Rif ^r , [F' <i>proAB+</i> <i>lac</i> ^q ZΔ <i>M15</i> Tn10 (Tet ^r) (Kan ^r)]	Stratagene
DH5-α	Cloning host for high efficiency transformation	New England Biolabs
IM01B	Plasmid propagation. SA08BΩP _{N25-} <i>hsdS</i> (CC ^a 1-1)	(Monk <i>et al.</i> , 2015)
IM30B	Plasmid propagation. SA30BΩP _{N25-} <i>hsdS</i> (CC30-1)	(Monk <i>et al.</i> , 2015)
<i>Staphylococcus aureus</i>		
Newman	NCTC 8178	(Duthie & Lorenz, 1952)
Newman <i>clfB</i>	Derivative of Newman deficient in <i>clfB</i> ::Tc ^r	(Ni Eidhin <i>et al.</i> , 1998)
RN4220	Restriction-deficient derivative of 8325-4	(Kreiwirth <i>et al.</i> , 1983)
SH1000	Derivative of lab strain 8325-4 with repaired defect in <i>rsbU</i>	(Horsburgh <i>et al.</i> , 2002)
SH1000 <i>clfA clfB fnbA fnbB</i>	Deficient in <i>clfA</i> ::Tn917_Ery ^r <i>clfB</i> ::Tc ^r <i>fnbA</i> ::Tc ^r <i>fnbB</i> ::Ery ^r	(O'Neill <i>et al.</i> , 2008)
MRSA252	HA-MRSA, SCC <i>mec</i> type II, MLST ^b type 36, CC ^a 30	(Holden <i>et al.</i> , 2004)
AD08	AD clinical isolate. CC ^a 1	(Fleury <i>et al.</i> , 2017)
AD08Δ <i>clfB</i>	Derivative of AD08 deficient in <i>clfB</i>	This study
AD08Δ <i>fnbA</i>	Derivative of AD08 deficient in <i>fnbA</i>	This study
AD08Δ <i>fnbA</i> Δ <i>fnbB</i>	Derivative of AD08 deficient in <i>fnbA fnbB</i>	This study

AD08 Δ <i>fnbB</i>	Derivative of AD08 Δ <i>fnbA</i> Δ <i>fnbB</i> with <i>fnbA</i> restored	This study
AD08 Δ <i>clfB</i> Δ <i>fnbA</i>	Derivative of AD08 Δ <i>fnbA</i> deficient in <i>clfB</i>	This study
AD08 Δ <i>clfB</i> Δ <i>fnbA</i> Δ <i>fnbB</i>	Derivative of AD08 Δ <i>fnbA</i> Δ <i>fnbB</i> deficient in <i>clfB</i>	This study
AD08 Δ <i>clfB</i> Δ <i>fnbB</i>	Derivative of AD08 Δ <i>clfB</i> Δ <i>fnbA</i> Δ <i>fnbB</i> with <i>fnbA</i> restored	This study
AD08 <i>ermAM</i>	Derivative of AD08 carrying <i>ermAM</i> . Ery ^r	This study
AD08 <i>aphA3</i>	Derivative of AD08 carrying <i>aphA3</i> . Kan ^r	This study
AD08 Δ <i>esaDG</i>	Derivative of AD08 deficient in <i>esaD</i> , <i>esaG</i> and 10 putative <i>esaG</i> -like genes	This study
AD17	AD clinical isolate. CC ^a 1	(Fleury <i>et al.</i> , 2017)
AD24	AD clinical isolate. CC ^a 1	(Fleury <i>et al.</i> , 2017)
AD36	AD clinical isolate. CC ^a 1	(Fleury <i>et al.</i> , 2017)
AD40	AD clinical isolate. CC ^a 1	(Fleury <i>et al.</i> , 2017)
AD41	AD clinical isolate. CC ^a 1	(Fleury <i>et al.</i> , 2017)
AD45	AD clinical isolate. CC ^a 1	(Fleury <i>et al.</i> , 2017)
TS1024	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
TS1055	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
TS1064	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
TS1064 <i>ermAM</i>	Derivative of TS1064 carrying <i>ermAM</i> . Ery ^r	This study
TS1064 <i>aphA3</i>	Derivative of TS1064 carrying <i>aphA3</i> . Kan ^r	This study
TS1157	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH

TS1157 <i>ermAM</i>	Derivative of TS1157 carrying <i>ermAM</i> . Ery ^r	This study
TS1157 <i>aphA3</i>	Derivative of TS1157 carrying <i>aphA3</i> . Kan ^r	This study
TS1270	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
TS1310	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
TS1319	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
TS1334	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
TS1343	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
TS1493	Nasal carriage isolate from a healthy child. CC ^a 30	Desiree Bennett, Temple Street CUH
Coagulase-Negative Staphylococci		
<i>S. scuri</i> CoNSA	Temple street hospital clinical isolate from patient body swab.	This study
<i>S. lugdenensis</i> CoNSB	Temple street hospital clinical isolate from an unknown site.	This study
<i>S. caprae</i> CoNSC	Temple street hospital clinical isolate from patient cerebrospinal fluid.	This study
<i>S. xylosus</i> CoNSD	Temple street hospital clinical isolate from patient cerebrospinal fluid.	This study
<i>S. simulans</i> CoNSE	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. saprophyticus</i> CoNSF	Temple street hospital clinical isolate from an unknown site.	This study
<i>S. auricularis</i> CoNSH	Temple street hospital clinical isolate from patient tissue swab.	This study

<i>S. epidermidis</i> CoNSG	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. epidermidis</i> CoNSP	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. epidermidis</i> CoNSQ	Temple street hospital clinical isolate from patient cerebrospinal fluid.	This study
<i>S. epidermidis</i> CoNST	Temple street hospital clinical isolate from unknown site.	This study
<i>S. capitis</i> CoNSI	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. capitis</i> CoNSJ	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. haemolyticus</i> CoNSK	Temple street hospital clinical isolate from unknown site.	This study
<i>S. haemolyticus</i> CoNSO	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. hominis</i> CoNSL	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. hominis</i> CoNSM	Temple street hospital clinical isolate from patient cerebrospinal fluid.	This study
<i>S. hominis</i> CoNSS	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. warneri</i> CoNSN	Temple street hospital clinical isolate from patient blood culture.	This study
<i>S. warneri</i> CoNSR	Temple street hospital clinical isolate from patient blood culture.	This study
<i>Staphylococcus pseudintermedius</i>		
ED99	Canine pyoderma clinical isolate	(Bannoehr <i>et al.</i> , 2007)
ED99 Δ <i>spsD</i>	Derivative of ED99 deficient in <i>spsD</i>	(Pietrocola <i>et al.</i> , 2015)

ED99 Δ <i>spsD</i> _{<i>spsD</i>Res}	Derivative ED99 Δ <i>spsD</i> with <i>spsD</i> restored	(Richards, unpublished)
ED99 Δ <i>spsL</i>	Derivative of ED99 deficient in <i>spsL</i>	(Pietrocola <i>et al.</i> , 2015)
ED99 Δ <i>spsL</i> _{<i>spsL</i>Res}	Derivative of ED99 Δ <i>spsL</i> with <i>spsL</i> restored	(Richards <i>et al.</i> , 2018)
ED99 Δ <i>spsD</i> Δ <i>spsL</i>	Derivative of ED99 deficient in <i>spsD</i> <i>spsL</i>	(Pietrocola <i>et al.</i> , 2015)

^a Clonal Complex

^r Resistance

Table 2.2 Plasmids

Plasmid	Description	Reference
pIMAY	Temperature sensitive plasmid vector for allelic exchange in <i>S. aureus</i> . Inducible <i>secY</i> antisense RNA. Cm ^r .	(Monk <i>et al.</i> , 2012)
pIMAY:: $\Delta clfB$	pIMAY carrying <i>clfB</i> deletion construct with 500 bp upstream and downstream regions of <i>clfB</i> . Cm ^r .	(Mulcahy <i>et al.</i> , 2012)
pIMAY:: $\Delta fnbA$	pIMAY carrying <i>fnbA</i> deletion construct with 500 bp upstream and 504 bp downstream regions of <i>fnbA</i> . Cm ^r .	This study
pIMAY:: $\Delta fnbA \Delta fnbB$	pIMAY carrying <i>fnbA fnbB</i> deletion construct with 500 bp upstream of <i>fnbA</i> and 500 bp downstream of <i>fnbB</i> . Cm ^r .	Zapotoczna and Geoghegan, unpublished
pIMAY:: <i>fnbAfnbB</i>	pIMAY carrying 500 bp upstream of <i>fnbA</i> , the <i>fnbA</i> gene, 680 bp intergenic region between <i>fnbA</i> and <i>fnbB</i> , the <i>fnbB</i> gene and 527 bp downstream of <i>fnbB</i> . Cm ^r .	This study
pIMAY:: <i>fnbA_restore</i>	pIMAY carrying 500 bp upstream of <i>fnbA</i> , the <i>fnbA</i> gene, 680 bp intergenic region between <i>fnbA</i> and <i>fnbB</i> and 527 bp downstream of <i>fnbB</i> . Cm ^r .	This study
pIMC:: <i>ermAM</i>	Site-specific Listerial integrative vector carrying <i>ermAM</i> . Cm ^r /Ery ^r .	(Monk <i>et al.</i> , 2008)
pIMC:: <i>aphA3</i>	Site-specific Listerial integrative vector carrying <i>aphA</i> . Cm ^r /Kan ^r .	(Monk <i>et al.</i> , 2008)

pIMAY::Int.S. <i>ermAM</i> _{CC1}	pIMAY carrying the <i>ermAM</i> _{CC1} insertion construct with 553 bp upstream and 586 bp downstream regions of the integration site amplified from AD08 and the <i>ermAM</i> cassette amplified from pIMC:: <i>ery</i> . Cm ^r /Ery ^r .	This study
pIMAY::Int.S. <i>ermAM</i> _{CC30}	pIMAY carrying the <i>ermAM</i> _{CC30} insertion construct with 553 bp upstream and 578 bp downstream regions of the integration site amplified from MRSA252 and the <i>ermAM</i> cassette amplified from pIMC:: <i>ery</i> . Cm ^r /Ery ^r .	This study
pIMAY::Int.S. <i>aphA3</i> _{CC1}	pIMAY carrying the <i>aphA3</i> _{CC1} insertion construct with 553 bp upstream and 586 bp downstream regions of the integration site amplified from AD08 and the <i>aphA3</i> cassette amplified from pIMC:: <i>kan</i> . Cm ^r /Kan ^r .	This study
pIMAY::Int.S. <i>aphA3</i> _{CC30}	pIMAY carrying the <i>aphA3</i> _{CC30} insertion construct with 553 bp upstream and 578 bp downstream regions of the integration site amplified from MRSA252 and the <i>aphA3</i> cassette amplified from pIMC:: <i>kan</i> . Cm ^r /Kan ^r .	This study
pGEX-4T2	<i>E. coli</i> vector for the expression of glutathione S-transferase tagged recombinant proteins. Ampicillin (Am) ^r .	GE Lifesciences

pGEX-4T2:: <i>CDSN</i> (CO)	pGEX-4T2 carrying <i>CDSN</i> gene codon optimised for <i>E. coli</i> encoding residues K33-P529. Am ^r .	This study
pGEX-4T2:: <i>CDSN</i>	pGEX-4T2 carrying <i>CDSN</i> gene codon optimised for <i>E. coli</i> encoding residues K33-P529 with C-terminal Strep-tag II. Am ^r .	This study
pGEX-4T2:: <i>CDSN</i> ΔNT	pGEX-4T2:: <i>CDSN</i> derivative where sequence encoding G60-G171 has been deleted. Am ^r .	This study
pGEX-4T2:: <i>CDSN</i> ΔCT	pGEX-4T2:: <i>CDSN</i> derivative where sequence encoding G376-G476 has been deleted. Am ^r .	This study
pGEX-4T2:: <i>CDSN</i> NTΩ	pGEX-4T2:: <i>CDSN</i> derivative carrying sequence encoding residues S62-G171. Am ^r .	This study
pGEX-4T2:: <i>L2v</i>	pGEX-4T2 carrying DNA encoding loricrin loop region 2. Am ^r .	(Mulcahy et al., 2012)
pGEX-4T2:: <i>HK10</i>	pGEX-4T2 carrying DNA encoding human cytokeratin 10 YY loop. Am ^r .	(Mulcahy et al., 2012)
pCU1	Multicopy shuttle plasmid. PC194 and PUC19. Am ^r /Cm ^r .	(Augustin et al., 1992)
pCU1:: <i>clfb</i>	pCU1 carrying DNA encoding CC ^a 8-type ClfB. Am ^r /Cm ^r .	(Ni Eidhin et al., 1998)
pFnBPA4	Plasmid carrying DNA encoding isotype I FnBPA. Cm ^r .	(Greene et al., 1995)
pFnBPB4	Plasmid carrying DNA encoding isotype I FnBPB. Cm ^r .	(Greene et al., 1995)
pQE-30:: <i>clfBN2N3</i>	<i>E. coli</i> vector for the expression of his tagged recombinant proteins. Derivative carrying DNA encoding CC ^a 1 type ClfB N2N3 subdomains. Am ^r .	(Fleury et al., 2017)

pQE-30:: <i>fnbAN2N3</i>	pQE-30 carrying DNA encoding isotype III FnBPA N2N3 subdomains. Am ^r .	(Loughman <i>et al.</i> , 2008a)
pQE-30:: <i>fnbBN2N3</i>	pQE-30 carrying DNA encoding isotype III FnBPB N2N3 subdomains. Am ^r .	(Burke <i>et al.</i> , 2011)

^a Clonal Complex

^r Resistance

Table 2.3 Primers

Primer	Sequence (5'-3')
pIMAY MCS F	TACATGTCAAGAATAAACTGCCAAAGC
pIMAY MCS R	AATACCTGTGACGGAAGATCACTTCG
ClfB A F	CCC <u>GTCGAC</u> ACAGTTTTTAACTATTCAACTCATGAG
ClfB D R	CCC <u>GAATTCCC</u> ATATCCTCCCATAGAGTGACCT
clfB OUT F	ATACGATCAAGATGAATTTTATGAAATC
clfB OUT R	CCTTGTGTTAATGCAAATTTAATTGTG
fnbA A	CCTCACTAAAGGGAACAAAAGCTG<u>GGTACCT</u> TCATTCTATTCAT CAGCTTATTTG
fnbA B	CACTATAATATCTCCCTTTAAATG
fnbA C	CATTAAAGGGAGATATTATAGTGGCATAATTAAACAAAAATT GACG
fnbA D	CGACTCACTATAGGGCGAATTG<u>GAGCTC</u> CTCTTGAATACCTT GATATAAC
fnbA_Res D	CGACTCACTATAGGGCGAATTG<u>GAGCTC</u> AGAACTAATCGCAT GCCCTAAAC
fnbB_Del F	*TAATCAATCTAAAATTGACAGG
fnbB_Del R	*CACTATAATATTCTCCCTTAAATG
FnBP OUT F	TTAACATGGTCATTATTAGAAAC
FnBP OUT R	GTCACCTAATAATACCGCAAATG
pIMAY Inv F	<u>GGTACCC</u> CAGCTTTTGTTCCTTTAGTGAGG
pIMAY Inv R	<u>GAGCTC</u> CAATTCGCCCTATAGTGAGTCG
CDSN F	GGG <u>GATCTA</u> AGAGCATTGGCACCTTCTC
CDSN R	TTT <u>GTCGACC</u> GGGCTGTTCAGCAGTTC
CDSN CO F	AAA <u>AGATCT</u> AAATCCATTGGGACATTC
CDSN CO R	TTT <u>GTCGACTT</u> ATGGACTGTCCAG
CDSN ST F	*GCTGCTTGGTCTCATCCTCAATTTGAAAAATAAGTCGACTCGA GCGGC
CDSN ST R	*TGGACTGTCCAGCAACTC
CDSN Δ NT F	*AATGGCAGTCTTCCAAC
CDSN Δ NT R	*ATCTCCTTTGCCGGTAAGG

CDSNΔCT F	*CCAGACCCGTCTGCGG
CDSNΔCT R	*TCCGGTACCCACTGGTTGAAAC
CDSNd ₃₃₋₆₁ F	*AGCTCTGGGTTCTCAAGCTAC
CDSNd ₃₃₋₆₁ R	*GGATCCACGCGGAACCAG
CDSNd ₁₇₂₋₅₂₉ F	*CCATGGTCTCATCCTCAATTTG
CDSNd ₁₇₂₋₅₂₉ R	*CCCAACTTGGAAACTGCTAC
pGEX MCS F	CTGGCAAGCCACGTTTGG
pGEX MCS R	GGAGCTGCATGTGTCACAG
fnbB N1 F	ACTACAGGAACTCAAGTTG
Int.S. A	CCTCACTAAAGGGAACAAAAGCTG <u>GGTACC</u> ACAACCTGAAGA TTCATGGAATG
Int.S. _{CC1} B	TCACTACTATACTTTTACAG
Int.S. _{CC30} B	TCACTATTATACTTTTACAG
Int.S. C	GCCAATGCACATAACAAC
Int.S. D	CGACTCACTATAGGGCGAATTG <u>GAGCTC</u> CACCGGCATAACCC AATAATG
Int.S. OUT F	ACGTACATTTGCTGCTATTG
Int.S. OUT R	CTAAAATACTAAAGTTTTG
Promoter _{cc1} F	*TTGTGCTGTAAAAGTATAGTAGTGACATTATGCTTTGGCAGTTT ATTC
Promoter _{cc30} F	*TTGTGCTGTAAAAGTATAATAGTGACATTATGCTTTGGCAGTTT ATTC
Erm R	*ATTTATTGTTGTTATGTGCATTGGCTTATTTCTCCCGTTAAAT AATAGATACC
Kan R	*ATTTATTGTTGTTATGTGCATTGGCTTAAAGCTTTTTAGACATC TAAATCTAGG
esaDG OUT F	TATGTATTTTGCACCATTTAGC
esaDG OUT R	CTTTATCATTGTGATGTTTGAAC

* Represents the presence of 5' phosphorylation

5' overhangs for primer extension are in bold

Restriction endonuclease recognition sequences are underlined

Chapter 3

Characterisation of the interaction between *Staphylococcus aureus* and corneodesmosin

3.1 Introduction

Staphylococcus aureus is not a typical member of the natural microflora of the skin, however the skin of over 70% of AD patients is colonised by *S. aureus* (Totte et al., 2016). AD skin exhibits a dysbiosis of the microbiota where there is a reduction in general microbial diversity and an increase in Firmicutes, particularly *S. aureus* (Leyden et al., 1974, Kong et al., 2012). During disease flares, *S. aureus* proliferates and dominates the skin microbiota. The density of *S. aureus* on AD skin correlates strongly with disease severity as it exacerbates AD disease symptoms (Tauber et al., 2016, Kong et al., 2012).

There are differences in the overall population structure of *S. aureus* strains colonising AD skin compared to commensal strains colonising the nares of healthy people. *S. aureus* strains from clonal complex (CC) 1 are more frequently isolated from the skin of Irish AD patients compared to the nasal cavity of healthy controls (Fleury et al., 2017), and carriage of CC1 strains has been linked to disease severity in one study (Yeung et al., 2011). Therefore a clinically relevant CC1 strain of *S. aureus*, AD08, was predominantly used for the research outlined in this chapter.

The AD skin environment is dramatically different to health skin which likely contributes to the ability of *S. aureus* to colonise AD skin and disrupt the normal microbiota. Characteristic of AD is a skin barrier defect with reduced structural integrity that is susceptible to penetration by allergens and microbes (Zheng et al., 2011). The skin protein filaggrin is broken down into amino acids including pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA) which contribute to the skin's natural moisturising factor (NMF) (Rawlings & Harding, 2004). In AD skin, levels of natural moisturising factor (NMF) are reduced, correlating with disease severity (Kezic et al., 2011). AD patients exhibit overexpression of proinflammatory cytokines IL-4, IL-13 and IL-31 in the skin which directly results in reduced expression of filaggrin (Howell et al., 2007a, Cornelissen et al., 2012) and this contributes to epithelial barrier defects and reduced NMF levels resulting in elevated skin pH and loss of hydration that are characteristic of AD skin (Kezic et al., 2008).

Low NMF levels also contribute to an altered corneum structure, where AD corneocytes have abnormal morphology with aberrant 'villus-like' projections (VPs) that protrude from the surface of the corneocytes. Corneodesmosomes consist of a complex of tight junction proteins that mediate corneocyte adherence in the stratum corneum. Present on the extracellular part of corneodesmosomes, is a glycoprotein called corneodesmosin (CDSN) which facilitates cell-cell adherence by homophilic interactions (Jonca *et al.*, 2011). In the stratum corneum of AD skin CDSN, normally confined to the tight junctions between cells, decorates the tips of the VPs on the corneocytes (Riethmuller *et al.*, 2015, Igawa *et al.*, 2013). CDSN contains two glycine/serine rich regions predicted to form omega loops, similar to those found in other cornified envelope proteins loricrin and cytokeratin 10 (K10) (Jonca *et al.*, 2011) both of which are ligands for *S. aureus* (Mulcahy *et al.*, 2012, Walsh *et al.*, 2004). Omega loops in proteins are so named as they resemble a loop pattern similar to the Greek letter omega (Ω). They consist of at least six consecutive amino acid residues that change direction in the polypeptide, and the residues at the beginning and end of the segment are very close together in space (Leszczynski & Rose, 1986).

S. aureus cell wall-anchored proteins (CWAPs) clumping factor B (ClfB) and fibronectin binding proteins A (FnBPA) and B (FnBPB) are involved primarily in ligand binding and share a similar domain structure with an N-terminal A domain followed by a repeat region that connects the A domain to a wall spanning region (Foster *et al.*, 2014). ClfB is critical for nasal colonisation where it mediates *S. aureus* adherence to the cornified envelope (CE) protein loricrin (Mulcahy *et al.*, 2012). Loricrin consists of 3-4 glycine/serine rich regions that form omega loops (Hohl *et al.*, 1991) and ClfB binds with the highest affinity to loop region 2 (L2v) of the protein (Mulcahy *et al.*, 2012). ClfB also binds to the skin protein K10, and the bacterial binding site has been localised to the glycine/serine rich C-terminal tail region of the protein that forms an omega loop (Walsh *et al.*, 2004). The third known ligand for ClfB is the plasma glycoprotein fibrinogen (Ni Eidhin *et al.*, 1998). ClfB binds to the glycine/serine rich α C domain at the C-terminus of the A α chain (Walsh *et al.*, 2008).

FnBPA and FnBPB are involved in binding to the extracellular matrix and plasma glycoprotein fibronectin. The genes *fnbA* and *fnbB* which encode FnBPA and FnBPB are located closely on the chromosome separated by a short intergenic region however the genes are independently transcribed (Jonsson *et al.*, 1991, Greene *et al.*, 1995). FnBPA and FnBPB also bind to the C-terminus of the γ -chain of fibrinogen (Wann *et al.*, 2000), elastin (Roche *et al.*, 2004) and plasminogen (Pietrocola *et al.*, 2016).

The A domain of ClfB, FnBPA and FnBPB is divided into subdomains N1 N2 and N3 where N2 N3 function in ligand binding primarily. ClfB binding to the glycine serine rich regions in Iorocrin, K10 and fibrinogen occurs by a mechanism known as 'dock, lock and latch' (DLL) which is facilitated by the N2 N3 subdomains (Mulcahy *et al.*, 2012, Walsh *et al.*, 2004, Ni Eidhin *et al.*, 1998). The N2 N3 subdomains in FnBPA and FnBPB bind to fibrinogen and elastin by DLL (Wann *et al.*, 2000, Roche *et al.*, 2004) and to plasminogen by an unknown mechanism that for FnBPB involves the N3 subdomain (Pietrocola *et al.*, 2016). Binding to fibronectin is accommodated by the fibronectin binding repeat region consisting of 10 (FnBPB) or 11 (FnBPA) tandem repeated motifs (Meenan *et al.*, 2007).

While the *clfB* gene is carried by all sequenced *S. aureus* strains (Lindsay *et al.*, 2006), there is nucleotide sequence variation between clonal complexes where the amino acid sequence of the ClfB protein is identical between strains of the same clonal complex (Murphy *et al.*, 2011). The majority of *S. aureus* strains sequenced carry both *fnbA* and *fnbB* genes however there are strains which only carry *fnbA* (Lower *et al.*, 2011, Peacock *et al.*, 2000). There have been seven allelic variants of FnBPA and FnBPB identified, some of which are associated with certain clonal complexes of *S. aureus*. CC1 strains have been associated with both isotype III FnBPA and FnBPB (Loughman *et al.*, 2008b, Burke *et al.*, 2010) and will now be referred to as CC1-type FnBPA and FnBPB.

ClfB has been shown to mediate *S. aureus* adherence to corneocytes isolated from AD patients. A mutant of *S. aureus* carrying a *clfB* null mutation had reduced binding to corneocytes isolated from low NMF AD patients. However binding was not completely diminished suggesting that adherence to

AD corneocytes is multifactorial and other *S. aureus* factors are involved (Feuillie *et al.*, 2018, Fleury *et al.*, 2017). *S. aureus* lacking both FnBPA and FnBPB show reduced adherence to AD skin compared to healthy skin. This suggests that one or both proteins are involved in mediating *S. aureus* adherence to AD skin (Cho *et al.*, 2001).

The precise role of ClfB and FnBPA and FnBPB in *S. aureus* colonisation of AD skin remains undescribed and the ligands for *S. aureus* in AD skin are unknown. Since CDSN is uncharacteristically localised on the tips of VPs which decorate the entire corneocyte surface in AD skin (Riethmuller *et al.*, 2015), it is potentially a highly accessible ligand for bacterial attachment. It is rational therefore to hypothesise that CDSN is a ligand for *S. aureus* and that the interaction is involved in *S. aureus* colonisation of AD skin.

Other staphylococcal spp., specifically the coagulase negative staphylococci (CoNS) are ubiquitous in the skin microbiome. The molecular mechanisms by which CoNS colonise skin are undescribed and the target skin ligands on corneocytes have not been identified. However CoNS are opportunistic pathogens, particularly in a nosocomial setting, where patients regularly have weakened immune responses and CoNS commensal strains including, but not limited to, *S. epidermidis* and *S. haemolyticus*, can enter the blood stream through indwelling medical devices such as catheters and intravenous lines (Becker *et al.*, 2014, Sievert *et al.*, 2013) therefore identifying the target ligand (s) for CoNS in the skin may be of benefit for future therapeutic strategies. *S. epidermidis* expresses eleven known cell wall-anchored proteins, many of which are uncharacterised. It is well established that the SD repeat protein SdrG promotes *S. epidermidis* adherence to human fibrinogen (Hartford *et al.*, 2001) and more recently, it's been shown that SdrF mediates bacterial adherence to human K10, keratinocytes and epithelial cells (Trivedi *et al.*, 2017). Aap, a homologue of the *S. aureus* protein SasG, is capable of mediating bacterial adherence to human corneocytes, where it interacts with an unidentified host ligand (Macintosh *et al.*, 2009). Given that several of these proteins are similar to *S. aureus* CWAPs, it is possible that they bind to similar host ligands.

In this chapter a novel interaction between *S. aureus* CWAPs ClfB, FnBPA, FnBPB and CDSN is described. Furthermore, using a mutant of *S. aureus* deficient in ClfB, FnBPA and FnBPB, it was shown that these proteins are involved in mediating *S. aureus* adherence to corneocytes taken from AD patients. Lastly, by blocking the *S. aureus* binding site in CDSN on AD corneocytes, CDSN was identified as a key ligand for *S. aureus* adherence in AD skin.

3.2 Results

3.2.1 *S. aureus* adheres to immobilized corneodesmosin

The aberrant distribution of CDSN on the surface of corneocytes from low NMF AD patients suggests that it could be an accessible ligand for bacterial adherence. Furthermore, the protein contains glycine/serine rich repeat regions that are predicted to form omega loops (Jonca *et al.*, 2011) characteristic of other ClfB ligands loricrin and K10. Therefore, we investigated whether CDSN was a ligand for *S. aureus*. Solid phase bacterial adherence assays were carried out where lab strain Newman was tested for adherence to commercially sourced C-terminally 6 x histidine-tagged CDSN (His-CDSN). Briefly, doubling concentrations of His-CDSN was coated onto the wells of a microtitre plate. Bacteria were grown to exponential phase ($OD_{600} = 0.35$), adjusted to an $OD_{600} = 1$ and incubated with the ligand on the plate. Unbound cells were removed by washing and adherent cells were stained using crystal violet. Absorbance measured at 570 nm correlates directly with the density of stained adherent bacteria. Newman adhered in a dose-dependent saturable manner (Figure 3.1) indicating that CDSN is indeed a ligand for *S. aureus*.

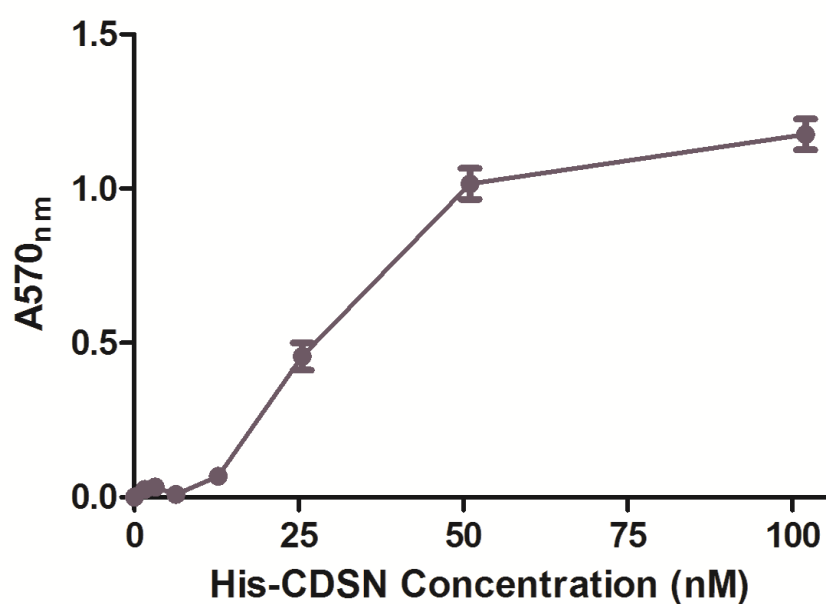


Figure 3.1. *S. aureus* Newman adheres to corneodesmosin. *S. aureus* Newman was grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of His-CDSN. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates.

3.2.2 Construction, expression and purification of recombinant GST-CDSN

To fully characterise the interaction between *S. aureus* and CDSN at the molecular level it was desirable to develop a system to allow genetic manipulation of the *CDSN* gene as well as expression of GST-tagged recombinant CDSN protein. Previous attempts at purification of recombinant CDSN from an *E. coli* expression system were not successful (Caubet *et al.*, 2004b). Truncated GST-tagged protein fragments were co-purified with the full-length GST-CDSN protein during affinity chromatography. The authors postulated that this was due to tRNA limitation in *E. coli*. Here, codon optimisation (CO) for *E. coli* was carried out on the *CDSN* gene encoding the entire mature protein (amino acid residues K33-P529) to alter the nucleotide sequence, while maintaining the desired amino acid sequence. The DNA was synthesised commercially and included 5' and 3' restriction sites BglII and SalI respectively, for subsequent subcloning. The DNA was amplified and cloned into the IPTG-inducible vector pGEX-4T2 for the expression of N-terminally GST-tagged recombinant protein [pGEX-4T2::*CDSN*(CO)]. Attempts to purify GST-tagged CDSN using glutathione affinity chromatography were not successful as a homogenous preparation of protein was not produced. The eluted protein was analysed by SDS-PAGE and a band corresponding full length GST-tagged CDSN (~75 kDa) was very faint. The protein preparation predominantly contained proteins with an apparent molecular weight lower than that predicted for full length GST-tagged CDSN (Figure 3.2A). Several different sized proteins in the sample reacted with monoclonal anti-GST IgG in a western immunoblot, suggesting they were GST-tagged recombinant polypeptides of CDSN (Figure 3.2B). Next, cultures carrying pGEX-4T2::*CDSN*(CO) were induced with IPTG, samples were harvested at multiple time points post induction and analysed by western immunoblot using anti-GST IgG (Figure 3.2C). Band intensity increased up until 180 min post-induction but not thereafter suggesting that 180 min of induction is sufficient to maximise protein yield. There was no timepoint however, where only full length GST-CDSN was produced.

To overcome this, a two-step purification process was employed where DNA encoding a Strep-tag II affinity tag was linked to the 3' end of the *CDSN* gene sequence in pGEX-4T2 using inverse PCR (Figure 3.3A). Purification by Strep-Tactin affinity chromatography (Figure 3.3B) followed by glutathione affinity chromatography (Figure 3.3C) allowed for the selective purification of full-length protein with an N-terminal GST-tag and a C-terminal Strep-tag II, hereafter known as rCDSN. SDS-PAGE analysis and western immunoblot with anti-GST IgG show successful purification of pure full length rCDSN (Figure 3.3D).

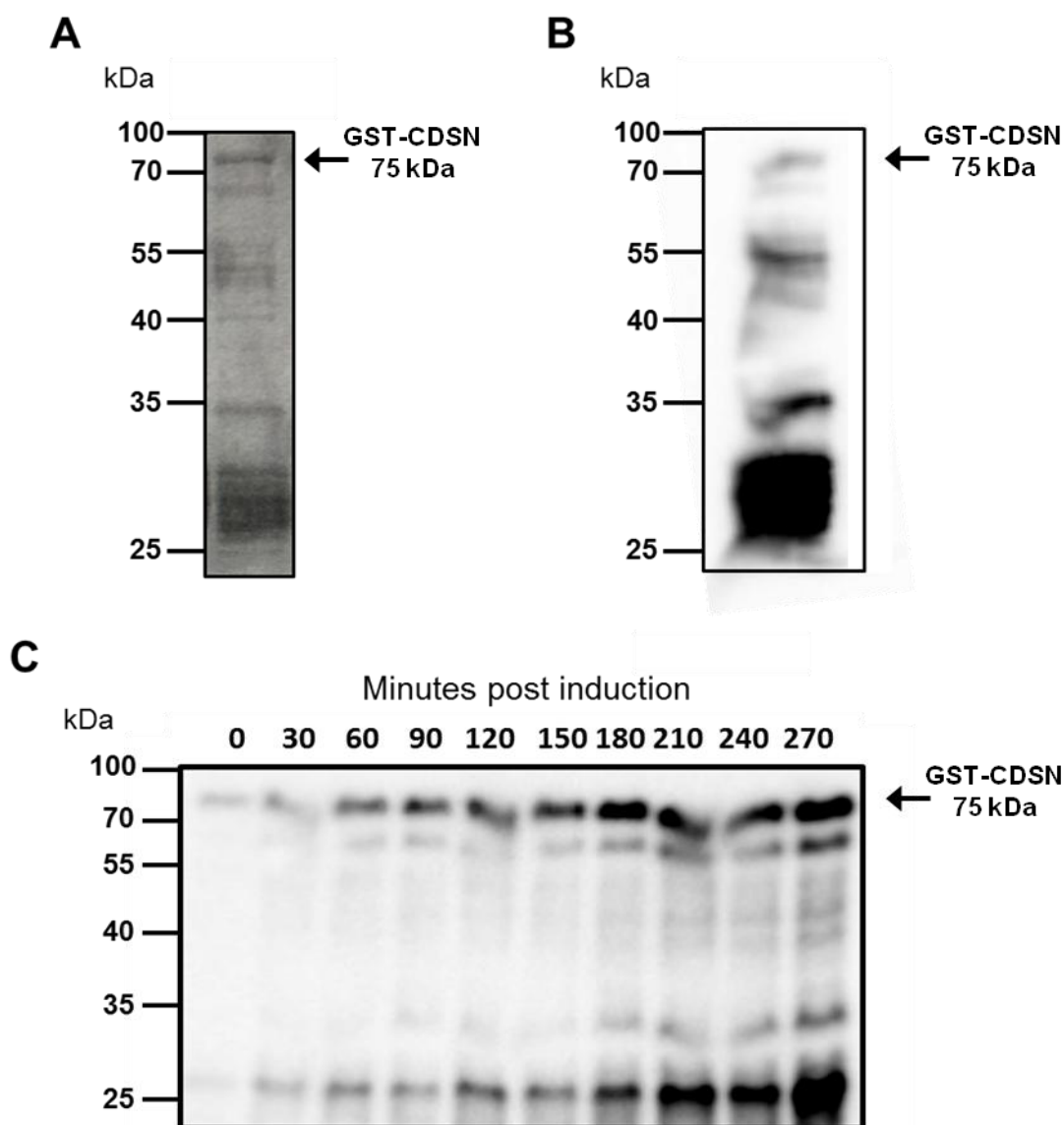


Figure 3.2. Purification of recombinant CDSN using glutathione affinity chromatography. **A)** Following purification by glutathione affinity chromatography, purified GST-CDSN protein was separated on a 10% SDS-PAGE gel and stained using InstantBlue protein stain. A faint band corresponding full length GST is seen at ~75 kDa. **B)** Western immunoblot of purified CDSN probed with mouse anti-GST IgG followed by rabbit anti-mouse IgG-HRP. **C)** *E. coli* pGEX-4T2::CDSN(CO) was grown to an $OD_{600} = 0.6$ and induced with 1 mM IPTG for 5 h. Samples were taken every 30 min, separated by SDS-PAGE, transferred to a PVDF membrane and probed using anti-GST IgG followed by rabbit anti-mouse IgG-HRP.

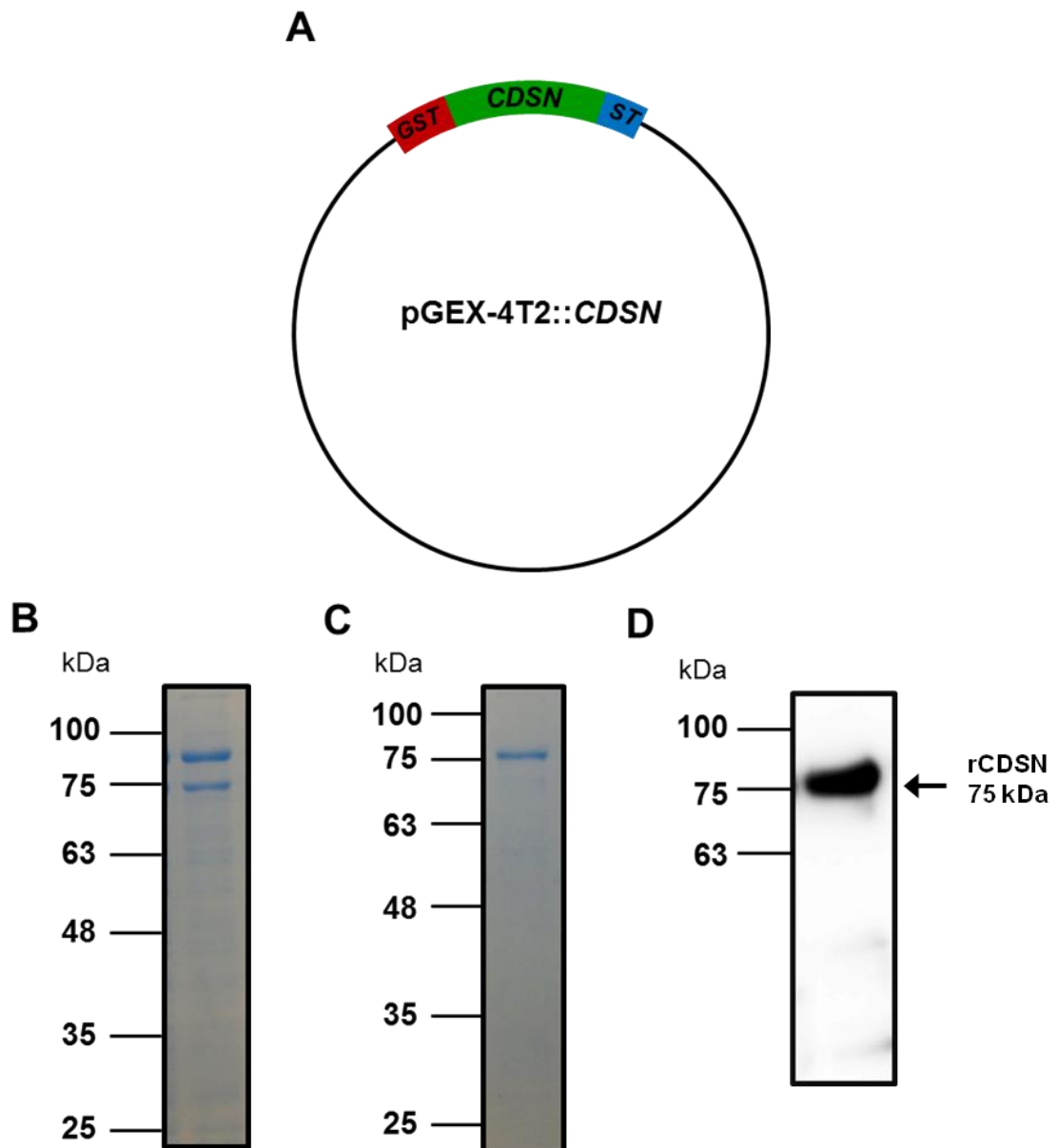


Figure 3.3. Purification of rCDSN **A)** Schematic diagram of pGEX-4T2::CDSN. Sequence encoding N-terminal GST-tag and C-terminal Strep (ST) tag is indicated. **B)** SDS-PAGE of rCDSN protein purification following Strep-Tactin affinity chromatography. Separated protein was stained with InstantBlue protein stain. **C)** SDS-PAGE of rCDSN purification following glutathione affinity chromatography. Separated protein was stained using InstantBlue. A single band representing full length rCDSN (~75 kDa) is seen. **D)** Western Immunoblot of rCDSN probed with mouse anti-GST IgG followed by rabbit anti-mouse IgG-HRP.

3.2.3 *S. aureus* AD_{CC1} isolates adhere more strongly to CDSN than commensal CC30 isolates

S. aureus CC1 isolates are associated with colonisation of the skin in AD patients (Fleury *et al.*, 2017) and it is therefore conceivable that AD strains from CC1 (AD_{CC1}) adhere strongly to CDSN. To investigate this, adherence of a collection of AD_{CC1} strains to a single concentration of immobilized rCDSN (40 nM) was measured. CC30 strains are established commensals in the nares of healthy individuals (Fleury *et al.*, 2017) therefore a collection of CC30 strains isolated from the nasal cavity of healthy children was used as a comparative control. Mean adherence of AD_{CC1} strains to rCDSN was significantly higher than the mean adherence of CC30 nasal carriage isolates ($p < 0.05$) (Figure 3.4). This data suggests that AD_{CC1} strains adhere more strongly to CDSN than CC30 nasal carriage strains.

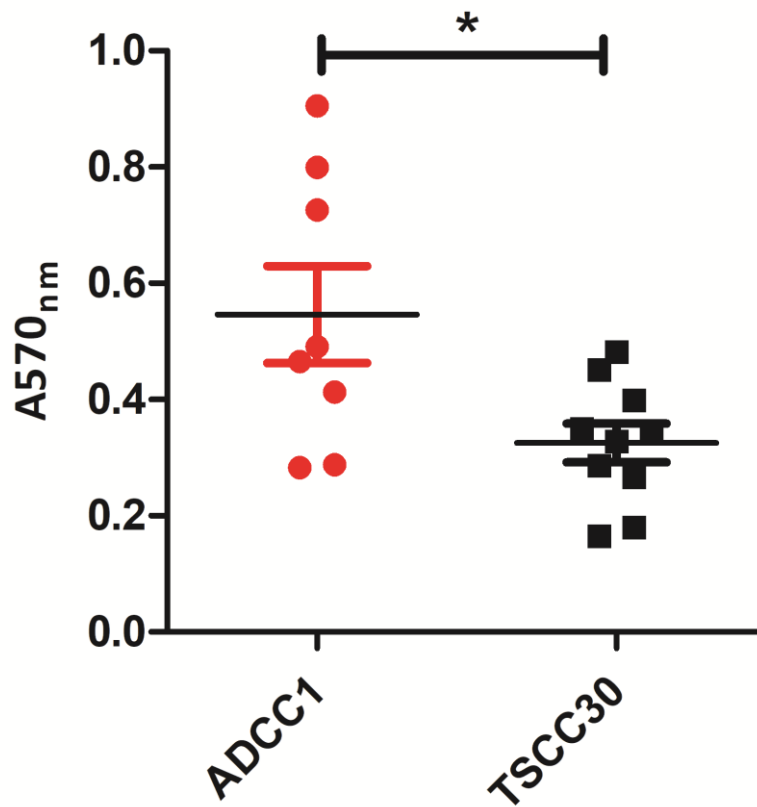


Figure 3.4. AD_{CC1} isolates adhere more strongly to CDSN than commensal CC30 isolates. *S. aureus* strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with a single concentration of rCDSN (40 nM). Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. ADCC1 represents eight AD_{CC1} strains and TSCC30 represents ten CC30 strains isolated from the nasal cavity of healthy children at Temple Street Children's Hospital Dublin. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. An unpaired t-test was performed to compare differences between groups, $p < 0.05$.

3.2.4 Construction and validation of a *clfB* null mutation in *S. aureus* AD08

Previously, the *S. aureus* CWAP ClfB was implicated in mediating bacterial adherence to corneocytes isolated from low NMF AD patients. In addition, ClfB binds to protein sequences harbouring glycine/serine rich omega loops. To investigate whether ClfB promotes the adherence of *S. aureus* to CDSN, an isogenic mutant of *S. aureus* strain AD08 deficient in ClfB was isolated. AD08 is a representative CC1 strain isolated from the infected skin lesion of a paediatric patient with low NMF AD (Fleury *et al.*, 2017). AD08 Δ *clfB* was constructed by allelic exchange using pIMAY:: Δ *clfB* (Mulcahy *et al.*, 2012). Plasmid pIMAY:: Δ *clfB* is a temperature sensitive plasmid containing a nucleotide sequence homologous to 500 bp upstream and 500 bp downstream of the *clfB* gene of Newman. Plasmid pIMAY:: Δ *clfB* was introduced into the *E. coli* strain IM01B. IM01B is an engineered strain that lacks the *E. coli dcm* gene so DNA is not cytosine methylated. Furthermore, IM01B expresses *hsdS1*, *hsdS2* and *hsdM2*, carried by *S. aureus* CC1 strains and consequently expresses a CC1-specific adenine-methylation profile (Monk *et al.*, 2015). Passage of plasmids through the restriction-deficient modification strain IM01B allows high-efficiency transfer of plasmid DNA to *S. aureus* CC1 strains.

pIMAY:: Δ *clfB* was isolated from IM01B and AD08 was transformed by electroporation. Allelic exchange was used to remove the *clfB* gene from the AD08 chromosome (Figure 3.5A) as described in section 1.5.1. The deletion was confirmed by PCR-amplification across the *clfB* gene region from genomic DNA. ClfB mutants of AD08 yielded a 1 kb product while the wild-type AD08 strain yielded a product of approximately 4 kb (Figure 3.5B). The mutation was verified by DNA sequencing of the PCR amplicon (Eurofins Genomics).

The *S. aureus* global regulator *agr* can undergo spontaneous mutations during passage *in vitro*. Mutations in *agr* can affect products of Agr regulation and incur reduced virulence (Traber & Novick, 2006, Traber *et al.*, 2008). Typically, *agr* activity can be assessed by examining the activity of δ -toxin as it is regulated by RNAIII, a transcript from the Agr two-component system. Haemolytic activity of δ -toxin on sheep blood agar is increased in synergy with β -haemolysin. The *S. aureus* strain RN4220 only produces β -haemolysin and cross-streaking *S. aureus* strains that express δ -toxin will show a clear zone of

haemolysis where the two interact (Tegmark *et al.*, 2000), giving an indication of a functional Agr system. Here, the haemolytic activity of AD08 and AD08 Δ *clfB* was assessed by cross-streaking each strain on sheep blood agar against RN4220. A clear zone of δ -toxin haemolysis is evident for both strains indicating that *agr* activity has not been affected during mutagenesis (Figure 3.5C). The growth profile of the mutant and the parental wild-type was assessed. Both strains had a similar growth profile and reached the same final OD₆₀₀ (Figure 3.5D). These data indicate that mutagenesis has not affected *agr* activity or δ -toxin activity.

3.2.5 Adherence of AD08 to CDSN is not solely dependent by ClfB

To investigate if ClfB is involved in mediating adherence of *S. aureus* to rCDSN, isogenic mutants of Newman and AD08 deficient in ClfB were tested. Solid-phase bacterial adherence assays were used where bacterial adherence to immobilized rCDSN was measured. Both wild-type strains adhered to rCDSN avidly and dose-dependently (Figure 3.6A, B). Newman *clfB* did not adhere to rCDSN (Figure 3.6A), indicating that the adherence of Newman to CDSN is mediated solely by ClfB. AD08 Δ *clfB*, however, adhered to rCDSN in a dose-dependent manner (Figure 3.6B) that was not significantly different to the WT. This suggests that there are other *S. aureus* factors involved in promoting AD08 adherence to CDSN.

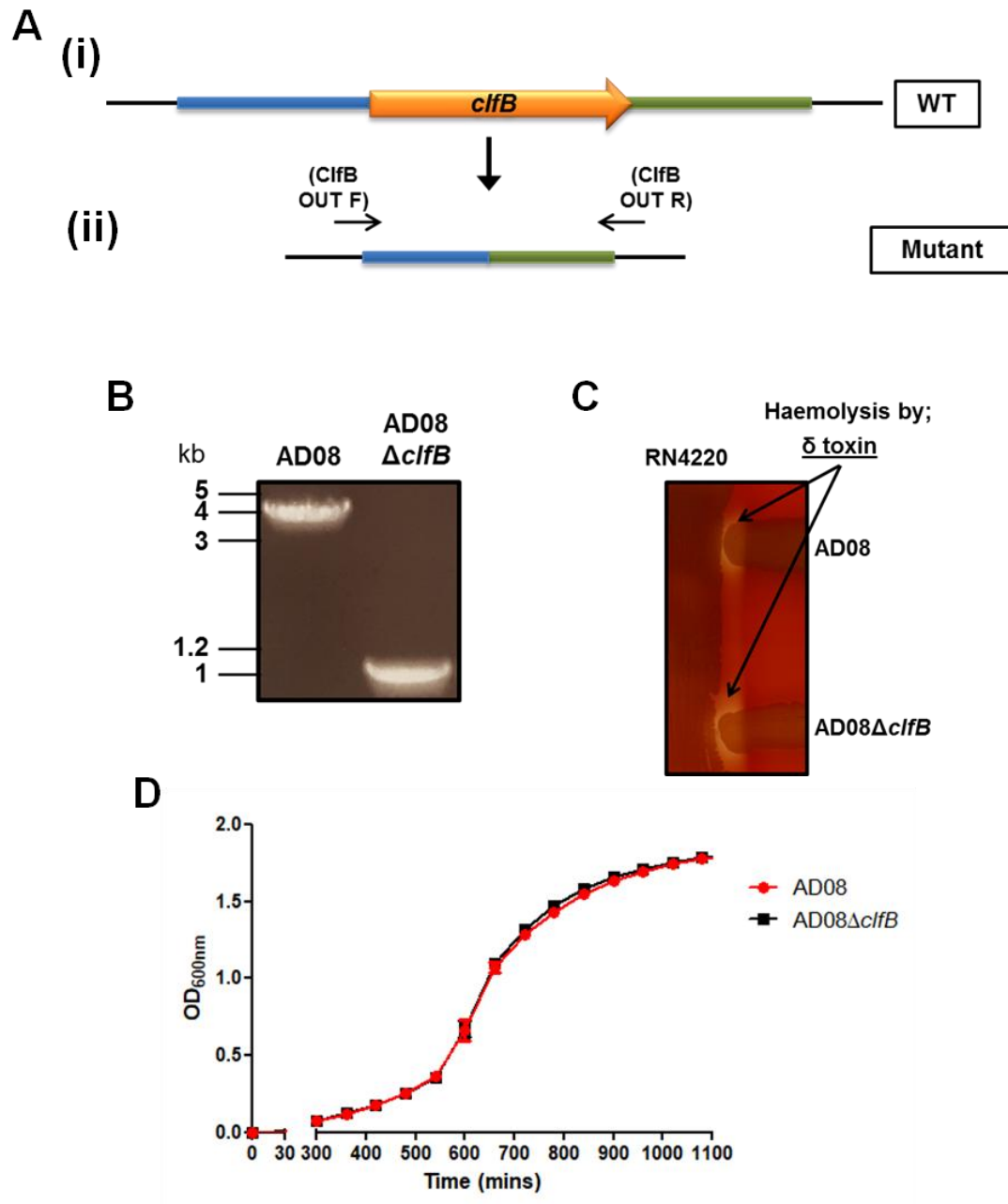


Figure 3.5. Schematic representation of the *clfB* mutation. **A)** The *clfB* gene was deleted from the AD08 chromosome by allelic exchange. **B)** The deletion was confirmed by PCR, with purified genomic DNA from AD08 and AD08 Δ *clfB* and primers ClfB OUT F and R. **C)** AD08 and AD08 Δ *clfB* were cross-streaked against RN4220 to examine the haemolytic profile. An arrow indicates haemolysis due to δ -toxin. **D)** OD₆₀₀ of AD08 and AD08 Δ *clfB* growing in TSB broth was recorded over an 18 h period. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates.

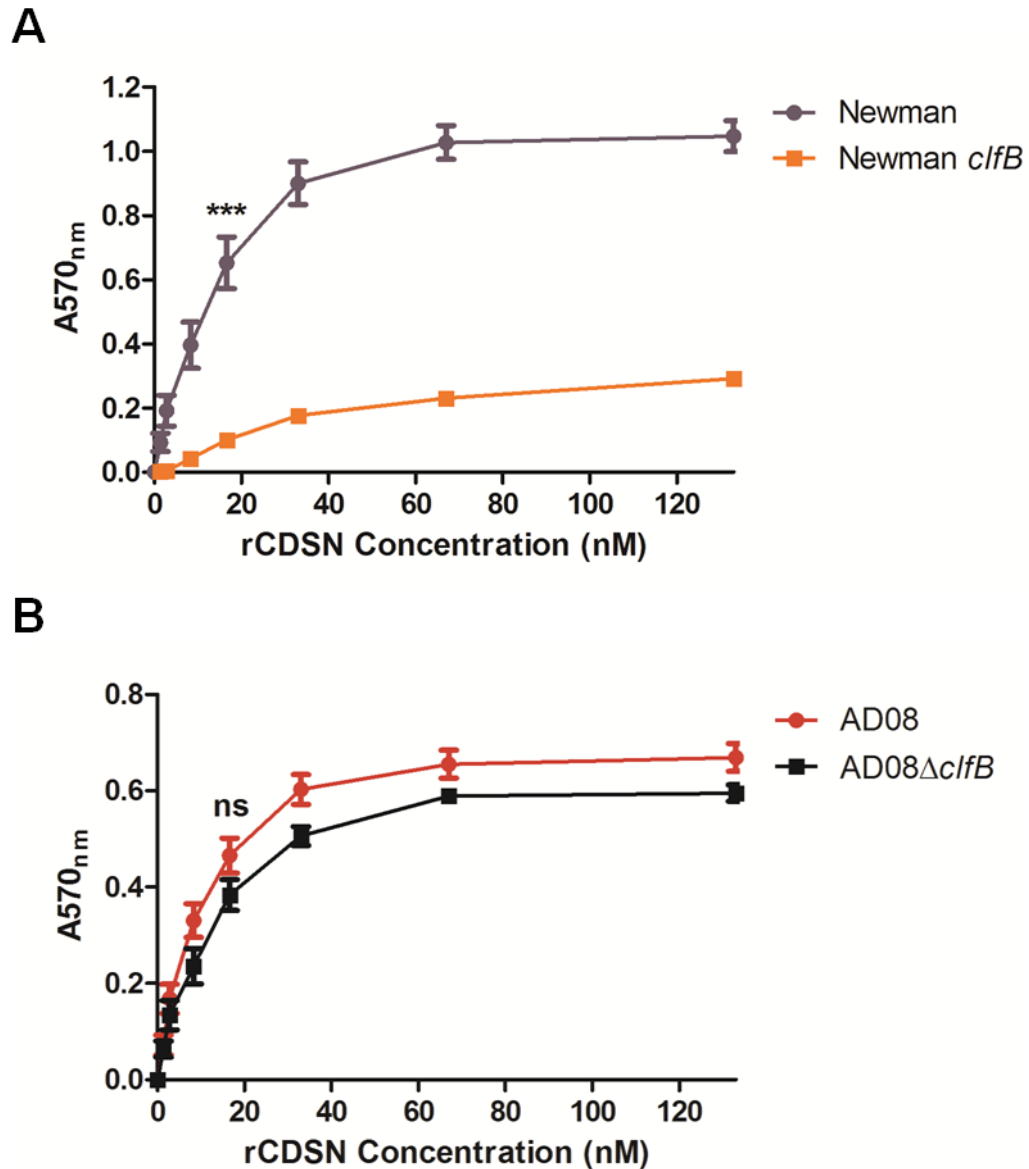


Figure 3.6. *S. aureus* adherence to immobilized rCDSN is not dependent on ClfB. *S. aureus* strains **A)** Newman, Newman *clfB* **B)** AD08 and AD08Δ*clfB* were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of rCDSN. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with a Bonferroni posttest was performed to compare variances between WT and ClfB-deficient mutants, **A)** $p < 0.001$, **B)** $p = \text{non-significant (ns)}$. Significance is represented at 16.60 nM rCDSN.

3.2.6 Construction and validation of FnBPA, FnBPB and ClfB deficient mutants of *S. aureus* AD08

In strain Newman, the genes encoding fibronectin binding proteins A (FnBPA) and B (FnBPB), *fnbA* and *fnbB*, contain a point mutation resulting in a premature stop codon in the sequence encoding the C-terminus of the protein. Both proteins are truncated and lack the C-terminal sorting signal, including the recognition sequence. Thus, FnBPA and FnBPB are not anchored to the *S. aureus* cell wall and Newman does not adhere to fibronectin (Grundmeier *et al.*, 2004). Previously *S. aureus* strains isolated from the skin of AD patients were found to have increased fibronectin binding compared to control commensal strains, suggesting that they express one or more fibronectin binding proteins (Fleury, 2017). Additionally, FnBPA and/or FnBPB have been implicated in colonisation of AD skin as lab strains of *S. aureus* deficient in *fnbA* and *fnbB* genes had reduced adherence to AD skin sections (Cho *et al.*, 2001).

To determine if FnBPA or FnBPB mediate adherence of AD08 to CDSN, mutants of AD08 deficient in FnBPA and/or FnBPB were constructed by allelic exchange using the plasmid vector pIMAY. Analysis of the genome sequence of AD08 showed that both *fnbA* and *fnbB* are carried on the AD08 chromosome. Consistent with AD08 being assigned to CC1, this strain carries CC1-type *fnbA* and *fnbB*. Plasmid pIMAY:: Δ *fnbA* was constructed using AD08 genomic DNA as template DNA. The regions 500 bp upstream and 504 bp downstream of the *fnbA* gene were amplified by PCR, where primers A and B annealed to the 5' and 3' upstream region and primers C and D annealed to the 5' and 3' region downstream of *fnbA*. Cloning of the AB and CD products into pIMAY was carried out using sequence and ligase independent cloning as described in section 2.2.4. Deletion of *fnbA* in AD08 was achieved by allelic exchange following the same procedure described in section 1.5.1. The resulting mutant strain was validated by PCR with primers that anneal outside of *fnbA* (*fnbA* OUT F and R) (Figure 3.7A) and comparing the product to the parental wild-type product (Figure 3.9A).

The genes *fnbA* and *fnbB* are located adjacent to each other on the chromosome with a 680bp intergenic region separating them (Jonsson *et al.*, 1991) and it was possible to delete both genes in a single round of

mutagenesis. The plasmid pIMAY:: $\Delta fnbA\Delta fnbB$ (Zapotoczna and Geoghegan, unpublished) was used to isolate AD08 $\Delta fnbA\Delta fnbB$ by homologous recombination. This plasmid contains sequence homology to 550 bp upstream of *fnbA* and 550 bp downstream of *fnbB*. AD08 $\Delta fnbA\Delta fnbB$ was isolated by the same procedure described in section 3.2.4. Again, the deletion was confirmed by PCR with OUT primers that anneal upstream of *fnbA* and downstream of *fnbB* (Figure 3.7B). A PCR product of approximately 1.1 kb (Figure 3.9A) was obtained using genomic DNA of the mutant strain as template, confirming that the mutation was introduced into the chromosome.

Given the importance of ClfB in mediating adherence to AD corneocytes (Feuillie *et al.*, 2018, Fleury *et al.*, 2017), null mutations in the *clfB* gene were introduced into the AD08 $\Delta fnbA$ and AD08 $\Delta fnbA\Delta fnbB$ mutants. The plasmid pIMAY:: $\Delta clfB$ (Mulcahy *et al.*, 2012) was used again to introduce the *clfB* null mutation into these strains by allelic exchange. The mutation was confirmed by amplifying across the *clfB* gene region with *clfB* OUT primers that anneal to regions outside of the *clfB* gene using AD08 $\Delta clfB\Delta fnbA$ and AD08 $\Delta clfB\Delta fnbA\Delta fnbB$ and wild-type AD08 genomic DNA as template. A 1 kb product indicates that the mutagenesis was successful and the mutant strains carry a *clfB* null mutation (Figure 3.9A).

To isolate AD08 $\Delta fnbB$ and AD08 $\Delta clfB\Delta fnbB$, the *fnbA* gene was restored onto the AD08 $\Delta fnbA\Delta fnbB$ and AD08 $\Delta clfB\Delta fnbA\Delta fnbB$ chromosome respectively using allelic exchange and the plasmid pIMAY::*fnbA_restore*. Primers *fnbA* A and *fnbA_Res* D were used to amplify the region 500 bp upstream of the *fnbA* gene and 527 bp downstream of the *fnbB* gene (Figure 3.8A). The product was cloned into pIMAY by SLIC to generate plasmid pIMAY::*fnbAfnbB*. An inverse PCR with plasmid pIMAY::*fnbAfnbB* as template and primers *fnbB_Del* F and *fnbB_Del* R as carried out to delete the *fnbB* gene and generate plasmid pIMAY::*fnbA_restore* (Figure 3.8B). This plasmid contains sequence homology to 500 bp upstream of *fnbA*, the *fnbA* gene, intergenic region between *fnbA* and *fnbB* and 527 bp downstream of *fnbB*. Plasmid pIMAY::*fnbA_restore* was transformed into AD08 $\Delta fnbA\Delta fnbB$ and AD08 $\Delta clfB\Delta fnbA\Delta fnbB$ and allelic exchange was carried out as previously described. The resulting strains AD08 $\Delta fnbB$ and AD08 $\Delta clfB\Delta fnbB$ carry *fnbA*

on the chromosome at its original location (Figure 3.8C) which was confirmed by amplifying across the gene region with FnBP OUT primers (Figure 3.9A).

Each mutation was confirmed by DNA sequencing of a PCR amplicon (Eurofins Genomics). The haemolytic profile of δ -toxin production was identical between wild-type and mutant strains suggesting that the Agr system is active in all mutants (Figure 3.9B). The growth profile of each mutant strain was similar to that of the wild-type indicating that growth was not been affected during mutagenesis (Figure 3.9C).

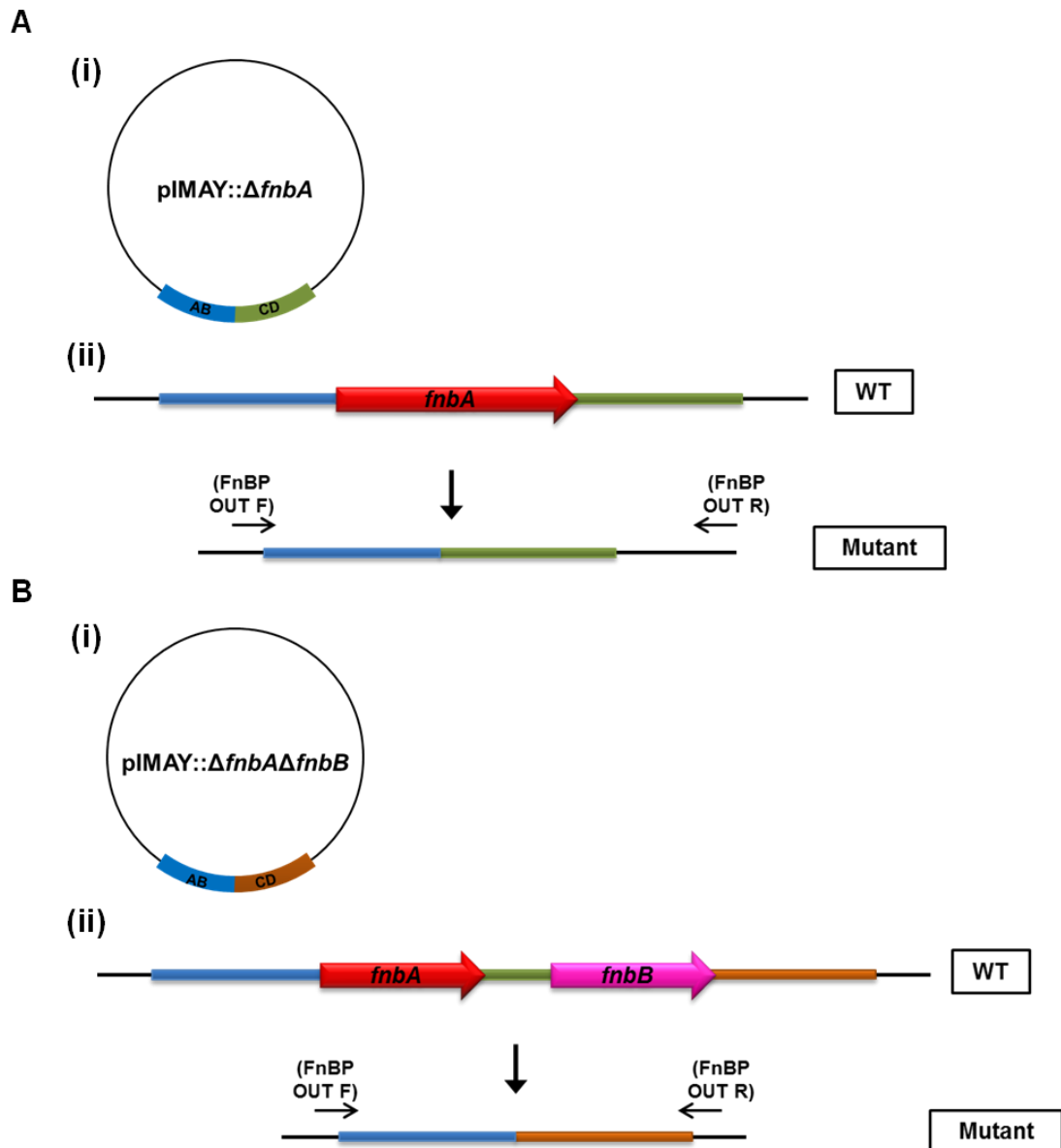


Figure 3.7. Schematic representation of *fnbA* and *fnbB* mutations. **A [i]** pIMAY:: $\Delta fnbA$ was used to delete the *fnbA* gene from the AD08 chromosome by allelic exchange. **B [i]** pIMAY:: $\Delta fnbA\Delta fnbB$ was used to delete both *fnbA* and *fnbB* genes and the intergenic region from the AD08 chromosome. **A[ii]** **B[ii]** The deletions were confirmed by PCR using genomic DNA and FnBP OUT F and R primers.

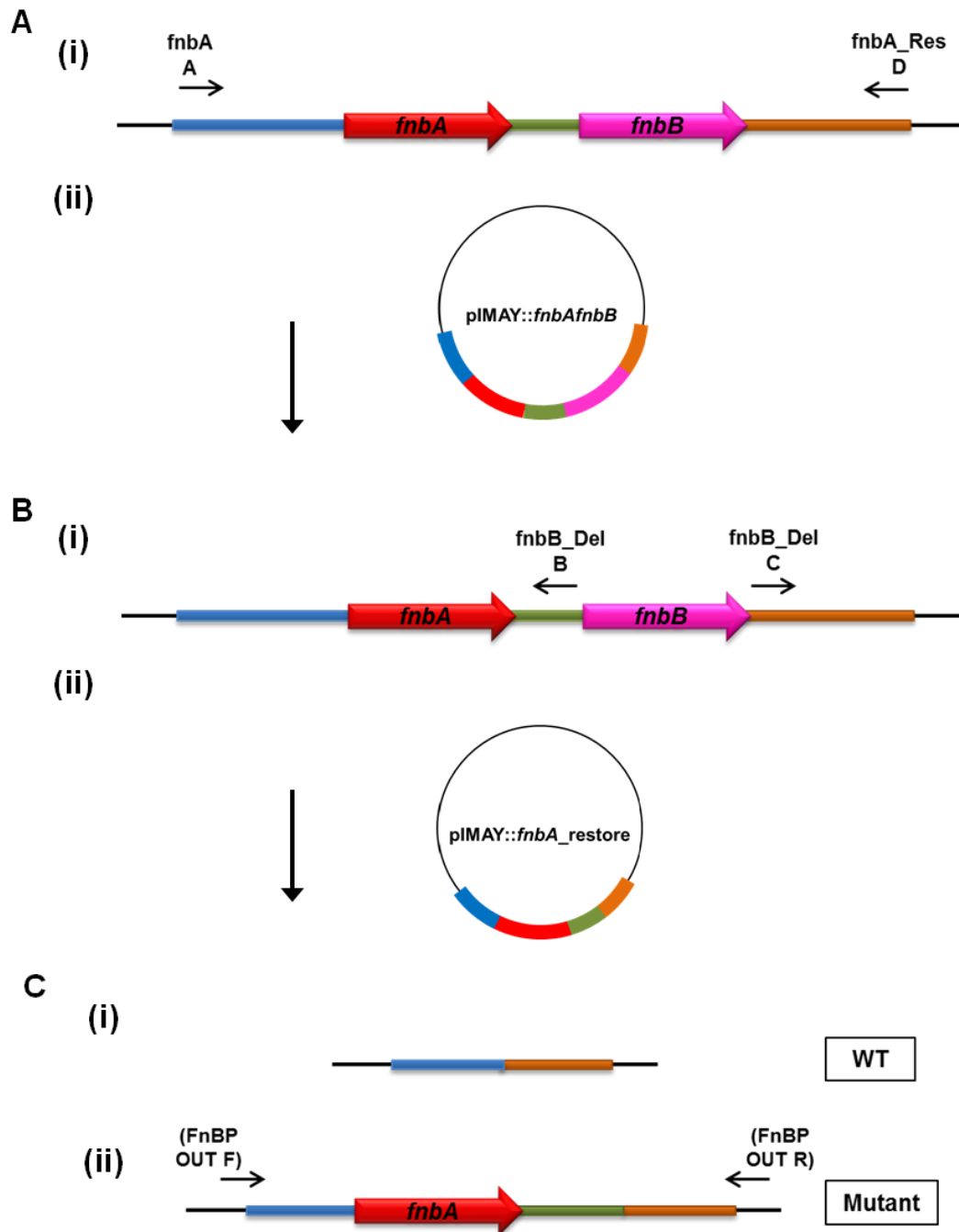


Figure 3.8. Schematic representation of the *fnbA* restoration. **A [i][ii]** The *fnbAfnB* construct was generated using AD08 genomic DNA as template and primers *fnbA* F A and *fnbA* R D to amplify 500 bp upstream *fnbA*, the *fnbA* gene, 680 bp intergenic region, the *fnbB* gene and 500 bp downstream of *fnbB*. **B[i][ii]** Inverse PCR with primers *fnbA* R B and *fnbA* F C was used to remove the *fnbB* gene from the construct. **C)** The mutation was confirmed by PCR using FnBP OUT F and R primers.

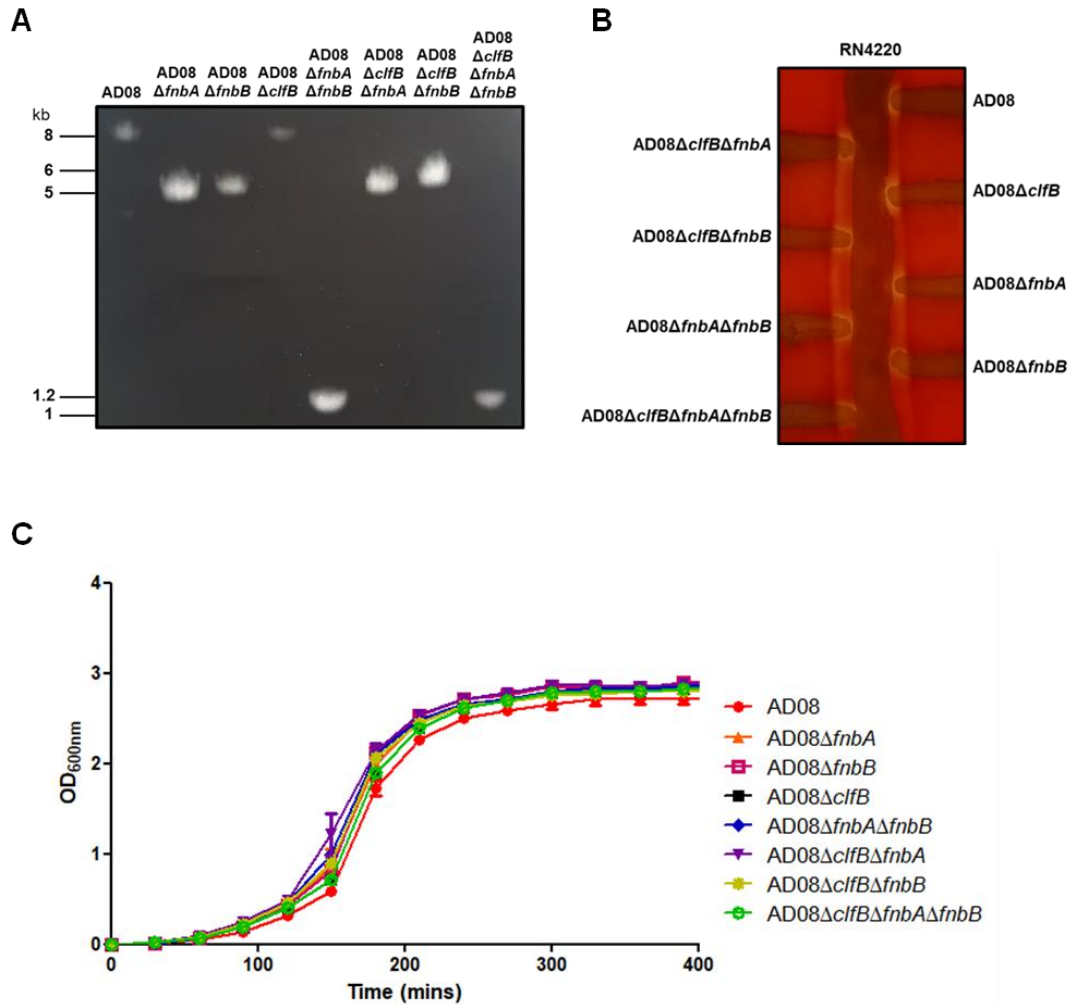


Figure 3.9. Validation of AD08 mutants. **A)** The *fnbA* and/or *fnbB* gene(s) were removed from the AD08 chromosome and the deletion(s) confirmed by PCR, with purified genomic DNA from each strain as template, using primers FnBP OUT F and R. **B)** Strains were cross-streaked against RN4220 to examine δ -toxin activity. **C)** OD₆₀₀ of AD08 and AD08 mutants growing in TSB broth was recorded over a 16 h period. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates.

3.2.7 ClfB and FnBPB mediate *S. aureus* adherence to CDSN

To elucidate the role of ClfB, FnBPA and FnBPB in mediating *S. aureus* adherence to CDSN, the adherence of AD08 mutants to rCDSN was measured using solid phase bacterial adherence assays (Figure 3.10A). There was no reduction in adherence of AD08 Δ *fnbA* or AD08 Δ *clfB* to rCDSN compared to WT AD08. However there was a significant reduction in adherence of AD08 Δ *fnbB*. Adherence to rCDSN was also reduced when more than one protein was absent. Adherence of AD08 Δ *clfB* Δ *fnbA* and AD08 Δ *fnbA* Δ *fnbB* was significantly reduced while adherence of AD08 Δ *clfB* Δ *fnbB* was completely abolished. AD08 Δ *clfB* Δ *fnbA* Δ *fnbB* did not adhere to rCDSN. These data suggest that ClfB, and FnBPB in particular, play an important role in mediating *S. aureus* adherence to CDSN under the conditions tested. The failure of AD08 Δ *clfB* Δ *fnbB* to adhere to rCDSN suggests that FnBPA is not contributing to adherence under the conditions tested.

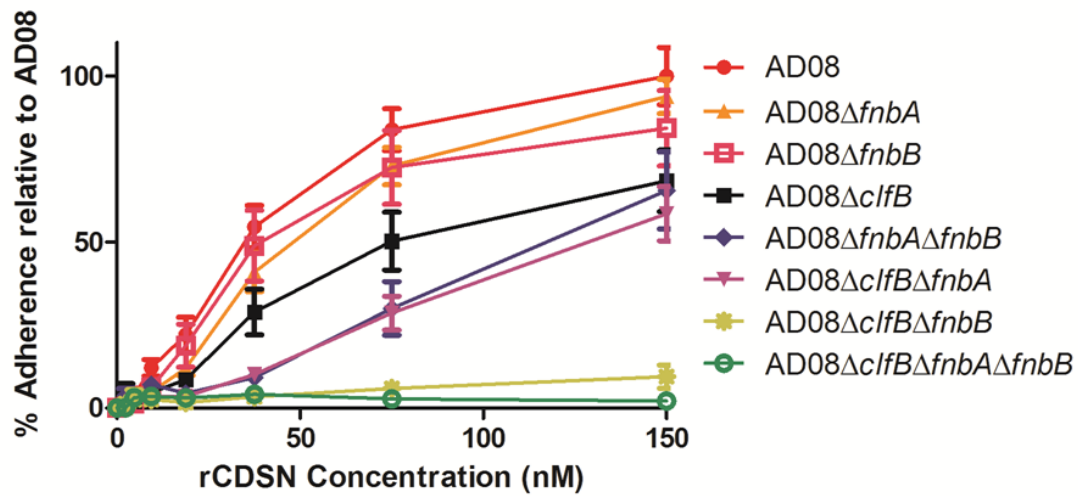
3.2.8 Investigating *S. aureus* adherence to rCDSN under different conditions

S. aureus expresses a wide repertoire of CWAPs which can be expressed in different phases of growth or in response to different environmental conditions. ClfA shares a similar domain structure to ClfB and also binds to fibrinogen (McDevitt *et al.*, 1997). In lab strains of *S. aureus*, expression of ClfA occurs mainly in stationary phase (Homerova *et al.*, 2004) while expression of ClfB, FnBPA and FnBPB is highest in the exponential phase of growth (Said-Salim *et al.*, 2003, McGavin *et al.*, 1997). In response to iron-limited conditions, *S. aureus* expresses a number of surface bound proteins that sequester haem from haemoglobin in blood, providing a source of iron *in vivo*. These include IsdA, IsdB and IsdH (Grigg *et al.*, 2010). IsdA has been implicated previously in skin colonisation as it confers bacterial resistance to host fatty acids and peptides expressed in human skin (Clarke *et al.*, 2007) and can promote *S. aureus* adherence to human desquamated nasal epithelial cells and mediates nasal colonisation to cotton rats (Clarke *et al.*, 2006).

To investigate whether *S. aureus* factors expressed under different *in vitro* growth conditions, such as iron limitation or in stationary phase, could

interact with CDSN, AD08 and AD08 Δ *clfB* Δ *fnbA* Δ *fnbB* were grown in the iron deficient and nutrient limited medium RPMI and in TSB to stationary phase and adherence to rCDSN was measured using solid phase bacterial adherence assays. AD08 grown in RPMI adhered to rCDSN while AD08 Δ *clfB* Δ *fnbA* Δ *fnbB* did not (Figure 3.11A). Furthermore, stationary phase AD08 did not adhere to rCDSN (Figure 3.11B). These data indicate that there are no other factors expressed by AD08 under the conditions tested that are sufficient to promote adherence to CDSN *in vitro*. These data also show that AD08 retains the ability to adhere to CDSN under iron limitation.

A



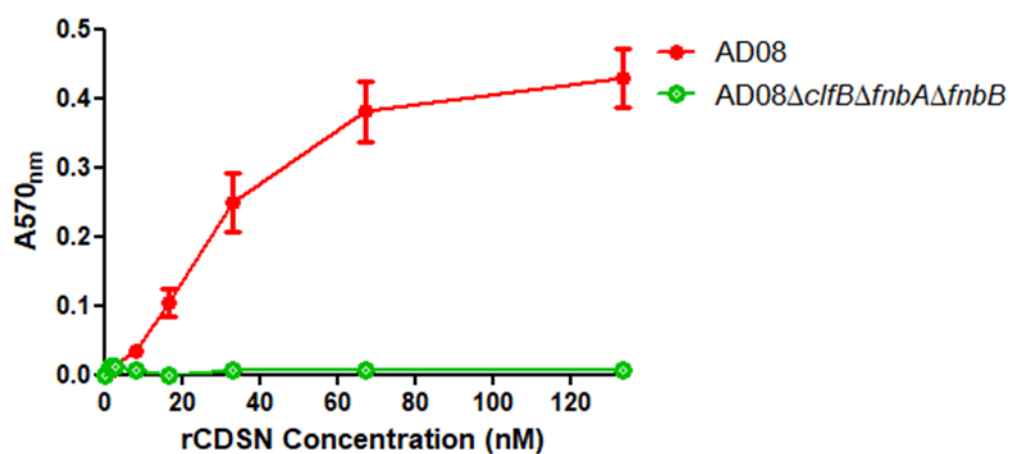
B

Compared to WT	Conc.		
AD08	37.5 μ M	75 μ M	150 μ M
$\Delta fnbA$	ns	ns	ns
$\Delta fnbB$	***	***	***
$\Delta clfB$	ns	ns	ns
$\Delta fnbA\Delta fnbB$	***	***	***
$\Delta clfB\Delta fnbA$	***	***	***
$\Delta clfB\Delta fnbB$	***	***	***
$\Delta clfB\Delta fnbA\Delta fnbB$	***	***	***

ns represents non-significant data
*** represents $p < 0.001$

Figure 3.10. ClfB, FnBPA and FnBPB mediate *S. aureus* adherence to CDSN. A) *S. aureus* strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of rCDSN. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. **B)** A two-way ANOVA with a Bonferroni posttest was performed to compare variances between WT and mutant strains.

A



B

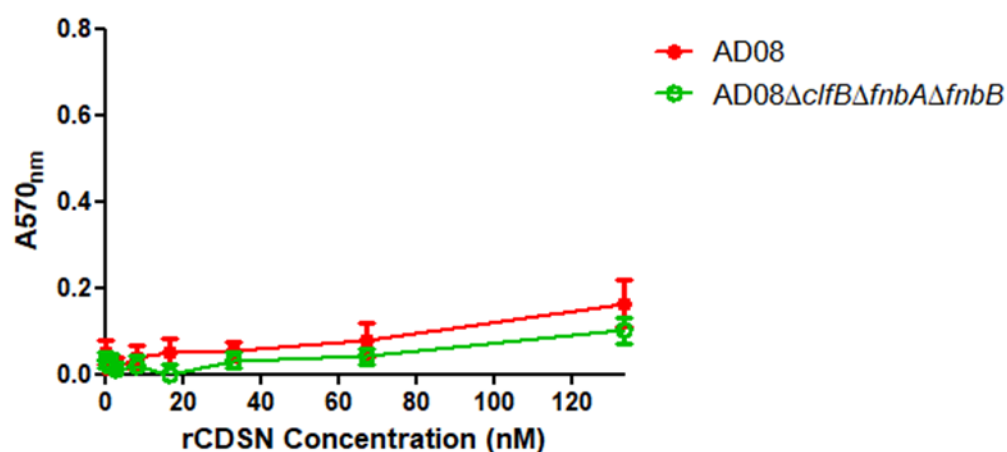


Figure 3.11. Adherence of *S. aureus* AD08 to rCDSN under variable bacterial growth conditions. *S. aureus* strains were grown to stationary phase in **A)** RPMI and **B)** TSB and added to wells of a microtitre plate coated with doubling dilutions of rCDSN. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates.

3.2.9 Carriage of *fnbB* in AD_{CC1} strains and nasal CC30 isolates

Section 3.2.7 describes the importance of FnBPB in mediating adherence of AD_{CC1} strain AD08 to CDSN. While all sequenced *S. aureus* strains carry *fnbA*, some strains do not carry *fnbB* (Lower *et al.*, 2011, Peacock *et al.*, 2000). To investigate *fnbB* carriage by AD_{CC1} strains, primers were designed to amplify the *fnbB* gene where the forward primer annealed within the gene at the end of the sequence encoding the N1 subdomain of the A domain (Figure 3.12A [i]). PCR reactions using genomic DNA from eight AD_{CC1} strains as template with primers *fnbB*_{CC1} N1 F and FnBP OUT R were carried out. Bands approximately 3.5 kb representing *fnbB* confirmed that each AD_{CC1} strain carry the *fnbB* gene (Figure 3.12A [ii]). DNA sequencing of the PCR amplicon (Eurofins Genomics) showed that the *fnbB* nucleotide sequence is conserved across the eight AD_{CC1} strains indicating that they express the same allelic variant of FnBPB. BLAST alignments with the *fnbB* sequence from MRSA476 (data not shown) confirmed that the AD_{CC1} strains tested here carry isotype III *fnbB* which has been previously associated with CC1 strains (Burke *et al.*, 2010).

To investigate *fnbB* carriage in CC30 nasal isolates, primers to amplify across the chromosomal region where *fnbB* is normally located were designed using the sequence of CC1 strain MRSA476, as publically available sequences of CC30 strains do not carry the *fnbB* gene. Primers FnBP OUT F and R anneal to regions 550 bp upstream of *fnbA* and 550 bp downstream of *fnbB* in MRSA476 (Figure 3.12B [i]). These primers anneal to regions 550 bp up and downstream of *fnbA* in the commonly used CC30 MRSA252 strain (Figure 3.12B [ii]). PCR reactions were carried out using genomic DNA from MRSA252 and two CC30 nasal isolates, TS1064 and TS1157, as template with primers FnBP OUT F and R. A 4 kb PCR product was identical for all three strains and represents the *fnbA* gene along with regions up and downstream of the gene (Figure 3.12B [iii]). This indicates that CC30 nasal isolates TS1064 and TS1157 do not carry the *fnbB* gene.

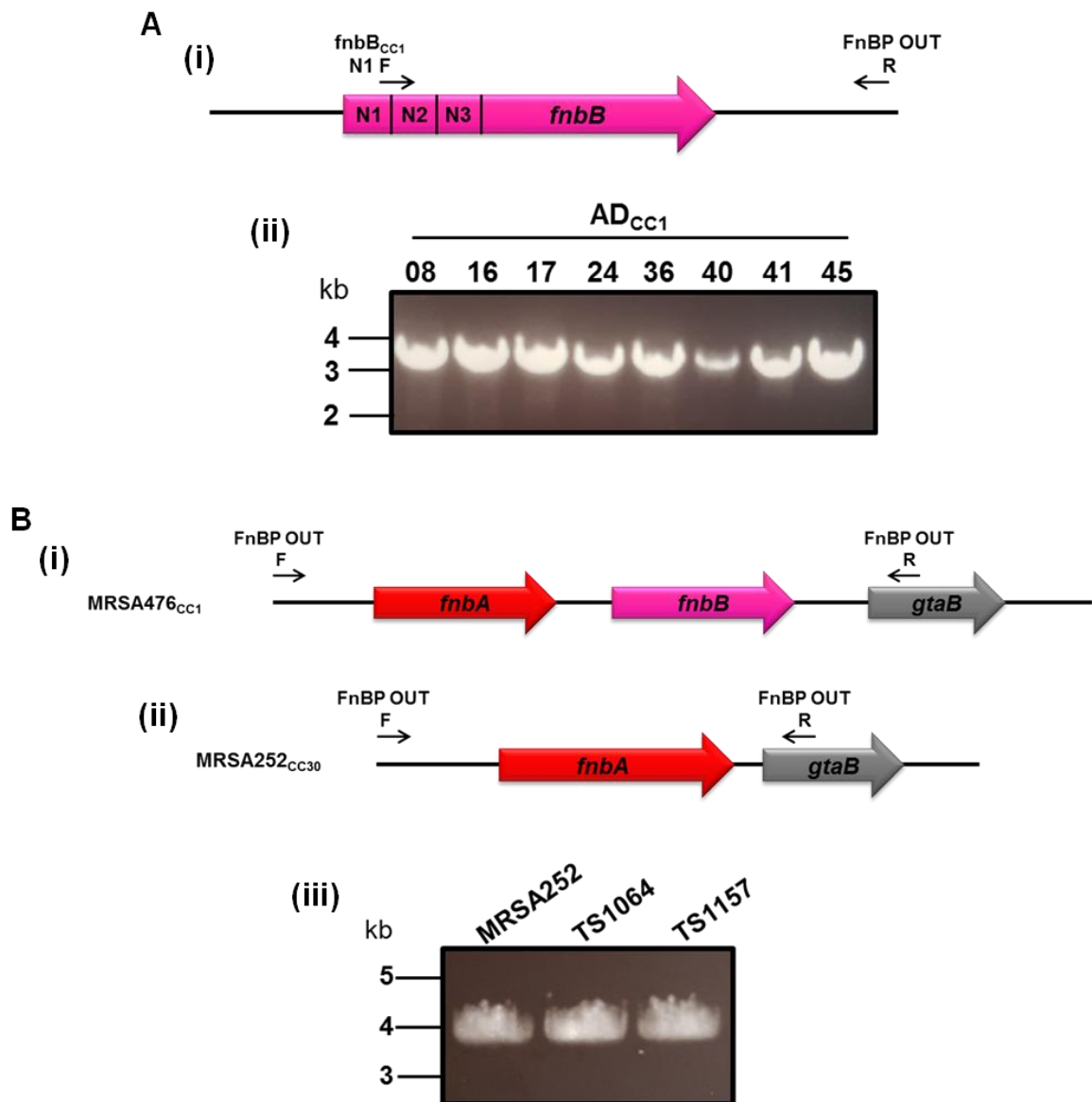


Figure 3.12. Carriage of *fnbB* in AD_{CC1} and CC30 nasal isolates. A i] ii] Primers *fnbB*_{CC1} N1 F and FnBP OUT R were used to amplify the *fnbB* gene in AD_{CC1} strains. **B) i]** Primers FnBP OUT F and FnBP OUT R were designed using MSSA476 nucleotide sequence to anneal to approximately 550 bp upstream of *fnbA* and downstream of *fnbB*. **ii] iii]** Primers FnBP OUT F and R and purified genomic DNA of *S. aureus* CC30 strains were used to amplify across the region encoding the *fnbA* gene.

3.2.10 Purification of CC1-type recombinant His-tagged ClfB N2N3, FnBPA N2N3 and FnBPB N2N3

It was evident that ClfB and FnBPB can mediate bacterial adherence to CDSN, therefore it was necessary to confirm a direct interaction between each protein and CDSN. Recombinant proteins are typically used to characterise protein-ligand interactions. ClfB, FnBPA and FnBPB share a common ligand fibrinogen (Ni Eidhin *et al.*, 1998, Keane *et al.*, 2007, Burke *et al.*, 2011). The ClfB binding site in fibrinogen is in the C-terminus of the α A chain (Walsh *et al.*, 2008) while the FnBPA and FnBPB binding site is in C-terminus of the γ -chain (Wann *et al.*, 2000), however all three proteins bind to fibrinogen by the same 'dock, lock and latch' mechanism (see section 1.4.2.1.4) facilitated by the N2 N3 subdomains (Ganesh *et al.*, 2011, Keane *et al.*, 2007). To determine if the N2 N3 subdomains of ClfB, FnBPA and FnBPB could support binding to CDSN, *E. coli* expression plasmids carrying the N2 N3 subdomains of CC1-type ClfB, FnBPA and FnBPB were used for the expression of recombinant His-tagged proteins (Fleury *et al.*, 2017, Loughman *et al.*, 2008b, Burke *et al.*, 2010). Recombinant rClfBN2N3_{CC1}, rFnBPAN2N3_{CC1} and rFnBPBN2N3_{CC1} were purified using Ni²⁺ chromatography and analysed by SDS-PAGE (Figure 3.13) which showed high level purity of recombinant proteins with apparent molecular weights of 38.5 kDa, 36 kDa and 38 kDa respectively.

3.2.11 Recombinant ClfB N2N3, FnBPA N2N3 and FnBPB N2N3 bind to human CDSN

The affinity of ClfB, FnBPA and FnBPB for CDSN was measured using surface plasmon resonance (SPR). This technique allows for the detection of a ligand-analyte binding interaction in real time. Here, the biacore system was used which utilises a sensor chip with a thin gold layer as a conducting film between the ligand coated onto the chip and the sample analyte in solution. Polarised light is focused on the gold surface of the chip and reflected onto a detector. At a specific angle, known as the resonance angle, electrons in the gold film are resonated forming surface plasmons which are sensitive to the surrounding environment. Free analyte is passed over the surface of the chip and any interaction between the analyte and the ligand coated onto the chip changes the mass on the surface of the chip, which results in a shift in

resonance angle. This shift is detected by the optical detector and plotted onto a sensorgram against time where one response unit (RU) corresponds to 0.0001 ° shift in SPR angle.

Here, a single-cycle kinetics assay was used to measure the affinity of ClfB-, FnBPA-, FnBPB- CDSN interactions. This involved coating the chip with the ligand and passing five increasing concentrations of analyte over the chip followed by a dissociation phase where buffer is passed over the ligand-analyte complex (Karlsson *et al.*, 2006, Onell & Andersson, 2005).

Previously, SPR analysis of GST-tagged proteins using the biacore system involved coating the sensor chip with an anti-GST IgG before capture of the GST-tagged ligand. A GST control was necessary to ensure that there was no interaction between the recombinant bacterial proteins (analyte) and the 26 kDa GST-tag (Mulcahy *et al.*, 2012, Fleury *et al.*, 2017). Thus using commercial His-tagged human CDSN (His-CDSN) was more desirable than rCDSN for this experiment.

His-CDSN was immobilized onto the sensor chip by amine coupling. A second flow cell remained uncoated and served as a reference surface. Increasing concentrations of rClfBN2N3_{CC1}, rFnBPAN2N3_{CC1} and rFnBPBN2N3_{CC1} were passed over the surface of the chip. The surface of the chip was regenerated between individual cycles. All sensorgram data were subtracted from the corresponding data of the reference flow cell and the response generated from passing buffer over the chip surface was also subtracted from each sensorgram (Figure 3.14A, 3.15A, 3.16A respectively). Data were analysed using BIAevaluation software (3.0). Each protein bound His-CDSN in a concentration-dependent manner (Figure 3.14B, 3.15B, 3.16B respectively). The dissociation constant (K_D) of each interaction was calculated by plotting the level of binding in response units (RU) against the concentration of rClfBN2N3_{CC1}, rFnBPAN2N3_{CC1} and rFnBPBN2N3_{CC1} (Table 3.1). These data indicate that rClfBN2N3_{CC1}, rFnBPAN2N3_{CC1} and rFnBPBN2N3_{CC1} bind CDSN independently in a mechanism facilitated by the N2 N3 subdomains. rClfBN2N3_{CC1} and rFnBPBN2N3_{CC1} bound CDSN with similar affinities ($44.6 \pm 2.29 \times 10^{-6}$ M and $49.1 \pm 2.36 \times 10^{-6}$ M). rFnBPAN2N3_{CC1} had a significantly higher affinity for CDSN than both other proteins ($6.91 \pm 1.35 \times 10^{-6}$ M, $p < 0.05$).

A one-way ANOVA with a Tukeys posttest was performed to compare variances between all proteins).

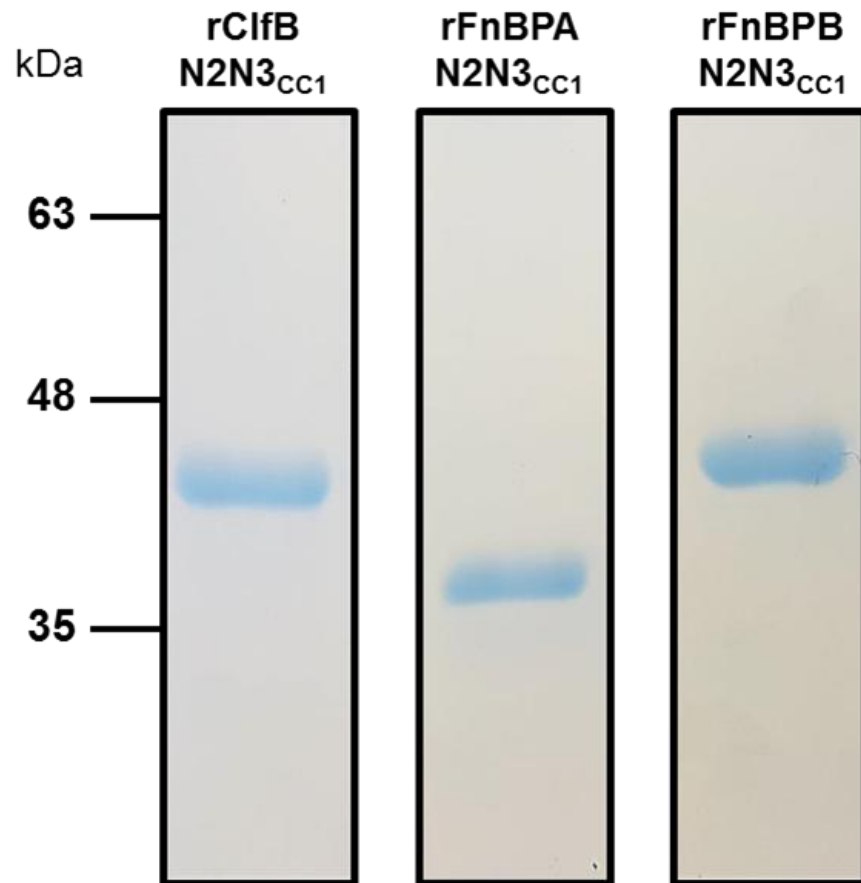


Figure 3.13. SDS-PAGE of rClfBN2N3_{CC1}, rFnBPAN2N3_{CC1} and rFnBPBN2N3_{CC1}. Following purification by nickel affinity chromatography, recombinant proteins (5 μ M) were separated on 10% SDS-PAGE gel, and stained using InstantBlue protein stain.

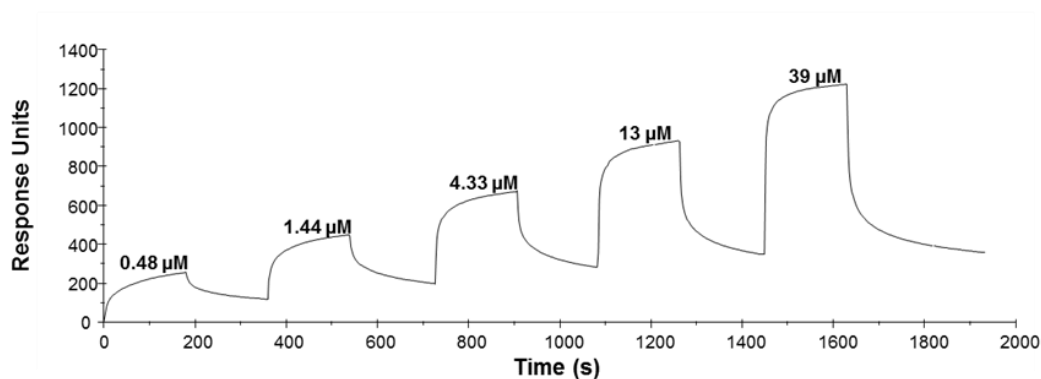
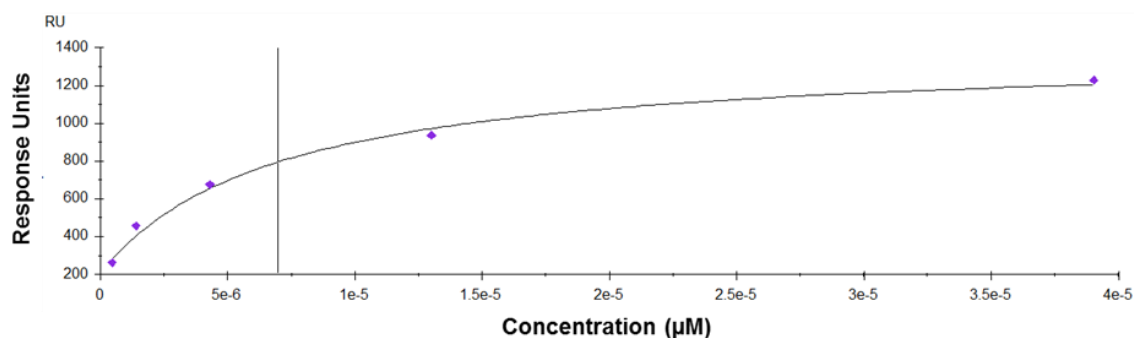
A**B**

Figure 3.14. rFnBPAN2N3_{CC1} binds directly to His-CDSN. **A)** Representative sensorgram displaying the binding of rFnBPAN2N3_{CC1} to and dissociation from His-CDSN. His-CDSN was coated onto a sensor chip by amine coupling and increasing concentrations of rFnBPAN2N3_{CC1} were passed over the chip. Binding interactions were plotted as response units against time. **B)** Representative binding curve showing response units plotted against rFnBPAN2N3_{CC1} concentration allowing for the calculation of the K_D of the interaction, represented here as a vertical line. Data shown is representative of five independent experiments.

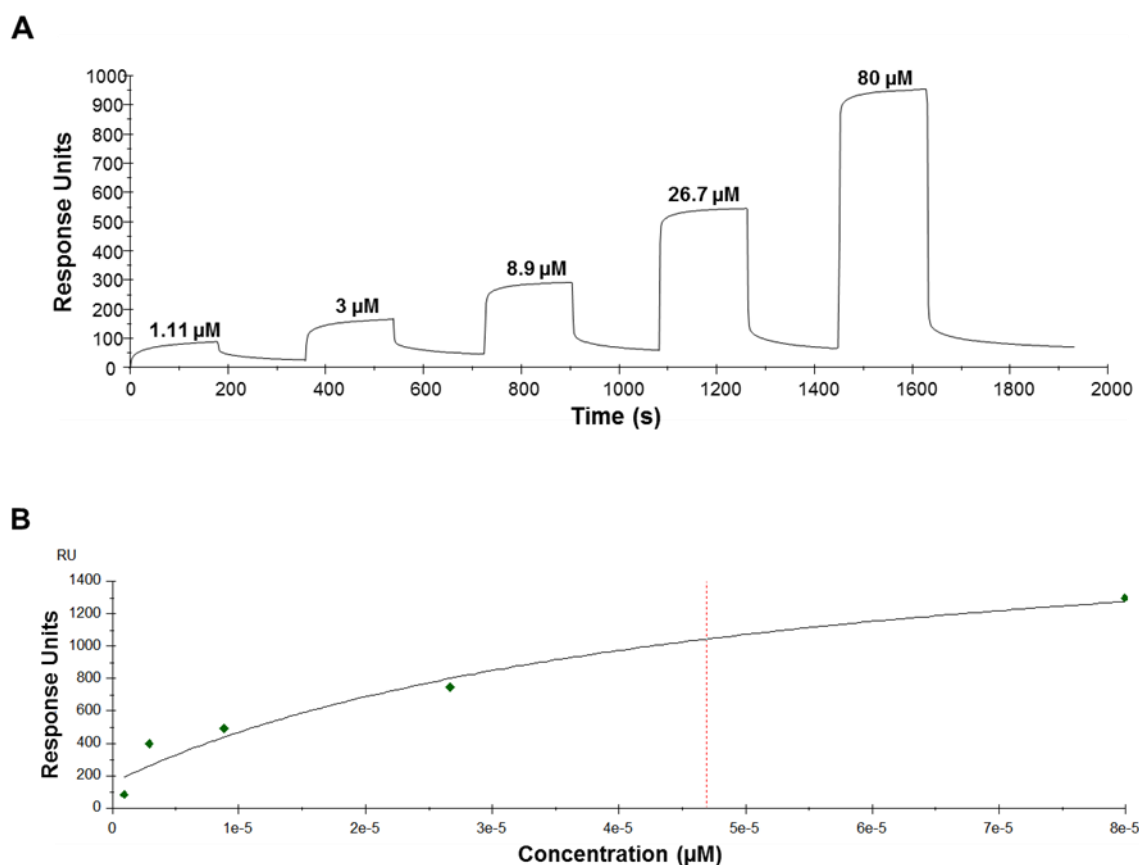


Figure 3.15. rFnBPBN2N3_{CC1} binds directly to His-CDSN. A) Representative sensorgram displaying the binding of rFnBPBN2N3_{CC1} to and dissociation from His-CDSN. His-CDSN was coated onto a sensor chip by amine coupling and increasing concentrations of rFnBPBN2N3_{CC1} were passed over the chip. Binding interactions were plotted as response units against time. **B)** Representative binding curve showing response units plotted against rFnBPBN2N3_{CC1} concentration allowing for the calculation of the K_D of the interaction, represented here as a vertical line. Data shown is representative of five independent experiments.

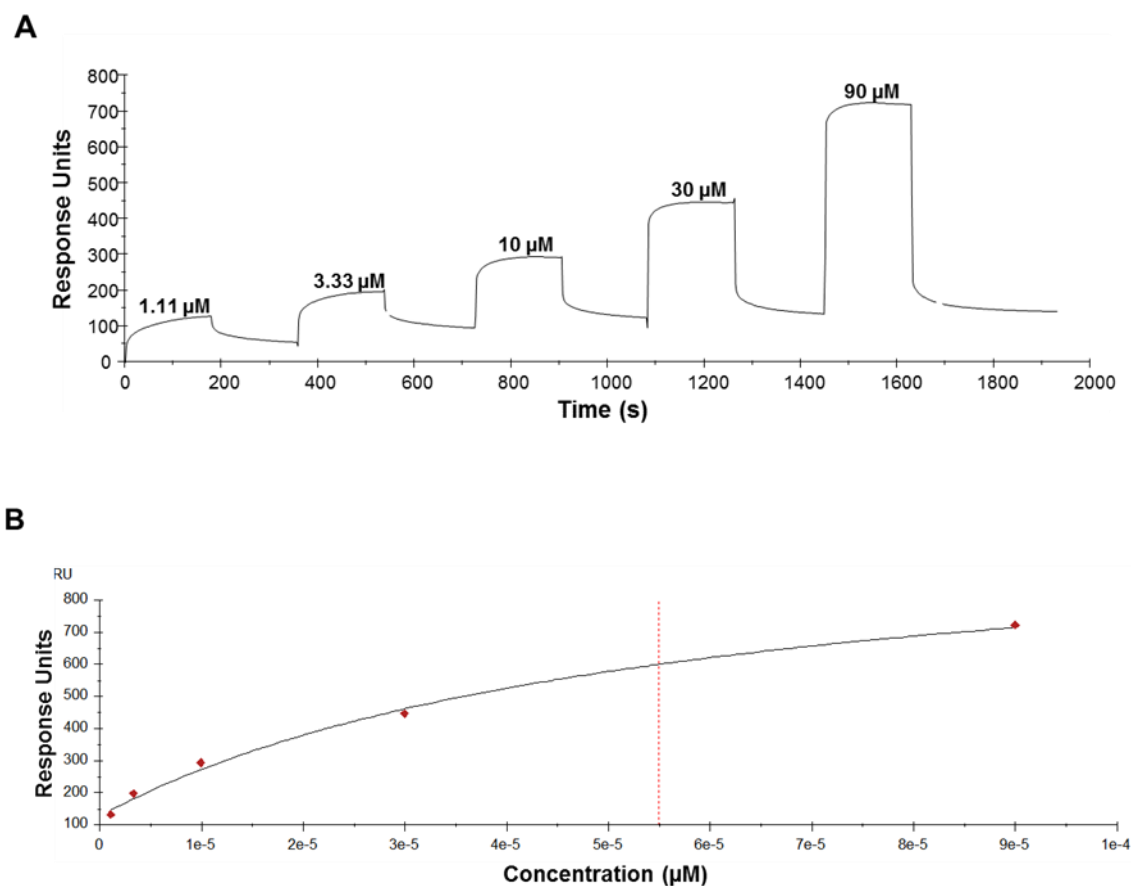


Figure 3.16. rClfBN2N3_{CC1} binds directly to His-CDSN. A) Representative sensorgram displaying the binding of rClfBN2N3_{CC1} to and dissociation from His-CDSN. His-CDSN was coated onto a sensor chip by amine coupling and increasing concentrations of rClfBN2N3_{CC1} were passed over the chip. Binding interactions were plotted as response units against time. **B)** Representative binding curve showing response units plotted against rClfBN2N3_{CC1} concentration allowing for the calculation of the K_D of the interaction, represented here as a vertical line. Data shown is representative of five independent experiments.

Protein	K_D (M) $\pm$ SD ^a
rFnBPAN2N3_{CC1}	6.91 $\pm$ 1.35 $\times 10^{-6}$
rFnBPBN2N3_{CC1}	44.6 $\pm$ 2.29 $\times 10^{-6}$
rClfBN2N3_{CC1}	49.1 $\pm$ 2.36 $\times 10^{-6}$

^aData represents the mean of 5 independent experiments

Table 3.1. Affinities of rFnBPAN2N3_{CC1}, rFnBPBN2N3_{CC1}, rClfBN2N3_{CC1} for His-CDSN determined using surface plasmon resonance.

3.2.12 ClfB, FnBPA and FnBPB mediate *S. aureus* adherence to AD corneocytes

Having elucidated a role for ClfB, FnBPA and FnBPB in binding to the skin protein CDSN *in vitro*, the role of these proteins in mediating bacterial adherence to AD skin was investigated. Fleury et al., (2017) measured bacterial adherence to low NMF corneocytes isolated from AD patients and showed reduced adherence in the absence of ClfB (Fleury *et al.*, 2017). Adherence was not completely abolished however indicating that other factors are involved in promoting the interaction. Using technologies that allow for the study of single-cell or single-protein interactions provides a more thorough analysis of the interaction. Atomic force microscopy (AFM) can be used in this manner as the binding force of single proteins or single cells can be detected and quantified (Formosa *et al.*, 2015). In Single-cell force spectroscopy (SCFS), a single bacterial cell is immobilised onto the tip of an AFM cantilever, brought into contact with a sample and retracted and the binding force of the interaction is detected and quantified (Dufrene, 2014).

Here, native corneocytes were isolated from the skin of AD children with low NMF levels using a tape-stripping method. The adherence of *S. aureus* AD08 and various mutants, to these AD corneocytes was measured by SCFS. Bacteria were grown to exponential phase (Figure 3.17) or stationary phase (Figure 3.18) and a single cell was isolated and immobilised onto the AFM cantilever. The probe was passed over the surface of a single corneocyte and the force of adhesion events between the bacteria and the corneocyte was recorded. For bacteria grown to exponential phase, strong adhesion peaks were detected for wild-type AD08, indicating that it adheres to the AD corneocytes, while adhesion force was significantly reduced for AD08 Δ clfB Δ fnbA Δ fnbB. There was a minor reduction in adhesion force for AD08 Δ fnbA Δ fnbB and a major reduction for AD08 Δ clfB. When bacteria were grown to stationary phase, wild-type AD08 adhered to the AD corneocytes while adhesion force was almost abolished in AD08 Δ clfB Δ fnbA Δ fnbB. Conversely however, there was a major reduction in adhesion force for AD08 Δ fnbA Δ fnbB and a minor reduction for AD08 Δ clfB. These data suggest that ClfB plays a greater role in adherence to low NMF corneocytes during

exponential growth and FnBPA and/or FnBPB are the more dominant adhesins on *S. aureus* grown to stationary phase.

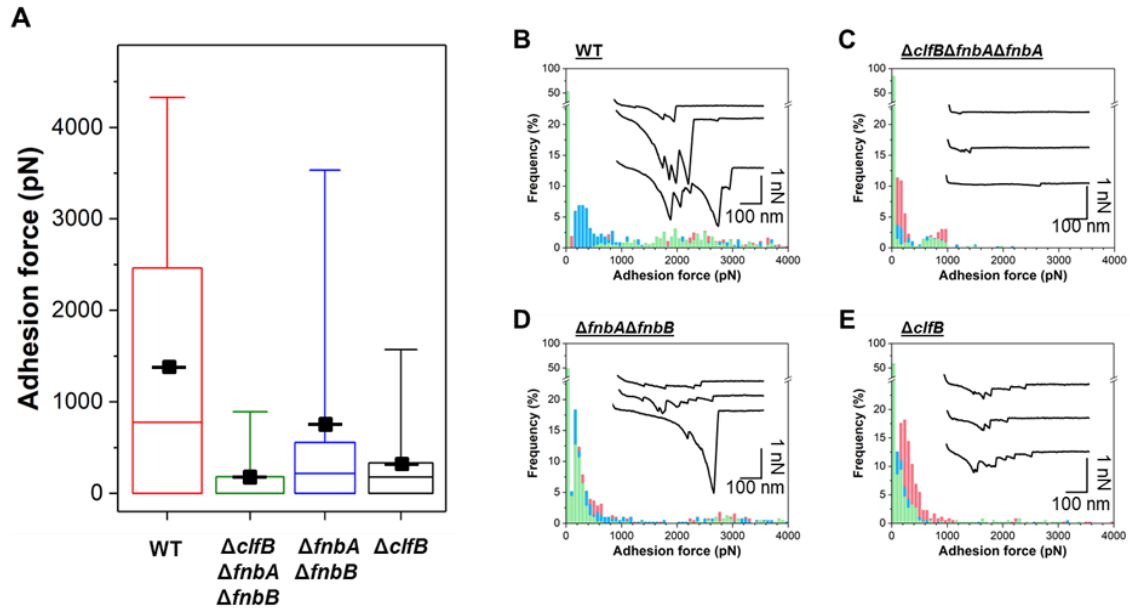


Figure 3.17. ClfB, FnBPA and/or FnBPB mediate adherence of exponential phase *S. aureus* to AD corneocytes. **A)** AD08 (red), AD08 $\Delta clfB$ (black), AD08 $\Delta fnbA \Delta fnbB$ (blue), AD08 $\Delta clfB \Delta fnbA \Delta fnbB$ (green) were grown to exponential phase and a single cell was immobilised onto a probe. Single cell force spectroscopy was used whereby the probe was passed over the surface of the corneocyte and the force of adhesion events between the bacteria and the corneocyte surface were recorded. Box plots show mean adhesion force (full square), median, first and third quartiles (box) and range of data without outliers (whiskers). Data was obtained from three independent experiments. **B), C), D)** and **E)** show force-distance curves of the three independent experiments and the % frequency of high-force adhesion events is shown. Red, blue and green bars indicate a different independent experiment. Single cell force spectroscopy was performed by Dr. Cécile Formosa-Dague at the Louvain Institute of Biomolecular Science and Technology, Université catholique de Louvain, Belgium.

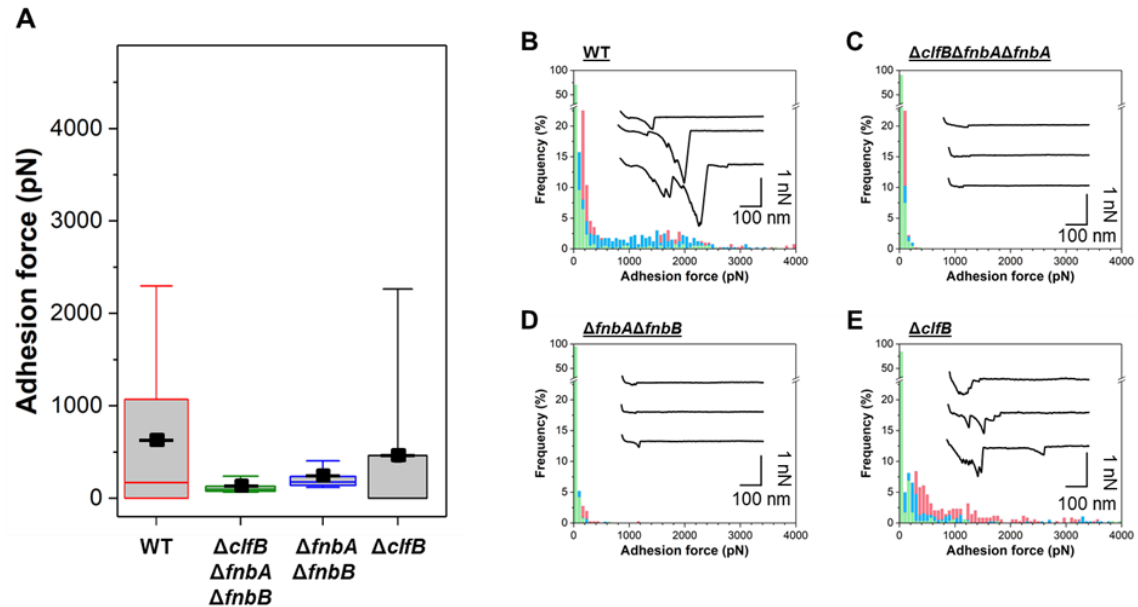


Figure 3.18. ClfB, FnBPA and/or FnBPB mediate adherence of stationary phase *S. aureus* to AD corneocytes. **A)** AD08 (red), AD08 ΔclfB (black), AD08 $\Delta\text{fnbA}\Delta\text{fnbB}$ (blue), AD08 $\Delta\text{clfB}\Delta\text{fnbA}\Delta\text{fnbB}$ (green) were grown to stationary phase and a single cell was immobilised onto a probe. Single cell force spectroscopy was used whereby the probe was passed over the surface of the corneocyte and the force of adhesion events between the bacteria and the corneocyte surface were recorded. Box plots show mean adhesion force (full square), median, first and third quartiles (box) and range of data without outliers (whiskers). Data was obtained from three independent experiments. **B), C), D)** and **E)** show force-distance curves of the three independent experiments and the % frequency of high-force adhesion events is shown. Red, blue and green bars indicate a different independent experiment. Single cell force spectroscopy was performed by Dr. Cécile Formosa-Dague at the Louvain Institute of Biomolecular Science and Technology, Université catholique de Louvain, Belgium.

3.2.13 Localising the *S. aureus* binding site in CDSN

CDSN contains two glycine/serine rich repeat regions, one at the N-terminus and one at the C-terminus of the protein, which are predicted to form Ω loops (Jonca *et al.*, 2011) (Figure 3.19A). Due to the high affinity of ClfB for the glycine/serine rich Ω loop regions in loricrin and K10 (Mulcahy *et al.*, 2012, Walsh *et al.*, 2004), we postulated that the ClfB binding site in CDSN may be present in one or both of the Ω loop regions. To study this, recombinant truncates of CDSN were purified lacking either the N-terminal Ω loop (Figure 3.19B[i]) or C-terminal Ω loop (Figure 3.19C[i]). Plasmid pGEX-4T2::CDSN was used to generate a variant plasmid where the DNA encoding the N-terminal Ω loop domain was deleted (Figure 3.19B[ii]) and a second plasmid where the DNA encoding the C-terminal Ω loop domain was deleted (Figure 3.19C[ii]), using inverse PCR. The deletions were confirmed by PCR-amplification of the gene products compared to the full-length CDSN gene and verified by DNA sequencing (Eurofins Genomics). Plasmids pGEX-4T2::CDSN Δ NT and pGEX-4T2::CDSN Δ CT were introduced into the protease deficient *E. coli* Topp3 for expression of GST-tagged recombinant proteins. GST-CDSN Δ NT (~66 kDa, Δ 60-171) and GST-CDSN Δ CT (~67 kDa, Δ 376-476) (hereafter known as Δ NT and Δ CT respectively) were purified from *E. coli* using glutathione affinity chromatography (Figure 3.20A, B). These proteins were sufficiently pure that Strep-Tactin affinity chromatography was not necessary.

Adherence of AD08 to purified rCDSN, Δ NT and Δ CT was measured by solid phase bacterial adherence assays. Recombinant GST was included to ensure that bacteria were not adhering to the 26 kDa GST-tag. AD08 adhered to CDSN and to Δ CT but not to Δ NT and GST (Figure 3.20C) implying that the C-terminal Ω loop is not required for AD08 adherence and that the binding site for *S. aureus* incorporates the N-terminal Ω loop.

To establish whether the N-terminal Ω loop alone could support *S. aureus* adhesion, GST-tagged N-terminal Ω loop was produced. Plasmid pGEX-4T2::CDSN was used as template for inverse PCR to delete DNA encoding residues 33-61 and 172-529 of CDSN, leaving the GST-tag, the N-terminal Ω loop and C-terminal Strep-tag II affinity tag (Figure 3.21A). Plasmid pGEX-4T2::CDSN Ω NT was introduced into *E. coli* and recombinant GST-

tagged CDSN NT-Ω loop (hereafter known as NT-Ω loop) was expressed and purified by glutathione affinity chromatography followed by Strep-Tactin affinity chromatography. A homogenous preparation of pure protein was not achieved however (Figure 3.21B). SDS-PAGE analysis of eluted protein showed a band corresponding full length GST-tagged NT-Ω loop (approximately 37 kDa) and several peptides of apparent lower molecular weight. These smaller proteins reacted with monoclonal anti-GST IgG. Nevertheless this protein preparation, containing truncates or breakdown products of NT-Ω loop, was used for further analysis.

To investigate whether the N-terminal Ω loop could facilitate binding to *S. aureus*, solid phase bacterial adherence assays were carried out, where AD08 adherence to NT-Ω loop, ΔNT and ΔCT was measured (Figure 3.22). AD08 adhered to NT-Ω loop and ΔCT but not to ΔNT. These data signify that the N-terminal Ω loop of CDSN comprises the *S. aureus* binding site.

3.2.14 ClfB and FnBPB facilitate AD08 adherence to the N-terminal Ω loop of CDSN

To investigate the role of ClfB, FnBPA and FnBPB in mediating bacterial adherence to the N-terminal Ω loop of CDSN independently, single point bacterial adherence assays were carried out where adherence of AD08 mutants expressing individual CWAPs to a single concentration of ΔNT and ΔCT (66 nM) was measured. There was a significant reduction in adherence of AD08Δ*fnbA*Δ*fnbB*, AD08Δ*clfB*Δ*fnbA* and AD08Δ*clfB*Δ*fnbB* to ΔCT compared to WT AD08 (Figure 3.23), indicating that removing one of the three CWAPs affects bacterial adherence. There was a detectable level of adherence of AD08Δ*fnbA*Δ*fnbB* to ΔCT which suggests that ClfB can mediate the interaction independently. AD08Δ*clfB*Δ*fnbB* adheres to ΔCT at a similar level to AD08Δ*clfB*Δ*fnbA*Δ*fnbB* which indicates that FnBPA is not capable of supporting the interaction in the absence of both ClfB and FnBPB. In contrast, the reduction in adherence of AD08Δ*clfB*Δ*fnbA* is minor, suggesting that FnBPB is the dominant protein involved in mediating AD08 adherence to ΔCT. In agreement with previous findings, bacteria did not adhere to ΔNT in any case (Figure 3.23).

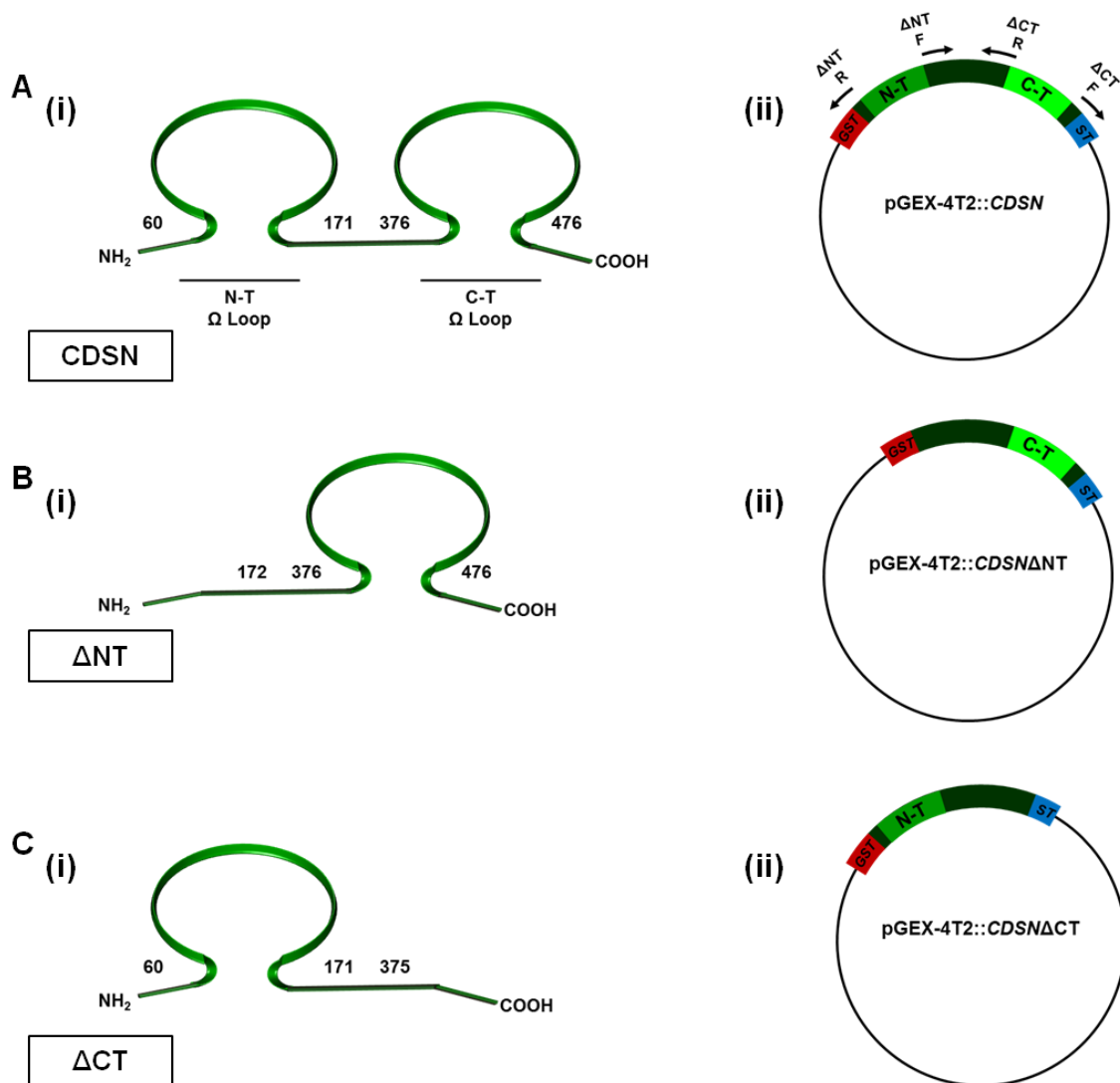


Figure 3.19. CDSN loop domain structure. A[i] Schematic diagram depicting omega loops present in the N-terminus and C-terminus of human CDSN. **A[ii]** Inverse PCR using pGEX-4T2::CDSN as template and primers ΔNT F and R and ΔCT F and R were used to construct plasmids pGEX-4T2::CDSNΔNT **B[ii]** and pGEX-4T2::CDSNΔCT **C[ii]** respectively. Recombinant proteins lacking the **B[i]** N-terminal loop or **C[i]** C-terminal loop were produced in this study.

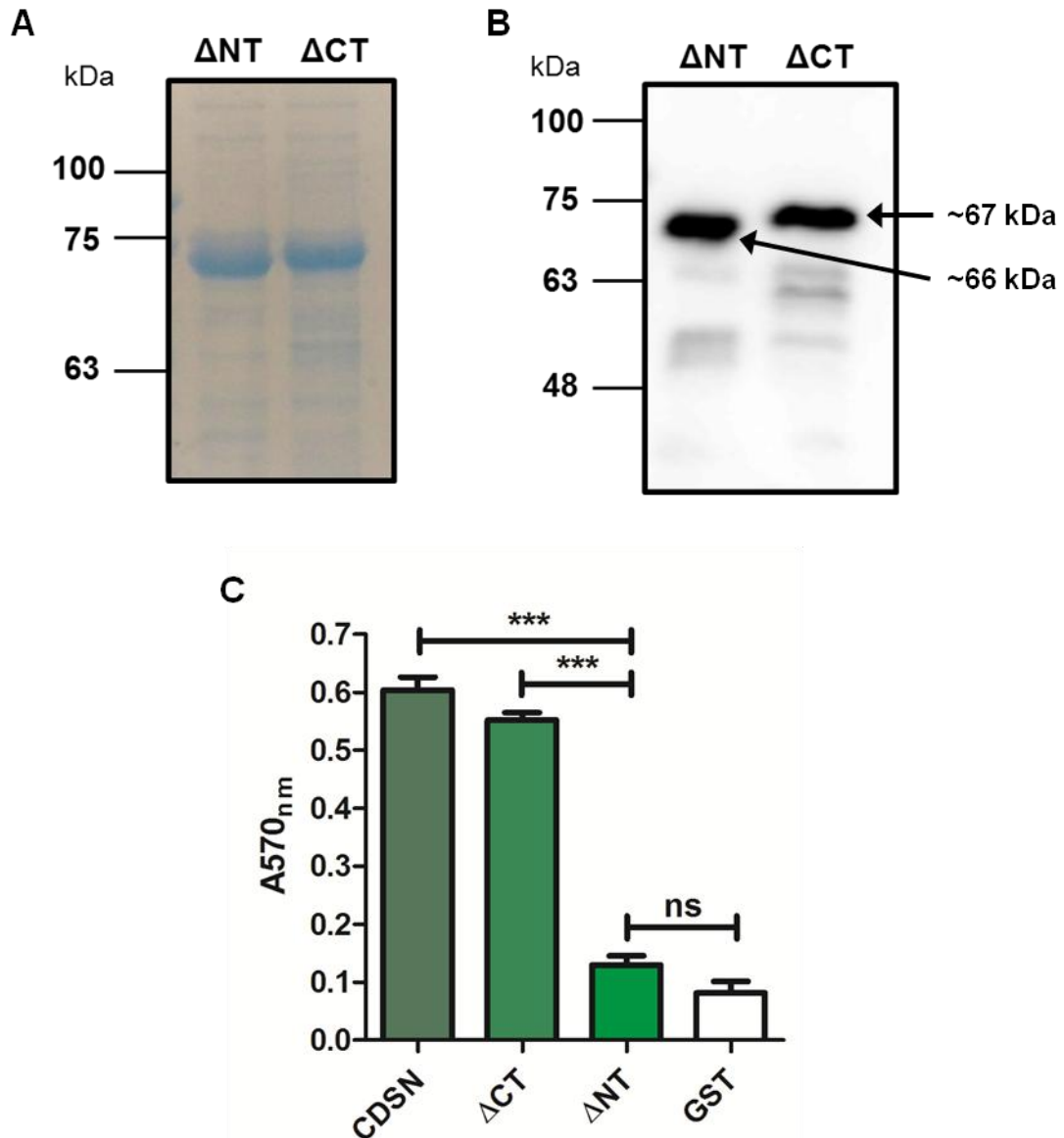


Figure 3.20. *S. aureus* does not adhere to CDSN in the absence of the N-terminal Ω loop domain. Following purification by glutathione affinity chromatography, **A)** protein was separated on a 10% SDS-PAGE gel and stained using InstantBlue protein stain. **B)** Separated protein was transferred onto a PVDF membrane and probed with mouse anti-GST IgG followed by rabbit anti-mouse HRP. **C)** AD08 was grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with 66 nM of recombinant proteins. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A one-way ANOVA with Tukeys posttest was carried out to compare differences between variant proteins.

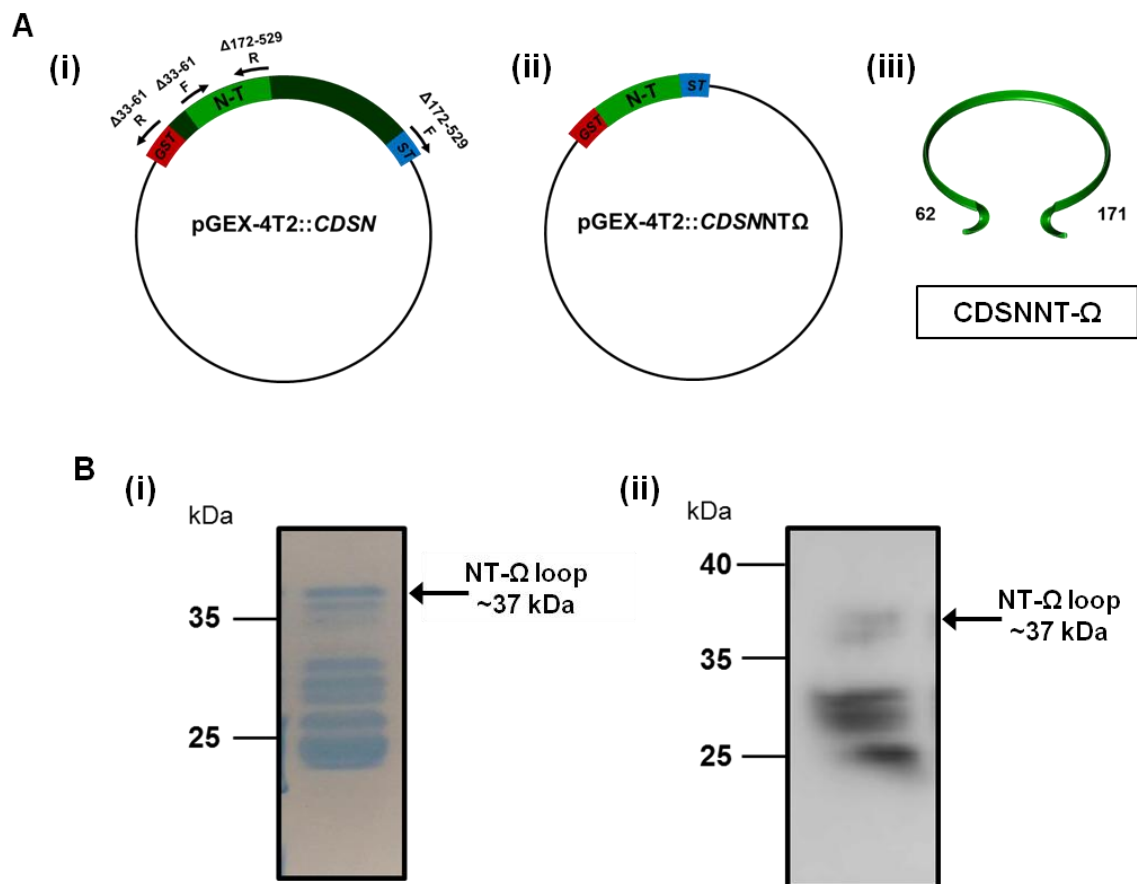


Figure 3.21 Construction of CDSN N-terminal Ω loop. **A [i]** Consecutive inverse PCR reactions using pGEX-4T2::CDSN as template with primers $\Delta 33-61$ F and R and $\Delta 172-529$ F and R, were used to construct **A[ii]** pGEX-4T2::CDSNNT Ω . **A[iii]** Schematic diagram depicting CDSN-NT Ω residues 62-171. **B [i]** Following purification by Strep-Tactin affinity chromatography and sequential glutathione affinity chromatography, separated protein was stained with InstantBlue protein stain. **B[ii]** Separated protein was transferred onto a PVDF membrane and probed with mouse anti-GST IgG followed by rabbit anti-mouse HRP.

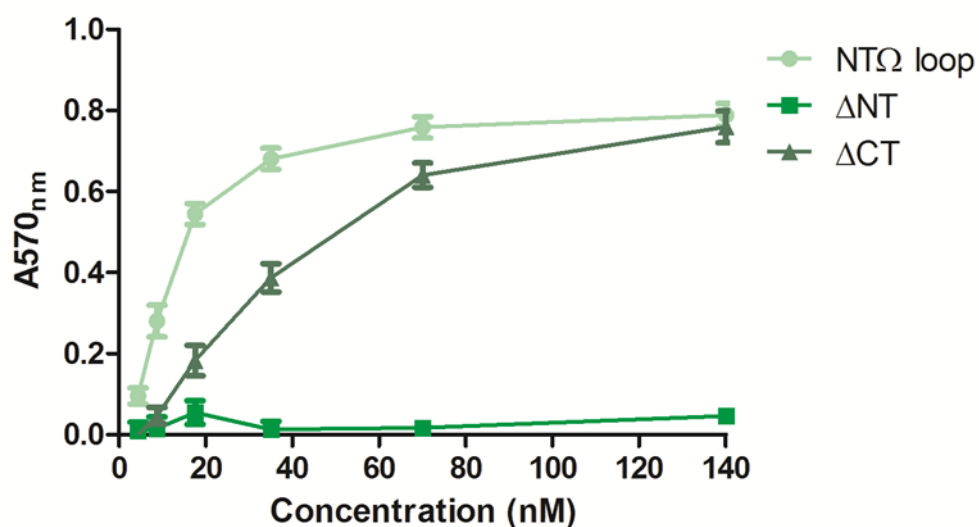


Figure 3.22. The N-terminal Ω loop of CDSN comprises the *S. aureus* binding site. *S. aureus* AD08 was grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of recombinant protein. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates.

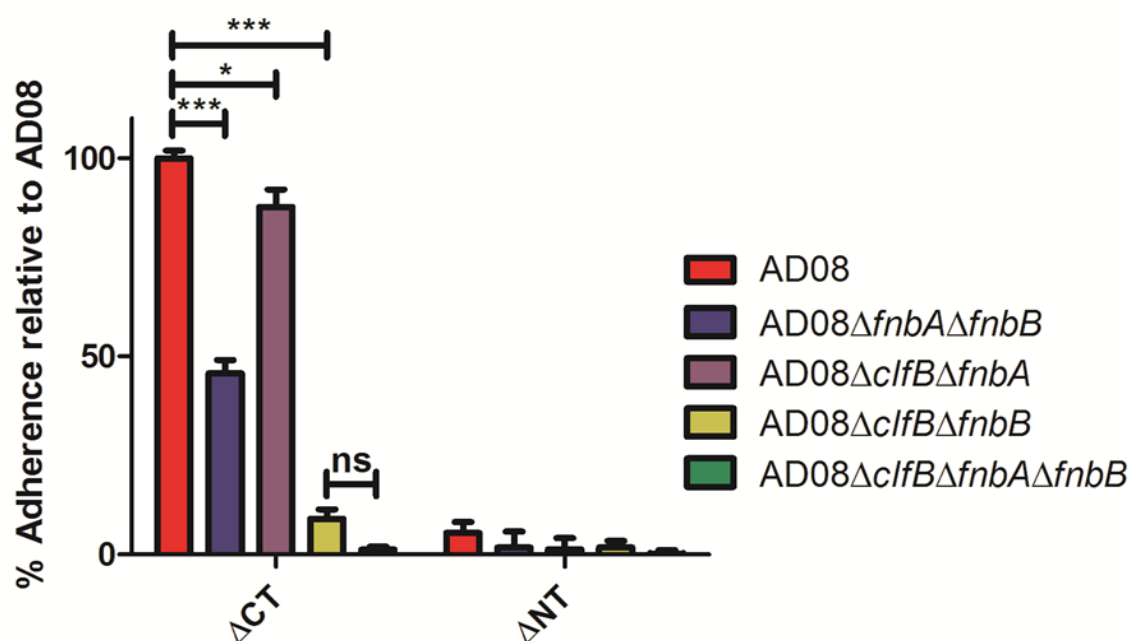


Figure 3.23. ClfB and FnBPB facilitate AD08 adherence to the N-terminal Ω loop of CDSN. AD08 and mutants strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with 100 nM of recombinant proteins. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A one-way ANOVA with Tukeys posttest was carried out to compare differences between mutants.

3.2.15 Generation of anti-CDSN F(ab)₂ fragments

To examine whether CDSN was a target ligand for *S. aureus* in AD skin, we attempted to block the *S. aureus* binding site in CDSN using a polyclonal rabbit anti-CDSN IgG that was raised against the N-terminal (residues 40-229) domain of CDSN. *S. aureus* expresses the protein SpA on its cell surface (Forsgren & Nordstrom, 1974) which binds avidly to the Fc region of IgG (Deisenhofer, 1981) capturing it at the bacterial cell surface. AD08 carries the *spa* gene (Fleury *et al.*, 2017) and therefore we assumed SpA is expressed by AD08. To overcome the possibility of SpA binding to anti-CDSN IgG, the Fc region of the anti-CDSN IgG was proteolytically cleaved to generate anti-CDSN F(ab)₂ fragments that would not bind to AD08 (Figure 3.24A). This was achieved using the endopeptidase pepsin to digest the IgG into light chain F(ab)₂ fragments and heavy chain Fc peptides. The F(ab)₂ preparation was purified by passage through a column containing immobilised SpA to capture Fc peptides and any remaining undigested IgG. SDS-PAGE analysis of the digest product prior to passage through the column showed bands representing the F(ab)₂ fragments and whole undigested IgG at an apparent molecular weight of 88 kDa and 135 kDa respectively while analysis of the purified product showed a single faint band representing purified F(ab)₂ fragments (Figure 3.24B).

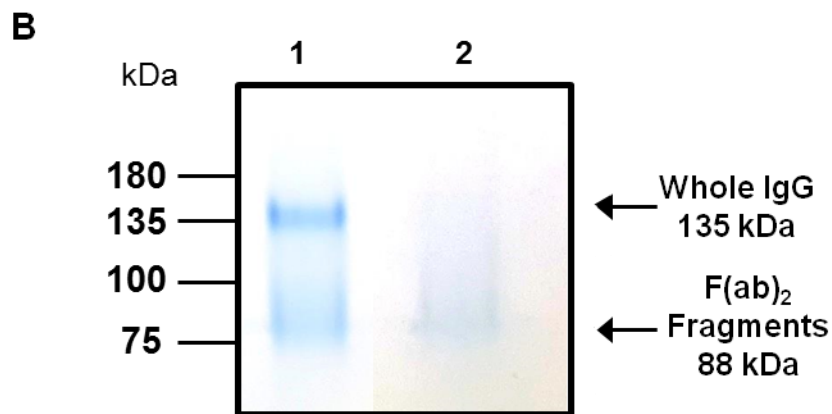
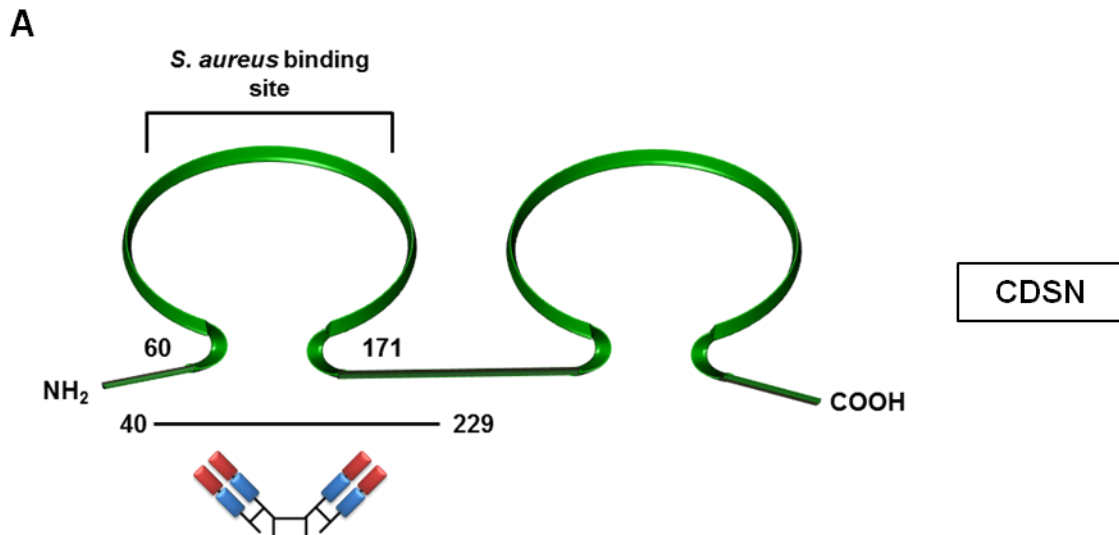


Figure 3.24. Generation of anti-CDSN F(ab)₂ fragments. **A)** Schematic diagram depicting polyclonal rabbit anti-CDSN F(ab)₂ IgG raised against the N-terminal region of CDSN (amino acid residues 40-229). **B)** SDS-PAGE of anti-CDSN IgG. Bands representing whole undigested IgG and F(ab)₂ fragments are displayed in lane 1. A band representing purified F(ab)₂ fragments is displayed in lane 2.

3.2.16 More CDSN is detected on the surface of low NMF corneocytes

A characteristic of AD is low NMF levels in the skin, presumably due to a decrease or absence of the skin protein filaggrin (Kezic *et al.*, 2008). Reduced NMF levels also correlate directly with disease severity (Kezic *et al.*, 2011) and abnormal corneocyte morphology (Riethmuller *et al.*, 2015), where 'villus-like' projections tipped with CDSN decorate the entire corneocyte surface. Thus we can hypothesise that patients with more severe AD disease symptoms, present with lower NMF levels in their skin and potentially more aberrant CDSN localisation in the stratum corneum. Here, anti-CDSN F(ab)₂ fragments were immobilized onto an AFM cantilever and scanned over the surface of corneocytes isolated from AD patients. The corneocytes tested were isolated from AD patients with low NMF levels and high NMF levels, termed low NMF corneocytes and high NMF corneocytes respectively from hereafter. The frequency of anti-CDSN F(ab)₂ fragments adhesion to low NMF corneocytes was significantly higher than to high NMF corneocytes (Figure 3.25). These data suggests the N-terminal domain of CDSN is more abundant or accessible in low NMF corneocytes.

3.2.17 *S. aureus* targets CDSN to adhere to AD corneocytes

It was recently shown that *S. aureus* AD08 adheres significantly more strongly to low NMF corneocytes than to high NMF corneocytes (Feuillie *et al.*, 2018). Given the correlation between CDSN abundance on low NMF corneocytes (section 3.2.16), strong *S. aureus* adherence to low NMF corneocytes and ability of ClfB and FnBPB to mediate adherence to CDSN (section 3.2.7), we hypothesised that CDSN was the target ligand for *S. aureus* adherence to AD corneocytes.

To investigate this, the N-terminal domain of the protein was blocked using anti-CDSN [F(ab)₂] and adherence of AD08 to low NMF corneocytes was measured by AFM. Corneocytes were isolated from an AD patient with low NMF levels and incubated with multiple concentrations of anti-CDSN N-terminal F(ab)₂ fragments. A single bacterial cell was immobilised onto the tip of an AFM cantilever and the adhesion force to a single corneocyte was quantified. There was a dose-dependent reduction in adhesion force to the corneocytes for each

bacterium-corneocyte pair tested (Figure 3.26), suggesting that the ability of AD08 to adhere to AD corneocytes is reduced when the *S. aureus* binding site in CDSN is blocked. These data indicate that *S. aureus* AD08 targets the skin ligand CDSN to adhere to AD corneocytes.

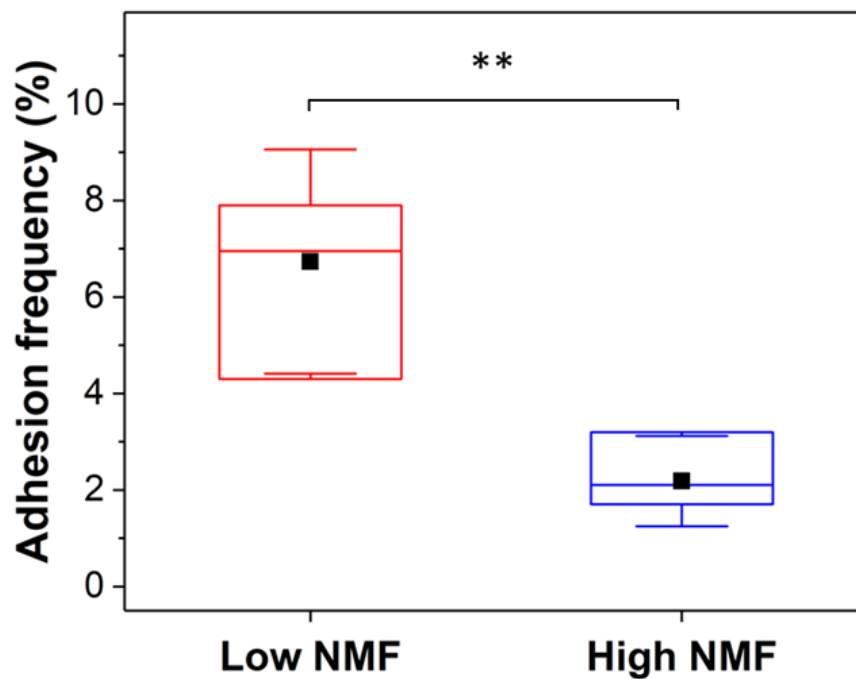


Figure 3.25. F(ab)₂ bind more strongly to low NMF corneocytes than to high NMF corneocytes. Corneocytes were isolated from AD patients with low NMF levels and high NMF levels. Atomic force microscopy was used to measure binding of anti-CDSN F(ab)₂ fragments to the low NMF corneocytes (red) and high NMF corneocytes (blue). Box plot shows mean % adhesion frequency (full square), median, first and third quartiles (box) and range of data without outliers (whiskers) from data obtained from three independent experiments. Atomic force microscopy was performed by Dr. Cécile Formosa-Dague at the Louvain Institute of Biomolecular Science and Technology, Université catholique de Louvain, Belgium.

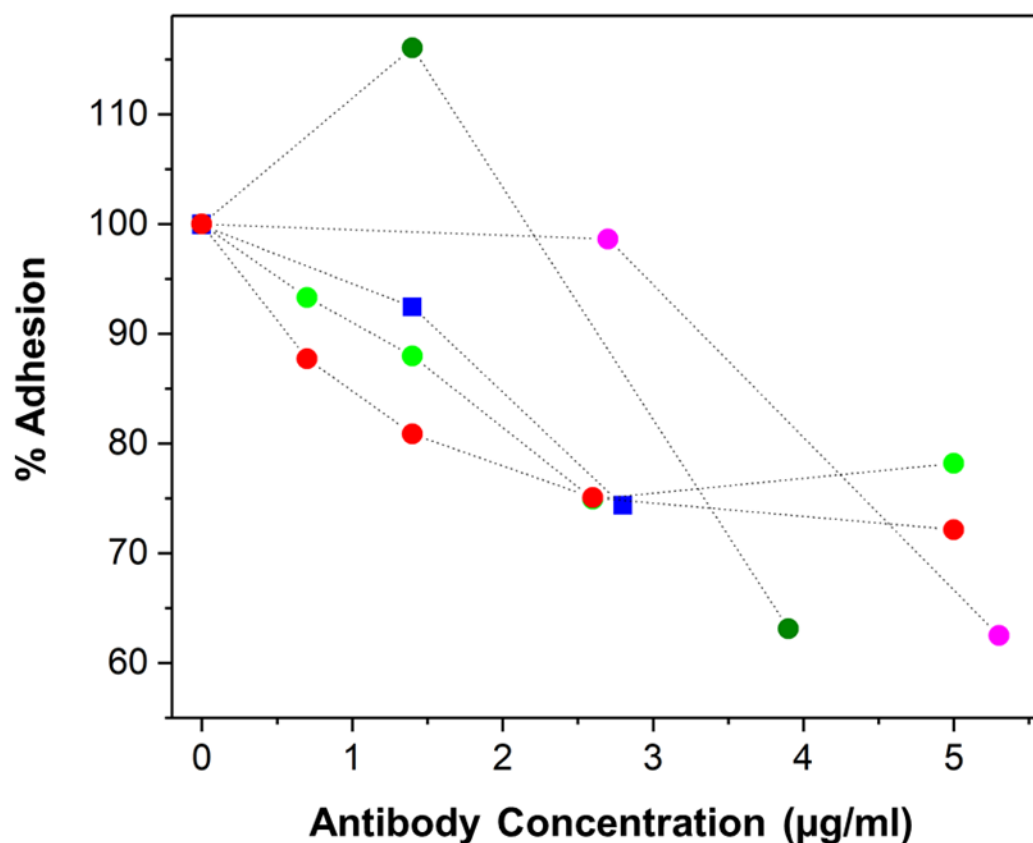


Figure 3.26. Blocking the bacterial binding site in CDSN reduces *S. aureus* adherence to AD corneocytes. Corneocytes were isolated from an AD patient with low NMF levels and incubated with multiple concentrations of anti-CDSN F(ab)₂ fragments. AD08 was grown to exponential phase and a single cell was immobilised onto a probe. Single cell force spectroscopy was used whereby the probe was passed over the surface of the corneocyte and the force of adhesion events between the bacteria and the corneocyte surface were recorded. The data was obtained from 5 independent experiments and each colour represents an independent experiment where a different bacterium-corneocyte pair was tested. Single cell force spectroscopy was performed by Dr. Cécile Formosa-Dague at the Louvain Institute of Biomolecular Science and Technology, Université catholique de Louvain, Belgium.

3.2.18 CDSN is not a ligand for coagulase-negative staphylococci

The skin of AD patients has increased colonisation by staphylococci, including some CoNS spp. (Kong *et al.*, 2012). CoNS are typical skin commensals in the healthy population, however there is very little known about the mechanisms by which they colonise skin. The target ligands in skin, healthy or otherwise, for CoNS spp. have not been identified. Some CoNS species share ligands with *S. aureus* including *S. epidermidis* which adheres to K10 and fibrinogen *in vitro* in an interaction mediated by CWAPs with similar domain structures to *S. aureus* proteins ClfB, FnBPA and FnBPB (Bowden *et al.*, 2005, Hartford *et al.*, 2001, Trivedi *et al.*, 2017). It is possible that CoNS species share other ligands with *S. aureus*.

To investigate CDSN as a potential ligand for CoNS in skin, a collection of CoNS strains isolated from patients at Temple Street Children's hospital, Dublin (Kindly provided by Dr. Des Bennett) was used and includes *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. warneri*, *S. capitis*, *S. auricularis*, *S. saprophyticus*, *S. simulans*, *S. xylosus*, *S. caprae* and *S. scuri* strains. The adherence of these strains and *S. aureus* AD08 to a single concentration of rCDSN (76 nM) was measured by solid phase bacterial adherence assays. The data obtained was standardised relative to the adherence of AD08 to rCDSN. None of the CoNS clinical isolates adhered to rCDSN (Figure 3.27). CoNSN, which is a *S. warneri* isolate, appears to have weak adherence to rCDSN, however the raw absorbance value at 570 nm ($A_{570} = 0.18$) was below 0.2 and is therefore not considered biologically significant. These data indicate that CDSN is not a ligand for CoNS, by the conditions tested here.

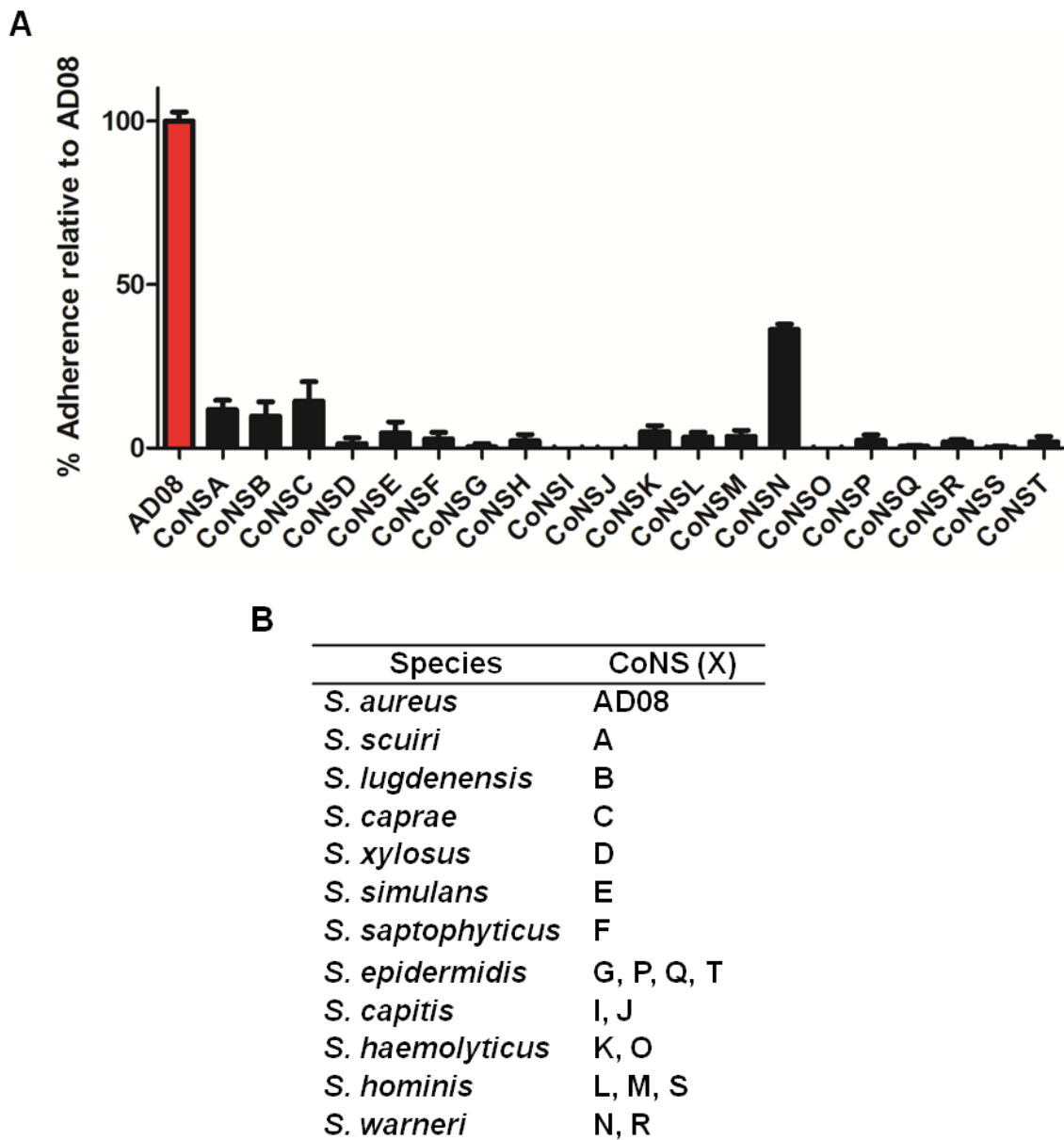


Figure 3.27. CDSN is not a ligand for coagulase-negative staphylococci.

A) Staphylococcal strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with a single concentration of rCDSN (76 nM). Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A one-way ANOVA with Dunnetts posttest was performed to compare differences between *S. aureus* AD08 and CoNS strains, $p > 0.05$. **B)** CoNS(A-T) represent CoNS isolates from Temple Street Children's Hospital Dublin.

3.3 Discussion

S. aureus colonisation of AD skin has been widely reported (Totte *et al.*, 2016) but poorly characterised as the bacterial factors responsible for AD skin colonisation have not been described. The first step of colonisation is bacterial attachment to a host cell or tissue typically mediated by a direct interaction between a bacterial protein and host ligand. The target ligand for *S. aureus* in AD skin has not been identified. In healthy skin corneocytes of the stratum corneum are densely packed in the matrix of cornified envelope proteins and tightly adhered together by corneodesmosome proteins including corneodesmosin (Candi *et al.*, 2005). AD skin however is characterised by a skin barrier defect with aberrant corneocyte morphology. Corneodesmosome products have abnormal displacement in the stratum corneum of AD skin and corneodesmosin covers the entire corneocyte surface (Riethmuller *et al.*, 2015, Igawa *et al.*, 2013). This chapter identified corneodesmosin (CDSN) as a novel ligand for *S. aureus* and showed that *S. aureus* CC1 strains, which are associated with AD skin colonisation, adhere stronger to CDSN than nasal isolates typical of the healthy population (Fleury *et al.*, 2017). It is presumable therefore to consider CDSN as the target ligand for *S. aureus* attachment to AD skin. CoNS spp. colonise healthy skin by unknown molecular mechanisms and it was shown here that CDSN is not a ligand for any of the CoNS strains in the conditions tested. This data emphasises the prominence of CDSN as an exclusive ligand in humans for *S. aureus*, perhaps giving it an advantage on AD skin by allowing it a greater opportunity to colonise compared to CoNS commensals.

To characterise host-microbe interactions at the molecular level, it is useful to generate a system that allows for manipulation of genes and purification of recombinant proteins. Typically this can be achieved using *E. coli* expression systems however previous attempts at purifying recombinant CDSN from bacteria have been unsuccessful (Caubet *et al.*, 2004b). In this chapter, a plasmid carrying the human *CDSN* gene was constructed and the recombinant protein expressed in *E. coli* and purified using consecutive Strep-Tactin affinity chromatography and glutathione affinity chromatography for production of a

GST-tagged recombinant CDSN protein. The purification of this protein allowed for the *in vitro* analysis of the interaction between *S. aureus* and CDSN.

S. aureus expresses a number of surface bound virulence factors that contribute to colonisation and infection by adherence to host cells and ligands (Foster *et al.*, 2014) including the CWAP ClfB, which has been implicated in colonisation of AD skin previously. In the absence of ClfB, *S. aureus* had reduced adherence to low NMF AD corneocytes (Feuillie *et al.*, 2018, Fleury *et al.*, 2017). ClfB binds to glycine/serine rich omega loop regions found in the skin proteins loricrin and K10 (Mulcahy *et al.*, 2012, Walsh *et al.*, 2004) and similar glycine/serine rich regions predicted to form omega loops are present in CDSN. In this chapter, adherence of lab strain Newman to CDSN was dependent on ClfB however a clinically relevant AD strain, AD08, expressed additional CDSN binding proteins.

CWAPs of *S. aureus* are functionally redundant in nature and colonisation is normally multifactorial (Foster *et al.*, 2014). Strain Newman is a well characterised *S. aureus* lab strain and has been used extensively to study *S. aureus* virulence however it is limited as it does not express functional fibronectin binding proteins (Grundmeier *et al.*, 2004). Previously FnBPA and FnBPB deficient mutants of *S. aureus* laboratory strain 8325-4 had reduced adherence to skin biopsies taken from AD patients implicating FnBPA and/or FnBPB in colonisation of AD skin. AD skin has higher levels of fibronectin and it was therefore assumed that fibronectin-binding activity was responsible for stronger adherence to AD skin biopsies (Cho *et al.*, 2001). Here, a mutant of AD08 deficient in FnBPB had significantly reduced adherence to CDSN. Using double mutants of AD08 deficient in two CWAPs gave insight into the CDSN-binding ability of a single factor in isolation on the *S. aureus* surface. ClfB and FnBPB supported adherence to CDSN independently. In the absence of both ClfB and FnBPB, AD08 did not adhere to CDSN under these conditions, which implies that FnBPA is not capable of independently supporting the interaction. Taken together this data indicates that ClfB, and predominantly FnBPB, mediate *S. aureus* adherence to CDSN. The role of FnBPA in binding to CDSN *in vivo*, where environmental conditions are different to those used *in vitro*, remains to be seen. The same allelic variant of the *fnbB* gene is carried by all

AD_{CC1} stains tested in this study and while it has not been tested here, it is possible that FnBPB isotypes have differing affinities for CDSN with CC1-type potentially having a higher affinity for CDSN, thus providing an adherence advantage over other *S. aureus* strains in colonising AD skin.

The transcription of genes encoding CWAPs in *S. aureus* is regulated by different global regulators and in some instances, is specific to growth phase. Studies using lab strains of *S. aureus* have shown that expression of ClfB, FnBPA and FnBPB primarily occurs in exponential phase due to negative transcriptional regulation by Agr, and degradation by protease activity in stationary phase (McAleese *et al.*, 2001, McGavin *et al.*, 1997, Saravia-Otten *et al.*, 1997). More recently, however, it was shown that some clinical isolates of *S. aureus* express FnBPA and FnBPB during stationary phase (Geoghegan *et al.*, 2013), therefore it was reasonable to question whether stationary phase AD08 would maintain ClfB, FnBPA and FnBPB activity. The CWAP ClfA has a similar domain structure to ClfB and shares a common ligand, fibrinogen, with ClfB, FnBPA and FnBPB (McDevitt *et al.*, 1997) and expression of ClfA is highest in stationary phase (Homerova *et al.*, 2004). Under the conditions tested here, stationary phase AD08 lost its ability to adhere to CDSN, indicating that ClfA, and indeed other *S. aureus* factors expressed in stationary phase, are not involved in mediating AD08 adherence to CDSN.

In this chapter, it was shown that the N2 N3 subdomains of FnBPA bound CDSN with an almost 10-fold higher affinity than both FnBPB and ClfB. FnBPA expressed on the bacterial cell surface by AD08 was not capable of independently supporting bacterial adherence to CDSN under the conditions tested however. While *fnbA* and *fnbB* lie adjacent to each other on the chromosome, the genes are independently transcribed (Greene *et al.*, 1995) and therefore it is possible that AD08 expresses an insufficient level of FnBPA to promote adherence to CDSN when grown under the conditions described in this chapter.

ClfB, FnBPA and FnBPB share similar domain structure. The fibronectin-binding repeat region of FnBPA and FnBPB supports binding to fibronectin (Schwarz-Linek *et al.*, 2003) however the SD repeat region of ClfB has not been shown to bind to any ligands. The N2 N3 subdomains of ClfB facilitate

binding to glycine serine rich regions in loricrin, K10 and fibrinogen by the 'dock, lock and latch' (DLL) mechanism while the N2 N3 subdomains of FnBPA and FnBPB bind to fibrinogen and elastin by the same DLL mechanism (Ni Eidhin *et al.*, 1998, Keane *et al.*, 2007, Burke *et al.*, 2011). CDSN contains glycine serine rich regions, similar to ClfB ligands loricrin, K10 and fibrinogen and it is likely that binding to CDSN occurs by the DLL mechanism.

Studying the interaction between CDSN and *S. aureus in vitro* is hindered however as a suitable growth medium to mimic conditions on human skin is lacking. Media such as TSB, used specifically for microbiological culture, is specialised to promote bacterial growth and contains an abundance of available nutrients. AD skin has an elevated pH that is closer to neutral pH (Eberlein-Konig *et al.*, 2000) similar to that in TSB. This is partly due to the absence of filaggrin breakdown products UCA and PCA. Nutrient availability on AD skin however is likely to be less plentiful than in TSB media. *S. aureus* has adapted mechanisms that allow the bacteria to survive in nutrient starved conditions. For example, in iron limited conditions, Isd proteins are expressed and sequester haem from haemoglobin in blood for bacterial survival (Grigg *et al.*, 2010). Grown in nutrient limited and iron deficient media, AD08 maintained its ability to adhere to CDSN in nutrient starved conditions which alludes to the potential capability of AD08 to adhere to CDSN *in vivo*. This was dependent on the presence of ClfB and/or FnBPA and/or FnBPB. The contribution of each individual protein in promoting AD08 adherence to CDSN in these conditions is unknown.

The importance of ClfB, FnBPA and FnBPB in colonisation of AD skin was also examined *ex vivo*, where native corneocytes were isolated from low NMF AD patients and adherence of a single bacterial cell to these corneocytes was measured by atomic force microscopy (AFM). Using this technique provides a more sensitive analysis of bacterial adherence to host cells and proteins. In the absence of ClfB, FnBPA and FnBPB, AD08 was unable adhere to AD corneocytes in any growth phase tested. Furthermore, during exponential phase growth, ClfB played a dominant role in adherence while the adherence of bacteria in stationary phase growth was dependent on FnBPA and/or FnBPB. This is significant as bacteria are present in different growth phases during

colonisation, therefore expressing adhesins capable of binding to skin ligands during all phases of bacterial growth would be beneficial for persistent colonisation. These data also illustrate that AD08 maintains FnBPA and/or FnBPB activity during stationary phase, as detected by the sensitive AFM technology. The contribution of FnBPA and FnBPB independently mediating adherence to AD corneocytes was not probed here. *In vitro* bacterial adherence assays described in this chapter suggest that there is low level FnBPA activity in AD08 and therefore it is reasonable to hypothesise that FnBPB is dominant in promoting attachment of stationary phase AD08 to AD corneocytes.

Binding sites for ClfB have been localised to glycine/serine rich regions, predicted for form Ω loops (Mulcahy *et al.*, 2012, Walsh *et al.*, 2004). In this chapter, the *S. aureus* binding site in CDSN was mapped to the N-terminal Ω loop region of CDSN. Binding to glycine/serine rich regions has been reported exclusively to ClfB however this chapter depicts the first report of FnBPA and FnBPB binding to glycine/serine rich Ω loop regions. The natural role of CDSN in the skin is to mediate cell to cell adherence by homophilic interactions facilitated by the glycine/serine rich N-terminal region of the protein. In healthy skin during desquamation, this region of CDSN is cleaved by proteolytic enzymes (Jonca *et al.*, 2002). AD skin however has atypical CDSN degradation, where intact CDSN is found more frequently in the stratum corneum of AD patients compared to healthy controls (Igawa *et al.*, 2017). This suggests that the *S. aureus* binding site in CDSN is potentially more abundant in AD skin compared to healthy skin. Detection of the N-terminal domain by anti-CDSN IgG specific for the N-terminus confirmed that this is the case on low NMF AD skin corneocytes. Importantly, *S. aureus* adherence to AD corneocytes was reduced when the bacterial binding site in CDSN was blocked with N-terminal specific anti-CDSN IgG.

In summary, this chapter identified the skin protein CDSN as a novel ligand for *S. aureus* where CWAPs ClfB and FnBPB mediated the interaction. Binding to CDSN by recombinant ClfB, FnBPA and FnBPB was facilitated by the N2 N3 subdomains and recombinant FnBPA bound CDSN with the highest affinity. Furthermore, ClfB, FnBPA and/or FnBPB were required for adherence to low NMF AD corneocytes. The majority of data presented in this chapter was

generated using only one strain of *S. aureus* however. A comprehensive analysis of the interaction between *S. aureus* and CDSN would include additional *S. aureus* isolates, tested across a range of conditions that closely mimic the *in vivo* scenario. The *S. aureus* binding site in CDSN was localised to the N-terminal Ω loop region of CDSN and this chapter provides evidence that *S. aureus* binding to this N-terminal Ω loop region of CDSN is necessary for the ability of *S. aureus* to adhere to AD corneocytes. Combined, this suggests that *S. aureus* targets CDSN to bind to AD corneocytes and alludes to the hypothesis that CDSN is a critical ligand for *S. aureus* colonisation of AD skin.

Chapter 4

Molecular analysis of the interaction between staphylococci and corneocyte proteins

4.1 Introduction

S. aureus is a commensal organism that typically colonises the moist squamous epithelium of the anterior nares (Cole *et al.*, 2001) where approximately 20% of the healthy adult population are persistently colonised and the remaining 80% are intermittent carriers (van Belkum *et al.*, 2009). *S. aureus* is also an opportunistic pathogen and carriage of *S. aureus* is a risk factor for infection (von Eiff *et al.*, 2001, Wertheim *et al.*, 2005). To establish colonisation a bacterium must first attach to a host cell or tissue, which is achieved by interactions between specific host ligands and adhesive factors expressed by the bacteria.

The upper layer of skin, the stratum corneum, consists of dead cells known as corneocytes. During formation of the stratum corneum, the cells are forced into a flattened shape by a complex of cytokeratin proteins embedded in a filaggrin matrix. The corneocytes are further supported by structural proteins of the cornified envelope including loricrin, involucrin and small proline-rich proteins, embedded in an insoluble lipid matrix (Candi *et al.*, 2005). Thus the skin acts as a reservoir of potential ligands for bacterial adherence. The majority of cornified envelope proteins are encoded for by a cluster of genes at human chromosome band 1q21, known as the epidermal differentiation complex (EDC) (Volz *et al.*, 1993, Mischke *et al.*, 1996). Several proteins encoded by genes of the EDC have undefined functions, and could potentially support bacterial adherence to the skin.

Known skin ligands for *S. aureus* present in human skin include the proteins loricrin and cytokeratin 10 (K10). *S. aureus* adherence to both loricrin and K10 is mediated by the cell wall-anchored protein (CWAP) ClfB, where ClfB binds to glycine/serine omega (Ω) loop regions present in both proteins (Mulcahy *et al.*, 2012, Walsh *et al.*, 2004). Adherence to loricrin in particular is vital for *S. aureus* colonisation of the anterior nares, as *S. aureus* colonisation was significantly reduced in loricrin knockout mice. Furthermore, a mutant of *S. aureus* deficient in ClfB had reduced ability to colonise wild-type mice but not to loricrin knockout mice, indicating that the interaction between loricrin and ClfB is important for nasal colonisation (Mulcahy *et al.*, 2012). Other *S. aureus* surface

factors, including IsdA, SdrC and SasG, have been shown to promote adherence to desquamated epithelial cells however the target ligand remains unidentified. SdrD has also been shown to contribute to *S. aureus* adherence to desquamated epithelial cells (Corrigan *et al.*, 2009) presumably by binding to the desmosome protein desmoglein-1 (Askarian *et al.*, 2016).

S. aureus does not typically colonise the skin of the healthy population however AD skin is frequently colonised by *S. aureus* (Totte *et al.*, 2016). Due to the skin barrier defect and reduced structural integrity of the stratum corneum associated with AD skin, it is conceivable to assume that proteins previously shielded by the tight formation of the skins layers, may be more accessible to bacteria in AD skin. K10 is located below the cornified layer and loricrin is present in the cornified envelope surrounded by a lipid envelope in healthy skin and as such, may not be wholly accessible to *S. aureus*. However, in AD skin there is an abnormal lipid profile, where the total level of ceramides is reduced (Janssens *et al.*, 2012). It is possible that the atypical protein conformation and reduced barrier integrity in AD skin may provide an environment of more accessible protein ligands for bacterial adherence.

The association of staphylococci and atopic inflammation in skin is not specific to humans. Canine pyoderma is an umbrella term for atopic inflammatory skin diseases in canines that includes atopic dermatitis (Richards *et al.*, 2018). While there is some evidence to suggest that a skin barrier defect is involved in pathogenesis of canine AD (Marsella *et al.*, 2011) a thorough analysis of the stratum corneum is lacking. Dogs with canine pyoderma frequently have increased colonisation by staphylococci (Mason, 1989) in particular the coagulase-positive *S. pseudintermedius*. This is a normal commensal organism in canines however increased colonisation by *S. pseudintermedius* is associated with canine atopic dermatitis. Indeed adherence of *S. pseudintermedius* to corneocytes isolated from dogs with atopic dermatitis is higher than to corneocytes from healthy controls (Simou *et al.*, 2005, McEwan, 2000).

The exact mechanism of how *S. pseudintermedius* colonises canine AD skin is poorly understood as the target ligand in canine skin has not been identified. Similar to *S. aureus*, adherence to host cells and proteins is

facilitated by CWAPs. SpsO and SpsD have been shown to contribute to adherence to canine corneocytes *ex vivo* (Bannoehr *et al.*, 2011) while SpsL and SpsD mediate *S. pseudintermedius* adherence to canine fibrinogen, fibronectin and for SdsD, K10. SpsD and SpsL have a similar domain structure to *S. aureus* MSCRAMM proteins ClfB, FnBPA and FnBPB with an N-terminal A domain followed by a repeat region and a C-terminal LPXTG motif for anchoring to the cell wall (Geoghegan *et al.*, 2009, Bannoehr *et al.*, 2011). Whether K10 is the target ligand for *S. pseudintermedius* in canine AD skin is unknown and it is possible that another unidentified ligand exists.

The aim of this chapter was to investigate adherence of *S. aureus* to skin proteins of the cornified envelope including LCE proteins, loricrin and K10. The role of FnBPA and FnBPB in mediating *S. aureus* adherence was examined using isogenic mutants of *S. aureus* deficient in ClfB and/or FnBPA and/or FnBPB and measuring adherence to immobilised human loricrin and K10. Finally, a novel interaction between *S. pseudintermedius* and human CDSN was identified using isogenic mutants of *S. pseudintermedius* deficient in CWAPs SpsD and SpsL.

4.2 Results

4.2.1 Late cornified envelope proteins are not ligands for *S. aureus*

Within the EDC locus there are subgroups of related genes including a group of eighteen genes expressing late cornified envelope (LCE) proteins. LCE proteins are some of the last to be expressed during cornification and are incorporated into cornified envelope very late in the process (Marshall *et al.*, 2001, Jackson *et al.*, 2005). It has been reported that loss of LCE3B and LCE3C is a risk factor for an inflammatory skin disease, psoriasis, (de Cid *et al.*, 2009, Bergboer *et al.*, 2011) however there is very little known about the function of these eighteen proteins. To investigate whether any of the LCE proteins are ligands for *S. aureus*, the adherence of *S. aureus* AD08 to eighteen recombinant GST-tagged LCE proteins (kindly supplied by P. Zeeuwen) was measured by solid phase bacterial adherence assays. A recombinant peptide of a loop region of loricrin linked to GST (GST-L2v) was included as a positive control as it is an established ligand for *S. aureus* (Mulcahy *et al.*, 2012) and recombinant GST was used as a negative control. Bacteria were grown to exponential phase ($OD_{600} = 0.35$) in TSB (Figure 4.1A) and to stationary phase in RPMI (Figure 4.1B) and incubated with a single concentration of recombinant GST-tagged protein. The data obtained were standardised relative to AD08 adherence to GST-L2v. AD08 grown in both TSB and under iron limited conditions in RPMI adhered to GST-L2v. Adherence of AD08 to the LCE proteins was at a similar level as adherence to the negative recombinant GST control, which indicates that *S. aureus* AD08 does not adhere to these eighteen LCE proteins when grown under either of the conditions tested.

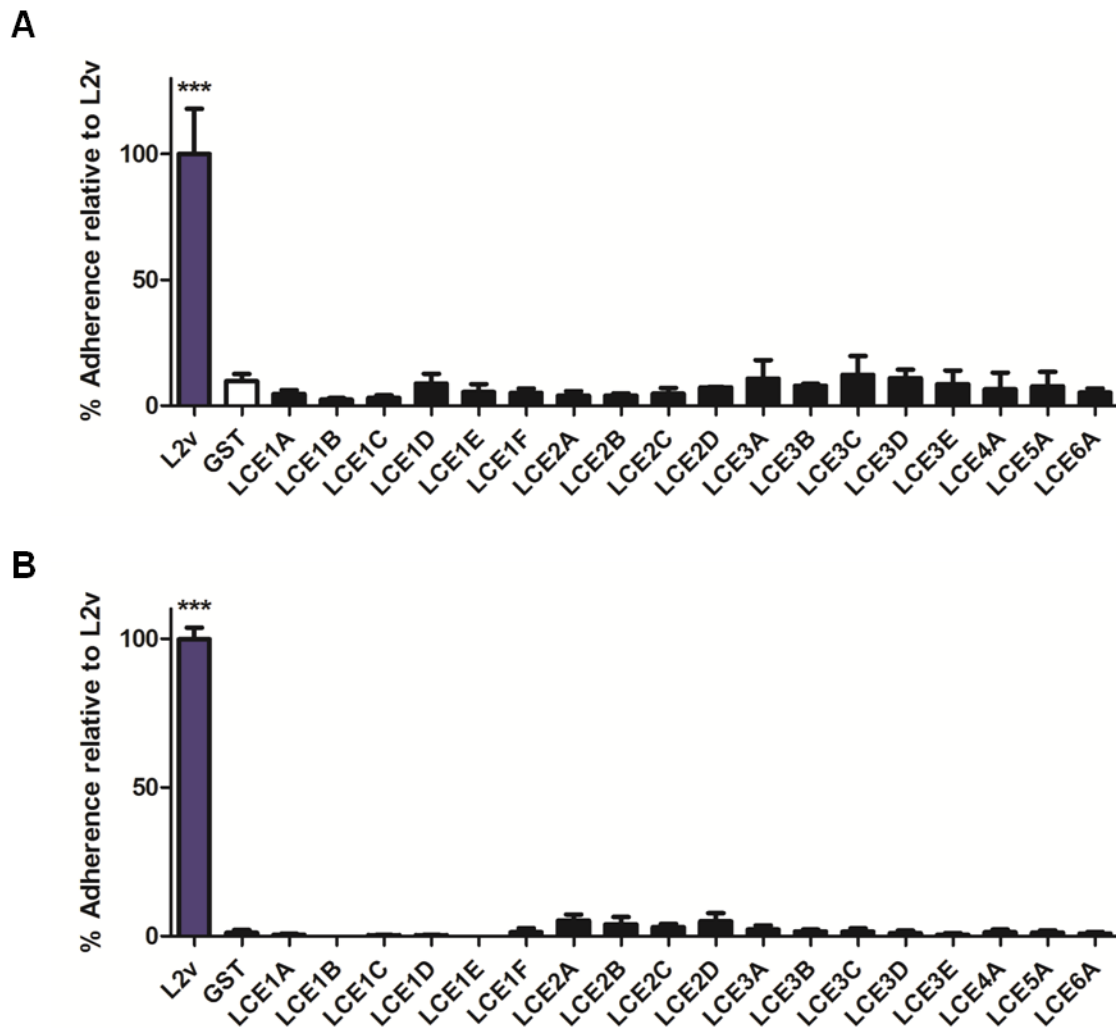


Figure 4.1. *S. aureus* AD08 does not adhere to recombinant LCE proteins. AD08 was grown to **A)** exponential phase ($OD_{600} = 0.35$) in TSB and **B)** stationary phase in RPMI and added to wells of a microtitre plate coated with 5 $\mu\text{g}/\text{ml}$ of recombinant protein. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A one-way ANOVA with Dunnetts posttest was carried out to compare differences between adherence to recombinant proteins and recombinant GST, $p > 0.05$ for LCE proteins, $p < 0.001$ for L2v.

4.2.2 Adherence of *S. aureus* to recombinant GST-tagged L2v

Loricrin constitutes approximately 75% of the total protein mass of the cornified envelope (Hohl *et al.*, 1991). The interaction between *S. aureus* and loricrin is well characterised and is necessary for colonisation of the anterior nares. Loricrin contains 3-4 glycine/serine rich omega (Ω) loop regions which *S. aureus* adheres to, mediated by the CWAP ClfB. ClfB binds with the highest affinity to loop region 2 (L2v) (Hohl *et al.*, 1991, Mulcahy *et al.*, 2012). Given the importance of loricrin as a ligand for *S. aureus* for nasal colonisation in the healthy population, and the importance of ClfB for binding of *S. aureus* to AD skin, it is possible that ClfB recognises loricrin as a ligand in AD skin. In strain Newman, adherence to loricrin is entirely dependent on ClfB. However Newman is a lab strain that does not express functional fibronectin binding proteins FnBPA and FnBPB (Grundmeier *et al.*, 2004). Therefore, the interaction between clinically relevant isolates of *S. aureus* and loricrin warrants further investigation.

Plasmid pGEX-4T2::L2v (Mulcahy *et al.*, 2012) was used for the expression and purification of recombinant GST-tagged L2v from *E. coli* using glutathione affinity chromatography. SDS-PAGE analysis of the purified GST-L2v revealed that the protein was pure (Figure 4.2A). *S. aureus* AD isolate AD08, CA-MRSA strain of the USA300 lineage LAC and lab strain Newman were examined for their ability to bind to GST-L2v. Solid phase bacterial adherence assays were used to measure *S. aureus* adherence to immobilised GST-L2v. It has been shown previously for strain Newman that adherence is ClfB dependent (Mulcahy *et al.*, 2012), therefore isogenic mutants deficient in ClfB were included. Newman adhered to GST-L2v in a ClfB dependent manner (Figure 4.2B). While there was a significant reduction in adherence of AD08 and LAC to GST-L2v in the absence of ClfB ($p < 0.001$) there was still a detectable level of adherence (Figure 4.2C, D), indicating that other factors expressed by AD08 and by LAC support the interaction.

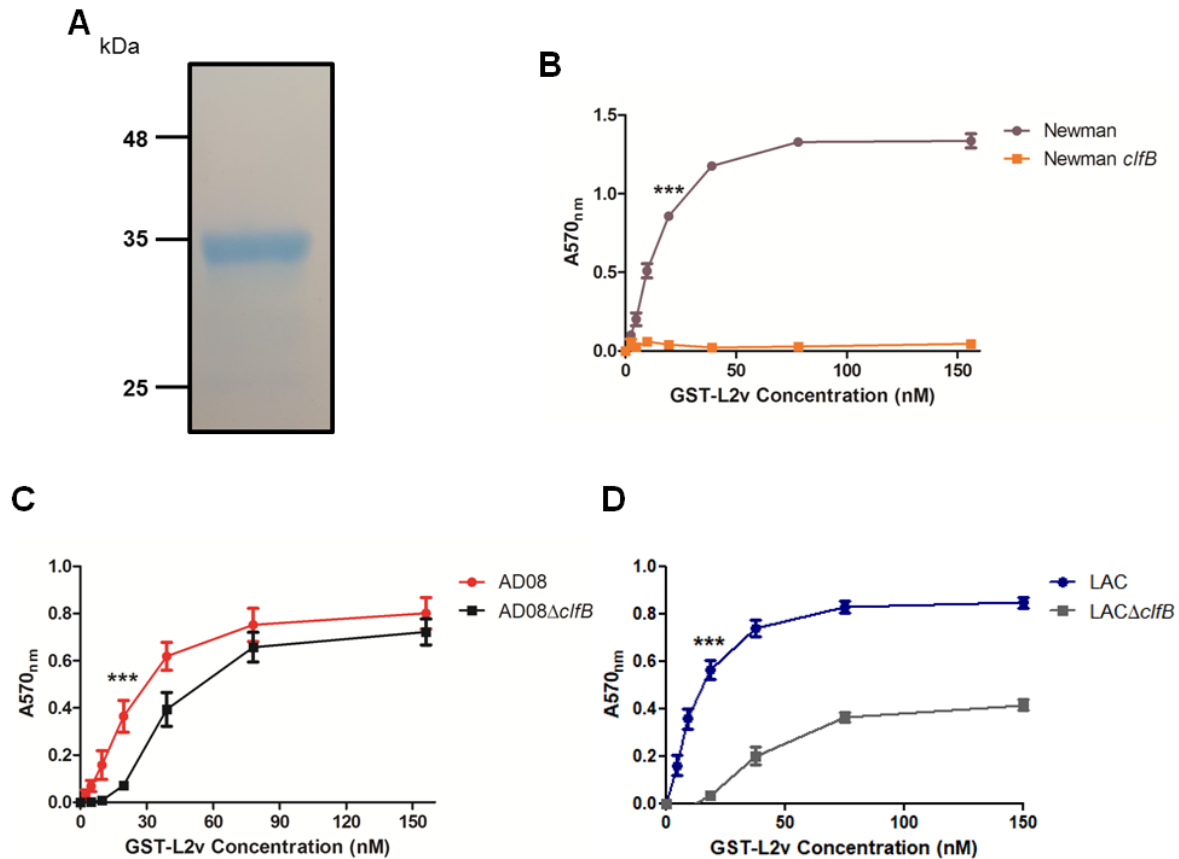


Figure 4.2. *S. aureus* adherence to loricrin is not solely mediated by ClfB.

A) Following purification by glutathione affinity chromatography, purified GST-L2v protein was separated on a 10% SDS-PAGE gel and stained using InstantBlue protein stain. **B-D)** *S. aureus* strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of GST-L2v. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with a Bonferroni posttest was performed to compare variances between WT and ClfB deficient mutants. Significance is represented at the 19.50 nM GST-L2v concentration.

4.2.3 FnBPA and FnBPB mediate adherence to loricrin

Having demonstrated that FnBPB and potentially FnBPA bind to a glycine/serine rich Ω loop region in the skin protein CDSN (section 3.2.13), we hypothesised that they may support binding to Ω loop regions in loricrin. To investigate this, a derivative of *S. aureus* SH1000 deficient in ClfA, ClfB, FnBPA and FnBPB (hereafter known as SH4X) was used as a surrogate host. Plasmids expressing ClfB, FnBPA and FnBPB (pCU1::*clfB*, pFnBPA4, pFnBPB4 respectively) as well as empty plasmid (pCU1) were introduced into SH4X to examine the ability of each protein to mediate bacterial adherence to loricrin independently. The adherence of SH4X (pCU1), SH4X (pCU1::*clfB*), SH4X (pFnBPA4) and SH4X (pFnBPB4) to immobilized recombinant GST-L2v was measured by solid phase bacterial adherence assays. In agreement with previous findings, SH4X (pCU1::*clfB*) adhered to GST-L2v while SH4X (pCU1) did not (Figure 4.3). SH4X (pFnBPA4) and SH4X (pFnBPB4) adhered to GST-L2v at a significantly higher level than SH4X (pCU1) ($p < 0.001$) indicating that both FnBPA and FnBPB are capable of independently facilitating *S. aureus* adherence to GST-L2v.

Having shown that FnBPA and FnBPB could support adhesion to GST-L2v when overexpressed in a lab strain, it was desirable to use a clinically relevant AD strain to investigate the role of FnBPA and FnBPB in mediating adherence to loricrin. Wild-type AD08 adhered to GST-L2v in a dose dependent manner while adherence of AD08 Δ *clfB* Δ *fnbA* Δ *fnbB* was abolished (Figure 4.4). There was no reduction in adherence of AD08 Δ *fnbA* and AD08 Δ *fnbB* but a significant reduction of AD08 Δ *clfB* ($p < 0.001$) indicating that ClfB is the dominant protein mediating AD08 adherence to GST-L2v. Adherence of AD08 Δ *clfB* was significantly higher than that of AD08 Δ *clfB* Δ *fnbA* Δ *fnbB* ($p < 0.001$) suggesting that FnBPA and/or FnBPB are capable of mediating the interaction. Furthermore, AD08 Δ *fnbA* Δ *fnbB* had a significant reduction in adherence compared to wild-type AD08 ($p < 0.001$). Similarly, adherence of AD08 Δ *clfB* Δ *fnbA* was significantly reduced compared to AD08 Δ *clfB* ($p < 0.01$) indicating that FnBPA is involved in mediating the interaction, however the level of adherence was significantly higher than for AD08 Δ *clfB* Δ *fnbA* Δ *fnbB* ($p < 0.01$) showing that FnBPB is also capable of mediating adherence to L2v

independently. Adherence of *AD08ΔclfBΔfnbB* to GST-L2v is almost abolished as there is no difference in adherence compared to *AD08ΔclfBΔfnbAΔfnbB*. This suggests that FnBPA is unable to maintain the interaction independently under the conditions tested here. Overall the data presented here implies that ClfB, FnBPA and FnBPB promote AD08 adherence to GST-L2v and that ClfB and FnBPB are the dominant proteins involved.

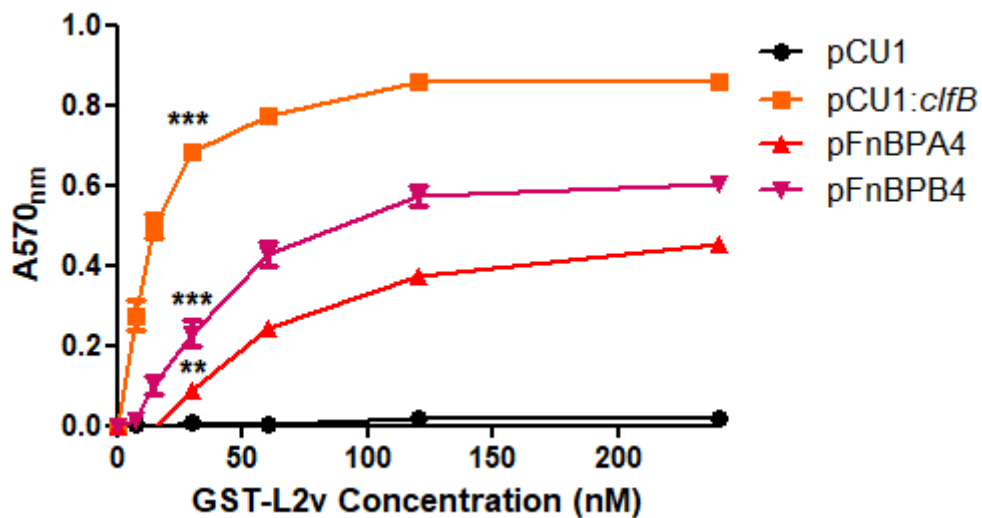


Figure 4.3. ClfB, FnBPA and FnBPB are capable of mediating *S. aureus* adherence to loricrin. *S. aureus* SH4X carrying pCU1, pCU1::clfB, pFnBPA4 and pFnBPB4 were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of GST-L2v. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with a Bonferroni posttest was performed to compare variances between SH4X (pCU1) and the strains carrying plasmids expressing ClfB, FnBPA and FnBPB. Significance is represented at the 30 nM GST-L2v concentration.

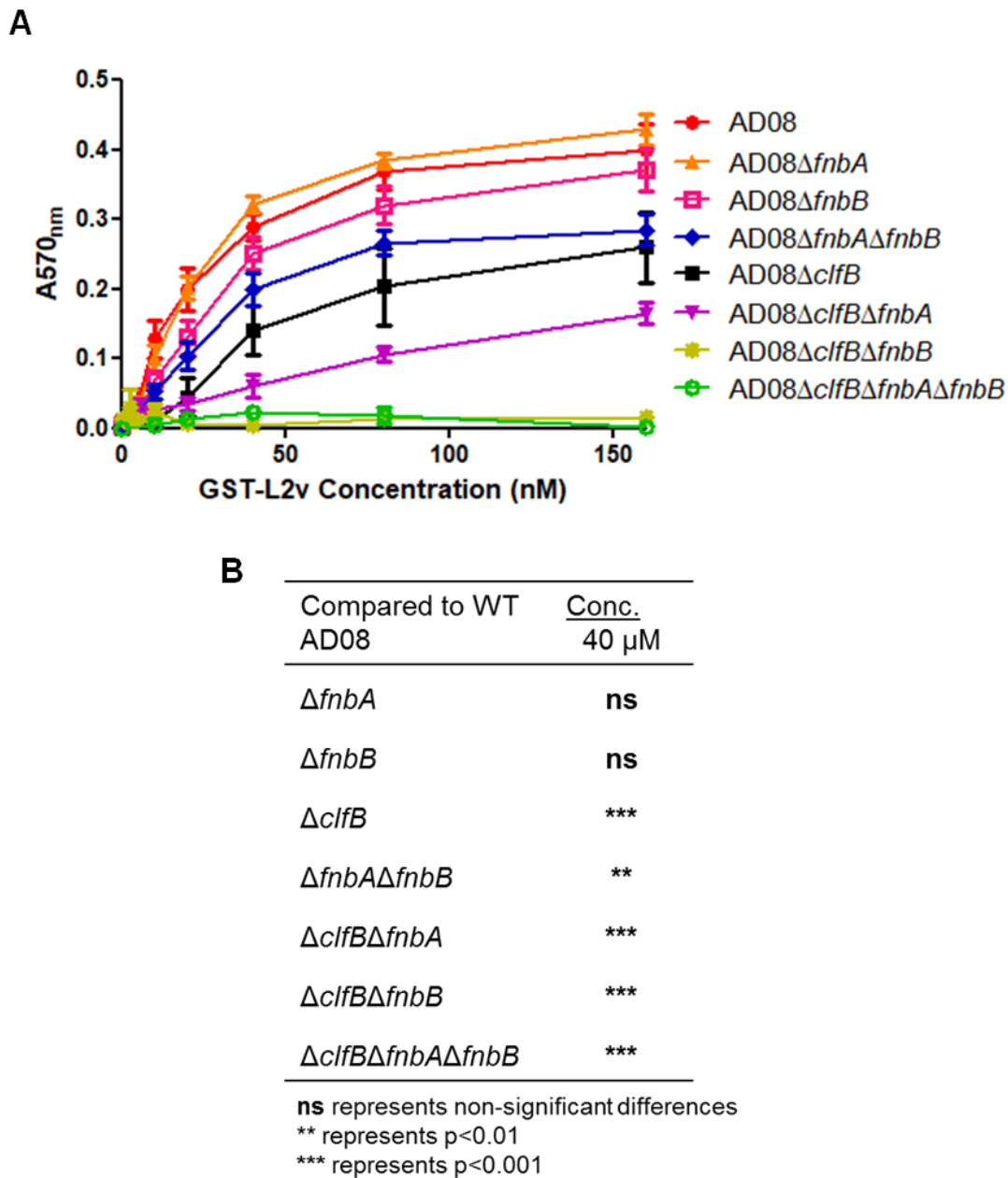


Figure 4.4. ClfB, FnBPA and FnBPB mediate AD08 adherence to loricrin.

A) *S. aureus* strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of GST-L2v. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. **B)** A two-way ANOVA with a Bonferroni posttest was performed to compare variances between WT and mutants.

4.2.4 Adherence of *S. aureus* to recombinant GST-HK10

The skin protein cytokeratin 10 (K10) is present below the cornified envelope and has been identified as a ligand for *S. aureus in vitro* (O'Brien *et al.*, 2002). Due to the barrier defect in AD skin, it is possible that K10 is more accessible to *S. aureus* in the skin of AD patients. Similar to loricrin, adherence to K10 is reported to be mediated by ClfB where it binds to a glycine/serine rich Ω loop region (Walsh *et al.*, 2004). Previous data describing the interaction between *S. aureus* and K10 used lab strains of *S. aureus* (Corrigan *et al.*, 2009, O'Brien *et al.*, 2002) and therefore binding to K10 by clinical AD isolates may depend on different bacterial factors. Plasmid pGEX-4T2::HK10 (Mulcahy *et al.*, 2012) was used for the expression and purification of recombinant GST-tagged human cytokeratin 10 peptide (hereafter known as HK10) from *E. coli* using glutathione affinity chromatography. SDS-PAGE analysis of the purified GST-HK10 revealed that the protein was pure (Figure 4.5A).

ClfB has been reported as the dominant CWAP mediating *S. aureus* adherence to K10 (Walsh *et al.*, 2004, Mulcahy *et al.*, 2012) using lab strains. However the possibility that there are other adhesins that recognise K10 has not been explored. The ability of FnBPA and/or FnBPB to mediate AD08 adherence to glycine/serine rich sequences in both CDSN and loricrin was described in section 3.2.7 and 4.2.3 respectively. The ClfB binding site in K10 has been mapped to an Ω loop region and therefore it is possible that FnBPA and FnBPB are also involved in promoting *S. aureus* adhesion to K10. To test this, the strains SH4X (pCU1), SH4X (pCU1::clfB), SH4X (pFnBPA4) and SH4X (pFnBPB4) described previously (see section 4.2.3) were used to study the ability of FnBPA and FnBPB to mediate *S. aureus* adherence to K10 independently. Solid phase bacterial adherence assays were performed to measure adherence to immobilised GST-HK10. SH4X (pCU1::clfB) adhered to GST-L2v while SH4X (pCU1) did not (Figure 4.5B). SH4X (pFnBPA4) and SH4X (pFnBPB4) adhered to GST-HK10 significantly stronger than SH4X (pCU1) ($p < 0.001$) indicating that both FnBPA and FnBPB are capable of facilitating adherence to K10 independently.

To investigate the role of FnBPA and FnBPB in a clinically relevant *S. aureus* strain, the adherence of AD08 to GST-HK10 was measured by solid

phase bacterial adherence assays. Similar to data presented for both CDSN (section 3.2.7) and L2v (section 4.2.3), wild-type AD08 adhered to GST-HK10 in a dose dependent manner. Adherence by *AD08ΔclfB* was significantly reduced however there was still a detectable level of adherence which suggests that AD08 expresses additional K10 binding protein(s) while adherence was abolished in the absence of ClfB, FnBPA and FnBPB (Figure 4.6). There was no reduction in adherence of *AD08ΔclfBΔfnbA* and *AD08ΔfnbAΔfnbB* which suggests that FnBPB and ClfB respectively are capable of mediating the interaction independently. Adherence of *AD08ΔclfBΔfnbB* to GST-HK10 is abolished indicating that FnBPA is unable to maintain the interaction in isolation under the conditions tested. These data signify that ClfB and FnBPB are involved in mediating AD08 adherence to GST-HK10. Under the conditions tested, FnBPA did not contribute to adherence.

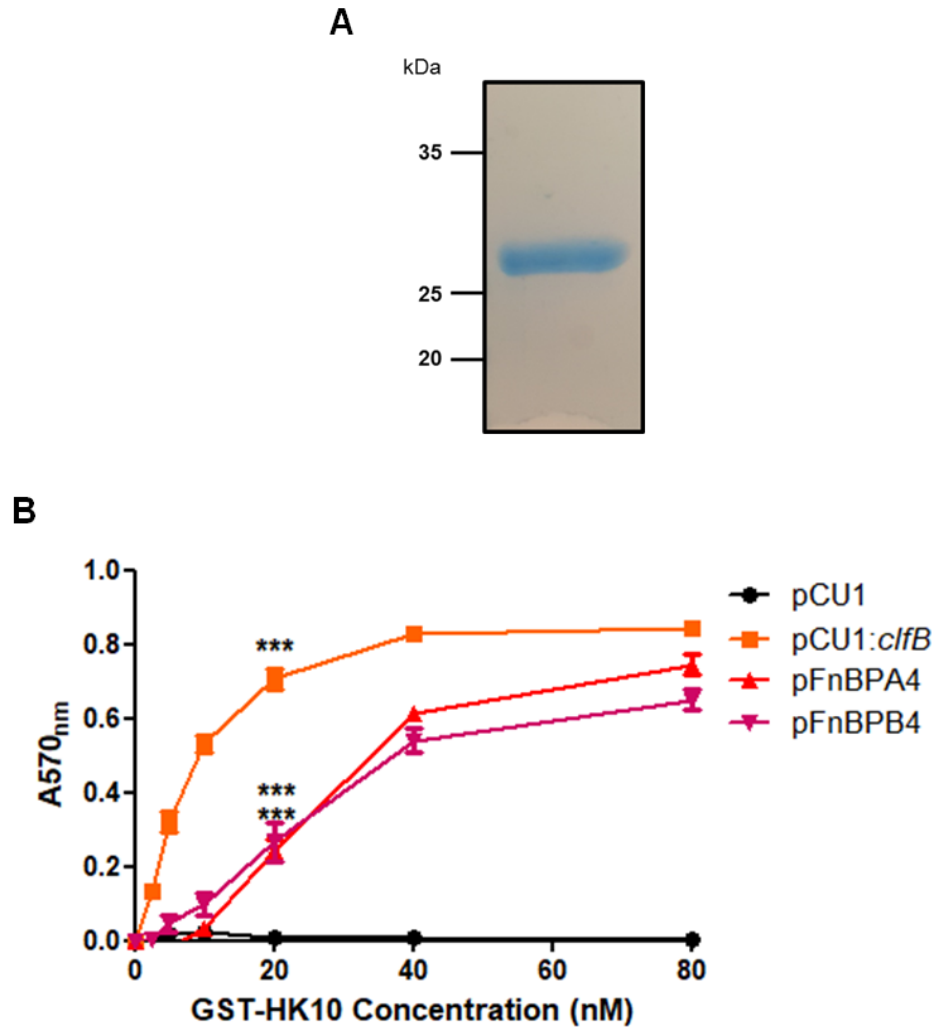
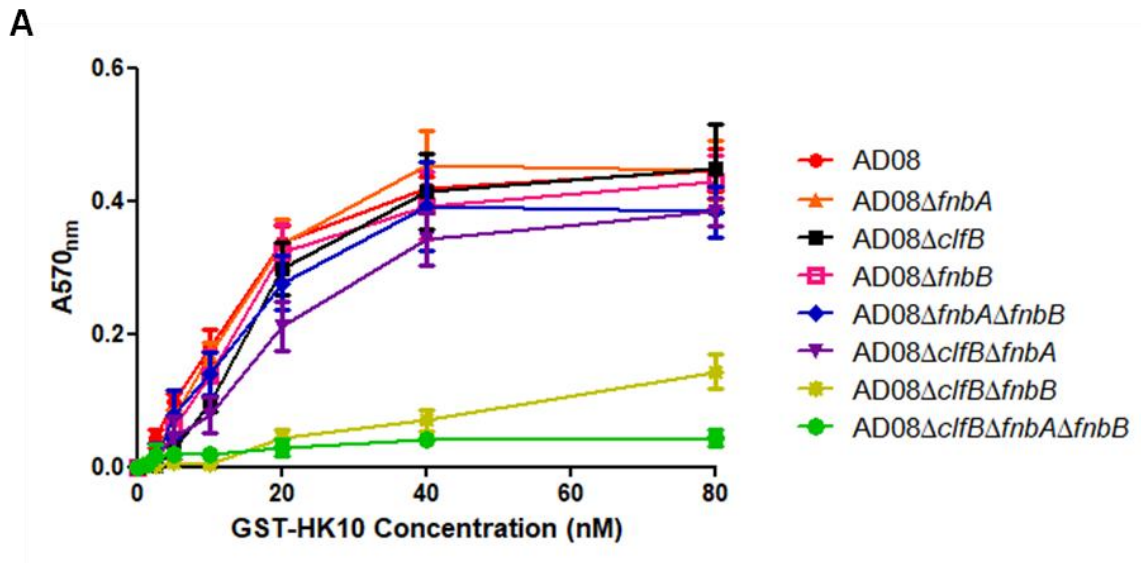


Figure 4.5. ClfB, FnBPA and FnBPB mediate adherence of *S. aureus* to cytokeratin 10. **A)** Following purification by glutathione affinity chromatography, purified GST-HK10 protein was separated on a 12% SDS-PAGE gel and stained using InstantBlue protein stain. **B)** *S. aureus* SH4X carrying pCU1, pCU1::*clfB*, pFnBPA4 and pFnBPB4 were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of GST-HK10. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with a Bonferroni posttest was performed to compare variances between SH4X (pCU1) and the strains carrying plasmids expressing ClfB, FnBPA and FnBPB. Significance is represented at the 20 nM GST-HK10 concentration.



B

Compared to WT AD08	Conc. 20 μ M
Δ <i>fnbA</i>	ns
Δ <i>fnbB</i>	ns
Δ <i>clfB</i>	ns
Δ <i>fnbA</i> Δ <i>fnbB</i>	ns
Δ <i>clfB</i> Δ <i>fnbA</i>	ns
Δ <i>clfB</i> Δ <i>fnbB</i>	***
Δ <i>clfB</i> Δ <i>fnbA</i> Δ <i>fnbB</i>	***

ns represents non-significant differences
 * represents $p < 0.05$
 *** represents $p < 0.001$

Figure 4.6. ClfB and FnBPB mediate AD08 adherence to cytokeratin 10. A) *S. aureus* strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of GST-HK10. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. **B)** A two-way ANOVA with a Bonferroni posttest was performed to compare variances between WT and mutants.

4.2.5 Adherence of *S. aureus* to fibrinogen

ClfB, FnBPA and FnBPB bind to several common ligands including CDSN, Ioricrin, K10 (section 3.2.7, 4.2.3, 4.2.4 respectively) and fibrinogen, where binding is dependent on the N2 N3 subdomains (Ni Eidhin *et al.*, 1998, Keane *et al.*, 2007, Burke *et al.*, 2010). The related protein, ClfA, is the archetypal fibrinogen binding protein and binds to the same site in fibrinogen as FnBPA and FnBPB (McDevitt *et al.*, 1997, Foster, 2016). Thus the potential ability of ClfA to promote *S. aureus* attachment to other ClfB, FnBPA and/or FnBPB ligands was considered. Solid phase bacterial adherence assays were used to measure adherence of AD08 and AD08 Δ clfB Δ fnbA Δ fnbB to immobilised fibrinogen. AD08 adhered to fibrinogen avidly and there was no reduction in the absence of ClfB, FnBPA and FnBPB (Figure 4.7). This suggests that AD08 expresses another fibrinogen binding protein under the growth conditions tested. AD08 Δ clfB Δ fnbA Δ fnbB did not adhere to GST-L2v or GST-HK10 however (Fig 4.4 and 4.6 respectively) under similar growth conditions, which indicates that the other fibrinogen binding protein(s) expressed by AD08, likely ClfA, does not contribute to adherence to GST-L2v or GST-HK10.

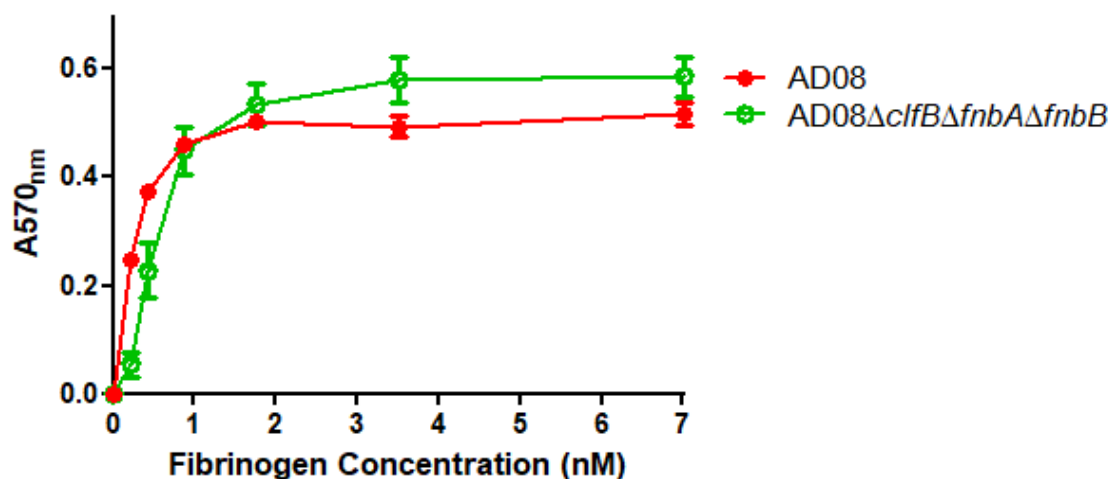


Figure 4.7. *S. aureus* adheres to fibrinogen in the absence of ClfB, FnBPA and FnBPB. *S. aureus* strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of fibrinogen. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with a Bonferroni posttest was performed to compare differences between WT and mutant, $p > 0.05$.

4.2.6 Identification of CDSN as a novel ligand for *S. pseudintermedius*

The coagulase-positive *S. pseudintermedius* is a typical canine commensal organism however increased colonisation by *S. pseudintermedius* has been associated with canine atopic dermatitis (Mason, 1989, McEwan, 2000). Similar to CoNS commensals, there is very little known about how *S. pseudintermedius* colonises skin. The surface of *S. pseudintermedius* harbours a number of proteins which bind to host ligands fibrinogen, fibronectin and K10 (Bannoehr *et al.*, 2011) however the target ligand of *S. pseudintermedius* in canine skin has not been identified. There is little known about the stratum corneum of canines however analysis of the published genome of a domestic boxer dog (Lindblad-Toh *et al.*, 2005) shows the presence of the *CDSN* gene. Purified canine CDSN is not commercially available however, and therefore human rCDSN was used for this analysis.

To investigate whether CDSN is a ligand for *S. pseudintermedius*, adherence of *S. pseudintermedius* ED99 and *S. aureus* AD08 to rCDSN was measured by solid phase bacterial adherence assay. ED99 adhered avidly to rCDSN (Figure 4.8) indicating that human CDSN is a ligand for *S. pseudintermedius*.

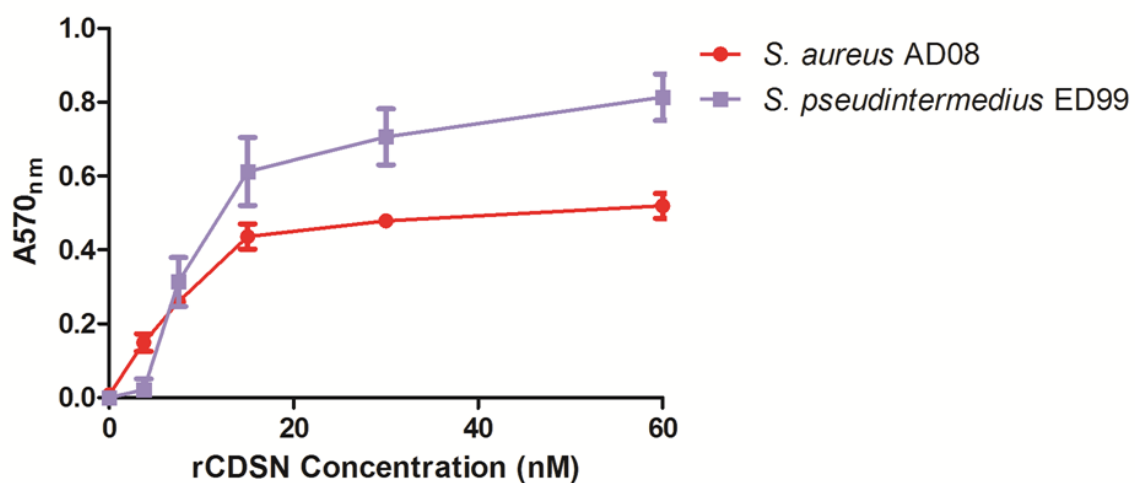


Figure 4.8. *S. pseudintermedius* ED99 adheres to rCDSN. Staphylococcal strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with doubling dilutions of rCDSN. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates.

4.2.7 Localising the *S. pseudintermedius* binding site in CDSN

CDSN contains two glycine/serine rich Ω loop regions, an N-terminal Ω loop region and a C-terminal Ω loop region. The N-terminal Ω loop region comprises the *S. aureus* binding site (see section 3.2.13). *S. pseudintermedius* has been shown to adhere to K10 (Bannoehr *et al.*, 2011) which is also a ligand for *S. aureus* and the *S. aureus* binding site in K10 is present in a glycine/serine-rich loop region. Therefore it was hypothesised that the glycine/serine rich loop regions in CDSN would incorporate the *S. pseudintermedius* binding site. Comparing the amino acid sequence of canine CDSN and human CDSN using Clustal Ω (Figure 4.9) showed that the proteins share 72% sequence identity. Analysis on the alignment shows that canine CDSN also has two glycine/serine rich regions. The C-terminal region (amino-acids 376-476 of human CDSN) is more highly conserved between human and canine CDSN with 78% sequence identity, while the N-terminal region (amino-acids 60-171) shares 68% sequence identity. Recombinant truncates of human CDSN lacking either the N-terminal predicted loop region (Δ NT) or the C-terminal loop region (Δ CT) were generated, expressed and purified (see section 3.2.13). To localise the *S. pseudintermedius* binding site in human CDSN, adherence of ED99 to rCDSN, Δ NT, Δ CT and recombinant GST was measured using solid phase bacterial adherence assays. ED99 adhered to full length rCDSN and Δ CT which suggests that the C-terminal Ω loop region is not important for *S. pseudintermedius* adherence (Figure 4.10). There was a significant reduction in adherence to Δ NT ($p < 0.01$) suggesting that the N-terminal domain incorporates a binding site for *S. pseudintermedius*. Adherence to CDSN in the absence of the N-terminal Ω loop was not completely abolished however and was significantly higher than adherence to recombinant GST ($p < 0.001$). This suggests that there is a second binding site elsewhere in the protein. Currently, the second *S. pseudintermedius* binding site in human CDSN has not been identified.

HumanCDSN	MGSSRAPWMGRVGGHGMALLLAGLLLPGLTAKSIGTFSDPCKDPTRITSPNDPCLTGK	60
CanineCDSN	MGSSQAPQEGHVGGGRGMMALLLAGLLLPGLAKSIATLSDPCKD-TRITSPSDPCLTGKS	59
	****:* * :***:*****:*****.*:***** *****.*****.	
HumanCDSN	DSSGFSSYSGSSSSGSSISS---ARSSGGGSSGSSSGSSIAQGGGAG--SFKPGTGYSQ	114
CanineCDSN	GSSSFSGHSGSSGSSSSSSSGSSGGLSGSGGSSGGSSGSSVAQGGSLGSSSLFKPGTGYSQ	119
	.**.*.*:****.*. ** * .*.*****.*****:***** * *****	
HumanCDSN	VSYSSGSGSSLQGASGSSQLGSSSSSHSGSSGSHSGSSSSSHSSSSSSSFQSSSSSFQVNGS	174
CanineCDSN	ISYSSGSSSSQSGS-----SSAQPGAGS	142
	:*****.*.*.*: ***** *	
HumanCDSN	ALPTNDNSYRGILNPSQPGQSSSSSQ-TFGVSSSGQSVSSNQRPCSSDIPDSPCSGGPIV	233
CanineCDSN	ALPISDDSSRLLISSSQSGGSSLSASPSSWISSSGQQRVNLRPCTSDVPDSPCSGGPIV	202
	.*:* * * :. ** * .** * . ** . * ***::*****	
HumanCDSN	SHSGPYIPSSHVSQGGQRPVVVVVDQHGSGAPGVVQGPFCNGLPGKPCPPITSVDKSY	293
CanineCDSN	SHSGSYISSSHVSQGGQRPVVVVVEQHGSGGPGGVQGAPCSTGGLPGKPCPPITSVDKSY	262
	**** * *****:*****.* * *** * ** *****	
HumanCDSN	GGYEVVGGSSDSYLVPGMTYSKGKIYPVGYFTKENPVKSGPVPSFAAGPPISEGKYFSS	353
CanineCDSN	GSYEVVGGSSDSYLVPGMTYSRGKIYPVGYFTKDNPVKSGPGAPSFAAGPPISEGKYFSS	322
	*.*****:*****:*****.*****	
HumanCDSN	NPIIPSQSA-----ASSAIAFPVGTGGVQLCGGGSTGSKGPCSPSSSRVPSSSSI	404
CanineCDSN	NPIIPSHSSSSSIYQSGASSAIVFPVGS GGVPCCGGGS--KGPCSLSGSGVHSSSV	379
	*****:*: *****.*****:*** ***** ***** *. * * * *:	
HumanCDSN	SSSSGLPYHPCGSASQSPCSPPGTGSFSSSSSQSSGKIILQPCGSKSSSSGHPCMSVSS	464
CanineCDSN	S--SSSFHPCGGVSQGPCSPPGTGTFGGSSSQSSGKIILQPCGSKSSSSGHPCISVSS	437
	* *. :****.*.*****:*. *****:****	
HumanCDSN	LTLTGGPDGSPHPDPSAGAKPCGSSSAGKIPCRSIRDILAQVKPLGPQLADPEVFLPQGE	524
CanineCDSN	-TLSGGPDGSPQPDPSAGAKSCGS--GKLPCRISIRDILAQVKPLGPQLADPEVFLPQGE	493
	** :*****:***** *** **:*****	
HumanCDSN	LLNSP	529
CanineCDSN	SLTSP	498
	*. **	

Figure 4.9. Clustal alignment of Human CDSN and Canine CDSN. * indicates positions which have a single conserved residue, : indicates conservation between residues of strongly similar properties, . Indicates conservation between residues of weakly similar properties. Residue coordinates are indicated. Residues corresponding to human N-terminal and C-terminal Ω loop regions and canine aligned residues are highlighted in yellow.

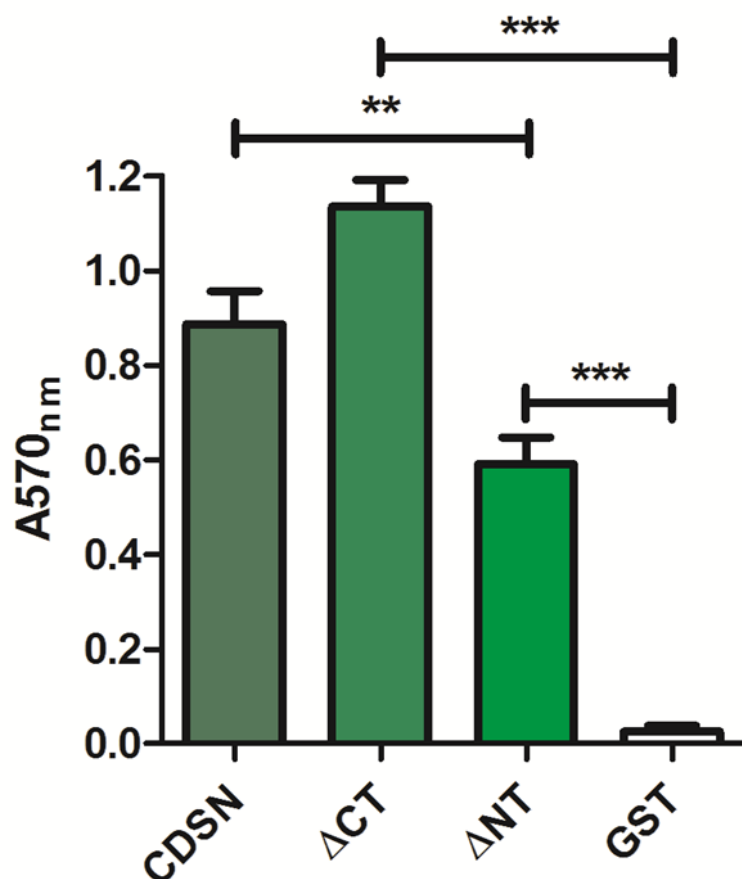


Figure 4.10. *S. pseudintermedius* ED99 adheres to the N-terminal Ω loop of CDSN and to another unidentified region. ED99 was grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with recombinant proteins (20 nM). Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. A one-way ANOVA with Tukeys posttest was carried out to compare differences between variant proteins.

4.2.8 SpsD and SpsL mediate *S. pseudintermedius* adherence to CDSN

S. pseudintermedius CWAPs SpsD and SpsL mediate bacterial adherence to canine fibrinogen, fibronectin and, for SpsD, K10. The N2 N3 subdomains of FnBPA and FnBPB bind to human fibrinogen (Foster, 2016) and CDSN (see section 3.2.11). Using ClustalΩ, the amino acid residues of SpsD and SpsL were aligned with residues of CC1-type FnBPA and FnBPB (data not shown). SpsD shares 37% and 44% identity with FnBPA and FnBPB N2 N3 subdomain residues while SpsL shares 34% and 32% identity with FnBPA and FnBPB N2 N3 subdomain residues. For comparison, a pairwise alignment of the N2 N3 subdomains of CC1-type FnBPA and FnBPB showed that they share 46% amino acid identity. Furthermore the N2 N3 subdomains of FnBPA and FnBPB bind to the same site in fibrinogen as ClfA (Foster, 2016) and pairwise alignments of both proteins with ClfA showed that the N2 N3 subdomains share only 24% and 29% identity respectively (data not shown). Therefore it is possible that SpsD and SpsL share other ligands with FnBPA and FnBPB and it was hypothesised that they could be involved in mediating adherence of *S. pseudintermedius* to human CDSN.

To examine this, the adherence of ED99 and isogenic mutants deficient in SpsD and/or SpsL to Δ CT was measured using solid phase bacterial adherence assays. In agreement with previous data, ED99 adhered to Δ CT in a dose dependent manner (Figure 4.11A). There was no reduction in the absence of SpsD and a significant reduction in the absence of SpsL ($p < 0.001$). A mutant deficient in both SpsD and SpsL did not adhere to Δ CT. These data suggest that both SpsD and SpsL promote adherence of ED99 to CDSN. The genes encoding SpsD and SpsL were re-introduced onto the chromosome of ED99 Δ spsD and ED99 Δ spsL respectively, restoring adherence to Δ CT (Figure 4.11B).

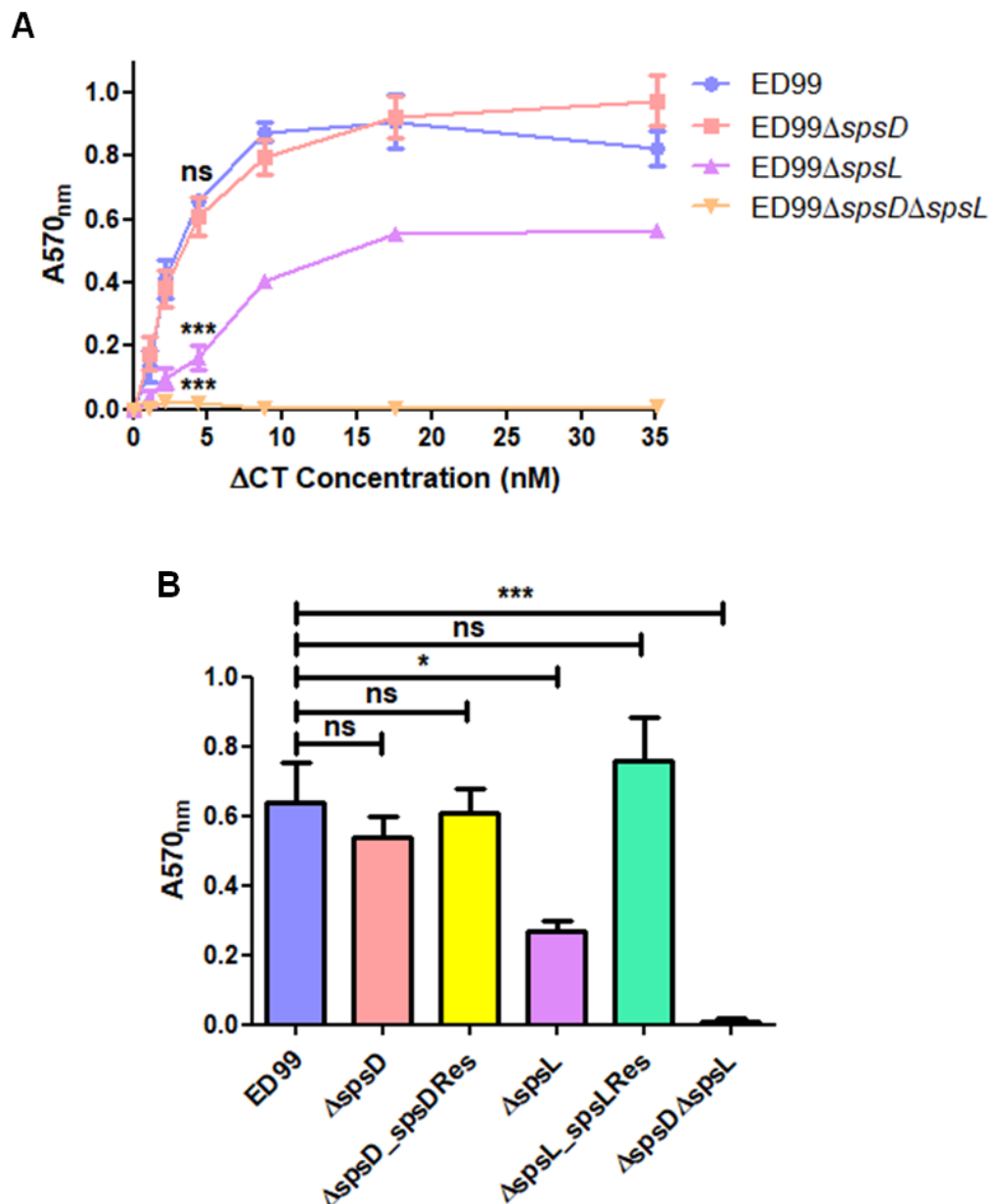


Figure 4.11. SpsD and SpsL mediate ED99 adherence to CDSN. Staphylococcal strains were grown to exponential phase ($OD_{600} = 0.35$) and added to wells of a microtitre plate coated with **A**) doubling dilutions or **B**) a single concentration (5 nM) of Δ CT. Adherent cells were stained using crystal violet and absorbance was measured at 570 nm. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates. **A**) A two-way ANOVA with a Bonferroni posttest was performed to compare variances between WT and mutants. Significance is represented at the 4.375 nM Δ CT concentration. **B**) A one-way ANOVA with Dunnetts posttest was carried out to compare differences between WT and mutants.

4.3 Discussion

The skin is the largest organ of the body and is made up of several layers. The outermost layer, the stratum corneum consists of dead corneocytes that are supported by structural proteins of the cornified envelope (Candi *et al.*, 2005). The skin therefore contains a plethora of proteins that bacteria may adhere to, to colonise this environment. Healthy skin is densely packed together and proteins may not be easily accessible for bacterial attachment. This chapter describes interactions between *staphylococci* and skin proteins of the stratum corneum.

Typically, colonisation of *S. aureus* in the host is multifactorial and depends on interactions between bacterial surface factors and host proteins. Eighteen late cornified envelope (LCE) proteins, expressed late during the cornification process, are localised to the cornified envelope last during formation however there is little else known of the function of these proteins (Jackson *et al.*, 2005). Furthermore, AD skin specifically has a defective skin barrier, with altered distribution and composition of certain stratum corneum proteins (Riethmuller *et al.*, 2015). This alludes to the possibility that some skin proteins may be more abundant or more available for bacterial adherence in AD skin compared to healthy skin. In this chapter, these LCE proteins were eliminated as potential ligands for a clinically relevant *S. aureus* AD strain AD08 under the conditions tested.

S. aureus adheres to two stratum corneum proteins loricrin and K10, both of which have been implicated in *S. aureus* adherence to desquamated epithelial cells and nasal colonisation. Several studies examining transcription of the genes encoding loricrin and K10 (*LOR* and *KRT10* respectively) in the skin of AD patients show that transcription of both genes is downregulated by the effects of Th2 cytokines IL-4 and IL-13 (Kim *et al.*, 2008, Bao *et al.*, 2017, Totsuka *et al.*, 2017). These studies relied on measuring gene expression at the level of transcription however and protein expression was not quantified. Loricrin constitutes roughly 75% of the protein content of the cornified envelope (Hohl *et al.*, 1991) and while a loricrin back-up system has been described in *LOR*^{-/-} mice (Jarnik *et al.*, 2002), a complete loricrin deficient phenotype has not

been reported in humans. K10 is part of the cytoskeleton of the stratum corneum and thus is involved in maintaining structural integrity (Candi *et al.*, 2005). Mutations in the *KRT10* gene causing skin disorders have been identified however a complete K10 deficiency has not been described in humans (Chamcheu *et al.*, 2011). As previously stated, AD skin has reduced structural integrity and it is possible that K10 and loricrin are more exposed in AD skin thus K10 and loricrin should not be dismissed as possible ligands for *S. aureus* in AD skin.

The interactions between *S. aureus* and loricrin and K10 were characterised previously using lab strain Newman, and ClfB was identified as the dominant protein mediating Newman adherence to both loricrin and K10 (Mulcahy *et al.*, 2012, Walsh *et al.*, 2004). This chapter describes the interaction between a clinically relevant *S. aureus* AD isolate, AD08, and loricrin and K10. A mutant of AD08 deficient in ClfB adhered to both loricrin and K10 indicating that AD08 expresses additional loricrin and K10 binding proteins. This first report of the existence of additional *S. aureus* loricrin and K10 binding proteins signifies that clinical isolates can display an altered ligand binding profile compared to lab strains. This strongly stresses the importance of using clinically relevant bacterial strains to completely describe host-ligand interactions.

Studies describing the interaction between *S. aureus* and glycine/serine rich regions have concluded that binding is exclusively facilitated by ClfB, however the previous chapter (Chapter 3) describes the ability of fibronectin binding proteins FnBPA and FnBPB to bind to a glycine/serine Ω loop region present in the skin protein CDSN (see section 3.2.13). The ability of FnBPB and potentially FnBPA to bind to other ClfB ligands was also described in this chapter. Experiments using double mutants of AD08 showed that ClfB and FnBPB are capable of mediating *S. aureus* attachment to loricrin and K10 independently however adherence is lost when ClfB and FnBPB are mutated. This signifies that ClfB and FnBPB are the dominant loricrin and K10 binding proteins expressed by AD08 and that FnBPA is unable to support the interaction independently under the conditions tested.

There is extensive sequence variation in the *clfB*, *fnbA* and *fnbB* genes between *S. aureus* and variants of each CWAP exist. Within a given clonal complex, the sequence of the *clfB* gene is highly conserved and the amino acid sequence of the N2 N3 subdomains of ClfB is identical (Murphy *et al.*, 2011). Similarly, allelic variants of *fnbA* and *fnbB* have been described. The sequences of the *clfB*, *fnbA* and *fnbB* genes carried by CC1 strains, including AD08, are referred to as CC1-type (in the case of FnBPA and FnBPB the CC1 variants are also called ‘isotype III FnBPA and FnBPB’) (Loughman *et al.*, 2008b, Burke *et al.*, 2010). CC30 and CC8 strains each carry their own unique *clfB*, *fnbA* and *fnbB* variant genes. Owing to the fact that the most commonly used strains for research such as Newman, 8325 derivatives and strains of the USA300 lineage are all from CC8, almost all research to date has been limited to the CC8-type CWAPs. The data described in this chapter indicate that CC1-type FnBPB can mediate AD08 adherence to both loricrin and K10, however CC1-type FnBPA was unable to support the interaction independently. Using a derivative of the CC8 CA-MRSA isolate LAC deficient in ClfB, indicated that CC8-type FnBPA and/or FnBPB can facilitate adherence to loricrin. Furthermore, using a plasmid (pFnBPA4) artificially expressing CC8-type FnBPA from *S. aureus* revealed that FnBPA is capable of mediating *S. aureus* adherence to both loricrin and K10 when overexpressed. It has been reported previously that CC1-type ClfB has a higher affinity for loricrin than CC30-type ClfB (Fleury *et al.*, 2017) which suggests that allelic variants of CWAPs may have different affinities for ligands. It is possible then, that CC1-type FnBPA has a lower affinity for loricrin and K10 compared to CC8-type and could not support AD08 adherence. However, the ability of AD08 to express *fnbA* under the conditions used in these experiments was not examined here.

Presumable differences in expression levels of ClfB, FnBPA and FnBPB across *S. aureus* strains should be considered. Expression of ClfB, FnBPA and FnBPB from SH4X (pCU1::*clfB*), (pFnBPA4) and (pFnBPB4) respectively was not measured and it is unknown whether protein levels on the bacterial cell surface were equal across strains. In each case the genes are under the control of their native promoters on multicopy plasmids. Therefore differences in adherence levels mediated by each CWAP reported here may be attributed to

differences in expression. Furthermore, the expression of ClfB, FnBPA and FnBPB by AD08 was not measured and it is conceivable therefore that AD08 expresses an insufficient quantity of FnBPA to facilitate bacterial adherence. Future experiments could compare the ability of CC1- and CC8-type FnBPA to support adhesion to ligands directly using a controlled system.

S. pseudintermedius is a coagulase-positive staphylococcal species associated with canine atopic dermatitis (Mason, 1989) and the target ligand for *S. pseudintermedius* in canine skin is unknown. *S. pseudintermedius* adheres to canine K10 and fibrinogen (Bannoehr *et al.*, 2011) mediated by CWAPs SpsD and SpsL. The corresponding human proteins are established ligands for *S. aureus* CWAPs ClfB, FnBPA and FnBPB (Foster *et al.*, 2014). The previous chapter describes the skin protein CDSN as a novel ligand for ClfB, FnBPA and FnBPB and highlights the potential importance of CDSN as target ligand for *S. aureus* in AD skin. Genomic analysis confirmed that domestic canines carry the *CDSN* gene which has 72% amino acid identity with human *CDSN*. This chapter identified human *CDSN* as a novel ligand for *S. pseudintermedius* with adherence mediated by two CWAPs SpsD and SpsL. Furthermore, the data presented show that *S. pseudintermedius* ED99 adheres to the N-terminal glycine/serine rich region of *CDSN* and to a second unidentified binding site. SpsD and SpsL have a similar domain structure to ClfB, FnBPA and FnBPB with an N-terminal A domain that facilitates binding to the *S. aureus* ligands fibrinogen and K10 (Geoghegan *et al.*, 2009, Bannoehr *et al.*, 2011). As ClfB binding to K10 and ClfB, FnBPA and FnBPB binding to fibrinogen and *CDSN* is facilitated by the N2 N3 subdomains in the A domain (Walsh *et al.*, 2004, Wann *et al.*, 2000)(This study, section 3.2.11) it is plausible to speculate that the A domain of SpsD and SpsL is responsible for binding to human *CDSN*. The N-terminal region is not highly conserved between human and canine *CDSN* however, and therefore it is feasible that the second unidentified binding site in human *CDSN* is conserved in canine *CDSN* and comprises the *S. pseudintermedius* binding site. Moreover, the role of *CDSN* in canine atopic dermatitis has not been elucidated and it is unknown whether the protein is expressed or indeed available for bacterial adherence in canine skin. Further analysis of the interaction between *S. pseudintermedius* and canine *CDSN* is

warranted to fully characterise the interaction and to elucidate CDSN as a possible ligand for *S. pseudintermedius* in canine atopic dermatitis.

In summary, this chapter examined the ability of staphylococci to adhere to corneocyte proteins. It was determined that LCE proteins of the cornified envelope are not ligands for *S. aureus* AD08, suggesting that these proteins do not play a role in *S. aureus* colonisation of AD skin. *S. aureus* adherence to loricrin and K10 was investigated and FnBPA and/or FnBPB binding to these glycine/serine rich proteins was reported. Both loricrin and K10 are established ligands for *S. aureus* ClfB and have been implicated in nasal colonisation. As the mechanisms promoting colonisation are multifactorial, it is conceivable that *S. aureus* adheres to multiple ligands in skin, healthy or AD, to establish persistent colonisation. The skin ligand CDSN was identified as a novel ligand for the canine AD commensal *S. pseudintermedius* mediated by CWAPs SpsD and SpsL. This emphasises the importance of CDSN as a ligand for coagulase positive staphylococci, particularly species associated with atopic dermatitis.

Chapter 5

Investigating the importance of *Staphylococcus aureus* intraspecies competition in colonisation of atopic dermatitis skin

5.1 Introduction

The ability of bacteria to colonise the human host relies on both host and bacterial factors. Bacterial mechanisms include adherence to host cells and tissues, evasion of host defences and competition for nutrient acquisition. *S. aureus* is an integral component of the human microbiome where it persistently colonises the nasal cavity of approximately 20% of healthy adults and the remaining 80% are intermittently colonised (Weidenmaier *et al.*, 2012, van Belkum *et al.*, 2009).

The microbiome of the skin consists of multiple genera of microorganisms including several staphylococcal subspecies, which make up approximately 17% of organisms isolated from the skin (Grice *et al.*, 2009). Staphylococci exhibit factors that contribute to survival on skin including resistance to desiccation, expression of factors to promote adherence to host proteins and nutrient acquisition, utilising urea as a nitrogen source and competing with other microbes (Byrd *et al.*, 2018). Coagulase-negative staphylococci in particular are adept at competing with other organisms in the skin. *S. epidermidis*, *S. lugdunensis* and *S. hominis* express antimicrobial peptides (AMPs) that inhibit growth of other organisms (Cogen *et al.*, 2010, Zipperer *et al.*, 2016, Nakatsuji *et al.*, 2017) and *S. caprae* expresses an autoinducing peptide that inhibits *S. aureus* quorum sensing, reducing *S. aureus* virulence (Paharik *et al.*, 2017).

Atopic dermatitis (AD) skin displays a microbiome dysbiosis where there is a reduction in microbial diversity and an increase in colonisation by Firmicutes (Leyden *et al.*, 1974, Kong *et al.*, 2012). Specifically, *S. aureus* persistently colonises the skin of over 70% of AD patients (Totte *et al.*, 2016). *S. aureus* dominates the microbiota in lesional skin during disease flares and the proliferation of *S. aureus* results in exacerbated AD disease symptoms (Tauber *et al.*, 2016, Kong *et al.*, 2012). Furthermore, CoNS species that typically exhibit anti-*S. aureus* activity in healthy skin are reduced in AD skin as CoNS strains isolated from AD patients rarely have antimicrobial activity (Nakatsuji *et al.*, 2017).

Within *S. aureus*, groups of genetically related strains known as clonal complexes (CC) are uniquely adapted to efficiently colonise different niches. Nasal carriage is associated with *S. aureus* CC30 in healthy individuals however in the skin of AD patients CC30 strains are underrepresented. Conversely, CC1 strains are enriched in AD skin populations (Fleury *et al.*, 2017, Yeung *et al.*, 2011, Harkins *et al.*, 2018, Clausen *et al.*, 2017) and rarely present in typical nasal carriage of the healthy population.

Persistent colonisation in a biotic environment such as AD skin inflicts a competitive pressure on microorganisms as they need to compete for nutrients and space to survive. Bacterial competition can be exploitative or interference based. Exploitative competition relies on fitness, where fitter bacteria have a faster growth rate, are efficient at obtaining and utilising nutrients and are adept at occupying space. Interference competition (hereafter known as direct competition) involves directly damaging cells of other strains which generally depends on the secretion of inhibitory molecules with antimicrobial or quorum-quenching effects (Hibbing *et al.*, 2010, Bauer *et al.*, 2018).

Studying bacterial competition can be facilitated by labelling the bacteria with a selective marker, such as an antibiotic resistance gene, to distinguish between competing strains. Introducing a multicopy plasmid expressing resistance can incur a fitness cost to the bacteria and plasmids are not usually retained stably in bacterial populations in the absence of selective pressure. Integration of the gene conferring antibiotic resistance onto the *S. aureus* chromosome overcomes this problem but the site of insertion of a gene and promoter onto the chromosome deserves special consideration. It is important to avoid disrupting or affecting transcription of known open-reading frames. Furthermore, sequences encoding small regulatory RNAs (sRNAs) that impact transcription or translation are sometimes located at intergenic regions on the *S. aureus* chromosome (Bohn *et al.*, 2010) and could be affected by the insertion of foreign DNA within an intergenic region. An untranscribed intergenic region with no known sRNAs has been identified in *S. aureus* where insertion of a foreign DNA sequence had no effect on transcription of the flanking genes, which suggests that this region may be a suitable site to introduce an antibiotic resistance cassette as a selective marker (Lei *et al.*, 2012).

Bacterial secretion systems play a pivotal role in virulence as they transport and secrete substrates across the bacterial cell envelope. The type VII secretion system (T7SS) in *S. aureus* secretes substrates that alter eukaryotic cells (e.g. EsxA) (Korea *et al.*, 2014) and a toxin (EsaD) that causes DNA damage in bacteria. Recently EsaD has been implicated in direct bacterial competition. Following biosynthesis the nuclease toxin EsaD is neutralised in the cytoplasm of the producer strain by the antitoxin EsaG. Secretion of EsaD is dependent on a putative membrane chaperone EsaE, and EsaG is likely removed from the EsaD/G complex during secretion (Cao *et al.*, 2016). There is sequence variation of *esaD* and *esaG* between strains of different clonal complexes and each *esaD* variant is associated with a different *esaG* variant. Carriage of *esaD* is not conserved across all *S. aureus* strains and genome sequence analysis has determined that approximately 50% of *S. aureus* strains carry *esaD*. Downstream of *esaG* are predicted *esaG* homologues. Each *S. aureus* sequence analysed carries at least two *esaG* homologues presumably to protect from variant EsaD activity of other *S. aureus* strains (Warne *et al.*, 2016). It is therefore possible that the T7SS in *S. aureus* plays a role in intraspecies bacterial competition.

In this chapter, *S. aureus* strains with an antibiotic resistant selective marker were constructed and used to investigate intraspecies competition. It was shown that some *S. aureus* CC1 AD isolates (AD_{CC1}) have an advantage over CC30 nasal control isolates in fitness and/or direct competition *in vitro*. Furthermore, the importance of the *esaDG* genes in AD_{CC1} isolate AD08 for outcompeting at least one CC30 nasal isolate was proposed.

5.2 Results

5.2.1 Construction of pIMAY::Int.S.*aphA3* and pIMAY::Int.S.*ermAM*

To establish whether CC1 strains of *S. aureus* from the infected skin of AD patients have a competitive advantage over CC30 nasal isolates, *in vitro* competitive index assays were carried out. This required labelling at least one competing strain with resistance to an antibiotic such as kanamycin or erythromycin so that strains could be distinguished by antibiotic selection. Functional *aphA3* and *ermAM* genes, encoding resistance to kanamycin (Kan^r) and erythromycin (Ery^r) respectively, have been integrated onto the chromosome of Gram-positive bacteria previously (Monk *et al.*, 2008). Here, the *aphA3* or *ermAM* genes were inserted at a specific untranscribed intergenic region on the *S. aureus* chromosome where DNA insertion was previously shown not to affect the transcription of flanking genes (Lei *et al.*, 2012).

DNA integration at the chromosomal target site involved constructing a DNA sequence with homology to nucleotides up- and down-stream of the integration site (Int.S.). Cloning different constructs into pIMAY for CC1 and for CC30 was necessary as the sequence upstream of Int.S. is not fully conserved between the two clonal complexes. Plasmids pIMAY::Int.S.*aphA3*_{CC1}, pIMAY::Int.S.*aphA3*_{CC30} (Figure 5.1), pIMAY::Int.S.*ermAM*_{CC1} and pIMAY::Int.S.*ermAM*_{CC30} were constructed using AD08 and MRSA252 genomic DNA as template for CC1 and CC30 sequences respectively. The regions 553 bp upstream and 586 or 578 bp downstream of Int.S. in AD08 and MRSA252 respectively were amplified by PCR, where primers A and B annealed upstream and C and D annealed downstream of Int.S. (Figure 5.1B). The sequences encoding the promoter and resistance genes *aphA3* and *ermAM* were amplified from pIMC::kan and pIMC::ery (Monk *et al.*, 2008) using primers Promoter F and Kan R/Erm R respectively (Figure 5.1A). The addition of complementary overhangs allowed the resistance gene product to be joined to the AB and CD products in a single overlap extension PCR reaction using primers Int.S. A and Int.S. D (Figure 5.1C). The resulting product was cloned into pIMAY using sequence and ligase independent cloning as described in section 2.2.4 (Figure 5.1D).

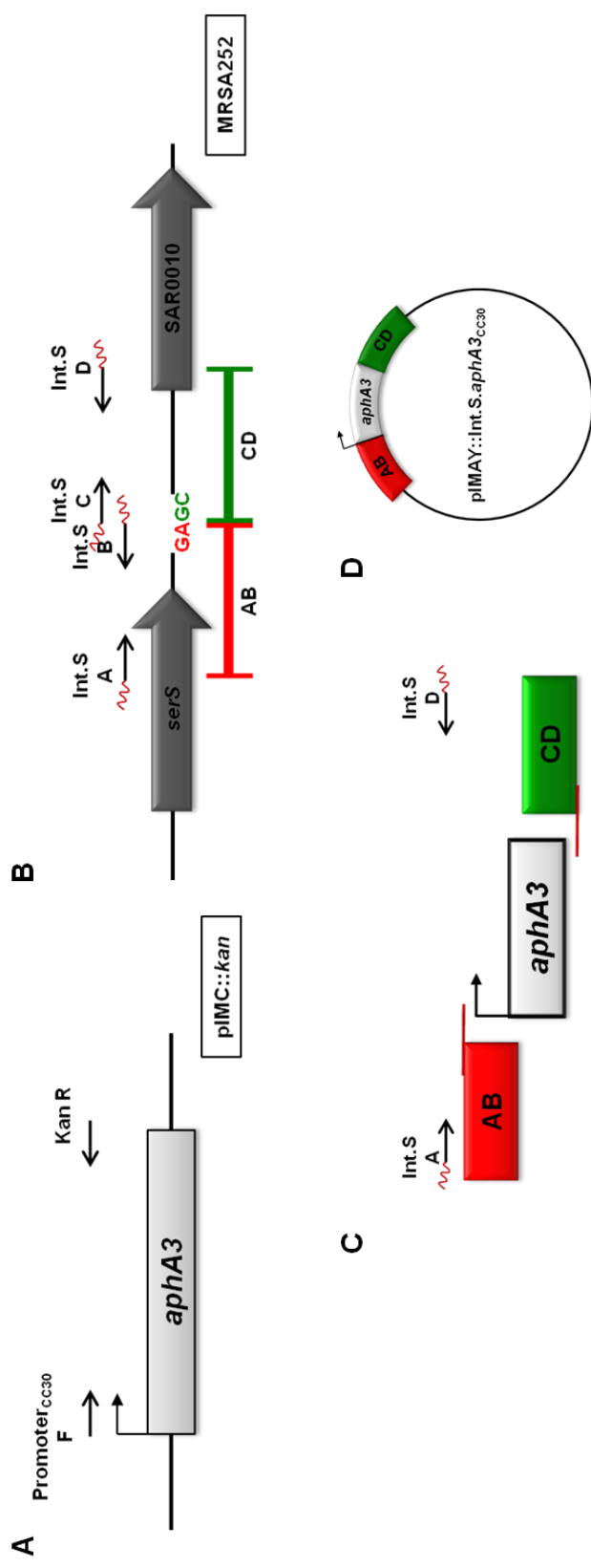


Figure 5.1. Schematic representation of pIMAY::Int.S.aphA3_{CC30} construction. **A)** Primers Promoter_{CC30} F and Kan R were used to amplify *aphA3* along with the 5' Phelp promoter sequence from plasmid pIMC3kan. **B)** MRSA252 genomic DNA was used as template with primers Int.S A, Int.S B, Int.S C and Int.S D to amplify approximately 550 bp up and downstream of the integration site to generate AB and CD products. **C)** The AB, CD products and *aphA3* gene construct were joined by PCR using primers Int.S. A and Int.S D where the complementary sequences (in red) in primers Promoter_{CC30} F and Kan R allowed the *aphA3* construct to anneal to the AB and CD products. **D)** Plasmid pIMAY::Int.S.aphA3_{CC30} was used to insert a kanamycin resistance gene onto the TS1064 and TS1157 chromosome by allelic exchange.

5.2.2 Generation and validation of Kan^r and Ery^r labelled strains

Kan^r and Ery^r tagged strains were generated in the AD_{CC1} isolate AD08, and two CC30 strains isolated from the nasal cavity of healthy children, TS1064 and TS1157, by allelic exchange using the plasmid vectors pIMAY::Int.S.*aphA3*_{CC1}, pIMAY::Int.S.*aphA3*_{CC30}, pIMAY::Int.S.*ermAM*_{CC1} and pIMAY::Int.S.*ermAM*_{CC30} following the same procedure described in section 1.5.1. The introduction of the antibiotic resistance (Ab^r) cassette on the chromosome was confirmed by amplifying across the Int.S. region with Int.S. OUT primers that anneal to regions 605 bp upstream and 635 bp downstream of the Int.S. using tagged strains and wild-type genomic DNA as template (Figure 5.2A). A 1.7 kb and 1.9 kb band representing insertion of *ermAM* and *aphA3* respectively indicated that tagging the strains with resistance genes was successful and tagged strains carried the resistance genes (Figure 5.2B [i,ii,iii]). The location of the Ab^r genes at the intended site on the chromosome was confirmed by DNA sequencing of a PCR amplicon (Eurofins Genomics). The tagged strains carrying *aphA3* and *ermAM* grew on agar containing kanamycin and erythromycin respectively (but not vice versa) and there was no growth of the parent strains, which confirmed that the genes conferring resistance were functional and expressed. δ -toxin production was identical between wild-type and tagged strains suggesting that the Agr system was active in all tagged strains and had not been affected during the insertion process (Figure 5.3A). The growth profile of each tagged strain was similar to that of the wild-type indicating that growth *in vitro* was not affected by carriage of Ab^r genes (Figure 5.3B [i,ii,iii]).

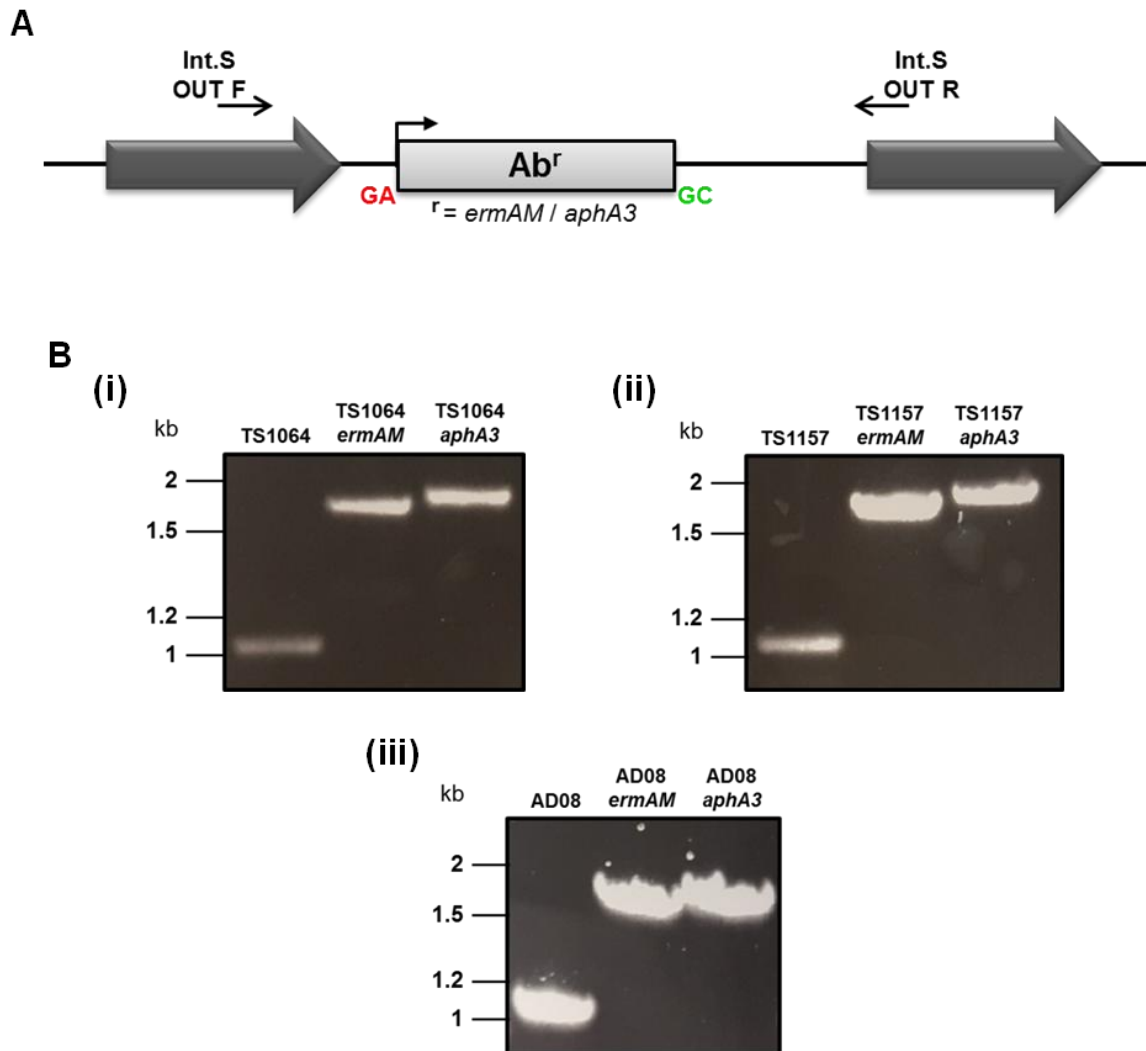


Figure 5.2. Generation of Kan^r and Ery^r *S. aureus* strains. **A** [i][ii] Int.S OUT F and R primers were designed to amplify across the insertion region to confirm the mutation. **B**) *ermAM* or *aphA3* were inserted onto the chromosome of **i]** TS1064 **ii]** TS1157 and **iii]** AD08. The insertion was confirmed by PCR with purified genomic DNA from each strain using primers Int.S OUT F and R.

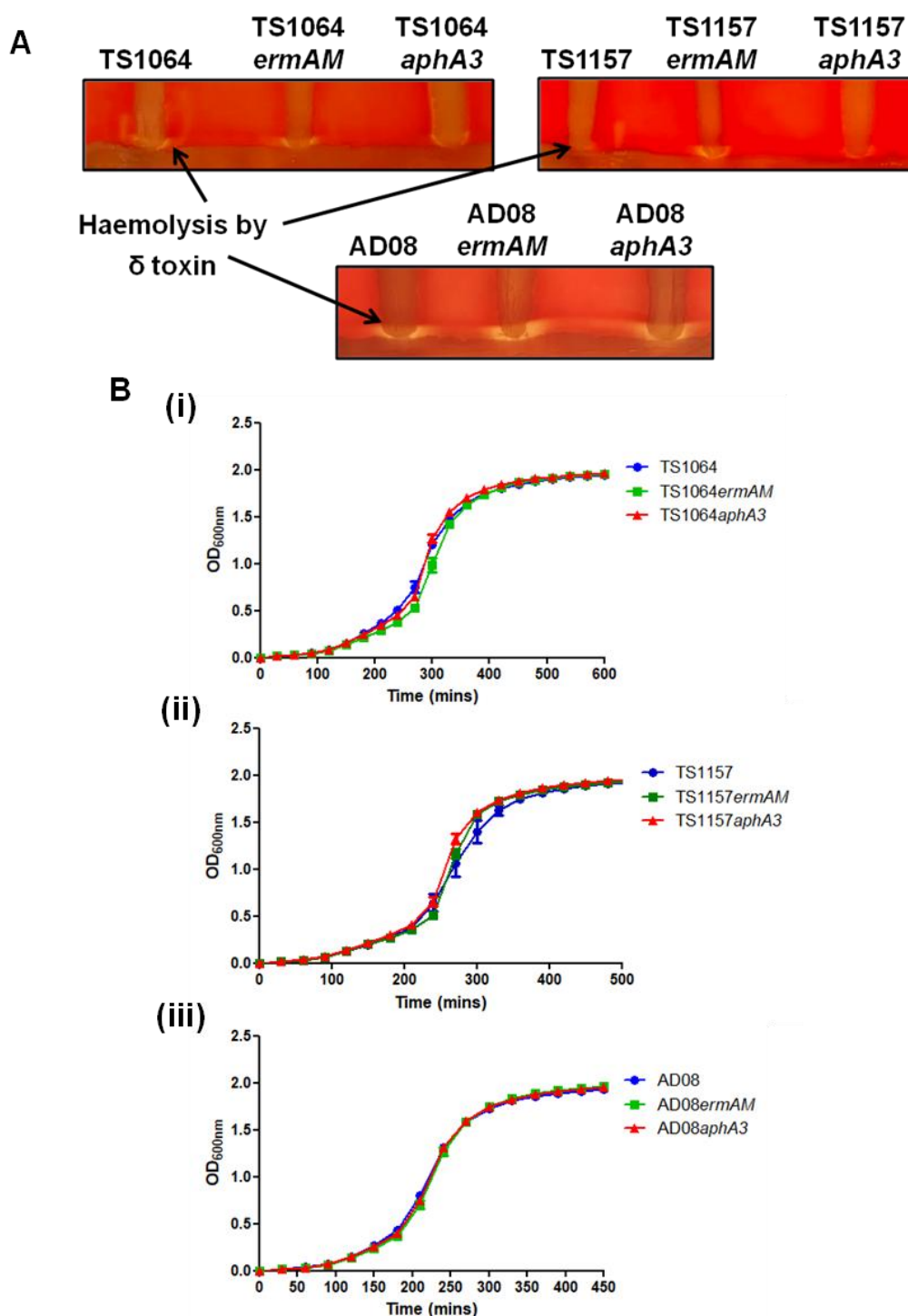


Figure 5.3. Validation of Kan^r and Ery^r *S. aureus* mutants. **A)** Wild-type and mutants were cross-streaked against RN4220 to examine the haemolytic profile. **B i) ii) iii)** OD₆₀₀ of wild-type and mutants growing in TSB broth was recorded over 16 h. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates.

5.2.3 Analysis of the effect of *aphA3* and *ermAM* carriage

ermAM encodes for an adenine methylase which modifies an adenine residue in the 50S ribosomal subunit that typically acts as the binding site for macrolide antibiotics (Leclercq & Courvalin, 2002) and *aphA3* encodes for a phosphotransferase (Gutierrez *et al.*, 1996) that modifies the aminoglycoside antibiotic to inhibit its binding ability, rather than altering the bacterial target (Munita & Arias, 2016). Given the difference in mode of action of each resistance enzyme, further validation of strains AD08*ermAM*, AD08*aphA3*, TS1064*ermAM*, TS1064*aphA3*, TS1157*ermAM* and TS1157*aphA3* was necessary to ensure that neither marker conferred a competitive advantage or deficit compared to the other marker. Furthermore, expression of an antibiotic resistance gene can incur a negative fitness cost on the bacteria (Andersson & Levin, 1999) therefore it was necessary to investigate potential effects of constitutive expression of *aphA3* and *ermAM* in the tagged strains.

To investigate if there was a fitness cost associated with carriage of *aphA3* or *ermAM* during growth in vitro, competitive growth assays were carried out competing AD08*ermAM* against AD08*aphA3* and AD08*ermAM* against AD08 wild-type (WT). Briefly, each *S. aureus* culture was diluted into a single vial of RPMI at a 1:1 ratio to give 1.5×10^4 CFU/ml. Co-cultures were incubated for a total of 7 h with samples taken of initial inoculum (T0), after 3 h incubation (T3), after 5 h incubation (T5) and after 7 h incubation (T7). Samples were plated onto TSA and following 12 h incubation, replica plated onto agar containing erythromycin (Ery) and/or kanamycin (Kan) to obtain CFU counts of resistant bacteria. In cases where a resistant strain was competed against a susceptible strain the CFU/ml of the susceptible strain was calculated by subtracting the number of resistant colonies from the number of total colonies grown on agar without antibiotic. To determine differences in growth between the competing strains, the CFU count for each individual strain at time zero (T0) was set to an arbitrary value of 100%. Results from experiments where the starting inoculum of two competing strains differed by more than 2-fold were not included. Viable counts at each of the timepoints were determined and expressed relative to the starting inoculum of 100%. AD08 grew efficiently in RPMI broth and the number of CFU at T5 increased by 1-log compared to CFU

at T0 and T3. The final viable count at T7 indicated a further 1-log increase in CFU (Figure 5.4B). Throughout the 7h co-incubation period there was no difference between the viable counts for AD08*ermAM* or AD08 WT, when grown in the same culture. Similarly, competing AD08*ermAM* against AD08*aphA3* showed no significant difference in viable counts at any of the timepoints tested (Figure 5.4A). Together, these data confirm that neither selective marker incurs a greater advantage or deficit in competitive growth. Furthermore it shows that constitutive expression of *ermAM* or *aphA3* has no effect on bacterial fitness under the conditions tested here.

Identical experiments were carried out to analyse the effect of *ermAM* and/or *aphA3* carriage in TS1064 and TS1157. Competing TS1064*ermAM* against TS1064*aphA3* indicated that there was no significant difference in viable counts between the two tagged strains (Figure 5.5A) and that carriage of either Ab^r cassette does not confer an advantage over the other. Measuring competitive growth of TS1064*aphA3* and TS1157*aphA3* against their respective parent strains showed no significant difference in viable counts (Figure 5.5B, C) confirming that carriage of *aphA3* has no effect on bacterial fitness under the conditions tested here.

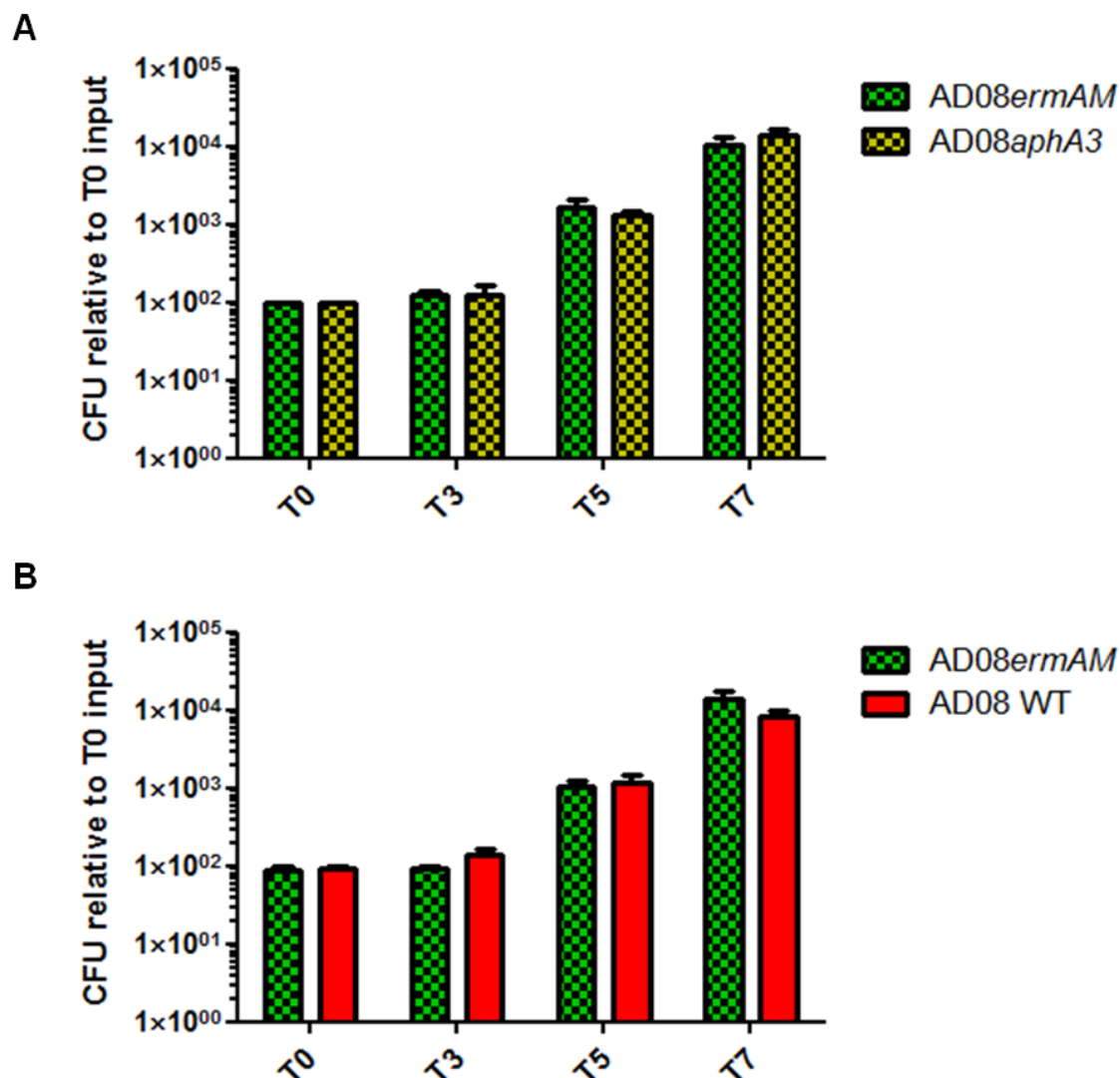


Figure 5.4. *ermAM* and *aphA3* carriage do not effect AD08 fitness. RPMI was inoculated with 1.5×10^4 CFU/ml of *S. aureus* cultures at a 1:1 ratio and grown for 7 h at 37 °C. Samples were taken at 0 h (T0), 3 h (T3), 5 h (T5) and 7 h (T7) and plated onto TSA. Ery or Kan resistant colonies were identified by replica plating onto selective agar. For each strain, CFU values at each timepoint were normalised relative to input CFU (T0). Presented are the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with bonferroni posttest was used to compare differences between strains at each timepoint, $p > 0.05$.

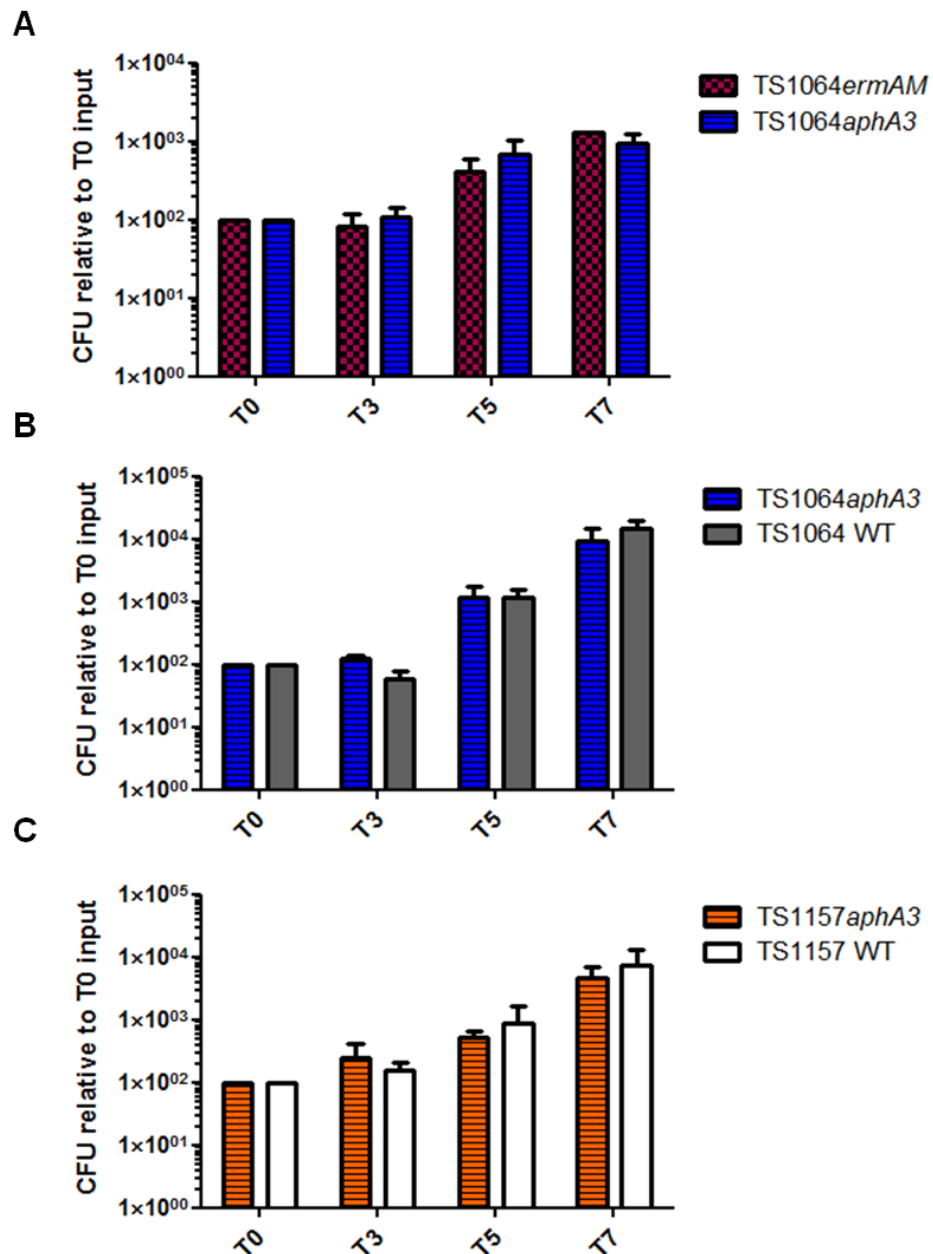


Figure 5.5. *ermAM* and *aphA3* carriage do not effect bacterial fitness of TS1064 and TS1157. RPMI was inoculated with 1.5×10^4 CFU/ml of *S. aureus* cultures at a 1:1 ratio and grown for 7 h at 37 °C. Samples were taken at 0 h (T0), 3 h (T3), 5 h (T5) and 7 h (T7) and plated onto TSA. Ery or Kan resistant colonies were identified by replica plating onto selective agar. For each strain, CFU values at each timepoint were normalised relative to input CFU (T0). Presented are the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with bonferroni posttest was used to compare differences between strains at each timepoint, $p > 0.05$.

5.2.4 Competitive growth of AD08_{CC1} against TS1064_{CC30} and TS1157_{CC30}

To investigate whether AD08 has a competitive growth advantage over TS1064 or TS1157, competitive growth assays were carried out with AD08*ermAM* against TS1064*aphA3* or TS1157*aphA3* as described in section 5.2.3. Viable counts for TS1064 and TS1157 were significantly reduced by greater than 1-log compared to AD08 in both co-cultures after 7 hours of co-incubation (Figure 5.6A, B) ($p < 0.001$). This suggests that AD08 is capable of outcompeting and/or inhibiting growth of both TS1064 and TS1157 *in vitro*.

To establish whether other AD_{CC1} strains could negatively affect TS1064 and TS1157, competitive growth assays were carried out using the AD_{CC1} isolates AD17 and AD24. AD17 and AD24 had no selective marker. Viable counts of both TS1064*aphA3* and TS1157*aphA3* were significantly lower than AD24 following 7 hours of co-culture (Figure 5.7A, B). This illustrates that AD24 is capable of outcompeting and/or inhibiting growth of both TS1064 and TS1157. During 7 hours of co-incubation, AD17 achieved similar CFUs as both TS1064 and TS1157 (Figure 5.8A, B) suggesting that AD17 does not have an advantage over TS1064 or TS1157 under the conditions tested here.

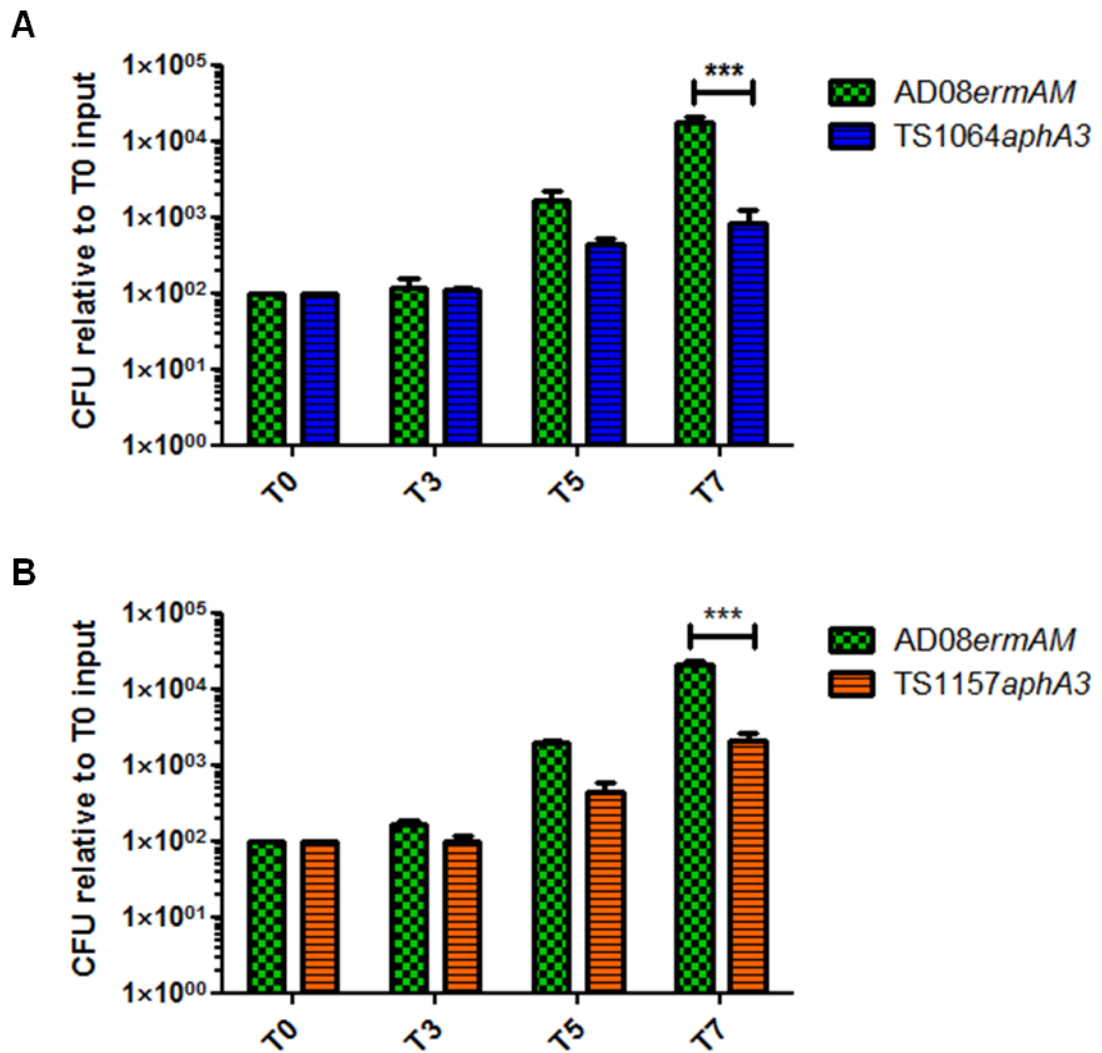


Figure 5.6. AD08 has increased growth compared to TS1064 and TS1157.

RPMI was inoculated with 1.5×10^4 CFU/ml of *S. aureus* cultures at a 1:1 ratio and grown for 7 h at 37 °C. Samples were taken at 0 h (T0), 3 h (T3), 5 h (T5) and 7 h (T7) and plated onto TSA. Ery or Kan resistant colonies were identified by replica plating onto selective agar. For each strain, CFU values at each timepoint were normalised relative to input CFU (T0). Presented are the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with bonferroni posttest was used to compare differences between strains at each timepoint, $p < 0.001$.

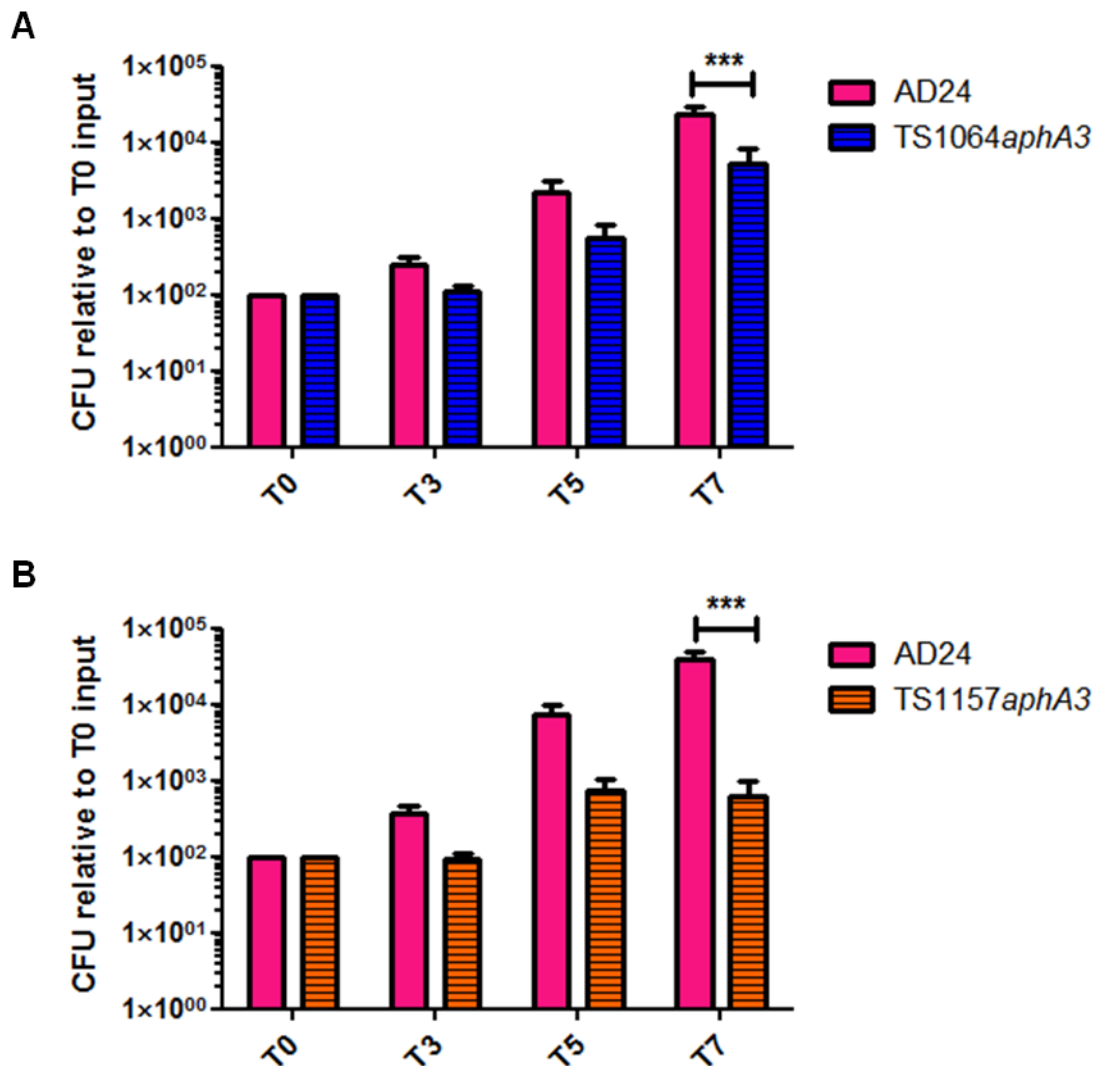


Figure 5.7. AD24 has increased growth compared to TS1064 and TS1157. RPMI was inoculated with 1.5×10^4 CFU/ml of *S. aureus* cultures at a 1:1 ratio and grown for 7 h at 37 °C. Samples were taken at 0 h (T0), 3 h (T3), 5 h (T5) and 7 h (T7) and plated onto TSA. Ery or Kan resistant colonies were identified by replica plating onto selective agar. For each strain, CFU values at each timepoint were normalised relative to input CFU (T0). Presented are the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with bonferroni posttest was used to compare differences between strains at each timepoint, $p < 0.001$.

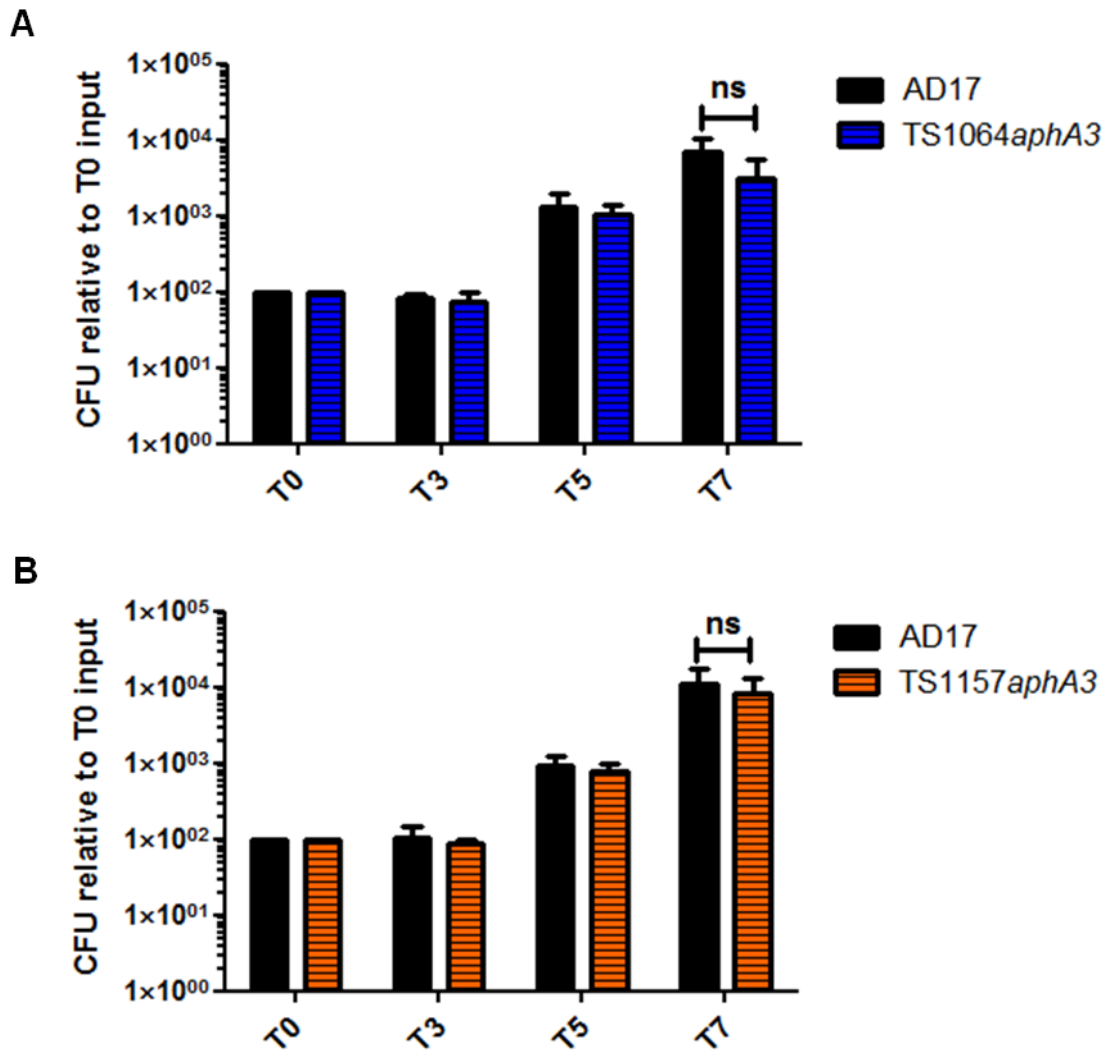


Figure 5.8. Growth of AD17 is similar to TS1064 and TS1157. RPMI was inoculated with 1.5×10^4 CFU/ml of *S. aureus* cultures at a 1:1 ratio and grown for 7 h at 37 °C. Samples were taken at 0 h (T0), 3 h (T3), 5 h (T5) and 7 h (T7) and plated onto TSA. Ery or Kan resistant colonies were identified by replica plating onto selective agar. For each strain, CFU values at each timepoint were normalised relative to input CFU (T0). Presented are the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with bonferroni posttest was used to compare differences between strains at each timepoint, $p > 0.05$.

5.2.5 Construction and Validation of AD08 Δ esaDG

The data presented thus far suggested that some AD_{CC1} strains have a competitive advantage over CC30 nasal isolates as two out of three AD_{CC1} strains outcompeted CC30 strains under the conditions tested. The underlying basis of this is unknown and there is limited evidence to suggest that *S. aureus* is capable of direct intraspecies competition. Cao et al., (2016) demonstrated that the T7SS secreted nuclease toxin EsaD inhibits growth of sensitive *S. aureus* strains (Cao et al., 2016). While this implies that the T7SS substrates are involved in competitively targeting other bacteria, the experiments were carried out using plasmid encoded *esaD* artificially overexpressing EsaD. Furthermore, bacteria overexpressing EsaD were grown with sensitive bacteria at a ratio of 20:1. Thus the significance of EsaD toxicity in direct *S. aureus* intraspecies competition remains unknown. Approximately 50% of *S. aureus* strains carry *esaD* and *esaG*, encoding EsaD and the cognate neutralising antitoxin EsaG respectively, while all *S. aureus* strains carry at least two *esaG*-like genes presumably to serve as a neutralising agent against variant EsaD toxins produced by other *S. aureus* strains (Cao et al., 2016, Warne et al., 2016).

While the majority of the *esaD* gene is conserved between *S. aureus* strains, there is extensive variation in the 3' end of the gene that gives rise to variant EsaD proteins found in different clonal complexes. *esaG* and *esaG*-like genes encode for small proteins of the DUF600 family (Warne et al., 2016, Cao et al., 2016). Using this information, BLAST alignments were carried out using an annotated *esaD* sequence from NCTC8325 (Warne et al., 2016) to identify *esaD* in the AD08 genome. Further analysis of the AD08 genome showed that it carries *esaG* and ten *esaG*-like genes (Figure 5.9A) linked with *esaD* on the chromosome.

To elucidate the potential inhibitory role of EsaD or indeed the protective role of EsaG and/or EsaG-like proteins in AD08 during direct intraspecies competition, a mutant of AD08 deficient in *esaD*, *esaG* and the *esaG*-like genes (hereafter termed *esaDG* locus) was generated by allelic exchange. The plasmid pIMAY:: Δ esaDG was constructed using commercially synthesised DNA that was homologous to 650 bp upstream of *esaD* and 500 bp downstream of

the final *esaG*-like gene (Figure 5.9B). The synthesised product was cloned into pIMAY by SLIC as described in section 2.2.4 (Figure 3.9C). pIMAY:: Δ *esaDG* was used to isolate a mutant of AD08 lacking the *esaDG* locus following the allelic exchange procedure described in section 1.5.1. Two genes encoding hypothetical transmembrane proteins of unknown function lie between *esaG* and the *esaG*-like genes in AD08, both of which were also deleted during mutagenesis. However Cao et. al., (2016) demonstrated that deleting these genes in NCTC8325, along with *esaD*, *esaG* and *esaG*-like genes, did not affect secretion of another T7SS substrate, EsxA, indicating that they are not required for type VII secretion (Cao et al., 2016). PCR with primers *esaDG* OUT F and R that anneal up and downstream of the *esaDG* deletion sequence (Figure 3.9B) were used to validate AD08 Δ *esaDG* by comparing the product to the parental wild-type product (Figure 3.10A). The mutation was confirmed by DNA sequencing of a PCR amplicon (Eurofins Genomics). The mutant was phenotypically identical to the wild-type in terms of δ -toxin activity on sheep blood agar (Figure 3.10B) and growth profile (Figure 3.11C) which indicates that *agr* and growth were not affected during mutagenesis.

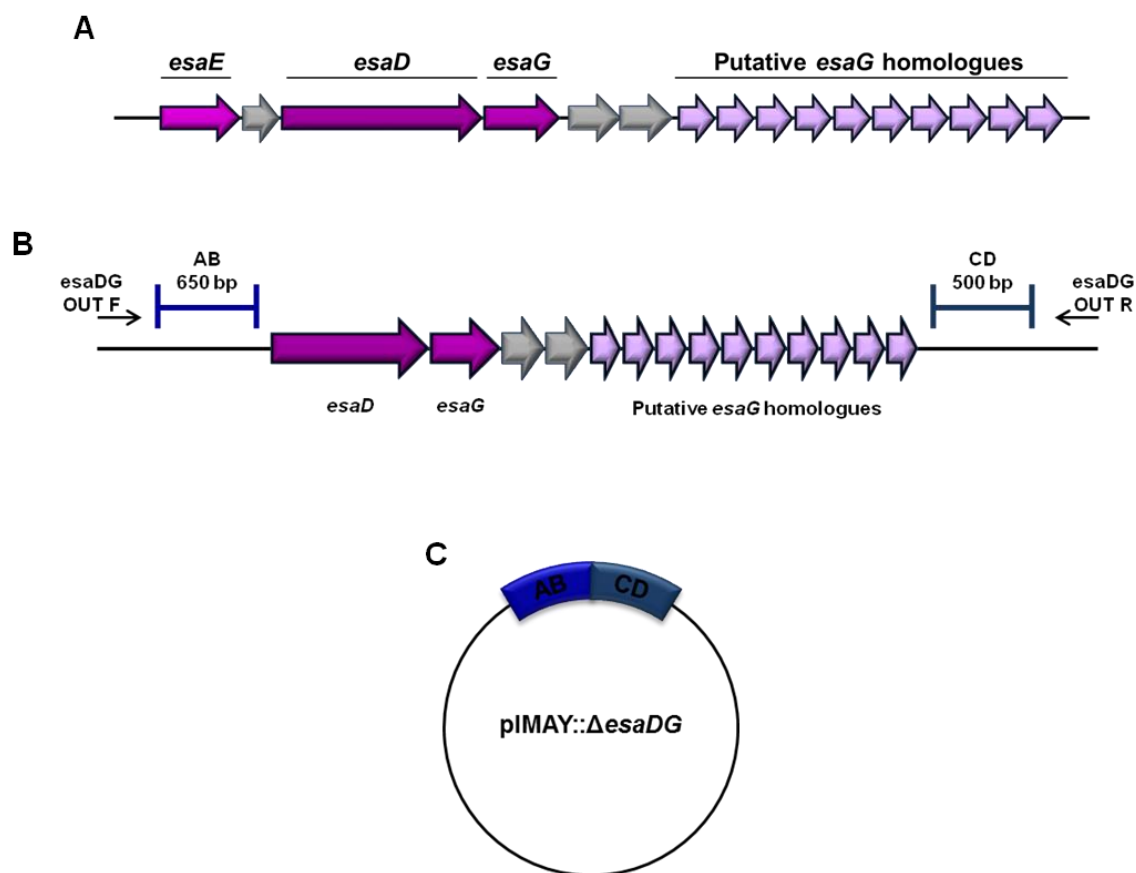


Figure 5.9. Schematic representation of pIMAY::Δ*esaDG* construction. **A)** Schematic of a predicted partial region of the *ess* locus in AD08. Genes encoding *esaE*, *esaD*, *esaG* and putative *esaG*-like genes are indicated. **B)** DNA homologous to 650 bp upstream of *esaD* and 500 bp downstream of the final *esaG*-like gene, as indicated, was synthesised commercially. *esaDG* OUT F and R primers were designed to amplify genomic DNA flanking the AB and CD regions as indicated and used to screen potential mutants. **C)** The synthesised DNA was cloned into the plasmid vector pIMAY to generate pIMAY::Δ*esaDG*. *esaD*, *esaG* and *esaG*-like genes were deleted from the AD08 chromosome by allelic exchange with pIMAY::Δ*esaDG*.

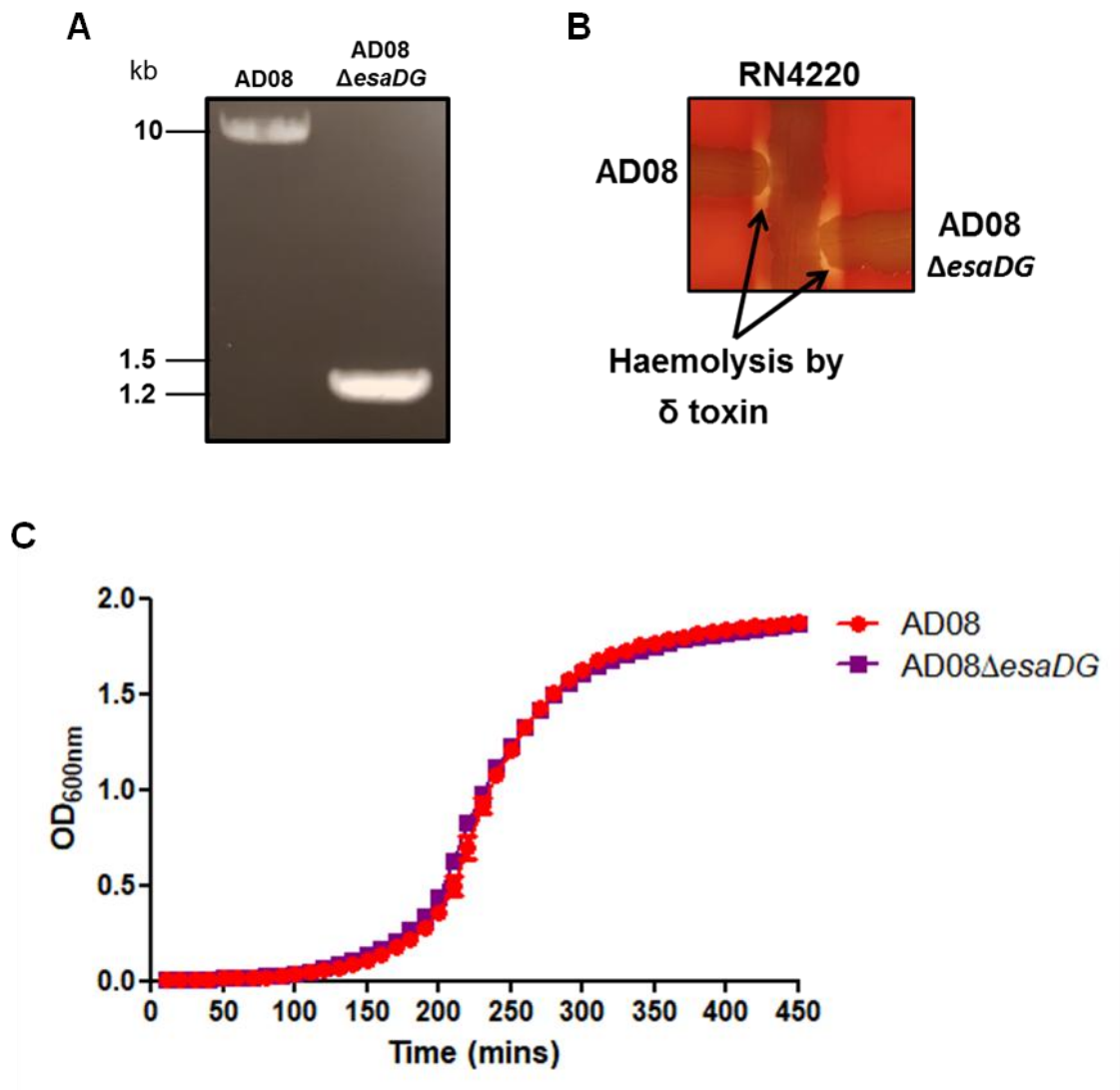


Figure 5.10. Validation of AD08 Δ esaDG. **A)** The deletion was confirmed by PCR with genomic DNA from AD08 and AD08 Δ esaDG using primers EsaDG OUT F and R. **B)** Wild-type and mutant were cross-streaked against RN4220 to examine the haemolytic profile. **C)** OD₆₀₀ of wild-type and mutant growing in TSB broth was recorded over 16 h. Each data point represents the mean values of three independent experiments and error bars show the SEM of the biological replicates.

5.2.6 Analysis of the inhibitory and/or protective effect of the *ess* locus in intraspecies competition

To investigate whether *EsaD*, *EsaG* and/or *EsaG*-like proteins contribute to AD08 fitness during direct competition, competitive growth assays were carried out as described in section 5.2.3 where AD08 Δ *esaDG* was competed against TS1064*aphA3* and TS1157*aphA3*. A further experiment competing AD08*ermAM* against TS1064*aphA3* was included as a control. AD08 Δ *esaDG* retained the ability to outcompete TS1157*aphA3* under the conditions tested (Figure 3.11A). However, there was no significant difference in viable counts between AD08 Δ *esaDG* and TS1064*aphA3* (Figure 3.11B) indicating that deletion of the *esaDG* locus eliminates the ability of AD08 to outcompete TS1064. In the same experiment, AD08*ermAM* significantly outcompeted and/or inhibited growth of TS1064*aphA3* ($p < 0.01$) (Figure 3.11C) which confirmed that the assay was functioning correctly. The data obtained here suggest that *esaD*, *esaG* and/or the *esaG*-like genes may contribute to direct competition between AD08 and TS1064 but not TS1157.

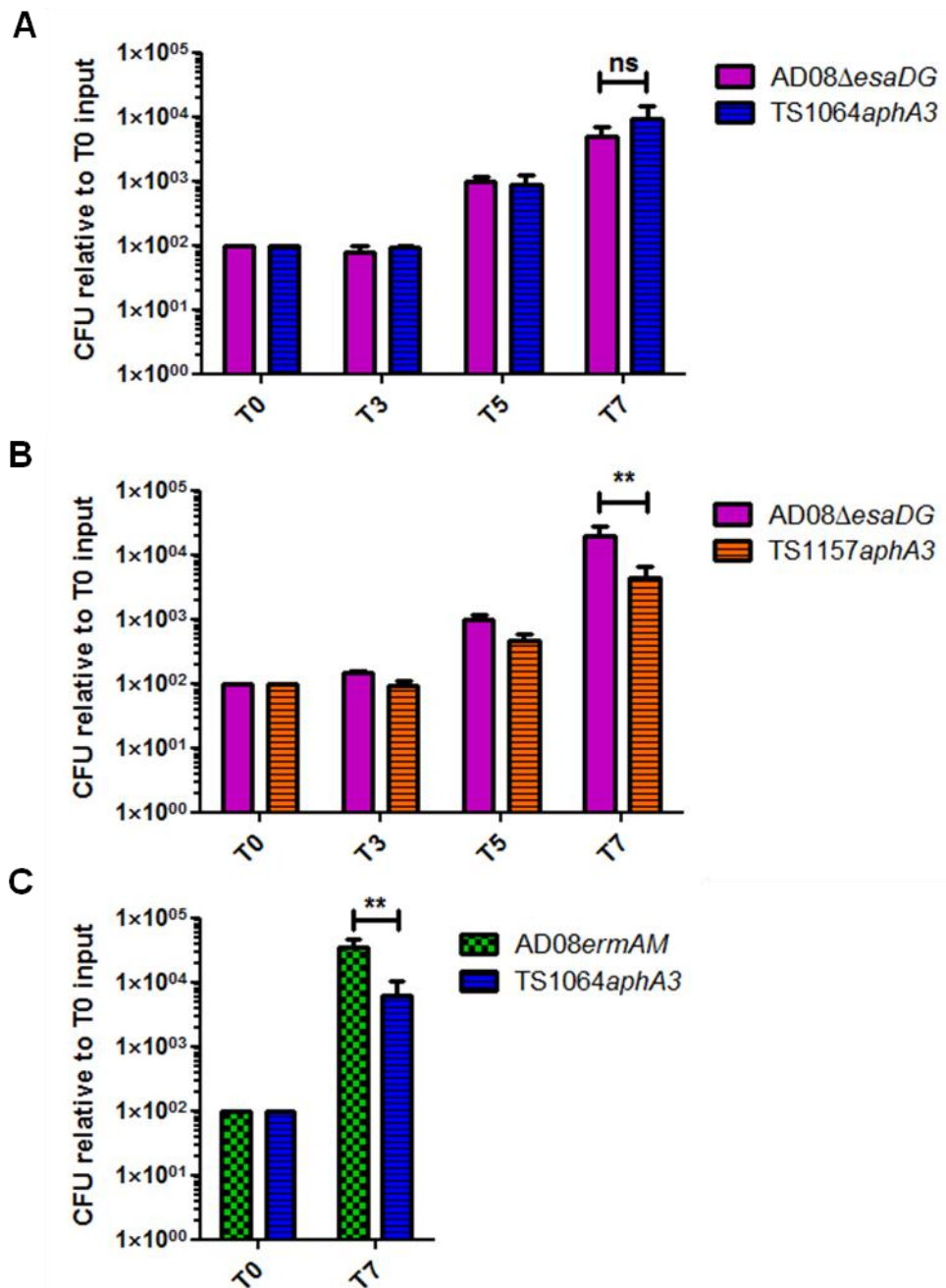


Figure 5.11. *esaD* and/or *esaG* and/or *esaG* homologues contribute to the competitive growth of AD08. RPMI was inoculated with 1.5×10^4 CFU/ml of *S. aureus* cultures at a 1:1 ratio and grown for 7 h at 37 °C. Samples were taken at 0 h (T0), 3 h (T3), 5 h (T5) and 7 h (T7) and plated onto TSA. Ery or Kan resistant colonies were identified by replica plating onto selective agar. For each strain, CFU values at each timepoint were normalised relative to input CFU (T0). Presented are the mean values of three independent experiments and error bars show the SEM of the biological replicates. A two-way ANOVA with bonferroni posttest was used to compare differences between strains at each timepoint, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

5.3 Discussion

S. aureus colonises the nasal cavity of the healthy population (van Belkum *et al.*, 2009) and while it is a frequent cause of soft skin and tissue infections (Bassetti *et al.*, 2014), it is not a dominant member of the skin microbiota (Oh *et al.*, 2014). It is well established that the skin of AD patients displays a microbiome dysbiosis, where there is a reduction in microbial diversity and an increase in staphylococci, particularly *S. aureus*. During disease flares especially, *S. aureus* dominates the microbiome which suggests that it is selected for over other organisms in this environment (Leyden *et al.*, 1974, Kong *et al.*, 2012).

Within the *S. aureus* species itself there exists a clonal association for colonising different niches where, in Irish and British patient cohorts, *S. aureus* CC1 strains are prevalent in AD skin (Fleury *et al.*, 2017, Harkins *et al.*, 2018) but not typically associated with colonisation of the nasal cavity of healthy individuals (Fleury *et al.*, 2017, Feil *et al.*, 2003). This suggests that AD_{CC1} strains may be adapted to colonise AD skin. Inversely, CC30 strains are ubiquitous within the nasal cavity of the healthy population (Feil *et al.*, 2003, Rasmussen *et al.*, 2013, Holtfreter *et al.*, 2007) but are underrepresented in the AD population (Fleury *et al.*, 2017). This chapter explored the hypothesis that AD_{CC1} strains have a competitive growth advantage over CC30 nasal isolates.

In vitro competitive growth assays can be used to compare both fitness and direct competition between strains. Fitter bacteria engaging in exploitation competition have a faster growth rate with rapid proliferation, assumedly occupying space and utilising nutrients quicker and more efficiently within an environment. Bacteria also engage in interference competition, where the release of antimicrobial peptides, toxins and quorum-quenching molecules inhibit growth of competing strains (Paharik *et al.*, 2017, Hibbing *et al.*, 2010). This is evident in healthy skin where natural skin commensals secrete antimicrobial peptides that directly inhibit growth of pathogenic bacteria on the skin (Cogen *et al.*, 2010, Shu *et al.*, 2013). In *S. aureus* autoinducing peptides involved in quorum sensing are capable of cross inhibiting *agr* function in other

strains (Jarraud *et al.*, 2000). However there is little other evidence to suggest that *S. aureus* participates in active intraspecies competition.

This chapter outlines the development of a competitive growth assay where both bacterial fitness and direct competition over time were considered, as competing strains were grown together in culture media. *In vitro* analysis of competitive growth is not optimal to mimic the *in vivo* scenario however elucidating bacterial mechanisms at the molecular level is difficult and time consuming in an *in vivo* model. Furthermore, animal models of AD are not sufficient to accurately mimic the human AD phenotype (Ewald *et al.*, 2017). Assumedly nutrient availability is less plentiful on skin than in commonly used enriched lab media, therefore the nutrient-limited media RPMI was chosen to measure comparative growth.

Three *S. aureus* strains including one AD_{CC1} isolate AD08 and two CC30 nasal carriage isolates TS1064 and TS1157 were labelled with an antibiotic selective marker conferring resistance to either kanamycin or erythromycin to distinguish between competing strains. *ermAM* confers resistance to macrolides and encodes for a methylase that modifies the ribosomal antibiotic target site in the bacteria (Leclercq & Courvalin, 2002) while *aphA3* offers resistance to aminoglycosides by a phosphotransferase that alters the active site on the antibiotic compound (Munita & Arias, 2016). Kanamycin and erythromycin were selected as none of the three chosen strains harbour natural resistance. Acquisition of an antibiotic resistance gene can incur a negative fitness cost on the bacteria and resistant bacteria are generally less fit than the otherwise identical susceptible counterpart population (Andersson & Levin, 1999, Moura de Sousa *et al.*, 2017). Here, Ab^r-tagged *S. aureus* strains were constructed and validated allowing for differentiation between two strains cultured in the same tube. The tagged strains had a similar growth profile to the parental wild-type strains indicating that constitutive expression of the genes conferring antibiotic resistance did not elicit a growth deficit.

To generate tagged strains that were otherwise isogenic, the genes were integrated onto the chromosome at a specific location identified by Lei *et al.*, (2012) where no known sRNAs are located and insertion of foreign DNA has no effect on transcription of the flanking genes (Lei *et al.*, 2012). Despite the

careful choice of target integration site used in this study, additional analysis of the tagged strains using a genome-wide approach would be desirable to ensure that there are no differences in gene expression elsewhere on the chromosome. Furthermore, despite rapid advances in genome sequencing and bioinformatic methods, identification and annotation of sRNAs has proven difficult (Liu *et al.*, 2018) and the possibility of an unidentified sRNA at the insertion site cannot be excluded.

Establishing and validating strains with distinguishable selective markers, as described here, greatly aids to the study of bacterial virulence, pathogenesis and microbial interactions. In this chapter, these tagged strains allowed for investigation into the intraspecies competitive fitness of AD_{CC1} strains against CC30 nasal isolates. An initial experiment (Figure 5.5A) indicated that growth of TS1064 in RPMI was slower than both AD08 and TS1157, however normal growth of TS1064 was consistently reported in subsequent experiments (Figure 5.5B) where the growth profile over 7 h was similar between the three strains. It is likely that the abnormal growth depicted in Figure 5.5A was due to broth inadequacy. Measuring growth of AD_{CC1} strains against CC30 nasal isolates using competitive growth assays showed that two AD_{CC1} isolates AD08 and AD24 are capable of outcompeting and/or inhibiting growth of TS1064 and TS1157. A third AD_{CC1} isolate AD17 did not compete with either CC30 strain under the conditions tested. Neither TS1064 nor TS1157 were capable of outcompeting any of the AD_{CC1} strains under the conditions tested. The data presented here is indicative that some AD_{CC1} strains have a competitive growth advantage which might contribute to their ability to efficiently colonise AD skin.

Based on the data presented here, it is unknown whether AD_{CC1} strains are generally fitter than CC30 nasal isolates or whether they are capable of directly inhibiting growth through interference competition. The recent identification of the nuclease toxin EsaD and cognate antitoxin EsaG, has implicated a potential role for the *S. aureus* T7SS in bacterial competition. The AD_{CC1} isolate AD08 carries the *esaD* and *esaG* genes encoding EsaD and EsaG respectively. Every *S. aureus* strain analysed to date carries at least two *esaG*-like genes but the exact number varies between strains within a clonal

complex (Warne *et al.*, 2016). The *esaG*-like genes are proposed to act as a defence mechanism, neutralising the EsaD toxin produced by other *S. aureus* strains. Ten *esaG*-like genes were identified in the AD08 genome sequence suggesting that AD08 may be protected against EsaD variants expressed by multiple *S. aureus* strains.

This chapter investigates the potential contribution of the *esaDG* locus to the competitive advantage of AD08 over CC30 nasal isolates. Specifically, a mutant of AD08 deficient in *esaD*, *esaG* and *esaG*-like genes was no longer able to outcompete TS1064 indicating that one or more of the proteins encoded by these genes are important for competitive fitness of AD08. Presumably then, the reduced growth of TS1064 in the presence of wild-type AD08 is suggestive of interference and not exploitative competition, mediated by the inhibitory activity of EsaD, however this is unconfirmed and the potential involvement of EsaG and EsaG homologues cannot be excluded. The absence of *esaD*, *esaG* and *esaG*-like genes did not affect the ability of AD08 to outcompete TS1157 which suggests that the gene products are not involved in competition against TS1157. The ability of AD08 to outcompete TS1157 may be related to general fitness or the presence of another unknown toxin. Together these data imply that *esaD*, *esaG* and/or the *esaG*-like genes are important for competition of at least one AD_{CC1} strain against at least one CC30 nasal carriage isolate.

Interestingly, *esaD* has not been detected in the genome sequence of any CC30 isolate analysed (Windmuller *et al.*, 2015, Warne *et al.*, 2016). TS1064 and TS1157 were unable to outcompete the AD08 mutant lacking genes encoding the protective antitoxins EsaG and EsaG homologues which strongly suggests that they do not express EsaD. In contrast, *esaD* has been identified in several CC1 strains (Burts *et al.*, 2008, Warne *et al.*, 2016), including AD08 and therefore analysis of the genome sequences of other AD_{CC1} strains for carriage of *esaD* is warranted. The data presented here is suggestive of EsaD activity however secretion of EsaD by AD08 was not confirmed. The obvious next step would be to generate an individual *esaD* null mutation to probe for the role of EsaD alone. Competing wild-type AD08 against an isogenic mutant deficient in the *esaDG* locus and a mutant deficient in *esaD* alone would further elucidate the importance of T7SS for AD08 intraspecies

competition. This chapter describes a small number strains tested, in one growth condition. Advancing this research would require confirming EsaD secretion and investigating its activity in a greater number of *S. aureus* isolates, grown under different conditions, particularly conditions similar to the *in vivo* environment. T7SS substrates are weakly detected when bacteria is grown in lab media, however T7SS activity is upregulated in the presence of host factors including haemoglobin or its cofactor haemin (Casabona *et al.*, 2017b) thus future experiments could be improved by the addition of haemin to the bacterial growth media.

Measuring competitive growth of AD_{CC1} strains cultured with CC30 nasal carriage isolates from healthy individuals strongly insinuates that AD_{CC1} strains are more competitively fit than CC30 nasal isolates which may contribute to their ability to successfully colonise AD skin. The data presented here documents the potential role of the *S. aureus* T7SS and substrates of the *ess* locus in intraspecies competition during host colonisation. However this raises the question as to why CC30 strains are more successful than CC1 in colonising other niches, such as the nasal cavity of the healthy human population, where carriage of CC1 is rare. It is probable that the competitive growth advantage of AD_{CC1} strains is only one aspect of their ability to colonise AD skin. Chapter 4, section 4.2.10 details the ability of AD_{CC1} strains to adhere more strongly to CDSN, which is the proposed target ligand for *S. aureus* attachment to AD skin. Here, equal numbers of competing strains were grown *in vitro* however in AD skin the abundance of CDSN presumably provides more adhesion sites for AD_{CC1} strains giving it a distinct advantage when colonising this environment. Once established on AD skin it can outcompete CC30 (and possibly other) isolates. The dynamics of *S. aureus* colonising AD skin are doubtlessly multifactorial and CC1 strains may be adapted to this niche with superior fitness, interference, and adherence abilities.

Chapter 6

Discussion

6.1 Discussion

Atopic dermatitis (AD) affects approximately 20% of people worldwide, causing itchy, red, inflamed skin lesions (Deckers *et al.*, 2012) which affects both the physical and emotional health of patients (Drucker *et al.*, 2017). In the US, it is estimated that the disease costs over \$ 5 billion annually between medical costs and lost productivity at work or school (Adamson, 2017). Elucidating the molecular factors contributing to the pathogenesis of AD is necessary to further the development of novel therapeutic strategies. This thesis examines *S. aureus* factors involved in the initial step of colonisation; adhesion to corneocyte proteins.

AD skin exhibits dramatic structural differences compared to healthy skin which alludes to the potential of unidentified novel ligands for *S. aureus* in AD skin. Recently it has been shown that *S. aureus* binds more strongly to low NMF AD corneocytes compared to high NMF corneocytes (Feuillie *et al.*, 2018) which proposes an association between *S. aureus* burden on AD skin and reduced NMF levels. Using mutants of *S. aureus* confirmed the role of ClfB and FnBPB and/or FnBPA in mediating adherence of a clinically relevant AD strain, AD08, to AD corneocytes presumably by binding to an exposed ligand on the corneocyte surface. The reduced NMF levels typical of AD skin correlate with aberrant corneocyte morphology and reduced tight junction integrity in AD skin. Nanoscale protrusions project from the corneocyte surface and the tight junction protein corneodesmosin (CDSN), normally confined to the peripheral edges of healthy corneocytes, is abnormally displaced on the tips of these protrusions in AD skin (Riethmuller *et al.*, 2015, Igawa *et al.*, 2013). This thesis identified CDSN as a novel ligand for *S. aureus* where FnBPB and ClfB were responsible for mediating adherence of AD08 to the N-terminal Ω loop region of CDSN. FnBPA was unable to support the adhesion of AD08 independently under the conditions tested *in vitro*.

To facilitate desquamation, the N-terminal glycine/serine rich region of CDSN is subject to proteolytic cleavage by the activity of protease KLK7 in the stratum corneum of healthy skin however degradation of CDSN is reduced in AD skin due to impaired secretion of KLK7 (Igawa *et al.*, 2017). The abnormal

displacement of CDSN (Riethmuller *et al.*, 2015) likely contributes to an increased accessibility of CDSN for *S. aureus* attachment in AD skin. Using an antibody specific for the N-terminal domain of CDSN, which contains the *S. aureus* binding site, showed that the N-terminal domain is intact on the surface of AD corneocytes. Blocking the *S. aureus* binding site in CDSN using this antibody reduced the ability of AD08 to adhere to AD corneocytes. Furthermore, coagulase-negative staphylococci, which are natural commensals of healthy skin, do not adhere to CDSN emphasising the importance of CDSN as an exclusive ligand for *S. aureus* in AD skin. Combined, this data strongly suggests that *S. aureus* targets CDSN to adhere to AD skin. Interestingly, the canine pathogen *S. pseudintermedius* also adhered to corneodesmosin. *S. pseudintermedius* causes canine pyoderma in dogs with underlying AD. Therefore a common ligand is targeted by two related skin pathogens.

Having identified that FnBPA and/or FnBPB promote adherence to the glycine/serine rich region in CDSN led us to revisit previously described *S. aureus* skin ligands including the glycine/serine rich proteins loricrin and K10. Previous investigation into the interaction between *S. aureus* and loricrin and K10 used lab strains of *S. aureus* and reported that binding was exclusively dependent on ClfB binding to the glycine/serine Ω loop regions in both proteins (Mulcahy *et al.*, 2012, Walsh *et al.*, 2004). However, functional assays using a mutant of AD08 deficient in ClfB allowed the detection of additional loricrin and K10 binding proteins expressed by AD08. This result is not entirely surprising as colonisation is multifactorial and *S. aureus* cell wall-anchored proteins (CWAPs) are functionally redundant in nature. Experiments using various mutants of AD08 identified a role for FnBPB in mediating *S. aureus* adherence to loricrin and K10 where adherence to both proteins was dependent on ClfB and FnBPB. Similar to CDSN, FnBPA was unable to facilitate the interaction independently under the conditions tested.

There are seven allelic variants of FnBPA, which have clonal associations (Loughman *et al.*, 2008b). Given that lab strains are usually from CC8 (e.g. Newman and 8325 derivatives), the majority of research investigating FnBPA function has focused on isotype I (CC8-type). AD CC1 strains (AD_{CC1}) express isotype III (CC1-type) FnBPA. Here, CC8-type FnBPA overexpressed

artificially from a plasmid supported *S. aureus* adherence to both loricrin and K10. It is possible that FnBPA variants have different affinities to glycine/serine rich regions in loricrin, K10 and CDSN. Interestingly, recombinant N2 N3 subdomains of CC1-type FnBPA had an almost 10-fold higher affinity for CDSN than both FnBPB and ClfB. It is possible therefore that FnBPA is expressed weakly by AD08 under the conditions tested here. Measuring mRNA levels of both FnBPA and FnBPB in AD08 would be useful to determine this. Examining the role of FnBPA in adherence to these ligands in more *in vivo* like conditions may contribute to increased FnBPA ligand-binding activity. Regardless, this thesis depicts the first report of fibronectin binding proteins FnBPA and FnBPB binding to glycine/serine rich Ω loop regions and implicates a potential role of FnBPA and/or FnBPB in colonisation. Previous work characterising the interaction between *S. aureus* and loricrin and K10 using lab strain Newman was flawed due to the absence of functional fibronectin-binding proteins (Grundmeier *et al.*, 2004). Therefore, this thesis highlights the importance of using clinically relevant bacterial strains as lab strains provide narrow insights into host-pathogen interactions. Here the interaction between *S. aureus* and CDSN was characterised using only one relevant *S. aureus* strain grown in a limited number of conditions which are likely not fully representative of *in vivo* conditions. While AD08, as a CC1 strain, is representative of 20% of AD isolates, a limitation of this thesis is that it excluded AD strains from other clonal complexes. A robust analysis of the interaction between *S. aureus* and corneocyte proteins would include several more *S. aureus* strains tested in various conditions.

The evidence presented strongly suggests that CDSN is a target for *S. aureus* adherence to AD corneocytes, however blocking the bacterial binding site in CDSN did not fully eliminate *S. aureus* adhering to the corneocytes. The reduction in adherence was dose-dependent therefore it is possible that the highest concentration of antibody used was not sufficient to block every available binding site in CDSN on the corneocytes. It is also possible that *S. aureus* adheres to other ligands on AD corneocytes. In healthy individuals, the interaction between loricrin and *S. aureus* is critical for colonisation in the nasal cavity however the gene *LOR* encoding loricrin is downregulated in AD skin by

IL-4 and IL-13 (Bao *et al.*, 2017, Totsuka *et al.*, 2017) and it is unknown whether it is entirely deficient in AD skin. The N2 N3 subdomains of ClfB facilitate binding to loricrin and CDSN, presumably by the same 'dock, lock and latch' mechanism so it is unlikely ClfB can bind to both ligands simultaneously. Fibronectin expression is induced by IL-4 and IL-13 in human fibroblasts (Postlethwaite *et al.*, 1992, Machino *et al.*, 2001) and increased deposition of fibronectin has been detected on AD skin (Cho *et al.*, 2001). Therefore fibronectin may be an additional ligand for *S. aureus* FnBPA and/or FnBPB in AD skin. Furthermore, AD patients are colonised by the same strain in the nasal cavity as the skin (Harkins *et al.*, 2018). While it is unlikely that CDSN is a ligand for *S. aureus* in the healthy population due to its confinement to the peripheral edges of corneocytes, it's entirely possible that CDSN is an exposed ligand in the nasal cavity of AD patients. Probing AD corneocytes or skin biopsies isolated from AD patients with antibodies specific for the regions in loricrin comprising *S. aureus* binding sites would help to determine whether this protein is a relevant ligand in AD skin.

Within *S. aureus*, clonal complexes (CC) comprise groups of strains adapted to colonise different niches. The nasal cavity of the healthy population is predominantly colonised by CC30 strains (Feil *et al.*, 2003, Melles *et al.*, 2004) however CC30 strains are underrepresented in AD skin (Fleury *et al.*, 2017). Conversely, CC1 strains are not typical in the nasal cavity of healthy individuals however are frequently isolated from AD patients (Fleury *et al.*, 2017, Yeung *et al.*, 2011, Harkins *et al.*, 2018, Clausen *et al.*, 2017). AD_{CC1} strains adhere stronger to CDSN *in vitro* compared to CC30 nasal carriage isolates from healthy controls. AD08 binding to CDSN is reliant on FnBPB however the *fnbB* gene is not usually carried by CC30 isolates (Lower *et al.*, 2011, Peacock *et al.*, 2000) whereas all AD_{CC1} strains used in this study carry *fnbB*. This is suggestive of a prominent role for FnBPB in mediating *S. aureus* adherence to AD skin and highlights the importance of the interaction between CDSN and FnBPB. It would be interesting to compare the affinities of the seven different allelic variants of FnBPB for CDSN, as all of the AD_{CC1} strains used in this thesis express the isotype III FnBPB variant.

Commensal CC30 strains retained the ability to adhere to CDSN albeit at a reduced level. This is indicative of other bacterial and/or host factors contributing to the ability of AD_{CC1} strains to colonise AD skin. Exploitation and/or interference competition for resources and space are commonplace between microbes in any microbiome (Hibbing *et al.*, 2010). This study identified a potential competitive growth advantage of AD_{CC1} strains. The ability of AD_{CC1} strains to outcompete and/or inhibit growth of CC30 nasal commensals *in vitro* may correspond to an *in vivo* scenario where AD_{CC1} strains are more competitively fit than other *S. aureus* strains thus contributing to their ability to successfully colonise AD skin. Furthermore, AD08 lost its ability to outcompete another *S. aureus* strain in the absence of T7SS substrates EsaD and EsaG. This proposes the potential role of the *S. aureus* T7SS in intraspecies interference competition where toxic activity of EsaD against competing strains may influence the enhanced survival of AD_{CC1} strains. However this phenotype was only observed using one AD_{CC1} strain grown in one condition *in vitro*. Thus a more thorough analysis of the role of EsaD in intraspecies interference competition is warranted.

Members of the microbiota of healthy skin, including several coagulase-negative staphylococcal (CoNS) spp. inhibit or reduce growth of *S. aureus* primarily through the release of peptides with antibacterial activity against *S. aureus* (Byrd *et al.*, 2018, Paharik *et al.*, 2017, Cogen *et al.*, 2010). CoNS strains with anti-*S. aureus* activity are less frequently isolated from AD skin however (Nakatsuji *et al.*, 2017) and this anomaly is entirely undescribed. The ability of *S. aureus* to proliferate and dominate the microbiome during AD disease flares suggests that *S. aureus* is capable of outcompeting and/or inhibiting growth of other bacterial species including typical skin commensals. Therefore the inhibitory role of EsaD as a nuclease toxin against other bacterial species should be considered. Several studies have identified host factors that stimulate upregulated T7SS activity including haemoglobin, blood serum, pulmonary surfactant and host fatty acids (Casabona *et al.*, 2017b, Burts *et al.*, 2008, Ishii *et al.*, 2014, Lopez *et al.*, 2017). It is possible that a host factor unique to AD skin regulates T7SS activity in AD strains of *S. aureus*, providing them with a competitive advantage over other microbes in AD skin. Measuring

competitive growth of *S. aureus* against CoNS strains isolated from AD patients would be useful to elucidate whether the *ess* locus is involved in *S. aureus* interspecies competition. Presumably, EsaD-mediated competitive advantage does not fully describe the dynamics contributing to the success of CC1 in AD skin as CC1 strains are not frequently recovered from in the nasal cavity of the healthy population. More likely, successful colonisation of AD skin by AD_{CC1} strains is dependent on multiple factors including fitness, interference and adherence abilities.

This thesis used corneocytes isolated from AD patients to investigate the molecular determinants responsible for *S. aureus* colonisation of AD skin however models that mimic other aspects of the *in vivo* scenario would be useful for further analysis. Murine models of AD are not accurately comparable to humans (Ewald *et al.*, 2017) therefore alternative models to study AD are necessary. 3D skin equivalents treated with IL-4 and IL-13 offer a reasonable alternative as they provide a model of the full stratified epidermis presented with an AD-like phenotype (Smits *et al.*, 2017, De Vuyst *et al.*, 2017, Popov *et al.*, 2014). These models are not wholly physiologically relevant however as they lack both immune cells and the natural skin microbiota. Extracting skin biopsies from AD patients would be a useful tool to investigate the relationship between *S. aureus* and AD.

Modern AD treatments have focused on reducing disease symptoms by decreasing the hyper immune response with monoclonal antibodies blocking IL-4 and IL-13 (Gooderham *et al.*, 2018, Simpson *et al.*, 2018, Wollenberg *et al.*, 2019) however it is unknown whether this will have any effect on colonisation by *S. aureus*. Colonisation is a risk factor for infection and carriage of *S. aureus* aggravates AD disease symptoms. Thus eliminating *S. aureus* from the skin of AD patients has therapeutic potential for AD treatment. Due to growing concerns over antibiotic resistance, novel eradication strategies are imperative. One method to avoid the selective pressure that leads to resistance is through the use of small molecules which competitively block protein-ligand interactions and inhibit a pathogen adhering to its cognate host ligand. This has been described for *E. coli*, where FimH antagonists competitively bind to the type-1 pilus adhesin FimH and block binding to the relative ligand on the surface of

bladder cells, thus preventing and treating urinary tract infections (Totsika *et al.*, 2013, Cusumano *et al.*, 2011, Kalas *et al.*, 2018). This has also been demonstrated for *S. aureus* where a peptide blocked homophillic interactions of CWAP SdrC and inhibited SdrC-mediated biofilm formation (Feuillie *et al.*, 2017). This thesis mapped the *S. aureus* binding site to the N-terminal Ω loop region in CDSN where binding is facilitated by the N2 N3 subdomains of ClfB and FnBPB. The N2 N3 subdomains of ClfB bind to glycine/serine rich regions in loricrin, K10 and α -chain of fibrinogen by the 'dock, lock and latch' (DLL) mechanism while FnBPB binds the γ -chain of fibrinogen by the DLL mechanism (Ni Eidhin *et al.*, 1998, Keane *et al.*, 2007, Burke *et al.*, 2011), therefore it can be hypothesised that binding to CDSN occurs by the same DLL mechanism. In theory identifying and/or synthesizing a compound to competitively bind to ClfB and FnBPB, and block the CDSN-binding site would inhibit the ability of *S. aureus* to adhere to AD skin and improve disease symptoms. Thus the data presented in this thesis provides insight for the future development of novel therapeutic options for the treatment of AD.

In summary, this thesis proposes a novel model for the interaction between *S. aureus* and AD skin (Figure 6.1). Reduced NMF levels correlate with altered cellular distribution of CDSN in AD skin (Riethmuller *et al.*, 2015), presenting CDSN as a more accessible and/or abundant ligand for *S. aureus* adherence in AD skin compared to healthy skin. Initial attachment is presumably achieved by adherence to CDSN, mediated primarily by FnBPB and ClfB binding to the N-terminal Ω loop domain of CDSN. The N-terminal domain, normally cleaved by proteases in healthy skin, is intact in AD skin (Igawa *et al.*, 2017). Thus, the abundance of adhesion sites for AD_{CC1} strains likely confers an advantage for adherence to AD skin. Following attachment, AD_{CC1} strains, which are probably more competitively fit than typical *S. aureus* commensal isolates, potentially engage in exploitative and/or interference competition through the activity of T7SS substrates such as EsaD, allowing them to proliferate and dominate this niche. Once colonisation has been established, *S. aureus* secreted factors and toxins stimulate the Th2-mediated immune response leading to inflammation which assumedly augments the skin barrier defect further, through IL-4 and IL-13 activity.

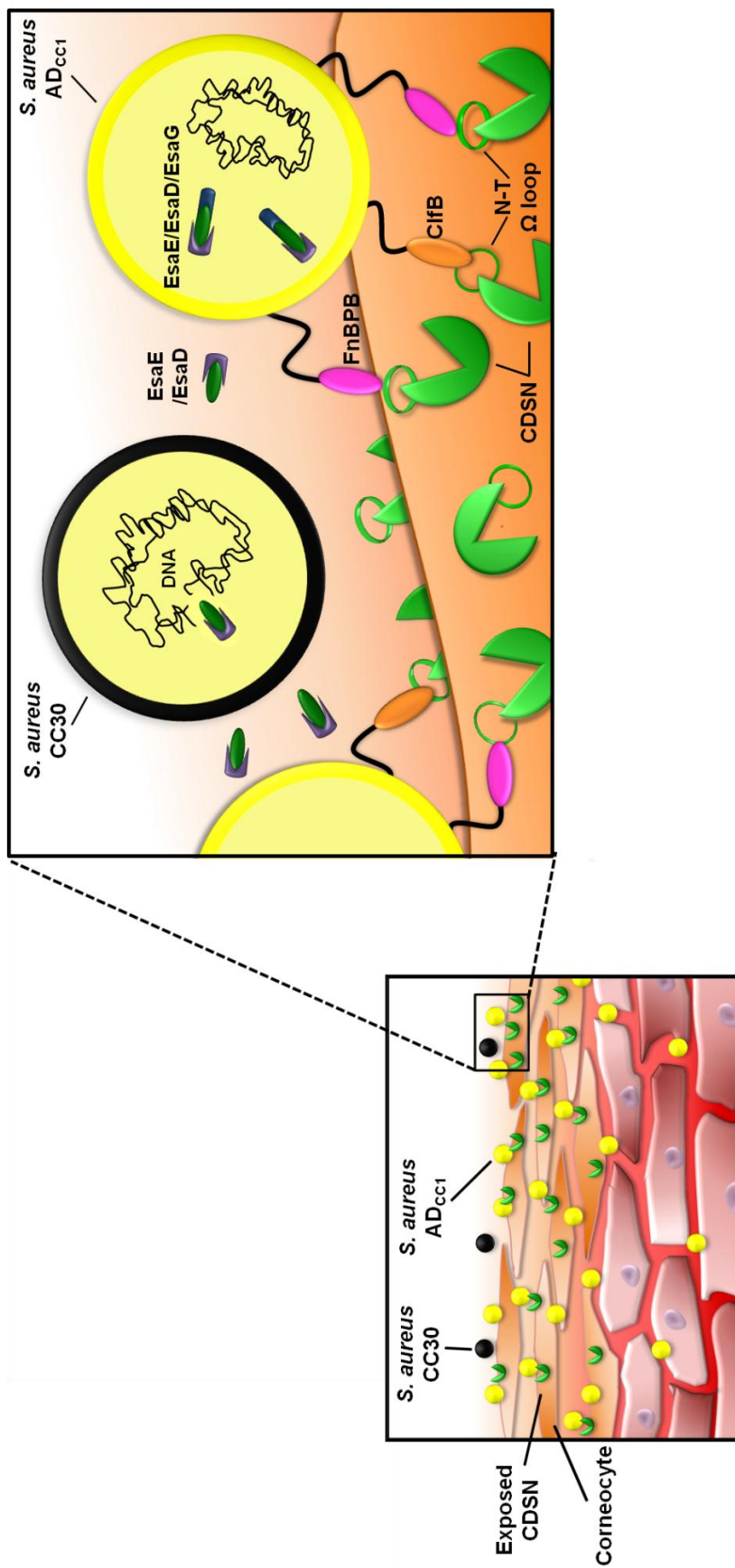


Figure 6.1. Proposed model of the molecular factors contributing to *S. aureus* colonisation of atopic dermatitis skin. *S. aureus* AD CC1 (AD_{cc1}) strains adhere to exposed corneodesmosin (CDSN) on AD corneocytes where FnBPB and ClfB facilitate binding to the N-terminal (N-T) Ω loop region in CDSN. Following initial attachment, AD_{cc1} strains outcompete and/or inhibit the growth of other *S. aureus* strains (e.g. CC30). In some cases this effect is partially due to the toxic activity of the type VII secretion system effector protein EsaD which degrades the DNA of competing bacteria.

References

- Abdallah, A.M., Gey van Pittius, N.C., Champion, P.A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C.M., Appelmek, B.J., and Bitter, W. (2007) Type VII secretion--mycobacteria show the way. *Nat Rev Microbiol* **5**: 883-891.
- Adamson, A.S. (2017) The Economics Burden of Atopic Dermatitis. *Adv Exp Med Biol* **1027**: 79-92.
- Agerer, F., Lux, S., Michel, A., Rohde, M., Ohlsen, K., and Hauck, C.R. (2005) Cellular invasion by *Staphylococcus aureus* reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. *J Cell Sci* **118**: 2189-2200.
- Albanesi, C., Fairchild, H.R., Madonna, S., Scarponi, C., De Pita, O., Leung, D.Y., and Howell, M.D. (2007) IL-4 and IL-13 negatively regulate TNF- α - and IFN- γ -induced beta-defensin expression through STAT-6, suppressor of cytokine signaling (SOCS)-1, and SOCS-3. *J Immunol* **179**: 984-992.
- Ali, S.M., and Yosipovitch, G. (2013) Skin pH: from basic science to basic skin care. *Acta Derm Venereol* **93**: 261-267.
- Alonzo, F., 3rd, and Torres, V.J. (2014) The bicomponent pore-forming leucocidins of *Staphylococcus aureus*. *Microbiol Mol Biol Rev* **78**: 199-230.
- Aly, K.A., Anderson, M., Ohr, R.J., and Missiakas, D. (2017) Isolation of a Membrane Protein Complex for Type VII Secretion in *Staphylococcus aureus*. *J Bacteriol* **199**: e00482-17.
- Anderson, M., Aly, K.A., Chen, Y.H., and Missiakas, D. (2013) Secretion of atypical protein substrates by the ESAT-6 secretion system of *Staphylococcus aureus*. *Mol Microbiol* **90**: 734-743.
- Andersson, D.I., and Levin, B.R. (1999) The biological cost of antibiotic resistance. *Curr Opin Microbiol* **2**: 489-493.
- Andrade, M.A., Ciccarelli, F.D., Perez-Iratxeta, C., and Bork, P. (2002) NEAT: a domain duplicated in genes near the components of a putative Fe³⁺ siderophore transporter from Gram-positive pathogenic bacteria. *Genome Biol* **3**: Research0047.
- Argudin, M.A., Argumosa, V., Mendoza, M.C., Guerra, B., and Rodicio, M.R. (2013) Population structure and exotoxin gene content of methicillin-susceptible *Staphylococcus aureus* from Spanish healthy carriers. *Microb Pathog* **54**: 26-33.
- Askarian, F., Ajayi, C., Hanssen, A.M., van Sorge, N.M., Pettersen, I., Diep, D.B., Sollid, J.U., and Johannessen, M. (2016) The interaction between *Staphylococcus aureus* SdrD and desmoglein 1 is important for adhesion to host cells. *Sci Rep* **6**: 22134.
- Atkins, K.L., Burman, J.D., Chamberlain, E.S., Cooper, J.E., Poutrel, B., Bagby, S., Jenkins, A.T., Feil, E.J., and van den Elsen, J.M. (2008) *S. aureus* IgG-binding proteins SpA and Sbi: host specificity and mechanisms of immune complex formation. *Mol Immunol* **45**: 1600-1611.

- Augustin, J., Rosenstein, R., Wieland, B., Schneider, U., Schnell, N., Engelke, G., Entian, K.D., and Gotz, F. (1992) Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis*. *Eur J Biochem* **204**: 1149-1154.
- Bae, T., and Schneewind, O. (2006) Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* **55**: 58-63.
- Bannoehr, J., Ben Zakour, N.L., Reglinski, M., Inglis, N.F., Prabhakaran, S., Fossum, E., Smith, D.G., Wilson, G.J., Cartwright, R.A., Haas, J., Hook, M., van den Broek, A.H., Thoday, K.L., and Fitzgerald, J.R. (2011) Genomic and surface proteomic analysis of the canine pathogen *Staphylococcus pseudintermedius* reveals proteins that mediate adherence to the extracellular matrix. *Infect Immun* **79**: 3074-3086.
- Bannoehr, J., Ben Zakour, N.L., Waller, A.S., Guardabassi, L., Thoday, K.L., van den Broek, A.H., and Fitzgerald, J.R. (2007) Population genetic structure of the *Staphylococcus intermedius* group: insights into agr diversification and the emergence of methicillin-resistant strains. *J Bacteriol* **189**: 8685-8692.
- Bao, L., Mohan, G.C., Alexander, J.B., Doo, C., Shen, K., Bao, J., and Chan, L.S. (2017) A molecular mechanism for IL-4 suppression of loricrin transcription in epidermal keratinocytes: implication for atopic dermatitis pathogenesis. *Innate Immun* **23**: 641-647.
- Barbu, E.M., Ganesh, V.K., Gurusiddappa, S., Mackenzie, R.C., Foster, T.J., Sudhof, T.C., and Hook, M. (2010) beta-Neurexin is a ligand for the *Staphylococcus aureus* MSCRAMM SdrC. *PLoS Pathog* **6**: e1000726.
- Barbu, E.M., Mackenzie, C., Foster, T.J., and Hook, M. (2014) SdrC induces staphylococcal biofilm formation through a homophilic interaction. *Mol Microbiol* **94**: 172-185.
- Barker, J.N., Palmer, C.N., Zhao, Y., Liao, H., Hull, P.R., Lee, S.P., Allen, M.H., Meggitt, S.J., Reynolds, N.J., Trembath, R.C., and McLean, W.H. (2007) Null mutations in the filaggrin gene (FLG) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. *J Invest Dermatol* **127**: 564-567.
- Bassetti, M., Baguneid, M., Bouza, E., Dryden, M., Nathwani, D., and Wilcox, M. (2014) European perspective and update on the management of complicated skin and soft tissue infections due to methicillin-resistant *Staphylococcus aureus* after more than 10 years of experience with linezolid. *Clin Microbiol Infect* **20 Suppl 4**: 3-18.
- Bauer, M.A., Kainz, K., Carmona-Gutierrez, D., and Madeo, F., (2018) Microbial wars: Competition in ecological niches and within the microbiome. In: *Microb Cell*. pp. 215-219.
- Baur, S., Rautenberg, M., Faulstich, M., Grau, T., Severin, Y., Unger, C., Hoffmann, W.H., Rudel, T., Autenrieth, I.B., and Weidenmaier, C. (2014) A nasal epithelial receptor for *Staphylococcus aureus* WTA governs adhesion to epithelial cells and modulates nasal colonization. *PLoS Pathog* **10**: e1004089.

- Baurecht, H., Irvine, A.D., Novak, N., Illig, T., Buhler, B., Ring, J., Wagenpfeil, S., and Weidinger, S. (2007) Toward a major risk factor for atopic eczema: meta-analysis of filaggrin polymorphism data. *J Allergy Clin Immunol* **120**: 1406-1412.
- Bayer, A.S., Sullam, P.M., Ramos, M., Li, C., Cheung, A.L., and Yeaman, M.R. (1995) *Staphylococcus aureus* induces platelet aggregation via a fibrinogen-dependent mechanism which is independent of principal platelet glycoprotein IIb/IIIa fibrinogen-binding domains. *Infect Immun* **63**: 3634-3641.
- Becker, K., Friedrich, A., Lubritz, M., Weilert, G., Peters, G., and von Eiff, C., (2011) *Staphylococcus*, *Micrococcus*, and Other Catalase-Positive Cocci. In: Manual of Clinical Microbiology, 10th Edition. American Society of Microbiology Washington, DC, pp. 308-330.
- Becker, K., Heilmann, C., and Peters, G. (2014) Coagulase-negative staphylococci. *Clin Microbiol Rev* **27**: 870-926.
- Belgrave, D.C., Granell, R., Simpson, A., Guiver, J., Bishop, C., Buchan, I., Henderson, A.J., and Custovic, A. (2014) Developmental profiles of eczema, wheeze, and rhinitis: two population-based birth cohort studies. *PLoS Med* **11**: e1001748.
- Berdyshev, E., Goleva, E., Bronova, I., Dyjack, N., Rios, C., Jung, J., Taylor, P., Jeong, M., Hall, C.F., Richers, B.N., Norquest, K.A., Zheng, T., Seibold, M.A., and Leung, D.Y. (2018) Lipid abnormalities in atopic skin are driven by type 2 cytokines. *JCI Insight* **3**: e98006.
- Bergboer, J.G., Tjabringa, G.S., Kamsteeg, M., van Vlijmen-Willems, I.M., Rodijk-Olthuis, D., Jansen, P.A., Thuret, J.Y., Narita, M., Ishida-Yamamoto, A., Zeeuwen, P.L., and Schalkwijk, J. (2011) Psoriasis risk genes of the late cornified envelope-3 group are distinctly expressed compared with genes of other LCE groups. *Am J Pathol* **178**: 1470-1477.
- Berube, B.J., and Bubeck-Wardenburg, J. (2013) *Staphylococcus aureus* alpha-toxin: nearly a century of intrigue. *Toxins (Basel)* **5**: 1140-1166.
- Besier, S., Ludwig, A., Brade, V., and Wichelhaus, T.A. (2003) Molecular analysis of fusidic acid resistance in *Staphylococcus aureus*. *Mol Microbiol* **47**: 463-469.
- Bieber, T. (2008) Atopic dermatitis. *N Engl J Med* **358**: 1483-1494.
- Bingham, R.J., Rudino-Pinera, E., Meenan, N.A., Schwarz-Linek, U., Turkenburg, J.P., Hook, M., Garman, E.F., and Potts, J.R. (2008) Crystal structures of fibronectin-binding sites from *Staphylococcus aureus* FnBPA in complex with fibronectin domains. *Proc Natl Acad Sci U S A* **105**: 12254-12258.
- Bobrovskyy, M., Willing, S.E., Schneewind, O., and Missiakas, D. (2018) EssH peptidoglycan hydrolase enables *Staphylococcus aureus* type VII secretion across the bacterial cell wall envelope. *J Bacteriol* **200**: e00268-18.
- Boguniewicz, M., and Leung, D.Y. (2011) Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* **242**: 233-246.

- Bohn, C., Rigoulay, C., Chabelskaya, S., Sharma, C.M., Marchais, A., Skorski, P., Borezee-Durant, E., Barbet, R., Jacquet, E., Jacq, A., Gautheret, D., Felden, B., Vogel, J., and Bouloc, P. (2010) Experimental discovery of small RNAs in *Staphylococcus aureus* reveals a riboregulator of central metabolism. *Nucleic Acids Res* **38**: 6620-6636.
- Bowden, M.G., Chen, W., Singvall, J., Xu, Y., Peacock, S.J., Valtulina, V., Speziale, P., and Hook, M. (2005) Identification and preliminary characterization of cell-wall-anchored proteins of *Staphylococcus epidermidis*. *Microbiology* **151**: 1453-1464.
- Bowden, M.G., Heuck, A.P., Ponnuraj, K., Kolosova, E., Choe, D., Gurusiddappa, S., Narayana, S.V., Johnson, A.E., and Hook, M. (2008) Evidence for the "dock, lock, and latch" ligand binding mechanism of the staphylococcal microbial surface component recognizing adhesive matrix molecules (MSCRAMM) SdrG. *J Biol Chem* **283**: 638-647.
- Brauweiler, A.M., Goleva, E., and Leung, D.Y.M. (2014) Th2 cytokines increase *Staphylococcus aureus* alpha toxin-induced keratinocyte death through the signal transducer and activator of transcription 6 (STAT6). *J Invest Dermatol* **134**: 2114-2121.
- Brown, S., Santa Maria, J.P., Jr., and Walker, S. (2013) Wall teichoic acids of gram-positive bacteria. *Annu Rev Microbiol* **67**: 313-336.
- Brown, S.J., Kroboth, K., Sandilands, A., Campbell, L.E., Pohler, E., Kezic, S., Cordell, H.J., McLean, W.H., and Irvine, A.D. (2012) Intragenic copy number variation within filaggrin contributes to the risk of atopic dermatitis with a dose-dependent effect. *J Invest Dermatol* **132**: 98-104.
- Burke, F.M., Di Poto, A., Speziale, P., and Foster, T.J. (2011) The A domain of fibronectin-binding protein B of *Staphylococcus aureus* contains a novel fibronectin binding site. *Febs j* **278**: 2359-2371.
- Burke, F.M., McCormack, N., Rindi, S., Speziale, P., and Foster, T.J. (2010) Fibronectin-binding protein B variation in *Staphylococcus aureus*. *BMC Microbiol* **10**: e00160.
- Burts, M.L., DeDent, A.C., and Missiakas, D.M. (2008) EsaC substrate for the ESAT-6 secretion pathway and its role in persistent infections of *Staphylococcus aureus*. *Mol Microbiol* **69**: 736-746.
- Burts, M.L., Williams, W.A., DeBord, K., and Missiakas, D.M. (2005) EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. *Proc Natl Acad Sci U S A* **102**: 1169-1174.
- Bycroft, B.W. (1985) The Molecular Basis for the Mode of Action of Beta-Lactam Antibiotics and Mechanisms of Resistance | SpringerLink. *Pharmaceutical Research* **2**: 3-14.
- Byrd, A.L., Belkaid, Y., and Segre, J.A. (2018) The human skin microbiome. *Nat Rev Microbiol* **16**: 143-155.
- Byrd, A.L., Deming, C., Cassidy, S.K.B., Harrison, O.J., Ng, W.I., Conlan, S., Belkaid, Y., Segre, J.A., and Kong, H.H. (2017) *Staphylococcus aureus*

- and *Staphylococcus epidermidis* strain diversity underlying pediatric atopic dermatitis. *Sci Transl Med* **9**: e04651.
- Callard, R.E., and Harper, J.I. (2007) The skin barrier, atopic dermatitis and allergy: a role for Langerhans cells? *Trends Immunol* **28**: 294-298.
- Candi, E., Schmidt, R., and Melino, G. (2005) The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol* **6**: 328-340.
- Cao, Z., Casabona, M.G., Kneuper, H., Chalmers, J.D., and Palmer, T. (2016) The Ess/Type VII secretion system of *Staphylococcus aureus* secretes a nuclease toxin that targets competitor bacteria. *Nat Microbiol* **2**: 16183.
- Casabona, M.G., Buchanan, G., Zoltner, M., Harkins, C.P., Holden, M.T.G., and Palmer, T. (2017a) Functional analysis of the EsaB component of the *Staphylococcus aureus* Type VII secretion system. *Microbiology* **163**: 1851-1863.
- Casabona, M.G., Kneuper, H., Alferes de Lima, D., Harkins, C.P., Zoltner, M., Hjerde, E., Holden, M.T.G., and Palmer, T. (2017b) Haem-iron plays a key role in the regulation of the Ess/type VII secretion system of *Staphylococcus aureus* RN6390. *Microbiology* **163**: 1839-1850.
- Caubet, C., Jonca, N., Brattsand, M., Guerrin, M., Bernard, D., Schmidt, R., Egelrud, T., Simon, M., and Serre, G. (2004a) Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. *J Invest Dermatol* **122**: 1235-1244.
- Caubet, C., Jonca, N., Lopez, F., Esteve, J.P., Simon, M., and Serre, G. (2004b) Homo-oligomerization of human corneodesmosin is mediated by its N-terminal glycine loop domain. *J Invest Dermatol* **122**: 747-754.
- Chambers, H.F. (1997) Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin Microbiol Rev* **10**: 781-791.
- Chamcheu, J.C., Siddiqui, I.A., Syed, D.N., Adhami, V.M., Liovic, M., and Mukhtar, H. (2011) Keratin Gene Mutations in Disorders of Human Skin and its Appendages. *Arch Biochem Biophys* **508**: 123-137.
- Chan, A., and Mauro, T. (2011) Acidification in the epidermis and the role of secretory phospholipases. *Dermatoendocrinol* **3**: 84-90.
- Cheng, A.G., Kim, H.K., Burts, M.L., Krausz, T., Schneewind, O., and Missiakas, D.M. (2009) Genetic requirements for *Staphylococcus aureus* abscess formation and persistence in host tissues. *Faseb j* **23**: 3393-3404.
- Chieosilapatham, P., Ogawa, H., and Niyonsaba, F. (2017) Current insights into the role of human beta-defensins in atopic dermatitis. *Clin Exp Immunol* **190**: 155-166.
- Cho, S.H., Strickland, I., Boguniewicz, M., and Leung, D.Y. (2001) Fibronectin and fibrinogen contribute to the enhanced binding of *Staphylococcus aureus* to atopic skin. *J Allergy Clin Immunol* **108**: 269-274.

- Cianferoni, A., and Spergel, J. (2014) The importance of TSLP in allergic disease and its role as a potential therapeutic target. *Expert Rev Clin Immunol* **10**: 1463-1474.
- Clancy, K.W., Melvin, J.A., and McCafferty, D.G. (2010) Sortase Transpeptidases: Insights into Mechanism, Substrate Specificity and Inhibition. *Biopolymers* **94**: 385-396.
- Clarke, S.R., Brummell, K.J., Horsburgh, M.J., McDowell, P.W., Mohamad, S.A., Stapleton, M.R., Acevedo, J., Read, R.C., Day, N.P., Peacock, S.J., Mond, J.J., Kokai-Kun, J.F., and Foster, S.J. (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, S.R., and Foster, S.J. (2008) IsdA protects *Staphylococcus aureus* against the bactericidal protease activity of apolactoferrin. *Infect Immun* **76**: 1518-1526.
- Clarke, S.R., Mohamed, R., Bian, L., Routh, A.F., Kokai-Kun, J.F., Mond, J.J., Tarkowski, A., and Foster, S.J. (2007) The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. *Cell Host Microbe* **1**: 199-212.
- Classen, A., Kalali, B.N., Schnopp, C., Andres, C., Aguilar-Pimentel, J.A., Ring, J., Ollert, M., and 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.
- Clausen, M.L., Edslev, S.M., Andersen, P.S., Clemmensen, K., Kroghfelt, K.A., and Agner, T. (2017) *Staphylococcus aureus* colonization in atopic eczema and its association with filaggrin gene mutations. *Br J Dermatol* **177**: 1394-1400.
- Cogen, A.L., Yamasaki, K., Sanchez, K.M., Dorschner, R.A., Lai, Y., MacLeod, D.T., Torpey, J.W., Otto, M., Nizet, V., Kim, J.E., and Gallo, R.L. (2010) Selective antimicrobial action is provided by phenol-soluble modulins derived from *Staphylococcus epidermidis*, a normal resident of the skin. *J Invest Dermatol* **130**: 192-200.
- Cole, A.M., Tahk, S., Oren, A., Yoshioka, D., Kim, Y.H., Park, A., and Ganz, T. (2001) Determinants of *Staphylococcus aureus* nasal carriage. *Clin Diagn Lab Immunol* **8**: 1064-1069.
- Collen, D. (1998) Staphylokinase: a potent, uniquely fibrin-selective thrombolytic agent. *Nat Med* **4**: 279-284.
- Cornelissen, C., Marquardt, Y., Czaja, K., Wenzel, J., Frank, J., Luscher-Firzlaff, J., Luscher, B., and Baron, J.M. (2012) IL-31 regulates differentiation and filaggrin expression in human organotypic skin models. *J Allergy Clin Immunol* **129**: 426-433.
- Corrigan, R.M., Abbott, J.C., Burhenne, H., Kaever, V., and 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, R.M., and Foster, T.J. (2009) An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. *Plasmid* **61**: 126-129.
- Corrigan, R.M., Miajlovic, H., and Foster, T.J. (2009) Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. *BMC Microbiol* **9**: 22.
- Corrigan, R.M., Rigby, D., Handley, P., and Foster, T.J. (2007) The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. *Microbiology* **153**: 2435-2446.
- Corvaglia, A.R., Francois, P., Hernandez, D., Perron, K., Linder, P., and 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.
- Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W., and Gotz, F. (1999) The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect Immun* **67**: 5427-5433.
- Cusumano, C.K., Pinkner, J.S., Han, Z., Greene, S.E., Ford, B.A., Crowley, J.R., Henderson, J.P., Janetka, J.W., and Hultgren, S.J. (2011) Treatment and Prevention of Urinary Tract Infection with Orally Active FimH Inhibitors. *Sci Transl Med* **3**: 109ra115.
- Dale, B.A., Presland, R.B., Lewis, S.P., Underwood, R.A., and Fleckman, P. (1997) Transient expression of epidermal filaggrin in cultured cells causes collapse of intermediate filament networks with alteration of cell shape and nuclear integrity. *J Invest Dermatol* **108**: 179-187.
- Dastgheyb, S., Parvizi, J., Shapiro, I.M., Hickok, N.J., and Otto, M. (2015) Effect of biofilms on recalcitrance of staphylococcal joint infection to antibiotic treatment. *J Infect Dis* **211**: 641-650.
- de Cid, R., Riveira-Munoz, E., Zeeuwen, P.L., Robarge, J., Liao, W., Dannhauser, E.N., Giardina, E., Stuart, P.E., Nair, R., Helms, C., Escaramís, G., Ballana, E., Martín-Ezquerro, G., den Heijer, M., Kamsteeg, M., Joosten, I., Eichler, E.E., Lázaro, C., Pujol, R.M., Armengol, L., Abecasis, G., Elder, J.T., Novelli, G., Armour, J.A., Kwok, P., Bowcock, A., Schalkwijk, J., and Estivill, X. (2009) Deletion of the late cornified envelope (LCE) 3B and 3C genes as a susceptibility factor for psoriasis. *Nat Genet* **41**: 211-215.
- De Vuyst, E., Salmon, M., Evrard, C., Lambert de Rouvroit, C., and Poumay, Y. (2017) Atopic Dermatitis Studies through In Vitro Models. *Front Med (Lausanne)* **4**: 119.
- De, Y., Chen, Q., Schmidt, A.P., Anderson, G.M., Wang, J.M., Wooters, J., Oppenheim, J.J., and Chertov, O. (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* **192**: 1069-1074.

- Deckers, I.A., McLean, S., Linssen, S., Mommers, M., van Schayck, C.P., and Sheikh, A. (2012) Investigating international time trends in the incidence and prevalence of atopic eczema 1990-2010: a systematic review of epidemiological studies. *PLoS One* **7**: e39803.
- 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-Å resolution. *Biochemistry* **20**: 2361-2370.
- Deivanayagam, C.C., Wann, E.R., Chen, W., Carson, M., Rajashankar, K.R., Höök, M., and Narayana, S.V., (2002) A novel variant of the immunoglobulin fold in surface adhesins of *Staphylococcus aureus*: crystal structure of the fibrinogen-binding MSCRAMM, clumping factor A. In: *EMBO J.* pp. 6660-6672.
- DeLeo, F.R., Kennedy, A.D., Chen, L., Wardenburg, J.B., Kobayashi, S.D., Mathema, B., Braughton, K.R., Whitney, A.R., Villaruz, A.E., Martens, C.A., Porcella, S.F., McGavin, M.J., Otto, M., Musser, J.M., and Kreiswirth, B.N., (2011) Molecular differentiation of historic phage-type 80/81 and contemporary epidemic *Staphylococcus aureus*. In: *Proc Natl Acad Sci U S A.* pp. 18091-18096.
- Denecker, G., Hoste, E., Gilbert, B., Hocheplied, T., Ovaere, P., Lippens, S., Van den Broecke, C., Van Damme, P., D'Herde, K., Hachem, J.P., Borgonie, G., Presland, R.B., Schoonjans, L., Libert, C., Vandekerckhove, J., Gevaert, K., Vandenabeele, P., and Declercq, W. (2007) Caspase-14 protects against epidermal UVB photodamage and water loss. *Nat Cell Biol* **9**: 666-674.
- Diep, B.A., and Otto, M. (2008) The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol* **16**: 361-369.
- Diep, B.A., Stone, G.G., Basuino, L., Graber, C.J., Miller, A., des Etages, S.A., Jones, A., Palazzolo-Ballance, A.M., Perdreau-Remington, F., Sensabaugh, G.F., DeLeo, F.R., and Chambers, H.F. (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.
- Drucker, A.M., Wang, A.R., Li, W.Q., Sevetson, E., Block, J.K., and Qureshi, A.A. (2017) The Burden of Atopic Dermatitis: Summary of a Report for the National Eczema Association. *J Invest Dermatol* **137**: 26-30.
- Dufrene, Y.F. (2014) Atomic force microscopy in microbiology: new structural and functional insights into the microbial cell surface. *MBio* **5**: e01363-01314.
- Duthie, E.S., and Lorenz, L.L. (1952) Staphylococcal coagulase; mode of action and antigenicity. *J Gen Microbiol* **6**: 95-107.
- Dziewanowska, K., Patti, J.M., Deobald, C.F., Bayles, K.W., Trumble, W.R., and Bohach, G.A. (1999) Fibronectin binding protein and host cell

- tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect Immun* **67**: 4673-4678.
- Eberlein-Konig, B., Schafer, T., Huss-Marp, J., Darsow, U., Mohrenschlager, M., Herbert, O., Abeck, D., Kramer, U., Behrendt, H., and Ring, J. (2000) Skin surface pH, stratum corneum hydration, trans-epidermal water loss and skin roughness related to atopic eczema and skin dryness in a population of primary school children. *Acta Derm Venereol* **80**: 188-191.
- Eckhart, L., Lippens, S., Tschachler, E., and Declercq, W. (2013) Cell death by cornification. *Biochim Biophys Acta* **1833**: 3471-3480.
- Edslev, S.M., Clausen, M.L., Agner, T., Stegger, M., and Andersen, P.S. (2018) Genomic analysis reveals different mechanisms of fusidic acid resistance in *Staphylococcus aureus* from Danish atopic dermatitis patients. *J Antimicrob Chemother* **73**: 856-861.
- Edwards, A.M., Potter, U., Meenan, N.A., Potts, J.R., and Massey, R.C. (2011) *Staphylococcus aureus* keratinocyte invasion is dependent upon multiple high-affinity fibronectin-binding repeats within FnBPA. *PLoS One* **6**: e18899.
- Edwards, A.M., Potts, J.R., Josefsson, E., and Massey, R.C. (2010) *Staphylococcus aureus* host cell invasion and virulence in sepsis is facilitated by the multiple repeats within FnBPA. *PLoS Pathog* **6**: e1000964.
- Eichenfield, L.F., Tom, W.L., Berger, T.G., Krol, A., Paller, A.S., Schwarzenberger, K., Bergman, J.N., Chamlin, S.L., Cohen, D.E., Cooper, K.D., Cordoro, K.M., Davis, D.M., Feldman, S.R., Hanifin, J.M., Margolis, D.J., Silverman, R.A., Simpson, E.L., Williams, H.C., Elmets, C.A., Block, J., Harrod, C.G., Smith Begolka, W., and Sidbury, R. (2014) Guidelines of care for the management of atopic dermatitis: section 2. Management and treatment of atopic dermatitis with topical therapies. *J Am Acad Dermatol* **71**: 116-132.
- Emonts, M., Uitterlinden, A.G., Nouwen, J.L., Kardys, I., Maat, M.P., Melles, D.C., Witteman, J., Jong, P.T., Verbrugh, H.A., Hofman, A., Hermans, P.W., and Belkum, A. (2008) Host polymorphisms in interleukin 4, complement factor H, and C-reactive protein associated with nasal carriage of *Staphylococcus aureus* and occurrence of boils. *J Infect Dis* **197**: 1244-1253.
- Enright, M.C., Day, N.P., Davies, C.E., Peacock, S.J., and Spratt, B.G. (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* **38**: 1008-1015.
- Ewald, D.A., Noda, S., Oliva, M., Litman, T., Nakajima, S., Li, X., Xu, H., Workman, C.T., Scheipers, P., Svitacheva, N., Labuda, T., Krueger, J.G., Suarez-Farinas, M., Kabashima, K., and Guttman-Yassky, E. (2017) Major differences between human atopic dermatitis and murine models, as determined by using global transcriptomic profiling. *J Allergy Clin Immunol* **139**: 562-571.

- Ewing, C.I., Ashcroft, C., Gibbs, A.C., Jones, G.A., Connor, P.J., and David, T.J. (1998) Flucloxacillin in the treatment of atopic dermatitis. *Br J Dermatol* **138**: 1022-1029.
- Fahlberg, W.J., and Marston, J. (1960) Coagulase production by *Staphylococcus aureus*. I. Factors influencing coagulase production. *J Infect Dis* **106**: 111-115.
- Falugi, F., Kim, H.K., Missiakas, D.M., Schneewind, O., and Gilmore, M.S. (2013) Role of Protein A in the Evasion of Host Adaptive Immune Responses by *Staphylococcus aureus*. *MBio* **4**: e00575-13.
- Feil, E.J., Cooper, J.E., Grundmann, H., Robinson, D.A., Enright, M.C., Berendt, T., Peacock, S.J., Smith, J.M., Murphy, M., Spratt, B.G., Moore, C.E., and Day, N.P. (2003) How clonal is *Staphylococcus aureus*? *J Bacteriol* **185**: 3307-3316.
- Feuillie, C., Formosa-Dague, C., Hays, L.M., Vervaeck, O., Derclaye, S., Brennan, M.P., Foster, T.J., Geoghegan, J.A., and Dufrene, Y.F. (2017) Molecular interactions and inhibition of the staphylococcal biofilm-forming protein SdrC. *Proc Natl Acad Sci U S A* **114**: 3738-3743.
- Feuillie, C., Vitry, P., McAleer, M.A., Kezic, S., Irvine, A.D., Geoghegan, J.A., Dufrêne, Y.F., Fleiszig, S.M.J., and Parsek, M.R. (2018) Adhesion of *Staphylococcus aureus* to Corneocytes from Atopic Dermatitis Patients Is Controlled by Natural Moisturizing Factor Levels. *MBio* **9**: e01184-18.
- Fleury, O.M., McAleer, M.A., Feuillie, C., Formosa-Dague, C., Sansevere, E., Bennett, D.E., Towell, A.M., McLean, W.H.I., Kezic, S., Robinson, D.A., Fallon, P.G., Foster, T.J., Dufrene, Y.F., Irvine, A.D., and Geoghegan, J.A. (2017) Clumping Factor B Promotes Adherence of *Staphylococcus aureus* to Corneocytes in Atopic Dermatitis. *Infect Immun* **85**: e00994-16.
- Flick, M.J., Du, X., Prasad, J.M., Raghu, H., Palumbo, J.S., Smeds, E., Hook, M., and Degen, J.L. (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.
- Formosa, C., Schiavone, M., Boisrame, A., Richard, M.L., Duval, R.E., and Dague, E. (2015) Multiparametric imaging of adhesive nanodomains at the surface of *Candida albicans* by atomic force microscopy. *Nanomedicine* **11**: 57-65.
- Forsgren, A., and Nordstrom, K. (1974) Protein A from *Staphylococcus aureus*: the biological significance of its reaction with IgG. *Ann N Y Acad Sci* **236**: 252-266.
- Foster, T., (1996) *Staphylococcus*. In *Medical Microbiology 4th edition*. Baron, S. (eds). University of Texas Medical Branch, Texas. Chapter 12: 1-22.
- Foster, T.J. (2009) Colonization and infection of the human host by staphylococci: adhesion, survival and immune evasion. *Vet Dermatol* **20**: 456-470.
- Foster, T.J. (2016) The remarkably multifunctional fibronectin binding proteins of *Staphylococcus aureus*. *Eur J Clin Microbiol Infect Dis* **35**: 1923-1931.

- Foster, T.J., Geoghegan, J.A., Ganesh, V.K., and Hook, M. (2014) Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat Rev Microbiol* **12**: 49-62.
- Foster, T.J., and Hook, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol* **6**: 484-488.
- Fowler, T., Wann, E.R., Joh, D., Johansson, S., Foster, T.J., and Hook, M. (2000) Cellular invasion by *Staphylococcus aureus* involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. *Eur J Cell Biol* **79**: 672-679.
- Gallo, R.L., and Hooper, L.V. (2012) Epithelial antimicrobial defence of the skin and intestine. *Nat Rev Immunol* **12**: 503-516.
- Ganesh, V.K., Barbu, E.M., Deivanayagam, C.C., Le, B., Anderson, A.S., Matsuka, Y.V., Lin, S.L., Foster, T.J., Narayana, S.V., and Hook, M. (2011) Structural and biochemical characterization of *Staphylococcus aureus* clumping factor B/ligand interactions. *J Biol Chem* **286**: 25963-25972.
- Ganesh, V.K., Rivera, J.J., Smeds, E., Ko, Y.P., Bowden, M.G., Wann, E.R., Gurusiddappa, S., Fitzgerald, J.R., and 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.
- Geoghegan, J.A., Corrigan, R.M., Gruszka, D.T., Speziale, P., O'Gara, J.P., Potts, J.R., and Foster, T.J. (2010) Role of surface protein SasG in biofilm formation by *Staphylococcus aureus*. *J Bacteriol* **192**: 5663-5673.
- Geoghegan, J.A., Monk, I.R., O'Gara, J.P., and Foster, T.J. (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.
- Geoghegan, J.A., Smith, E.J., Speziale, P., and Foster, T.J. (2009) *Staphylococcus pseudintermedius* expresses surface proteins that closely resemble those from *Staphylococcus aureus*. *Vet Microbiol* **138**: 345-352.
- Ghuysen, J.M., and Strominger, J.L. (1963) STRUCTURE OF THE CELL WALL OF *STAPHYLOCOCCUS AUREUS*, STRAIN COPENHAGEN. II. SEPARATION AND STRUCTURE OF DISACCHARIDES. *Biochemistry* **2**: 1119-1125.
- Ghuysen, J.M., Tipper, D.J., and Strominger, J.L. (1965) STRUCTURE OF THE CELL WALL OF *STAPHYLOCOCCUS AUREUS*, STRAIN COPENHAGEN. IV. THE TEICHOIC ACID-GLYCOPEPTIDE COMPLEX. *Biochemistry* **4**: 474-485.
- Gilani, S.J., Gonzalez, M., Hussain, I., Finlay, A.Y., and Patel, G.K. (2005) *Staphylococcus aureus* re-colonization in atopic dermatitis: beyond the skin. *Clin Exp Dermatol* **30**: 10-13.
- Gomez, M.I., Lee, A., Reddy, B., Muir, A., Soong, G., Pitt, A., Cheung, A., and Prince, A. (2004) *Staphylococcus aureus* protein A induces airway

- epithelial inflammatory responses by activating TNFR1. *Nat Med* **10**: 842-848.
- Gomez, M.I., O'Seaghdha, M., Magargee, M., Foster, T.J., and Prince, A.S. (2006) *Staphylococcus aureus* protein A activates TNFR1 signaling through conserved IgG binding domains. *J Biol Chem* **281**: 20190-20196.
- Gomez, M.I., Seaghdha, M.O., and Prince, A.S. (2007) *Staphylococcus aureus* protein A activates TACE through EGFR-dependent signaling. *Embo j* **26**: 701-709.
- Gooderham, M.J., Hong, H.C., Eshtiaghi, P., and Papp, K.A. (2018) Dupilumab: A review of its use in the treatment of atopic dermatitis. *J Am Acad Dermatol* **78**: S28-s36.
- Goodyear, C.S., and Silverman, G.J. (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.
- Greene, C., McDevitt, D., Francois, P., Vaudaux, P.E., Lew, D.P., and Foster, T.J. (1995) Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of fnb genes. *Mol Microbiol* **17**: 1143-1152.
- Grice, E.A., Kong, H.H., Conlan, S., Deming, C.B., Davis, J., Young, A.C., Bouffard, G.G., Blakesley, R.W., Murray, P.R., Green, E.D., Turner, M.L., and Segre, J.A. (2009) Topographical and Temporal Diversity of the Human Skin Microbiome. *Science* **324**: 1190-1192.
- Grigg, J.C., Ukpabi, G., Gaudin, C.F., and Murphy, M.E. (2010) Structural biology of heme binding in the *Staphylococcus aureus* Isd system. *J Inorg Biochem* **104**: 341-348.
- Grigg, J.C., Vermeiren, C.L., Heinrichs, D.E., and Murphy, M.E. (2007) Haem recognition by a *Staphylococcus aureus* NEAT domain. *Mol Microbiol* **63**: 139-149.
- Gruber, R., Elias, P.M., Crumrine, D., Lin, T.K., Brandner, J.M., Hachem, J.P., Presland, R.B., Fleckman, P., Janecke, A.R., Sandilands, A., McLean, W.H., Fritsch, P.O., Mildner, M., Tschachler, E., and Schmuth, M. (2011) Filaggrin genotype in ichthyosis vulgaris predicts abnormalities in epidermal structure and function. *Am J Pathol* **178**: 2252-2263.
- Grundmeier, M., Hussain, M., Becker, P., Heilmann, C., Peters, G., and Sinha, B., (2004) Truncation of Fibronectin-Binding Proteins in *Staphylococcus aureus* Strain Newman Leads to Deficient Adherence and Host Cell Invasion Due to Loss of the Cell Wall Anchor Function. In: *Infect Immun*. pp. 7155-7163.
- Guerrin, M., Simon, M., Montezin, M., Haftek, M., Vincent, C., and Serre, G. (1998) Expression cloning of human corneodesmosin proves its identity with the product of the S gene and allows improved characterization of its processing during keratinocyte differentiation. *J Biol Chem* **273**: 22640-22647.

- Gutierrez, J.A., Crowley, P.J., Brown, D.P., Hillman, J.D., Youngman, P., and Bleiweis, A.S. (1996) Insertional mutagenesis and recovery of interrupted genes of *Streptococcus mutans* by using transposon Tn917: preliminary characterization of mutants displaying acid sensitivity and nutritional requirements. *J Bacteriol* **178**: 4166-4175.
- Hair, P.S., Echague, C.G., Sholl, A.M., Watkins, J.A., Geoghegan, J.A., Foster, T.J., and Cunnion, K.M. (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, P.S., Foley, C.K., Krishna, N.K., Nyalwidhe, J.O., Geoghegan, J.A., Foster, T.J., and Cunnion, K.M. (2013) Complement regulator C4BP binds to *Staphylococcus aureus* surface proteins SdrE and Bbp inhibiting bacterial opsonization and killing. *Results Immunol* **3**: 114-121.
- Hair, P.S., Ward, M.D., Semmes, O.J., Foster, T.J., and Cunnion, K.M. (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.
- Harkins, C.P., Pettigrew, K.A., Oravcova, K., Gardner, J., Hearn, R.M.R., Rice, D., Mather, A.E., Parkhill, J., Brown, S.J., Proby, C.M., and Holden, M.T.G. (2018) The Microevolution and Epidemiology of *Staphylococcus aureus* Colonization during Atopic Eczema Disease Flare. *J Invest Dermatol* **138**: 336-343.
- Harkins, C.P., Pichon, B., Doumith, M., Parkhill, J., Westh, H., Tomasz, A., de Lencastre, H., Bentley, S.D., Kearns, A.M., and Holden, M.T.G. (2017) Methicillin-resistant *Staphylococcus aureus* emerged long before the introduction of methicillin into clinical practice. *Genome Biol* **18**: 130.
- Hartford, O., O'Brien, L., Schofield, K., Wells, J., and Foster, T.J. (2001) The Fbe (SdrG) protein of *Staphylococcus epidermidis* HB promotes bacterial adherence to fibrinogen. *Microbiology* **147**: 2545-2552.
- Hata, T.R., Kotel, P., Boguniewicz, M., Taylor, P., Paik, A., Jackson, M., Nguyen, M., Kabigting, F., Miller, J., Gerber, M., Zaccaro, D., Armstrong, B., Dorschner, R., Leung, D.Y., and Gallo, R.L. (2010) History of eczema herpeticum is associated with the inability to induce human beta-defensin (HBD)-2, HBD-3 and cathelicidin in the skin of patients with atopic dermatitis. *Br J Dermatol* **163**: 659-661.
- Hatano, Y., Adachi, Y., Elias, P.M., Crumrine, D., Sakai, T., Kurahashi, R., Katagiri, K., and Fujiwara, S. (2013) The Th2 cytokine, interleukin-4, abrogates the cohesion of normal stratum corneum in mice: implications for pathogenesis of atopic dermatitis. *Exp Dermatol* **22**: 30-35.
- Herman, P., El-Kirat-Chatel, S., Beaussart, A., Geoghegan, J.A., Foster, T.J., and Dufrene, Y.F. (2014) The binding force of the staphylococcal adhesin SdrG is remarkably strong. *Mol Microbiol* **93**: 356-368.
- Herman-Bausier, P., El-Kirat-Chatel, S., Foster, T.J., Geoghegan, J.A., and Dufrene, Y.F. (2015) *Staphylococcus aureus* Fibronectin-Binding Protein

A Mediates Cell-Cell Adhesion through Low-Affinity Homophilic Bonds. *MBio* **6**: e00413-00415.

- Herman-Bausier, P., Labate, C., Towell, A.M., Derclaye, S., Geoghegan, J.A., and Dufrene, Y.F. (2018) *Staphylococcus aureus* clumping factor A is a force-sensitive molecular switch that activates bacterial adhesion. *Proc Natl Acad Sci U S A* **115**: 5564-5569.
- Hibbing, M.E., Fuqua, C., Parsek, M.R., and Peterson, S.B. (2010) Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Microbiol* **8**: 15-25.
- Higgins, J., Loughman, A., van Kessel, K.P., van Strijp, J.A., and Foster, T.J. (2006) Clumping factor A of *Staphylococcus aureus* inhibits phagocytosis by human polymorphonuclear leucocytes. *FEMS Microbiol Lett* **258**: 290-296.
- Hohl, D., Mehrel, T., Lichti, U., Turner, M.L., Roop, D.R., and Steinert, P.M. (1991) Characterization of human loricrin. Structure and function of a new class of epidermal cell envelope proteins. *J Biol Chem* **266**: 6626-6636.
- Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R., Barron, A., Bason, N., Bentley, S.D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K.D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall, K., Ormond, D., Quail, M.A., Rabinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrell, B.G., Spratt, B.G., and Parkhill, J. (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* **101**: 9786-9791.
- Holtfreter, S., and Broker, B.M. (2005) Staphylococcal superantigens: do they play a role in sepsis? *Arch Immunol Ther Exp (Warsz)* **53**: 13-27.
- Holtfreter, S., Grumann, D., Schmudde, M., Nguyen, H.T., Eichler, P., Strommenger, B., Kopron, K., Kolata, J., Giedrys-Kalemba, S., Steinmetz, I., Witte, W., and Broker, B.M. (2007) Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *J Clin Microbiol* **45**: 2669-2680.
- Homerova, D., Bischoff, M., Dumolin, A., and Kormanec, J. (2004) Optimization of a two-plasmid system for the identification of promoters recognized by RNA polymerase containing *Staphylococcus aureus* alternative sigma factor sigmaB. *FEMS Microbiol Lett* **232**: 173-179.
- Horn, J., Stelzner, K., Rudel, T., and Fraunholz, M. (2018) Inside job: *Staphylococcus aureus* host-pathogen interactions. *Int J Med Microbiol* **308**: 607-624.
- Horsburgh, M.J., Aish, J.L., White, I.J., Shaw, L., Lithgow, J.K., and Foster, S.J. (2002) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* **184**: 5457-5467.

- Howell, M.D., Gallo, R.L., Boguniewicz, M., Jones, J.F., Wong, C., Streib, J.E., and Leung, D.Y. (2006) Cytokine milieu of atopic dermatitis skin subverts the innate immune response to vaccinia virus. *Immunity* **24**: 341-348.
- Howell, M.D., Kim, B.E., Gao, P., Grant, A.V., Boguniewicz, M., DeBenedetto, A., Schneider, L., Beck, L.A., Barnes, K.C., and Leung, D.Y. (2007a) Cytokine Modulation of AD Filaggrin Skin Expression. *J Allergy Clin Immunol* **120**: 150-155.
- Howell, M.D., Kim, B.E., Gao, P., Grant, A.V., Boguniewicz, M., DeBenedetto, A., Schneider, L., Beck, L.A., Barnes, K.C., and Leung, D.Y. (2009) Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* **124**: R7-r12.
- Howell, M.D., Kim, B.E., Gao, P., Grant, A.V., Boguniewicz, M., DeBenedetto, A., Schneider, L., Beck, L.A., Barnes, K.C., and Leung, D.Y.M. (2007b) Cytokine Modulation of AD Filaggrin Skin Expression. *The Journal of allergy and clinical immunology* **120**: 150-155.
- Huang, C.M., Lara-Corrales, I., and Pope, E. (2018) Effects of Vitamin D levels and supplementation on atopic dermatitis: A systematic review. *Pediatr Dermatol* **35**: 754-760.
- Huang, R., Ning, H., Shen, M., Li, J., Zhang, J., and Chen, X. (2017) Probiotics for the Treatment of Atopic Dermatitis in Children: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Front Cell Infect Microbiol* **7**: 392.
- Hvid, M., Johansen, C., Deleuran, B., Kemp, K., Deleuran, M., and Vestergaard, C. (2011) Regulation of caspase 14 expression in keratinocytes by inflammatory cytokines--a possible link between reduced skin barrier function and inflammation? *Exp Dermatol* **20**: 633-636.
- Igawa, S., Kishibe, M., Honma, M., Murakami, M., Mizuno, Y., Suga, Y., Seishima, M., Ohguchi, Y., Akiyama, M., Hirose, K., Ishida-Yamamoto, A., and Iizuka, H. (2013) Aberrant distribution patterns of corneodesmosomal components of tape-stripped corneocytes in atopic dermatitis and related skin conditions (ichthyosis vulgaris, Netherton syndrome and peeling skin syndrome type B). *J Dermatol Sci* **72**: 54-60.
- Igawa, S., Kishibe, M., Minami-Hori, M., Honma, M., Tsujimura, H., Ishikawa, J., Fujimura, T., Murakami, M., and Ishida-Yamamoto, A. (2017) Incomplete KLK7 Secretion and Upregulated LEKTI Expression Underlie Hyperkeratotic Stratum Corneum in Atopic Dermatitis. *J Invest Dermatol* **137**: 449-456.
- Illi, S., von Mutius, E., Lau, S., Nickel, R., Gruber, C., Niggemann, B., and Wahn, U. (2004) The natural course of atopic dermatitis from birth to age 7 years and the association with asthma. *J Allergy Clin Immunol* **113**: 925-931.
- Ishida-Yamamoto, A., Furio, L., Igawa, S., Honma, M., Tron, E., Malan, V., Murakami, M., and Hovnanian, A. (2014) Inflammatory peeling skin syndrome caused by homozygous genomic deletion in the PSORS1 region encompassing the CDSN gene. *Exp Dermatol* **23**: 60-63.

- Ishida-Yamamoto, A., and Igawa, S. (2015) The biology and regulation of corneodesmosomes. *Cell Tissue Res* **360**: 477-482.
- Ishida-Yamamoto, A., Igawa, S., Kishibe, M., and Honma, M. (2018) Clinical and molecular implications of structural changes to desmosomes and corneodesmosomes. *J Dermatol* **45**: 385-389.
- Ishida-Yamamoto, A., Senshu, T., Eady, R.A., Takahashi, H., Shimizu, H., Akiyama, M., and Iizuka, H. (2002) Sequential reorganization of cornified cell keratin filaments involving filaggrin-mediated compaction and keratin 1 deimination. *J Invest Dermatol* **118**: 282-287.
- Ishii, K., Adachi, T., Yasukawa, J., Suzuki, Y., Hamamoto, H., and Sekimizu, K. (2014) Induction of virulence gene expression in *Staphylococcus aureus* by pulmonary surfactant. *Infect Immun* **82**: 1500-1510.
- Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., Agata, T., and Mizunoe, Y. (2010) *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. *Nature* **465**: 346-349.
- Jackson, B., Tilli, C.M., Hardman, M.J., Avilion, A.A., MacLeod, M.C., Ashcroft, G.S., and Byrne, C. (2005) Late cornified envelope family in differentiating epithelia--response to calcium and ultraviolet irradiation. *J Invest Dermatol* **124**: 1062-1070.
- Jager, F., Kneuper, H., and Palmer, T. (2018) EssC is a specificity determinant for *Staphylococcus aureus* type VII secretion. *Microbiology* **164**: 816-820.
- Jager, F., Zoltner, M., Kneuper, H., Hunter, W.N., and Palmer, T. (2016) Membrane interactions and self-association of components of the Ess/Type VII secretion system of *Staphylococcus aureus*. *FEBS Lett* **590**: 349-357.
- Janssens, M., van Smeden, J., Gooris, G.S., Bras, W., Portale, G., Caspers, P.J., Vreeken, R.J., Hankemeier, T., Kezic, S., Wolterbeek, R., Lavrijsen, A.P., and Bouwstra, J.A. (2012) Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *J Lipid Res* **53**: 2755-2766.
- Jarnik, M., de Viragh, P.A., Scharer, E., Bundman, D., Simon, M.N., Roop, D.R., and Steven, A.C. (2002) Quasi-normal cornified cell envelopes in loricrin knockout mice imply the existence of a loricrin backup system. *J Invest Dermatol* **118**: 102-109.
- Jarraud, S., Lyon, G.J., Figueiredo, A.M., Lina, G., Vandenesch, F., Etienne, J., Muir, T.W., and Novick, R.P. (2000) Exfoliatin-producing strains define a fourth agr specificity group in *Staphylococcus aureus*. *J Bacteriol* **182**: 6517-6522.
- Ji, Y., Zhang, B., Van, S.F., Horn, Warren, P., Woodnutt, G., Burnham, M.K., and Rosenberg, M. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science* **293**: 2266-2269.

- Jonca, N., Guerrin, M., Hadjiolova, K., Caubet, C., Gallinaro, H., Simon, M., and Serre, G. (2002) Corneodesmosin, a component of epidermal corneocyte desmosomes, displays homophilic adhesive properties. *J Biol Chem* **277**: 5024-5029.
- Jonca, N., Leclerc, E.A., Caubet, C., Simon, M., Guerrin, M., and Serre, G. (2011) Corneodesmosomes and corneodesmosin: from the stratum corneum cohesion to the pathophysiology of genodermatoses. *Eur J Dermatol* **21 Suppl 2**: 35-42.
- Jonsson, I.M., Mazmanian, S.K., Schneewind, O., Verdrengh, M., Bremell, T., and 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.
- Jonsson, K., Signas, C., Muller, H.P., and Lindberg, M. (1991) Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterization of the second gene. *Eur J Biochem* **202**: 1041-1048.
- Josefsson, E., Hartford, O., O'Brien, L., Patti, J.M., and Foster, T. (2001) Protection against experimental *Staphylococcus aureus* arthritis by vaccination with clumping factor A, a novel virulence determinant. *J Infect Dis* **184**: 1572-1580.
- Josefsson, E., Higgins, J., Foster, T.J., and Tarkowski, A. (2008) Fibrinogen binding sites P336 and Y338 of clumping factor A are crucial for *Staphylococcus aureus* virulence. *PLoS One* **3**: e2206.
- Jung, M.Y., Chung, J.Y., Lee, H.Y., Park, J., Lee, D.Y., and Yang, J.M. (2015) Antibiotic Susceptibility of *Staphylococcus aureus* in Atopic Dermatitis: Current Prevalence of Methicillin-Resistant *Staphylococcus aureus* in Korea and Treatment Strategies. *Ann Dermatol* **27**: 398-403.
- Kalas, V., Hibbing, M.E., Maddirala, A.R., Chugani, R., Pinkner, J.S., Mydock-McGrane, L.K., Conover, M.S., Janetka, J.W., and Hultgren, S.J. (2018) Structure-based discovery of glycomimetic FmlH ligands as inhibitors of bacterial adhesion during urinary tract infection. *Proc Natl Acad Sci U S A* **115**: E2819-e2828.
- Kamata, Y., Taniguchi, A., Yamamoto, M., Nomura, J., Ishihara, K., Takahara, H., Hibino, T., and Takeda, A. (2009) Neutral cysteine protease bleomycin hydrolase is essential for the breakdown of deiminated filaggrin into amino acids. *J Biol Chem* **284**: 12829-12836.
- Kang, M., Ko, Y.P., Liang, X., Ross, C.L., Liu, Q., Murray, B.E., and 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, R., Katsamba, P.S., Nordin, H., Pol, E., and Myszka, D.G. (2006) Analyzing a kinetic titration series using affinity biosensors. *Anal Biochem* **349**: 136-147.

- Keane, F.M., Loughman, A., Valtulina, V., Brennan, M., Speziale, P., and Foster, T.J. (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.
- Kezic, S., Kemperman, P.M., Koster, E.S., de Jongh, C.M., Thio, H.B., Campbell, L.E., Irvine, A.D., McLean, W.H., Puppels, G.J., and Caspers, P.J. (2008) Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum. *J Invest Dermatol* **128**: 2117-2119.
- Kezic, S., O'Regan, G.M., Yau, N., Sandilands, A., Chen, H., Campbell, L.E., Kroboth, K., Watson, R., Rowland, M., McLean, W.H., and Irvine, A.D. (2011) Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. *Allergy* **66**: 934-940.
- Kiedrowski, M.R., Paharik, A.E., Ackermann, L.W., Shelton, A.U., Singh, S.B., Starner, T.D., and Horswill, A.R. (2016) Development of an in vitro colonization model to investigate *Staphylococcus aureus* interactions with airway epithelia. *Cell Microbiol* **18**: 720-732.
- Kim, B.E., Leung, D.Y., Boguniewicz, M., and Howell, M.D. (2008) Loricrin and involucrin expression is down-regulated by Th2 cytokines through STAT-6. *Clin Immunol* **126**: 332-337.
- Kim, G., and Bae, J.H. (2016) Vitamin D and atopic dermatitis: A systematic review and meta-analysis. *Nutrition* **32**: 913-920.
- Kneuper, H., Cao, Z.P., Twomey, K.B., Zoltner, M., Jager, F., Cargill, J.S., Chalmers, J., van der Kooi-Pol, M.M., van Dijk, J.M., Ryan, R.P., Hunter, W.N., and Palmer, T. (2014) Heterogeneity in ess transcriptional organization and variable contribution of the Ess/Type VII protein secretion system to virulence across closely related *Staphylococcus aureus* strains. *Mol Microbiol* **93**: 928-943.
- Kong, H.H., Oh, J., Deming, C., Conlan, S., Grice, E.A., Beatson, M.A., Nomicos, E., Polley, E.C., Komarow, H.D., Murray, P.R., Turner, M.L., and Segre, J.A. (2012) Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res* **22**: 850-859.
- Korea, C.G., Balsamo, G., Pezzicoli, A., Merakou, C., Tavarini, S., Bagnoli, F., Serruto, D., and Unnikrishnan, M. (2014) Staphylococcal Esx Proteins Modulate Apoptosis and Release of Intracellular *Staphylococcus aureus* during Infection in Epithelial Cells. *Infect Immun* **82**: 4144-4153.
- Krakauer, T., Pradhan, K., and Stiles, B.G. (2016) Staphylococcal Superantigens Spark Host-Mediated Danger Signals. *Front Immunol* **7**: 23.
- Kreiswirth, B.N., Lofdahl, S., Betley, M.J., O'Reilly, M., Schlievert, P.M., Bergdoll, M.S., and Novick, R.P. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* **305**: 709-712.

- Lacey, K.A., Mulcahy, M.E., Towell, A.M., Geoghegan, J.A., and McLoughlin, R.M. (2019) Clumping factor B is an important virulence factor during *Staphylococcus aureus* skin infection and a promising vaccine target. *PLoS Pathog* **15**: e1007713.
- Leclerc, E.A., Huchencq, A., Mattiuzzo, N.R., Metzger, D., Chambon, P., Ghyselinck, N.B., Serre, G., Jonca, N., and Guerrin, M. (2009) Corneodesmosin gene ablation induces lethal skin-barrier disruption and hair-follicle degeneration related to desmosome dysfunction. *J Cell Sci* **122**: 2699-2709.
- Leclercq, R., and Courvalin, P. (2002) Resistance to macrolides and related antibiotics in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* **46**: 2727-2734.
- Lei, M.G., Cue, D., Alba, J., Junecko, J., Graham, J.W., and Lee, C.Y. (2012) A single copy integration vector that integrates at an engineered site on the *Staphylococcus aureus* chromosome. *BMC Res Notes* **5**: e005.
- Leszczynski, J.F., and Rose, G.D. (1986) Loops in globular proteins: a novel category of secondary structure. *Science* **234**: 849-855.
- Leung, D.Y., Boguniewicz, M., Howell, M.D., Nomura, I., and Hamid, Q.A. (2004) New insights into atopic dermatitis. *J Clin Invest* **113**: 651-657.
- Leung, D.Y., Harbeck, R., Bina, P., Reiser, R.F., Yang, E., Norris, D.A., Hanifin, J.M., and Sampson, H.A. (1993) Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis. Evidence for a new group of allergens. *J Clin Invest* **92**: 1374-1380.
- Levy-Nissenbaum, E., Betz, R.C., Frydman, M., Simon, M., Lahat, H., Bakhan, T., Goldman, B., Bygum, A., Pierick, M., Hillmer, A.M., Jonca, N., Toribio, J., Kruse, R., Dewald, G., Cichon, S., Kubisch, C., Guerrin, M., Serre, G., Nothen, M.M., and Pras, E. (2003) Hypotrichosis simplex of the scalp is associated with nonsense mutations in CDSN encoding corneodesmosin. *Nat Genet* **34**: 151-153.
- Leyden, J.J., Marples, R.R., and Kligman, A.M. (1974) *Staphylococcus aureus* in the lesions of atopic dermatitis. *Br J Dermatol* **90**: 525-530.
- Li, M.Z., and Elledge, S.J. (2012) SLIC: a method for sequence- and ligation-independent cloning. *Methods Mol Biol* **852**: 51-59.
- Liang, X., Garcia, B.L., Visai, L., Prabhakaran, S., Meenan, N.A., Potts, J.R., Humphries, M.J., and Hook, M. (2016) Allosteric Regulation of Fibronectin/alpha5beta1 Interaction by Fibronectin-Binding MSCRAMMs. *PLoS One* **11**: e0159118.
- Lindblad-Toh, K., Wade, C.M., Mikkelsen, T.S., Karlsson, E.K., Jaffe, D.B., Kamal, M., Clamp, M., Chang, J.L., III, E.J.K., Zody, M.C., Mauceli, E., Xie, X., Breen, M., Wayne, R.K., Ostrander, E.A., Ponting, C.P., Galibert, F., Smith, D.R., deJong, P.J., Kirkness, E., Alvarez, P., Biagi, T., Brockman, W., Butler, J., Chin, C.-W., Cook, A., Cuff, J., Daly, M.J., DeCaprio, D., Gnerre, S., Grabherr, M., Kellis, M., Kleber, M., Bardeleben, C., Goodstadt, L., Heger, A., Hitte, C., Kim, L., Koepfli, K.-P., Parker, H.G., Pollinger, J.P., Searle, S.M.J., Sutter, N.B., Thomas,

- R., Webber, C., members, B.S.P., Baldwin, J., Abebe, A., Abouelleil, A., Aftuck, L., Ait-zahra, M., Aldredge, T., Allen, N., An, P., Anderson, S., Antoine, C., Arachchi, H., Aslam, A., Ayotte, L., Bachantsang, P., Barry, A., Bayul, T., Benamara, M., Berlin, A., Bessette, D., Blitshteyn, B., Bloom, T., Blye, J., Boguslavskiy, L., Bonnet, C., Boukhgalter, B., Brown, A., Cahill, P., Calixte, N., Camarata, J., Cheshatsang, Y., Chu, J., Citroen, M., Collymore, A., Cooke, P., Dawoe, T., Daza, R., Decktor, K., DeGray, S., Dhargay, N., Dooley, K., Dooley, K., Dorje, P., Dorjee, K., Dorris, L., Duffey, N., Dupes, A., Egbiremolen, O., Elong, R., Falk, J., Farina, A., Faro, S., Ferguson, D., Ferreira, P., Fisher, S., *et al.* (2005) Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* **438**: 803.
- Lindsay, J.A., Moore, C.E., Day, N.P., Peacock, S.J., Witney, A.A., Stabler, R.A., Husain, S.E., Butcher, P.D., and Hinds, J. (2006) Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *J Bacteriol* **188**: 669-676.
- Liu, J., Chen, D., Peters, B.M., Li, L., Li, B., Xu, Z., and Shirliff, M.E. (2016) Staphylococcal chromosomal cassettes mec (SCCmec): A mobile genetic element in methicillin-resistant *Staphylococcus aureus*. *Microb Pathog* **101**: 56-67.
- Liu, W., Rochat, T., Toffano-Nioche, C., Le Lam, T.N., Boulloc, P., and Morvan, C. (2018) Assessment of Bona Fide sRNAs in *Staphylococcus aureus*. *Front Microbiol* **9**: 228.
- Löffler, B., Hussain, M., Grundmeier, M., Bruck, M., Holzinger, D., Varga, G., Roth, J., Kahl, B.C., Proctor, R.A., and Peters, G. (2010) *Staphylococcus aureus* panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog* **6**: e1000715.
- Lopez, M.S., Tan, I.S., Yan, D., Kang, J., McCreary, M., Modrusan, Z., Austin, C.D., Xu, M., and Brown, E.J. (2017) Host-derived fatty acids activate type VII secretion in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **114**: 11223-11228.
- Loughman, A., Sweeney, T., Keane, F.M., Pietrocola, G., Speziale, P., and Foster, T.J. (2008a) Sequence diversity in the A domain of *Staphylococcus aureus* fibronectin-binding protein A. *BMC Microbiology* **8**: 74.
- Loughman, A., Sweeney, T., Keane, F.M., Pietrocola, G., Speziale, P., and Foster, T.J. (2008b) Sequence diversity in the A domain of *Staphylococcus aureus* fibronectin-binding protein A. *BMC Microbiol* **8**: 74.
- Lower, S.K., Lamlerthton, S., Casillas-Ituarte, N.N., Lins, R.D., Yongsunthon, R., Taylor, E.S., DiBartola, A.C., Edmonson, C., McIntyre, L.M., Reller, L.B., Que, Y.A., Ros, R., Lower, B.H., and Fowler, V.G., Jr. (2011) Polymorphisms in fibronectin binding protein A of *Staphylococcus aureus* are associated with infection of cardiovascular devices. *Proc Natl Acad Sci U S A* **108**: 18372-18377.

- Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N Engl J Med* **339**: 520-532.
- Lowy, F.D. (2003) Antimicrobial resistance: the example of *Staphylococcus aureus*. *J Clin Invest* **111**: 1265-1273.
- Ludwig, W., Seewaldt, E., Kilpper-Balz, R., Schleifer, K.H., Magrum, L., Woese, C.R., Fox, G.E., and Stackebrandt, E. (1985) The phylogenetic position of *Streptococcus* and *Enterococcus*. *J Gen Microbiol* **131**: 543-551.
- Luong, T., Sau, S., Gomez, M., Lee, J.C., and Lee, C.Y. (2002) Regulation of *Staphylococcus aureus* capsular polysaccharide expression by *agr* and *sarA*. *Infect Immun* **70**: 444-450.
- Machino, T., Hashimoto, S., Gon, Y., Kujime, K., Maruoka, S., and Horie, T. (2001) Interleukin-4 and interleukin-13 induce fibronectin production by human lung fibroblasts. *Allergy International* **50**: 197 - 202.
- Macintosh, R.L., Brittan, J.L., Bhattacharya, R., Jenkinson, H.F., Derrick, J., Upton, M., and Handley, P.S. (2009) The Terminal A Domain of the Fibrillar Accumulation-Associated Protein (Aap) of *Staphylococcus epidermidis* Mediates Adhesion to Human Corneocytes. *J Bacteriol* **191**: 7007-7016.
- Mallbris, L., Carlen, L., Wei, T., Heilborn, J., Nilsson, M.F., Granath, F., and Stahle, M. (2010) Injury downregulates the expression of the human cathelicidin protein hCAP18/LL-37 in atopic dermatitis. *Exp Dermatol* **19**: 442-449.
- Manting, E.H., and Driessen, A.J. (2000) Escherichia coli translocase: the unravelling of a molecular machine. *Mol Microbiol* **37**: 226-238.
- Marenholz, I., Nickel, R., Ruschendorf, F., Schulz, F., Esparza-Gordillo, J., Kerscher, T., Gruber, C., Lau, S., Worm, M., Keil, T., Kurek, M., Zaluga, E., Wahn, U., and Lee, Y.A. (2006) Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march. *J Allergy Clin Immunol* **118**: 866-871.
- Marraffini, L.A., Dedent, A.C., and Schneewind, O. (2006) Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. *Microbiol Mol Biol Rev* **70**: 192-221.
- Marsella, R., Olivry, T., and Carlotti, D.N. (2011) Current evidence of skin barrier dysfunction in human and canine atopic dermatitis. *Vet Dermatol* **22**: 239-248.
- Marshall, D., Hardman, M.J., Nield, K.M., and Byrne, C. (2001) Differentially expressed late constituents of the epidermal cornified envelope. *Proc Natl Acad Sci U S A* **98**: 13031-13036.
- Marshall, J.H., and Wilmoth, G.J. (1981) Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids. *J Bacteriol* **147**: 900-913.
- Mason, I.S., and Lloyd, D.H. (1989) The role of allergy in the development of canine pyoderma. *J Small Anim Pract* **30**: 216-218.
- Massey, R.C., Kantzanou, M.N., Fowler, T., Day, N.P., Schofield, K., Wann, E.R., Berendt, A.R., Hook, M., and Peacock, S.J. (2001) Fibronectin-

- binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. *Cell Microbiol* **3**: 839-851.
- Matsumoto, M., Zhou, Y., Matsuo, S., Nakanishi, H., Hirose, K., Oura, H., Arase, S., Ishida-Yamamoto, A., Bando, Y., Izumi, K., Kiyonari, H., Oshima, N., Nakayama, R., Matsushima, A., Hirota, F., Mouri, Y., Kuroda, N., Sano, S., and Chaplin, D.D. (2008) Targeted deletion of the murine corneodesmosin gene delineates its essential role in skin and hair physiology. *Proc Natl Acad Sci U S A* **105**: 6720-6724.
- Mazmanian, S.K., Ton-That, H., and Schneewind, O. (2001) Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. *Mol Microbiol* **40**: 1049-1057.
- Mazmanian, S.K., Ton-That, H., Su, K., and 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, H.K., Dedent, A.C., Hendrickx, A.P., Schneewind, O., and Missiakas, D.M. (2011) Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood. *PLoS Pathog* **7**: e1002307.
- McAleese, F.M., Walsh, E.J., Sieprawska, M., Potempa, J., and Foster, T.J. (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.
- McCormack, N., Foster, T.J., and Geoghegan, J.A. (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, D.P., McCarthy, H., O'Gara, J.P., and Geoghegan, J.A. (2014) Fibronectin-binding proteins are required for biofilm formation by community-associated methicillin-resistant *Staphylococcus aureus* strain LAC. *FEMS Microbiol Lett* **353**: 157-164.
- McDevitt, D., Nanavaty, T., House-Pompeo, K., Bell, E., Turner, N., McIntire, L., Foster, T., and Hook, M. (1997) Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. *Eur J Biochem* **247**: 416-424.
- McEwan, N.A. (2000) Adherence by *Staphylococcus intermedius* to canine keratinocytes in atopic dermatitis. *Res Vet Sci* **68**: 279-283.
- McGavin, M.J., Zahradka, C., Rice, K., and Scott, J.E. (1997) Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease. *Infect Immun* **65**: 2621-2628.
- McGuinness, W.A., Kobayashi, S.D., and DeLeo, F.R. (2016) Evasion of Neutrophil Killing by *Staphylococcus aureus*. *Pathogens* **5**: e0032.
- Meenan, N.A., Visai, L., Valtulina, V., Schwarz-Linek, U., Norris, N.C., Gurusiddappa, S., Hook, M., Speziale, P., and Potts, J.R. (2007) The

- tandem beta-zipper model defines high affinity fibronectin-binding repeats within *Staphylococcus aureus* FnBPA. *J Biol Chem* **282**: 25893-25902.
- Melles, D.C., Gorkink, R.F., Boelens, H.A., Snijders, S.V., Peeters, J.K., Moorhouse, M.J., van der Spek, P.J., van Leeuwen, W.B., Simons, G., Verbrugh, H.A., and van Belkum, A. (2004) Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. *J Clin Invest* **114**: 1732-1740.
- Meng, J., Moriyama, M., Feld, M., Buddenkotte, J., Buhl, T., Szollosi, A., Zhang, J., Miller, P., Ghatti, A., Fischer, M., Reeh, P.W., Shan, C., Wang, J., and Steinhoff, M. (2018) New mechanism underlying IL-31-induced atopic dermatitis. *J Allergy Clin Immunol* **141**: 1677-1689.e1678.
- Miajlovic, H., Zapotoczna, M., Geoghegan, J.A., Kerrigan, S.W., Speziale, P., and Foster, T.J. (2010) Direct interaction of iron-regulated surface determinant IsdB of *Staphylococcus aureus* with the GPIIb/IIIa receptor on platelets. *Microbiology* **156**: 920-928.
- Mildner, M., Jin, J., Eckhart, L., Kezic, S., Gruber, F., Barresi, C., Stremnitzer, C., Buchberger, M., Mlitz, V., Ballaun, C., Sterniczky, B., Fodinger, D., and Tschachler, E. (2010) Knockdown of filaggrin impairs diffusion barrier function and increases UV sensitivity in a human skin model. *J Invest Dermatol* **130**: 2286-2294.
- Milles, L.F., Schulten, K., Gaub, H.E., and Bernardi, R.C. (2018) Molecular mechanism of extreme mechanostability in a pathogen adhesin. *Science* **359**: 1527-1533.
- Mischke, D., Korge, B.P., Marenholz, I., Volz, A., and Ziegler, A. (1996) Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ("epidermal differentiation complex") on human chromosome 1q21. *J Invest Dermatol* **106**: 989-992.
- Moll, R., Divo, M., and Langbein, L. (2008) The human keratins: biology and pathology. *Histochem Cell Biol* **129**: 705-733.
- Monk, I.R., Casey, P.G., Cronin, M., Gahan, C.G., and Hill, C. (2008) Development of multiple strain competitive index assays for *Listeria monocytogenes* using pIMC; a new site-specific integrative vector. *BMC Microbiol* **8**: 96.
- Monk, I.R., Shah, I.M., Xu, M., Tan, M.W., and Foster, T.J. (2012) Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *MBio* **3**.
- Monk, I.R., Tree, J.J., Howden, B.P., Stinear, T.P., and Foster, T.J. (2015) Complete Bypass of Restriction Systems for Major *Staphylococcus aureus* Lineages. *MBio* **6**: e00308-00315.
- Moreillon, P., Entenza, J.M., Francioli, P., McDevitt, D., Foster, T.J., Francois, P., and Vaudaux, P. (1995) Role of *Staphylococcus aureus* coagulase

- and clumping factor in pathogenesis of experimental endocarditis. *Infect Immun* **63**: 4738-4743.
- Moura de Sousa, J., Balbontin, R., Durao, P., and Gordo, I. (2017) Multidrug-resistant bacteria compensate for the epistasis between resistances. *PLoS Biol* **15**: e2001741.
- Mulcahy, M.E., Geoghegan, J.A., Monk, I.R., O'Keeffe, K.M., Walsh, E.J., Foster, T.J., and McLoughlin, R.M. (2012) Nasal colonisation by *Staphylococcus aureus* depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. *PLoS Pathog* **8**: e1003092.
- Munita, J.M., and Arias, C.A. (2016) Mechanisms of Antibiotic Resistance. *Microbiol Spectr* **4**: VMBF-0016-2015.
- Munoz, P., Hortal, J., Giannella, M., Barrio, J.M., Rodriguez-Creixems, M., Perez, M.J., Rincon, C., and 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.
- Murphy, E., Lin, S.L., Nunez, L., Andrew, L., Fink, P.S., Dilts, D.A., Hoiseth, S.K., Jansen, K.U., and Anderson, A.S. (2011) Challenges for the evaluation of *Staphylococcus aureus* protein based vaccines: monitoring antigenic diversity. *Hum Vaccin* **7 Suppl**: 51-59.
- Myles, I.A., Earland, N.J., Anderson, E.D., Moore, I.N., Kieh, M.D., Williams, K.W., Saleem, A., Fontecilla, N.M., Welch, P.A., Darnell, D.A., Barnhart, L.A., Sun, A.A., Uzel, G., and Datta, S.K. (2018) First-in-human topical microbiome transplantation with *Roseomonas mucosa* for atopic dermatitis. *JCI Insight* **3**: 120608.
- Myles, I.A., Williams, K.W., Reckhow, J.D., Jammeh, M.L., Pincus, N.B., Sastalla, I., Saleem, D., Stone, K.D., and Datta, S.K. (2016) Transplantation of human skin microbiota in models of atopic dermatitis. *JCI Insight* **1**: 86955.
- Nagy, I., Pivarcsi, A., Kis, K., Koreck, A., Bodai, L., McDowell, A., Seltsmann, H., Patrick, S., Zouboulis, C.C., and Kemeny, L. (2006) Propionibacterium acnes and lipopolysaccharide induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines in human sebocytes. *Microbes Infect* **8**: 2195-2205.
- Nakamura, Y., Oscherwitz, J., Cease, K.B., Chan, S.M., Munoz-Planillo, R., Hasegawa, M., Villaruz, A.E., Cheung, G.Y., McGavin, M.J., Travers, J.B., Otto, M., Inohara, N., and Nunez, G. (2013) Staphylococcus delta-toxin induces allergic skin disease by activating mast cells. *Nature* **503**: 397-401.
- Nakatsuji, T., Chen, T.H., Narala, S., Chun, K.A., Two, A.M., Yun, T., Shafiq, F., Kotol, P.F., Bouslimani, A., Melnik, A.V., Latif, H., Kim, J.N., Lockhart, A., Artis, K., David, G., Taylor, P., Streib, J., Dorrestein, P.C., Grier, A., Gill, S.R., Zengler, K., Hata, T.R., Leung, D.Y., and Gallo, R.L. (2017) Antimicrobials from human skin commensal bacteria protect against *Staphylococcus aureus* and are deficient in atopic dermatitis. *Sci Transl Med* **9**.

- Nakatsuji, T., Chen, T.H., Two, A.M., Chun, K.A., Narala, S., Geha, R.S., Hata, T.R., and Gallo, R.L. (2016) *Staphylococcus aureus* Exploits Epidermal Barrier Defects in Atopic Dermatitis to Trigger Cytokine Expression. *J Invest Dermatol* **136**: 2192-2200.
- Ni Eidhin, D., Perkins, S., Francois, P., Vaudaux, P., Hook, M., and Foster, T.J. (1998) Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Mol Microbiol* **30**: 245-257.
- Niyonsaba, F., Iwabuchi, K., Someya, A., Hirata, M., Matsuda, H., Ogawa, H., and Nagaoka, I. (2002) A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology* **106**: 20-26.
- Niyonsaba, F., Ushio, H., Nagaoka, I., Okumura, K., and Ogawa, H. (2005) The human beta-defensins (-1, -2, -3, -4) and cathelicidin LL-37 induce IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes. *J Immunol* **175**: 1776-1784.
- Nomura, I., Goleva, E., Howell, M.D., Hamid, Q.A., Ong, P.Y., Hall, C.F., Darst, M.A., Gao, B., Boguniewicz, M., Travers, J.B., and Leung, D.Y. (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* **171**: 3262-3269.
- O'Brien, L., Kerrigan, S.W., Kaw, G., Hogan, M., Penades, J., Litt, D., Fitzgerald, D.J., Foster, T.J., and Cox, D. (2002) Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. *Mol Microbiol* **44**: 1033-1044.
- O'Keeffe, K.M., Wilk, M.M., Leech, J.M., Murphy, A.G., Laabei, M., Monk, I.R., Massey, R.C., Lindsay, J.A., Foster, T.J., Geoghegan, J.A., and McLoughlin, R.M. (2015) Manipulation of Autophagy in Phagocytes Facilitates *Staphylococcus aureus* Bloodstream Infection. *Infect Immun* **83**: 3445-3457.
- O'Neill, A.J., McLaws, F., Kahlmeter, G., Henriksen, A.S., and Chopra, I. (2007a) Genetic basis of resistance to fusidic acid in staphylococci. *Antimicrob Agents Chemother* **51**: 1737-1740.
- O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D.A., Loughman, A., Foster, T.J., and O'Gara, J.P. (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, D.A., and O'Gara, J.P. (2007b) Association between methicillin susceptibility and biofilm regulation in *Staphylococcus aureus* isolates from device-related infections. *J Clin Microbiol* **45**: 1379-1388.
- O'Regan, G.M., Sandilands, A., McLean, W.H., and Irvine, A.D. (2008) Filaggrin in atopic dermatitis. *J Allergy Clin Immunol* **122**: 689-693.
- O'Riordan, K., and Lee, J.C. (2004) *Staphylococcus aureus* capsular polysaccharides. *Clin Microbiol Rev* **17**: 218-234.

- Oh, J., Byrd, A.L., Deming, C., Conlan, S., Kong, H.H., and Segre, J.A. (2014) Biogeography and individuality shape function in the human skin metagenome. *Nature* **514**: 59-64.
- Oh, J., Conlan, S., Polley, E.C., Segre, J.A., and Kong, H.H. (2012) Shifts in human skin and nares microbiota of healthy children and adults. *Genome Med* **4**: 77.
- Onell, A., and Andersson, K. (2005) Kinetic determinations of molecular interactions using Biacore--minimum data requirements for efficient experimental design. *J Mol Recognit* **18**: 307-317.
- Ong, P.Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R.L., and Leung, D.Y. (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* **347**: 1151-1160.
- Otto, M. (2008) Staphylococcal biofilms. *Curr Top Microbiol Immunol* **322**: 207-228.
- Otto, M., Echner, H., Voelter, W., and Gotz, F. (2001) Pheromone cross-inhibition between *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect Immun* **69**: 1957-1960.
- Paharik, A.E., Parlet, C.P., Chung, N., Todd, D.A., Rodriguez, E.I., Van Dyke, M.J., Cech, N.B., and Horswill, A.R. (2017) Coagulase-Negative Staphylococcal Strain Prevents *Staphylococcus aureus* Colonization and Skin Infection by Blocking Quorum Sensing. *Cell Host Microbe* **22**: 746-756.e745.
- Palazzolo-Ballance, A.M., Reniere, M.L., Braughton, K.R., Sturdevant, D.E., Otto, M., Kreiswirth, B.N., Skaar, E.P., and DeLeo, F.R. (2008) Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus*. *J Immunol* **180**: 500-509.
- Pallen, M.J. (2002) The ESAT-6/WXG100 superfamily -- and a new Gram-positive secretion system? *Trends Microbiol* **10**: 209-212.
- Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J., O'Regan, G.M., Watson, R.M., Cecil, J.E., Bale, S.J., Compton, J.G., DiGiovanna, J.J., Fleckman, P., Lewis-Jones, S., Arseculeratne, G., Sergeant, A., Munro, C.S., El Houate, B., McElreavey, K., Halkjaer, L.B., Bisgaard, H., Mukhopadhyay, S., and McLean, W.H. (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* **38**: 441-446.
- Parfentjev, I.A., and Catelli, A.R. (1964) TOLERANCE OF *STAPHYLOCOCCUS AUREUS* TO SODIUM CHLORIDE. *Journal of Bacteriology* **88**: 1-3.
- Peacock, S.J., Day, N.P., Thomas, M.G., Berendt, A.R., and Foster, T.J. (2000) Clinical isolates of *Staphylococcus aureus* exhibit diversity in *fnb* genes and adhesion to human fibronectin. *J Infect* **41**: 23-31.
- Peacock, S.J., de Silva, I., and Lowy, F.D. (2001) What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiol* **9**: 605-610.

- Peacock, S.J., Foster, T.J., Cameron, B.J., and Berendt, A.R. (1999) Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. *Microbiology* **145** (Pt 12): 3477-3486.
- Pellerin, L., Henry, J., Hsu, C.Y., Balica, S., Jean-Decoster, C., Mechin, M.C., Hansmann, B., Rodriguez, E., Weindinger, S., Schmitt, A.M., Serre, G., Paul, C., and Simon, M. (2013) Defects of filaggrin-like proteins in both lesional and nonlesional atopic skin. *J Allergy Clin Immunol* **131**: 1094-1102.
- Pendaries, V., Malaisse, J., Pellerin, L., Le Lamer, M., Nachat, R., Kezic, S., Schmitt, A.M., Paul, C., Poumay, Y., Serre, G., and Simon, M. (2014) Knockdown of filaggrin in a three-dimensional reconstructed human epidermis impairs keratinocyte differentiation. *J Invest Dermatol* **134**: 2938-2946.
- Periasamy, S., Joo, H.S., Duong, A.C., Bach, T.H., Tan, V.Y., Chatterjee, S.S., Cheung, G.Y., and Otto, M. (2012) How *Staphylococcus aureus* biofilms develop their characteristic structure. *Proc Natl Acad Sci U S A* **109**: 1281-1286.
- Perlman, R.L. (2016) Mouse models of human disease: An evolutionary perspective. *Evol Med Public Health* **2016**: 170-176.
- Peschel, A., and Otto, M. (2013) Phenol-soluble modulins and staphylococcal infection. *Nat Rev Microbiol* **11**: 667-673.
- Peschel, A., Otto, M., Jack, R.W., Kalbacher, H., Jung, G., and Gotz, F. (1999) Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J Biol Chem* **274**: 8405-8410.
- Peters, A.S., Kellberger, J., Vogelberg, C., Dressel, H., Windstetter, D., Weinmayr, G., Genuneit, J., Nowak, D., von Mutius, E., and Radon, K. (2010) Prediction of the incidence, recurrence, and persistence of atopic dermatitis in adolescence: a prospective cohort study. *J Allergy Clin Immunol* **126**: 590-595.e591-593.
- Pietrocola, G., Gianotti, V., Richards, A., Nobile, G., Geoghegan, J.A., Rindi, S., Monk, I.R., Bordt, A.S., Foster, T.J., Fitzgerald, J.R., and Speziale, P., (2015) Fibronectin Binding Proteins SpsD and SpsL Both Support Invasion of Canine Epithelial Cells by *Staphylococcus pseudintermedius*. In: *Infect Immun.* pp. 4093-4102.
- Pietrocola, G., Nobile, G., Alfeo, M.J., Foster, T.J., Geoghegan, J.A., De Filippis, V., and Speziale, P. (2019) Fibronectin-binding protein B (FnBPB) from *Staphylococcus aureus* protects against the antimicrobial activity of histones. *J Biol Chem*.
- Pietrocola, G., Nobile, G., Gianotti, V., Zapotoczna, M., Foster, T.J., Geoghegan, J.A., and Speziale, P. (2016) Molecular Interactions of Human Plasminogen with Fibronectin-binding Protein B (FnBPB), a Fibrinogen/Fibronectin-binding Protein from *Staphylococcus aureus*. *J Biol Chem* **291**: 18148-18162.

- Planet, P.J., LaRussa, S.J., Dana, A., Smith, H., Xu, A., Ryan, C., Uhlemann, A.C., Boundy, S., Goldberg, J., Narechania, A., Kulkarni, R., Ratner, A.J., Geoghegan, J.A., Kolokotronis, S.O., and Prince, A. (2013) Emergence of the Epidemic Methicillin-Resistant *Staphylococcus aureus* Strain USA300 Coincides with Horizontal Transfer of the Arginine Catabolic Mobile Element and speG-mediated Adaptations for Survival on Skin. *MBio* **4**: e00889-13.
- Ponnuraj, K., Bowden, M.G., Davis, S., Gurusiddappa, S., Moore, D., Choe, D., Xu, Y., Hook, M., and Narayana, S.V. (2003) A "dock, lock, and latch" structural model for a staphylococcal adhesin binding to fibrinogen. *Cell* **115**: 217-228.
- Popov, L., Kovalski, J., Grandi, G., Bagnoli, F., and Amieva, M.R. (2014) Three-Dimensional Human Skin Models to Understand *Staphylococcus aureus* Skin Colonization and Infection. *Front Immunol* **5**: 41.
- Postlethwaite, A.E., Holness, M.A., Katai, H., and Raghov, R. (1992) Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest* **90**: 1479-1485.
- Purves, J., Thomas, J., Riboldi, G.P., Zapotoczna, M., Tarrant, E., Andrew, P.W., Londono, A., Planet, P.J., Geoghegan, J.A., Waldron, K.J., and Morrissey, J.A. (2018) A horizontally gene transferred copper resistance locus confers hyper-resistance to antibacterial copper toxicity and enables survival of community acquired methicillin resistant *Staphylococcus aureus* USA300 in macrophages. *Environ Microbiol* **20**: 1576-1589.
- Que, Y.A., Haeffliger, J.A., Piroth, L., François, P., Widmer, E., Entenza, J.M., Sinha, B., Herrmann, M., Francioli, P., Vaudaux, P., and Moreillon, P., (2005) Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis. In: *J Exp Med*. pp. 1627-1635.
- Raap, U., Wichmann, K., Bruder, M., Stander, S., Wedi, B., Kapp, A., and Werfel, T., (2008) Correlation of IL-31 serum levels with severity of atopic dermatitis. In: *J Allergy Clin Immunol*. United States, pp. 421-423.
- Ramsey, M.M., Freire, M.O., Gabriliska, R.A., Rumbaugh, K.P., and Lemon, K.P. (2016) *Staphylococcus aureus* Shifts toward Commensalism in Response to *Corynebacterium* Species. *Front Microbiol* **7**: 1230.
- Rasmussen, G., Monecke, S., Ehricht, R., and Soderquist, B. (2013) Prevalence of clonal complexes and virulence genes among commensal and invasive *Staphylococcus aureus* isolates in Sweden. *PLoS One* **8**: e77477.
- Rather, I.A., Bajpai, V.K., Kumar, S., Lim, J., Paek, W.K., and Park, Y.H. (2016) Probiotics and Atopic Dermatitis: An Overview. *Front Microbiol* **7**: 507.
- Rawlings, A.V., and Harding, C.R. (2004) Moisturization and skin barrier function. *Dermatol Ther* **17 Suppl 1**: 43-48.

- Reinholz, M., Ruzicka, T., and Schaubert, J. (2012) Cathelicidin LL-37: an antimicrobial peptide with a role in inflammatory skin disease. *Ann Dermatol* **24**: 126-135.
- Richards, A.C., O'Shea, M., Beard, P.M., Goncheva, M.I., Tuffs, S.W., Fitzgerald, J.R., and Lengeling, A. (2018) *Staphylococcus pseudintermedius* Surface Protein L (SpsL) Is Required for Abscess Formation in a Murine Cutaneous Infection Model. *Infect Immun* **86**: e00631-18.
- Riethmuller, C., McAleer, M.A., Koppes, S.A., Abdayem, R., Franz, J., Haftek, M., Campbell, L.E., MacCallum, S.F., McLean, W.H., Irvine, A.D., and Kezic, S. (2015) Filaggrin breakdown products determine corneocyte conformation in patients with atopic dermatitis. *J Allergy Clin Immunol* **136**: 1573-1580.e1571-1572.
- Roberts, G.A., Houston, P.J., White, J.H., Chen, K., Stephanou, A.S., Cooper, L.P., Dryden, D.T., and Lindsay, J.A. (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.
- Roche, F.M., Downer, R., Keane, F., Speziale, P., Park, P.W., and Foster, T.J. (2004) The N-terminal A domain of fibronectin-binding proteins A and B promotes adhesion of *Staphylococcus aureus* to elastin. *J Biol Chem* **279**: 38433-38440.
- Roche, F.M., Meehan, M., and Foster, T.J. (2003) The *Staphylococcus aureus* surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells. *Microbiology* **149**: 2759-2767.
- Rodriguez, E., Baurecht, H., Herberich, E., Wagenpfeil, S., Brown, S.J., Cordell, H.J., Irvine, A.D., and Weidinger, S. (2009) Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. *J Allergy Clin Immunol* **123**: 1361-1370.e1367.
- Roider, E., Ruzicka, T., and Schaubert, J. (2013) Vitamin d, the cutaneous barrier, antimicrobial peptides and allergies: is there a link? *Allergy Asthma Immunol Res* **5**: 119-128.
- Rojo, A., Aguinaga, A., Monecke, S., Yuste, J.R., Gastaminza, G., and Espana, A. (2014) *Staphylococcus aureus* genomic pattern and atopic dermatitis: may factors other than superantigens be involved? *Eur J Clin Microbiol Infect Dis* **33**: 651-658.
- Rooijackers, S.H., van Wamel, W.J., Ruyken, M., van Kessel, K.P., and van Strijp, J.A. (2005) Anti-opsonic properties of staphylokinase. *Microbes Infect* **7**: 476-484.
- Rudkin, J.K., Edwards, A.M., Bowden, M.G., Brown, E.L., Pozzi, C., Waters, E.M., Chan, W.C., Williams, P., O'Gara, J.P., and Massey, R.C. (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.

- Ruimy, R., Angebault, C., Djossou, F., Dupont, C., Epelboin, L., Jarraud, S., Lefevre, L.A., Bes, M., Lixandru, B.E., Bertine, M., El Miniai, A., Renard, M., Bettinger, R.M., Lescat, M., Clermont, O., Peroz, G., Lina, G., Tavakol, M., Vandenesch, F., van Belkum, A., Rousset, F., and Andremont, A. (2010) Are host genetics the predominant determinant of persistent nasal *Staphylococcus aureus* carriage in humans? *J Infect Dis* **202**: 924-934.
- Said-Salim, B., Dunman, P.M., McAleese, F.M., Macapagal, D., Murphy, E., McNamara, P.J., Arvidson, S., Foster, T.J., Projan, S.J., and Kreiswirth, B.N. (2003) Global regulation of *Staphylococcus aureus* genes by Rot. *J Bacteriol* **185**: 610-619.
- Sandilands, A., Terron-Kwiatkowski, A., Hull, P.R., O'Regan, G.M., Clayton, T.H., Watson, R.M., Carrick, T., Evans, A.T., Liao, H., Zhao, Y., Campbell, L.E., Schmuth, M., Gruber, R., Janecke, A.R., Elias, P.M., van Steensel, M.A., Nagtzaam, I., van Geel, M., Steijlen, P.M., Munro, C.S., Bradley, D.G., Palmer, C.N., Smith, F.J., McLean, W.H., and Irvine, A.D. (2007) Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* **39**: 650-654.
- SanMiguel, A., and Grice, E.A. (2015) Interactions between host factors and the skin microbiome. *Cell Mol Life Sci* **72**: 1499-1515.
- Saravia-Otten, P., Muller, H.P., and Arvidson, S. (1997) Transcription of *Staphylococcus aureus* fibronectin binding protein genes is negatively regulated by agr and an agr-independent mechanism. *J Bacteriol* **179**: 5259-5263.
- Sawada, E., Yoshida, N., Sugiura, A., and Imokawa, G. (2012) Th1 cytokines accentuate but Th2 cytokines attenuate ceramide production in the stratum corneum of human epidermal equivalents: an implication for the disrupted barrier mechanism in atopic dermatitis. *J Dermatol Sci* **68**: 25-35.
- Schaffer, A.C., Solinga, R.M., Cocchiaro, J., Portoles, M., Kiser, K.B., Risley, A., Randall, S.M., Valtulina, V., Speziale, P., Walsh, E., Foster, T., and Lee, J.C. (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.
- Schitteck, B., Hipfel, R., Sauer, B., Bauer, J., Kalbacher, H., Stevanovic, S., Schirle, M., Schroeder, K., Blin, N., Meier, F., Rassner, G., and Garbe, C. (2001) Dermcidin: a novel human antibiotic peptide secreted by sweat glands. *Nat Immunol* **2**: 1133-1137.
- Schlag, M., Biswas, R., Krismer, B., Kohler, T., Zoll, S., Yu, W., Schwarz, H., Peschel, A., and Gotz, F. (2010) Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. *Mol Microbiol* **75**: 864-873.
- Schleifer, K.H., and Kandler, O. (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**: 407-477.

- Schneewind, O., Mihaylova-Petkov, D., and Model, P. (1993) Cell wall sorting signals in surface proteins of gram-positive bacteria. *EMBO J* **12**: 4803-4811.
- Schulthess, B., Bloes, D.A., and Berger-Bachi, B. (2012) Opposing roles of sigmaB and sigmaB-controlled SpoVG in the global regulation of *esxA* in *Staphylococcus aureus*. *BMC Microbiol* **12**: 17.
- Schwarz-Linek, U., Werner, J.M., Pickford, A.R., Gurusiddappa, S., Kim, J.H., Pilka, E.S., Briggs, J.A., Gough, T.S., Hook, M., Campbell, I.D., and Potts, J.R. (2003) Pathogenic bacteria attach to human fibronectin through a tandem beta-zipper. *Nature* **423**: 177-181.
- Scott, I.R., and Harding, C.R. (1986) Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water activity of the environment. *Dev Biol* **115**: 84-92.
- Serre, G., Mils, V., Haftek, M., Vincent, C., Croute, F., Reano, A., Ouhayoun, J.P., Bettinger, S., and Soleilhavoup, J.P. (1991) Identification of late differentiation antigens of human cornified epithelia, expressed in re-organized desmosomes and bound to cross-linked envelope. *J Invest Dermatol* **97**: 1061-1072.
- Shah, M., and Mohanraj, M. (2003) High levels of fusidic acid-resistant *Staphylococcus aureus* in dermatology patients. *Br J Dermatol* **148**: 1018-1020.
- Sharp, J.A., Echague, C.G., Hair, P.S., Ward, M.D., Nyalwidhe, J.O., Geoghegan, J.A., Foster, T.J., and Cunnion, K.M. (2012) *Staphylococcus aureus* surface protein SdrE binds complement regulator factor H as an immune evasion tactic. *PLoS One* **7**: e38407.
- Shu, M., Wang, Y., Yu, J., Kuo, S., Coda, A., Jiang, Y., Gallo, R.L., and Huang, C.M. (2013) Fermentation of *Propionibacterium acnes*, a commensal bacterium in the human skin microbiome, as skin probiotics against methicillin-resistant *Staphylococcus aureus*. *PLoS One* **8**: e55380.
- Shukla, S.K., Pantrangi, M., Stahl, B., Briska, A.M., Stemper, M.E., Wagner, T.K., Zentz, E.B., Callister, S.M., Lovrich, S.D., Henkhaus, J.K., and Dykes, C.W. (2012) Comparative whole-genome mapping to determine *Staphylococcus aureus* genome size, virulence motifs, and clonality. *J Clin Microbiol* **50**: 3526-3533.
- Siboo, I.R., Chambers, H.F., and Sullam, P.M. (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., and Potempa, J. (2004) Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrob Agents Chemother* **48**: 4673-4679.
- Sievert, D.M., Ricks, P., Edwards, J.R., Schneider, A., Patel, J., Srinivasan, A., Kallen, A., Limbago, B., and Fridkin, S. (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.
- Signas, C., Raucci, G., Jonsson, K., Lindgren, P.E., Anantharamaiah, G.M., Hook, M., and Lindberg, M. (1989) Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. *Proc Natl Acad Sci U S A* **86**: 699-703.
- Silverberg, J.I., and Hanifin, J.M. (2013) Adult eczema prevalence and associations with asthma and other health and demographic factors: a US population-based study. *J Allergy Clin Immunol* **132**: 1132-1138.
- Simon, M., Jonca, N., Guerrin, M., Haftek, M., Bernard, D., Caubet, C., Egelrud, T., Schmidt, R., and Serre, G. (2001) Refined characterization of corneodesmosin proteolysis during terminal differentiation of human epidermis and its relationship to desquamation. *J Biol Chem* **276**: 20292-20299.
- Simon, M., Montezin, M., Guerrin, M., Durieux, J.J., and Serre, G. (1997) Characterization and purification of human corneodesmosin, an epidermal basic glycoprotein associated with corneocyte-specific modified desmosomes. *J Biol Chem* **272**: 31770-31776.
- Simou, C., Thoday, K.L., Forsythe, P.J., and Hill, P.B. (2005) Adherence of *Staphylococcus intermedius* to corneocytes of healthy and atopic dogs: effect of pyoderma, pruritus score, treatment and gender. *Vet Dermatol* **16**: 385-391.
- Simpson, C.L., Patel, D.M., and Green, K.J. (2011) Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. *Nat Rev Mol Cell Biol* **12**: 565-580.
- Simpson, E.L., Flohr, C., Eichenfield, L.F., Bieber, T., Sofen, H., Taieb, A., Owen, R., Putnam, W., Castro, M., DeBusk, K., Lin, C.Y., Voulgari, A., Yen, K., and Omachi, T.A. (2018) Efficacy and safety of lebrikizumab (an anti-IL-13 monoclonal antibody) in adults with moderate-to-severe atopic dermatitis inadequately controlled by topical corticosteroids: A randomized, placebo-controlled phase II trial (TREBLE). *J Am Acad Dermatol* **78**: 863-871.e811.
- Sinha, B., Francois, P.P., Nusse, O., Foti, M., Hartford, O.M., Vaudaux, P., Foster, T.J., Lew, D.P., Herrmann, M., and Krause, K.H. (1999) Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin $\alpha 5 \beta 1$. *Cell Microbiol* **1**: 101-117.
- Smits, J.P.H., Niehues, H., Rikken, G., van Vlijmen-Willems, I., van de Zande, G., Zeeuwen, P., Schalkwijk, J., and van den Bogaard, E.H. (2017) Immortalized N/TERT keratinocytes as an alternative cell source in 3D human epidermal models. *Sci Rep* **7**: 11838.
- Sollberger, G., Tilley, D.O., and Zychlinsky, A. (2018) Neutrophil Extracellular Traps: The Biology of Chromatin Externalization. *Dev Cell* **44**: 542-553.

- Song, C., Weichbrodt, C., Salnikov, E.S., Dynowski, M., Forsberg, B.O., Bechinger, B., Steinem, C., de Groot, B.L., Zachariae, U., and Zeth, K. (2013) Crystal structure and functional mechanism of a human antimicrobial membrane channel. *Proc Natl Acad Sci U S A* **110**: 4586-4591.
- Spaulding, A.R., Salgado-Pabon, W., Kohler, P.L., Horswill, A.R., Leung, D.Y., and Schlievert, P.M. (2013) Staphylococcal and streptococcal superantigen exotoxins. *Clin Microbiol Rev* **26**: 422-447.
- Speziale, P., Pietrocola, G., Foster, T.J., and Geoghegan, J.A. (2014) Protein-based biofilm matrices in Staphylococci. *Front Cell Infect Microbiol* **4**: 171.
- Stackebrandt, E., and Teuber, M. (1988) Molecular taxonomy and phylogenetic position of lactic acid bacteria. *Biochimie* **70**: 317-324.
- Staubitz, P., Neumann, H., Schneider, T., Wiedemann, I., and Peschel, A. (2004) MprF-mediated biosynthesis of lysylphosphatidylglycerol, an important determinant in staphylococcal defensin resistance. *FEMS Microbiol Lett* **231**: 67-71.
- Steinert, P.M. (1993) Structure, function, and dynamics of keratin intermediate filaments. *J Invest Dermatol* **100**: 729-734.
- Steinert, P.M., Mack, J.W., Korge, B.P., Gan, S.Q., Haynes, S.R., and Steven, A.C. (1991) Glycine loops in proteins: their occurrence in certain intermediate filament chains, loricrins and single-stranded RNA binding proteins. *Int J Biol Macromol* **13**: 130-139.
- Stemberk, V., Jones, R.P.O., Moroz, O., Atkin, K.E., Edwards, A.M., Turkenburg, J.P., Leech, A.P., Massey, R.C., and Potts, J.R. (2014) Evidence for Steric Regulation of Fibrinogen Binding to *Staphylococcus aureus* Fibronectin-binding Protein A (FnBPA)*. *J Biol Chem* **289**: 12842-12851.
- Sugimoto, S., Iwamoto, T., Takada, K., Okuda, K., Tajima, A., Iwase, T., and Mizunoe, Y. (2013) *Staphylococcus epidermidis* Esp degrades specific proteins associated with *Staphylococcus aureus* biofilm formation and host-pathogen interaction. *J Bacteriol* **195**: 1645-1655.
- Sun, F., Li, C., Jeong, D., Sohn, C., He, C., and Bae, T. (2010) In the *Staphylococcus aureus* two-component system sae, the response regulator SaeR binds to a direct repeat sequence and DNA binding requires phosphorylation by the sensor kinase SaeS. *J Bacteriol* **192**: 2111-2127.
- Syed, A.K., Reed, T.J., Clark, K.L., Boles, B.R., and Kahlenberg, J.M. (2015) *Staphylococcus aureus* phenol-soluble modulins stimulate the release of proinflammatory cytokines from keratinocytes and are required for induction of skin inflammation. *Infect Immun* **83**: 3428-3437.
- Tauber, M., Balica, S., Hsu, C.Y., Jean-Decoster, C., Lauze, C., Redoules, D., Viode, C., Schmitt, A.M., Serre, G., Simon, M., and Paul, C.F. (2016) *Staphylococcus aureus* density on lesional and nonlesional skin is

- strongly associated with disease severity in atopic dermatitis. *J Allergy Clin Immunol* **137**: 1272-1274.e1271-1273.
- Tegmark, K., Karlsson, A., and Arvidson, S. (2000) Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. *Mol Microbiol* **37**: 398-409.
- Thakker, M., Park, J.S., Carey, V., and Lee, J.C. (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, J.W., Missiakas, D.M., and Schneewind, O. (2009) *Staphylococcus aureus* synthesizes adenosine to escape host immune responses. *J Exp Med* **206**: 2417-2427.
- Ton-That, H., Liu, G., Mazmanian, S.K., Faull, K.F., and 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.
- Tong, S.Y., Davis, J.S., Eichenberger, E., Holland, T.L., and Fowler, V.G., Jr. (2015a) *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* **28**: 603-661.
- Tong, S.Y., Schaumburg, F., Ellington, M.J., Corander, J., Pichon, B., Leendertz, F., Bentley, S.D., Parkhill, J., Holt, D.C., Peters, G., and Giffard, P.M. (2015b) Novel staphylococcal species that form part of a *Staphylococcus aureus*-related complex: the non-pigmented *Staphylococcus argenteus* sp. nov. and the non-human primate-associated *Staphylococcus schweitzeri* sp. nov. *Int J Syst Evol Microbiol* **65**: 15-22.
- Totsika, M., Kostakioti, M., Hannan, T.J., Upton, M., Beatson, S.A., Janetka, J.W., Hultgren, S.J., and Schembri, M.A. (2013) A FimH inhibitor prevents acute bladder infection and treats chronic cystitis caused by multidrug-resistant uropathogenic *Escherichia coli* ST131. *J Infect Dis* **208**: 921-928.
- Totsuka, A., Omori-Miyake, M., Kawashima, M., Yagi, J., and Tsunemi, Y. (2017) Expression of keratin 1, keratin 10, desmoglein 1 and desmocollin 1 in the epidermis: possible downregulation by interleukin-4 and interleukin-13 in atopic dermatitis. *Eur J Dermatol* **27**: 247-253.
- Totte, J.E., van der Feltz, W.T., Hennekam, M., van Belkum, A., van Zuuren, E.J., and Pasmans, S.G. (2016) Prevalence and odds of *Staphylococcus aureus* carriage in atopic dermatitis: a systematic review and meta-analysis. *Br J Dermatol* **175**: 687-695.
- Totte, J.E., van Doorn, M.B., and Pasmans, S. (2017) Successful Treatment of Chronic *Staphylococcus aureus*-Related Dermatoses with the Topical Endolysin Staphefekt SA.100: A Report of 3 Cases. *Case Rep Dermatol* **9**: 19-25.

- Traber, K., and Novick, R. (2006) A slipped-mispairing mutation in AgrA of laboratory strains and clinical isolates results in delayed activation of agr and failure to translate delta- and alpha-haemolysins. *Mol Microbiol* **59**: 1519-1530.
- Traber, K.E., Lee, E., Benson, S., Corrigan, R., Cantera, M., Shopsin, B., and Novick, R.P. (2008) agr function in clinical *Staphylococcus aureus* isolates. *Microbiology* **154**: 2265-2274.
- Trivedi, S., Uhlemann, A.C., Herman-Bausier, P., Sullivan, S.B., Sowash, M.G., Flores, E.Y., Khan, S.D., Dufrene, Y.F., and Lowy, F.D. (2017) The Surface Protein SdrF Mediates *Staphylococcus epidermidis* Adherence to Keratin. *J Infect Dis* **215**: 1846-1854.
- Tzagoloff, H., and Novick, R. (1977) Geometry of cell division in *Staphylococcus aureus*. *J Bacteriol* **129**: 343-350.
- Unnikrishnan, M., Constantinidou, C., Palmer, T., and Pallen, M.J. (2017) The Enigmatic Esx Proteins: Looking Beyond Mycobacteria. *Trends Microbiol* **25**: 192-204.
- Valeva, A., Walev, I., Pinkernell, M., Walker, B., Bayley, H., Palmer, M., and 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, N.J., de Vogel, C.P., Boelens, H.A., Verveer, J., Nouwen, J.L., Verbrugh, H.A., and Wertheim, H.F. (2009) Reclassification of *Staphylococcus aureus* nasal carriage types. *J Infect Dis* **199**: 1820-1826.
- van den Akker, E.L., Nouwen, J.L., Melles, D.C., van Rossum, E.F., Koper, J.W., Uitterlinden, A.G., Hofman, A., Verbrugh, H.A., Pols, H.A., Lamberts, S.W., and van Belkum, A. (2006) *Staphylococcus aureus* nasal carriage is associated with glucocorticoid receptor gene polymorphisms. *J Infect Dis* **194**: 814-818.
- van Smeden, J., Janssens, M., Kaye, E.C., Caspers, P.J., Lavrijsen, A.P., Vreeken, R.J., and Bouwstra, J.A. (2014) The importance of free fatty acid chain length for the skin barrier function in atopic eczema patients. *Exp Dermatol* **23**: 45-52.
- Vaudaux, P.E., Francois, P., Proctor, R.A., McDevitt, D., Foster, T.J., Albrecht, R.M., Lew, D.P., Wabers, H., and Cooper, S.L. (1995) Use of adhesion-defective mutants of *Staphylococcus aureus* to define the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. *Infect Immun* **63**: 585-590.
- Vazquez, V., Liang, X., Horndahl, J.K., Ganesh, V.K., Smeds, E., Foster, T.J., and Hook, M. (2011) Fibrinogen is a ligand for the *Staphylococcus aureus* microbial surface components recognizing adhesive matrix molecules (MSCRAMM) bone sialoprotein-binding protein (Bbp). *J Biol Chem* **286**: 29797-29805.

- Villareal, V.A., Pilpa, R.M., Robson, S.A., Fadeev, E.A., and Clubb, R.T. (2008) The IsdC protein from *Staphylococcus aureus* uses a flexible binding pocket to capture heme. *J Biol Chem* **283**: 31591-31600.
- Visai, L., Yanagisawa, N., Josefsson, E., Tarkowski, A., Pezzali, I., Rooijakkers, S.H., Foster, T.J., and Speziale, P. (2009) Immune evasion by *Staphylococcus aureus* conferred by iron-regulated surface determinant protein IsdH. *Microbiology* **155**: 667-679.
- Volz, A., Korge, B.P., Compton, J.G., Ziegler, A., Steinert, P.M., and Mischke, D. (1993) Physical mapping of a functional cluster of epidermal differentiation genes on chromosome 1q21. *Genomics* **18**: 92-99.
- von Eiff, C., Becker, K., Machka, K., Stammer, H., and Peters, G. (2001) Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. *N Engl J Med* **344**: 11-16.
- Waldron, D.E., and Lindsay, J.A. (2006) Sau1: 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.
- Walker, J.N., Crosby, H.A., Spaulding, A.R., Salgado-Pabon, W., Malone, C.L., Rosenthal, C.B., Schlievert, P.M., Boyd, J.M., and Horswill, A.R. (2013) The *Staphylococcus aureus* ArlRS two-component system is a novel regulator of agglutination and pathogenesis. *PLoS Pathog* **9**: e1003819.
- Walsh, E.J., Miajlovic, H., Gorkun, O.V., and Foster, T.J. (2008) Identification of the *Staphylococcus aureus* MSCRAMM clumping factor B (ClfB) binding site in the α C-domain of human fibrinogen. *Microbiology* **154**: 550-558.
- Walsh, E.J., O'Brien, L.M., Liang, X., Hook, M., and Foster, T.J. (2004) Clumping factor B, a fibrinogen-binding MSCRAMM (microbial surface components recognizing adhesive matrix molecules) adhesin of *Staphylococcus aureus*, also binds to the tail region of type I cytokeratin 10. *J Biol Chem* **279**: 50691-50699.
- Wang, Y., Hu, M., Liu, Q., Qin, J., Dai, Y., He, L., Li, T., Zheng, B., Zhou, F., Yu, K., Fang, J., Liu, X., Otto, M., and Li, M. (2016) Role of the ESAT-6 secretion system in virulence of the emerging community-associated *Staphylococcus aureus* lineage ST398. *Sci Rep* **6**: 25163.
- Wann, E.R., Gurusiddappa, S., and Hook, M. (2000) The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *J Biol Chem* **275**: 13863-13871.
- Warne, B., Harkins, C.P., Harris, S.R., Vatsiou, A., Stanley-Wall, N., Parkhill, J., Peacock, S.J., Palmer, T., and Holden, M.T. (2016) The Ess/Type VII secretion system of *Staphylococcus aureus* shows unexpected genetic diversity. *BMC Genomics* **17**: 222.
- Weidenmaier, C., Goerke, C., and Wolz, C. (2012) *Staphylococcus aureus* determinants for nasal colonization. *Trends Microbiol* **20**: 243-250.
- Weidenmaier, C., and Peschel, A. (2008) Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nat Rev Microbiol* **6**: 276-287.

- Weidinger, S., Illig, T., Baurecht, H., Irvine, A.D., Rodriguez, E., Diaz-Lacava, A., Klopp, N., Wagenpfeil, S., Zhao, Y., Liao, H., Lee, S.P., Palmer, C.N., Jenneck, C., Maintz, L., Hagemann, T., Behrendt, H., Ring, J., Nothen, M.M., McLean, W.H., and Novak, N. (2006) Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *J Allergy Clin Immunol* **118**: 214-219.
- Weidinger, S., and Novak, N. (2016) Atopic dermatitis. *Lancet* **387**: 1109-1122.
- Weiss, W.J., Lenoy, E., Murphy, T., Tardio, L., Burgio, P., Projan, S.J., Schneewind, O., and 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.
- Wertheim, H.F., Melles, D.C., Vos, M.C., van Leeuwen, W., van Belkum, A., Verbrugh, H.A., and Nouwen, J.L. (2005) The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis* **5**: 751-762.
- Wertheim, H.F., Vos, M.C., Ott, A., van Belkum, A., Voss, A., Kluytmans, J.A., van Keulen, P.H., Vandenbroucke-Grauls, C.M., Meester, M.H., and Verbrugh, H.A. (2004) Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet* **364**: 703-705.
- Wertheim, H.F., Walsh, E., Choudhury, R., Melles, D.C., Boelens, H.A., Miajlovic, H., Verbrugh, H.A., Foster, T., and van Belkum, A. (2008) Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. *PLoS Med* **5**: e17.
- Windmuller, N., Witten, A., Block, D., Bunk, B., Sproer, C., Kahl, B.C., and Mellmann, A. (2015) Transcriptional adaptations during long-term persistence of *Staphylococcus aureus* in the airways of a cystic fibrosis patient. *Int J Med Microbiol* **305**: 38-46.
- Wollenberg, A., Howell, M.D., Guttman-Yassky, E., Silverberg, J.I., Kell, C., Ranade, K., Moate, R., and van der Merwe, R. (2019) Treatment of atopic dermatitis with tralokinumab, an anti-IL-13 mAb. *J Allergy Clin Immunol* **143**: 135-141.
- Xia, G., Kohler, T., and Peschel, A. (2010) The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. *Int J Med Microbiol* **300**: 148-154.
- Xiang, H., Feng, Y., Wang, J., Liu, B., Chen, Y., Liu, L., Deng, X., and Yang, M. (2012) Crystal structures reveal the multi-ligand binding mechanism of *Staphylococcus aureus* ClfB. *PLoS Pathog* **8**: e1002751.
- Xu, S., Corvaglia, A.R., Chan, S.H., Zheng, Y., and Linder, P., (2011) A type IV modification-dependent restriction enzyme SauUSI from *Staphylococcus aureus* subsp. *aureus* USA300. *Nucleic Acids Res* **39**: 5597-5610.
- Yamasaki, K., and Gallo, R.L. (2008) Antimicrobial peptides in human skin disease. *Eur J Dermatol* **18**: 11-21.
- Yang, D., Chertov, O., Bykovskaia, S.N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schroder, J.M., Wang, J.M., Howard, O.M., and

- Oppenheim, J.J. (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **286**: 525-528.
- Yang, Y.H., Jiang, Y.L., Zhang, J., Wang, L., Bai, X.H., Zhang, S.J., Ren, Y.M., Li, N., Zhang, Y.H., Zhang, Z., Gong, Q., Mei, Y., Xue, T., Zhang, J.R., Chen, Y., and Zhou, C.Z. (2014) Structural insights into SraP-mediated *Staphylococcus aureus* adhesion to host cells. *PLoS Pathog* **10**: e1004169.
- Yeung, M., Balma-Mena, A., Shear, N., Simor, A., Pope, E., Walsh, S., and McGavin, M.J. (2011) Identification of major clonal complexes and toxin producing strains among *Staphylococcus aureus* associated with atopic dermatitis. *Microbes Infect* **13**: 189-197.
- Yoneda, K., McBride, O.W., Korge, B.P., Kim, I.G., and Steinert, P.M. (1992) The cornified cell envelope: loricrin and transglutaminases. *J Dermatol* **19**: 761-764.
- Zanger, P., Nurjadi, D., Vath, B., and Kremsner, P.G. (2011) Persistent nasal carriage of *Staphylococcus aureus* is associated with deficient induction of human beta-defensin 3 after sterile wounding of healthy skin in vivo. *Infect Immun* **79**: 2658-2662.
- Zapotoczna, M., Jevnikar, Z., Miajlovic, H., Kos, J., and Foster, T.J. (2013) Iron-regulated surface determinant B (IsdB) promotes *Staphylococcus aureus* adherence to and internalization by non-phagocytic human cells. *Cell Microbiol* **15**: 1026-1041.
- Zapotoczna, M., McCarthy, H., Rudkin, J.K., O'Gara, J.P., and O'Neill, E. (2015) An Essential Role for Coagulase in *Staphylococcus aureus* Biofilm Development Reveals New Therapeutic Possibilities for Device-Related Infections. *J Infect Dis* **212**: 1883-1893.
- Zapotoczna, M., Riboldi, G.P., Moustafa, A.M., Dickson, E., Narechania, A., Morrissey, J.A., Planet, P.J., Holden, M.T.G., Waldron, K.J., and Geoghegan, J.A. (2018) Mobile-Genetic-Element-Encoded Hypertolerance to Copper Protects *Staphylococcus aureus* from Killing by Host Phagocytes. *MBio* **9**: e00550-18.
- Zheng, T., Yu, J., Oh, M.H., and Zhu, Z. (2011) The Atopic March: Progression from Atopic Dermatitis to Allergic Rhinitis and Asthma. *Allergy, Asthma & Immunology Research* **3**: 67-73.
- Zhou, X.M., Idler, W.W., Steven, A.C., Roop, D.R., and Steinert, P.M. (1988) The complete sequence of the human intermediate filament chain keratin 10. Subdomainal divisions and model for folding of end domain sequences. *J Biol Chem* **263**: 15584-15589.
- Zipperer, A., Konnerth, M.C., Laux, C., Berscheid, A., Janek, D., Weidenmaier, C., Burian, M., Schilling, N.A., Slavetinsky, C., Marschal, M., Willmann, M., Kalbacher, H., Schitteck, B., Brötz-Oesterheld, H., Grond, S., Peschel, A., and Krismer, B. (2016) Human commensals producing a novel antibiotic impair pathogen colonization. *Nature* **535**: 511-516.

Zong, Y., Xu, Y., Liang, X., Keene, D.R., Hook, A., Gurusiddappa, S., Hook, M., and Narayana, S.V. (2005) A 'Collagen Hug' model for *Staphylococcus aureus* CNA binding to collagen. *Embo j* **24**: 4224-4236.