



**The characterisation of the cell surface of *Staphylococcus aureus* in the  
search for new therapeutic targets.**

Kate Harvey

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy: Science

May 2018





CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Kate Harvey, certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except where fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of student:

Production Note:

Signature removed prior to publication.

Date: May 2018

## Acknowledgements

Firstly, I would like to thank Ian Charles who gave me the opportunity to start and be part of this project. Having a supervisor with such a strong calibre of research output and industry connections was an amazing opportunity in itself. Thank you for having enough belief in me to take me on as one of your first PhD students at UTS. You also helped facilitate my research time at UCL, London, an experience that was one of the highlights of my PhD and one I will carry with me through life.

I would like to acknowledge Steven Djordjevic for taking on the responsibility of supervision when Ian could no longer take on this role. Your deep knowledge of all things protein is amazing.

To my longest-lasting supervisor, Matt Padula, I cannot express how grateful I am that you were part of this team throughout my time at UTS. Your faith in me in simply supporting my decision to do a PhD and recommending me to Ian Charles in the first instance is something I will be forever grateful for. You were always willing to lend a helping hand and answer any questions I had. Further, your enthusiasm for teaching me the more mechanical aspects of mass spectrometry was contagious. I cannot express how much I appreciated your presence during this time, thank you.

To my newest co-supervisor, Benjamin Raymond, thank you for all your support. I admire you as a researcher, and the quality of your work, and it was a pleasure having someone on board like yourself. Thank you for all the hours spent reading various drafts and questions answered. Nothing was ever too much of a hassle and I am truly grateful for all your help.

To all my students throughout the year, especially the undergraduate, honours, and masters students I was able to supervise and mentor in the lab. Anna, Dean, Joel, and Nat thank you for this opportunity as I truly enjoyed it and found the experience thoroughly rewarding. I am glad for the friendships I have made and the unforgettable stories that this has provided.

The PhD crew: Jacqueline, thank you for being an absolutely amazing Charles lab buddy. We have been through so much in this PhD together, and I am glad I have made a new friend from this experience. Thank you for all your help, support, drinking encouragement, and all round bad influence. Mike, I'm glad I had someone in the lab that I could chat to and was always happy to provide a helping hand. You have been a huge help and influence throughout my PhD and I truly thank you for this. Also, thanks for all the venting time, laughs, and editing help! Isa, thanks for all the gym chatting session where we could de-stress about our PhDs and for all the laughs along the way. To everyone else in the lab, tea room, and office who has provided a smile, food, some great advice, and is always down for a good chat: Samira, Megan, Krish, Dan, Louisa, Ethan, Jess, Ronnie, Iain, Jerran, and Jess T. Having people to talk to who were along the same path was invaluable, and you have all helped me maintain what little sanity I had left while keeping a smile on my face.

I would also like to thank everyone who is behind the scenes at UTS who have helped my research; Shannon, Harry, Ian, Sarah, and Mercedes thank you for all the work you do.

To my amazing friends outside of UTS, Steph, Brenda, Katherine, Jess, Viv, Marissa, Judy, and Vanessa, thank you for dealing with me over these many long years. I have been grateful for all the downtime and support. I am lucky to count each of you as a friend.

Anthony, you have been incredible. I could not have asked for someone more supportive or encouraging to be with me on this final leg. Thank you for your "outside voice", always encouraging the mad scientist in me, and all the food!

Lastly, I need to thank all my family for their tremendous support over these years. To my dear Jeda for always asking how my "book" was going and maintained an unwavering interest in my studies. To my dad for all the words of encouragement. And finally, to my mother who has supported me in this academic journey and has had to put up with a PhD student living with her. This alone is an amazing feat, and not for the faint of heart. I thank you all for your love and support throughout the years.

**Declaration**

All data collected, analysed, and presented in this thesis is the work of the author unless clearly specified. Chapters 2-4 have been prepared for publication as described at the beginning of the relevant chapter. Chapter 4 is written verbatim from the papers it is made up of. The author declares no conflict of interest in this thesis or the data it contains.

## Table of Contents

<b>Abstract.....</b>	<b>xvii</b>
<b>Chapter 1. Introduction to <i>Staphylococcus aureus</i> proteomics.....</b>	<b>1</b>
1.1 <i>Staphylococcus aureus</i> .....	1
1.1.1 General background .....	1
1.1.2 Pathogenicity.....	2
1.1.2.1. Virulence factors.....	3
1.1.2.2. Adhesins .....	5
1.1.2.3. Glycosaminoglycans .....	6
1.1.3 Moonlighting proteins.....	7
1.1.3.1. Protein degradation.....	12
1.2 <i>Staphylococcus aureus</i> proteomics.....	16
1.2.1 <i>S. aureus</i> surface proteome.....	17
1.3 The resistance apocalypse .....	24
1.3.1 Lack of current therapeutics .....	24
1.3.2 Beyond antibiotics: the vaccine race.....	25
1.4 Project Aims .....	28
Aim 1: Characterise surface proteins in <i>S. aureus</i> .....	28
Aim 2: Determine the repertoire of proteins <i>S. aureus</i> possesses that bind heparin.....	28
Aim 3: Characterise the novel <i>S. aureus</i> surface moonlighting protein, Ef-Tu.....	28
Overall investigation techniques.....	29
Overarching aims .....	34
<b>Chapter 2. The surface proteome of <i>Staphylococcus aureus</i> .....</b>	<b>35</b>
2.1 Introduction .....	35
2.2 Material and methods .....	42
2.2.1 Cell growth .....	42
2.2.2 Live/dead staining .....	42
2.2.3 Cell harvest .....	43
2.2.4 Surface trypsin shaving.....	43
2.2.5 Solid phase extraction of peptides .....	44
2.2.6 Preparation of surface biotinylated proteins .....	44
2.2.7 Cell disruption of biotinylated cells.....	44
2.2.8 Affinity chromatography (streptavidin).....	45

2.2.9	Protein Preparation for Gel Electrophoresis.....	46
2.2.10	1D-PAGE of surface biotinylated proteins.....	46
2.2.11	2D-PAGE of surface biotinylated proteins.....	47
2.2.12	Avidin Western blot.....	47
2.2.13	Protein Preparation for LC-MS/MS.....	49
2.2.13.1.	Trypsin shaved peptides.....	49
2.2.13.2.	In gel protein digestion from 1D-SDS-PAGE.....	49
2.2.13.3.	In gel protein digestion from 2D-PAGE.....	50
2.2.14	LC-MS/MS.....	50
2.2.14.1.	LC-MS/MS for 1D biotinylation samples.....	51
2.2.15	Protein identification.....	51
2.2.16	DnaK antibody growth assays.....	52
2.2.17	Bioinformatic Analysis.....	53
2.3	Results.....	54
2.3.1	Live-Dead staining.....	54
2.3.2	Cell surface trypsin shaving.....	55
2.3.2.1.	Thirty min trypsin shaving protocol.....	55
2.3.2.2.	One min trypsin shaving protocol.....	55
2.3.2.3.	Comparison of trypsin incubation times.....	55
2.3.3	Cell surface Biotinylation.....	58
2.3.3.1.	No natively biotinylated proteins were identified in <i>S. aureus</i> SH1000.....	58
2.3.3.2.	1D-SDS-PAGE analysis of surface biotinylated proteins.....	58
2.3.3.3.	2D- PAGE analysis of surface biotinylated proteins.....	92
2.3.4	A large number of predicted cytosolic proteins are consistently found on the surface of <i>S. aureus</i> .....	92
2.3.5	Proteins derived from essential genes were surface exposed.....	96
2.3.5.1.	DnaK.....	97
2.3.5.2.	Confirmation that DnaK resides on the surface of <i>S. aureus</i> .....	103
2.4	Discussion.....	104
2.5	Conclusion.....	113
<b>Chapter 3. Putative heparin-binding proteins of <i>Staphylococcus aureus</i>.....</b>		<b>115</b>
3.1	Introduction.....	116
3.2	Material and methods.....	123
3.2.1	Cell Growth.....	123
3.2.2	Cell Lysis and Protein Harvest.....	123



3.2.3	Affinity Chromatography .....	123
3.2.4	Protein Preparation for Gel Electrophoresis .....	124
3.2.5	1D-SDS-PAGE One-Dimensional Gel Electrophoresis .....	124
3.2.6	Two-Dimensional Gel Electrophoresis .....	125
3.2.7	Protein Preparation for LC-MS/MS .....	125
3.2.8	Protein identification.....	126
3.2.9	Bioinformatic Analysis .....	127
3.3	Results.....	128
3.3.1	Potential heparin-binding proteins identified in <i>S. aureus</i> .....	128
3.3.2	Novel potential <i>S. aureus</i> adhesins identified .....	131
3.3.3	Identification of cleavage products binding heparin.....	132
3.3.4	Cleaved proteins bind heparin at high salt concentrations.....	135
3.4	Discussion.....	145
3.4.1	<i>S. aureus</i> retains high functional redundancy in adhesin proteins .....	145
3.4.2	Proteins from key metabolic pathways have moonlighting capabilities as potential adhesins .....	146
3.4.3	Cleaved protein products of <i>S. aureus</i> were retained by heparin affinity chromatography.... .....	147
3.4.4	Novel cleavage sites in ribosomal proteins identified.....	148
3.4.5	Implications of results on biofilm formation .....	149
3.5	Conclusion.....	154
<b>Chapter 4. Elongation factor Tu is highly processed and surface exposed.....</b>		<b>155</b>
4.1	Introduction .....	157
4.2	Methods.....	163
4.2.1	Bacterial growth .....	163
4.2.2	Enrichment of <i>S. aureus</i> surface proteins .....	163
4.2.2.1.	Biotinylation .....	163
4.2.2.2.	Trypsin shaving .....	164
4.2.3	Preparation and separation of whole cell lysates for one- and two-dimensional gel electrophoresis.....	164
4.2.3.1.	Whole cell lysis preparation .....	164
4.2.3.2.	1D -SDS-PAGE protein separation .....	164
4.2.3.3.	2D-PAGE protein separation.....	165
4.2.4	Heparin affinity chromatography.....	165

4.2.5	Liquid chromatography tandem mass spectrometry (LC-MS/MS) and MS/MS data analysis..	165
4.2.6	Dimethyl labelling and LC-MS/MS analysis of <i>S. aureus</i> proteins .....	166
4.2.6.1.	Dimethyl labelling of proteins .....	166
4.2.6.2.	LC-MS/MS (Sciex 5600) of dimethyl labelled proteins .....	166
4.2.6.3.	LC-MS/MS (Thermo Scientific Q Exactive™) of dimethyl labelled proteins .....	167
4.2.7	Bioinformatic analysis of Ef-Tu .....	168
4.3	Results .....	170
4.3.1	<i>Staphylococcus aureus</i> Ef-Tu (Sa <sub>Ef-Tu</sub> ) is accessible on the bacterial surface.....	170
4.3.2	Sa <sub>Ef-Tu</sub> is one of the most highly processed proteins in <i>S. aureus</i> .....	174
4.3.3	Sa <sub>Ef-Tu</sub> and Sa <sub>Ef-Tu</sub> fragments are retained by heparin affinity chromatography.....	177
4.3.4	Bioinformatic analysis of Sa <sub>Ef-Tu</sub> .....	177
4.3.4.1.	Bioinformatic analysis of heparin-binding site in Sa <sub>Ef-Tu</sub> .....	177
4.3.4.2.	Molecular modelling of Sa <sub>Ef-Tu</sub> .....	178
4.3.4.3.	Protein alignment of Ef-Tu from bacterial species.....	181
4.3.4.4.	Processing events expose new predicted surface macromolecule interaction sites.	185
4.4	Discussion .....	187
4.5	Conclusion .....	190
<b>Chapter 5. General Discussion .....</b>		<b>193</b>
5.1	Concluding remarks.....	209

## List of Figures

Figure 1.1 Potential mechanisms for the acquisition of multifunctional properties in proteins. .....	10
Figure 1.2 Schematic description of proteoforms. ....	14
Figure 1.3 Gel electrophoresis for separation of a protein sample. ....	31
Figure 1.4 Difference between what portion of a surface protein can be isolated using different methodologies. ....	33
Figure 2.1 Association of proteins with the (Gram positive) bacterial cell surface. ....	37
Figure 2.2 Graph representing the amount cytosolic proteins identified on the surface of <i>S. aureus</i> . ....	39
Figure 2.3 Live-dead staining results after incubation periods used in these experiments. ....	54
Figure 2.4 Venn diagram comparing proteins identified in the 30 min and 1 min trypsin shaving experiment. ....	56
Figure 2.5 Venn diagram including all proteins identified in 2 or more replicates ....	57
Figure 2.6 Venn diagram showing overlap of protein identifications across the three experiments. ....	59
Figure 2.7 PSORTb predicted locations of proteins identified in the 30 min trypsin shave experiment. ....	94
Figure 2.8 PSORTb predicted locations of proteins identified in the 1 min trypsin shave experiment. ....	94
Figure 2.9 PSORTb predicted locations of proteins identified in the biotinylation experiment. ....	95
Figure 2.10 PSORTb predicted locations of proteins identified across all surface methodologies. ....	95
Figure 2.11 3-10 2D-PAGE of <i>S. aureus</i> surface biotinylated proteins. ....	99
Figure 2.12 4-7 2D-PAGE of <i>S. aureus</i> WCL proteins. ....	100
Figure 2.13 DnaK protein sequence showing tryptic peptides generated from spot 210. ....	101
Figure 2.14 Small scale growth assays with antibodies to DnaK. ....	103
Figure 2.15 Possible causes for the presence of classically cytosolic proteins on the surface of <i>S. aureus</i> , and controls used in surface proteome analysis. ....	108
Figure 3.1 Structure of the heparan sulfate and heparin disaccharide units. ....	118
Figure 3.2 Diagram is depicting bacterial surface proteins interacting with proteoglycans (PG) on the host cell surface. ....	119
Figure 3.3 1D-SDS-PAGE gel of <i>S. aureus</i> putative heparin-binding proteins. ....	130

Figure 3.4 Venn diagram showing the overlap of <i>S. aureus</i> proteins identified across the three elutions (0-0.5 M, 0.5-1 M and 1 M+ NaCl). .....	131
Figure 3.5 Predicted cellular locations of identified proteins. ....	133
Figure 3.6 Functions of cleaved proteins identified. ....	134
Figure 3.7 Cleavage sites identified in extracellular matrix-binding protein ebh (hypothetical protein SAOUHSC_01447). ....	136
Figure 3.8 Cleavage maps of ribosomal proteins S7 and S9 which have fragments retained in the heparin column. ....	140
Figure 3.9 Diagram showing the ability of eDNA and GAGs (such as HP) aiding in the attachment of bacteria to host surfaces. ....	151
Figure 4.1 Sa <sub>Ef-Tu</sub> strain NCTC 8325 sequence showing regions identified in surface proteome analysis. ....	171
Figure 4.2 Cleavage map of Sa <sub>Ef-Tu</sub> . ....	172
Figure 4.3 Predicted 3-D ribbon and space-filling structures of Sa <sub>Ef-Tu</sub> showing cleavage sites. ....	179
Figure 4.4 Predicted 3-D space-filling structures of Sa <sub>Ef-Tu</sub> with putative heparin-binding motifs. ....	180
Figure 4.5 Alignments of Ef-Tu functional sites. ....	184
Figure 5.1 Immunogold labelling of <i>S. aureus</i> FbaA (aldolase) and detection by transmission electron microscopy (TEM). ....	200

## List of Tables

Table 1.1 Surface proteome studies of <i>S. aureus</i> .....	21
Table 2.1 787 Proteins identified as surface exposed in <i>S. aureus</i> SH1000.....	60
Table 2.2 Proteolytic clipping evidence in the C-terminus of DnaK.....	101
Table 2.3 Predicted MW and pI of possible fragments in spot 210.....	102
Table 2.4 Previous <i>S. aureus</i> surface studies that identified DnaK.....	112
Table 3.1 30S and 50S ribosomal proteins found in the 1 M+ NaCl elution. ....	138
Table 3.2 N-termini identified that maps to cleavage products of 30S ribosomal protein S7 and 30S ribosomal protein S9 found during gel electrophoresis analysis. ....	141
Table 3.3 Putative heparin-binding motifs found in 30S ribosomal protein S7 and 30S ribosomal protein S9.....	142
Table 4.1 List of moonlighting functions published for Ef-Tu in prokaryotes.....	159
Table 4.2 Dimethyl-labelled and semi-tryptic N-terminal peptides identified in Sa <sub>Ef-Tu</sub> .....	175
Table 4.3 Seven putative heparin-binding motifs identified in Sa <sub>Ef-Tu</sub> .....	178
Table 4.4 Number of binding sites in full length Sa <sub>Ef-Tu</sub> and fragments of Sa <sub>Ef-Tu</sub> .....	186

## List of papers

Widjaja M<sup>†</sup>, **Harvey KL**<sup>†</sup>, Hagemann L<sup>†</sup>, Berry IJ, Jarocki VM, Raymond BBA, Tacchi JL, Gründel A, Steele JR, Padula MP, Charles IG, Dumke R\*, & Djordjevic SP\*. (2017) Elongation factor Tu is a multifunctional and processed moonlighting protein. *Sci Rep* 7: doi:10.1038/s41598-017-10644-z

<sup>†</sup>These authors contributed equally to this work

\*These contributors share senior authorship

Macha IJ, Cazalbou S, Ben-Nissan B, **Harvey KL**, & Milthorpe B. (2015) Marine Structure Derived Calcium Phosphate–Polymer Biocomposites for Local Antibiotic Delivery. *Mar Drugs* 13: doi :10.3390/md13010666

**List of conference proceedings**

Poster presentation 17th International Symposium on Staphylococci and Staphylococcal Infections. Seoul, Korea.	2016
Poster presentation 21st Lorne proteomics symposium. Lorne, Australia.	2016
Poster presentation New Horizons conference. Sydney, Australia.	2015
Poster presentation BacPath 13- Molecular Analysis of Bacterial Pathogens. Phillip Island, Australia.	2015
Poster presentation Proteomics and beyond symposium. Sydney, Australia.	2014
Poster presentation 19th Lorne proteomics symposium. Lorne, Australia.	2014
Oral presentation and poster presentation The 5th Congress of European Microbiologists (FEMS 2013). Leipzig, Germany.	2013
Poster presentation 18th Lorne proteomics symposium. Lorne, Australia.	2013
Lightening talk and poster presentation Proteomics and beyond symposium. Sydney, Australia.	2012

## List of Abbreviations

### General

Methicillin resistant <i>Staphylococcus aureus</i>	MRSA
Hospital-acquired methicillin resistant <i>Staphylococcus aureus</i>	HA-MRSA
Community-acquired methicillin resistant <i>Staphylococcus aureus</i>	CA-MRSA
Vancomycin intermediate <i>Staphylococcus aureus</i>	VISA
Vancomycin resistant <i>Staphylococcus aureus</i>	VRSA
Hospital acquired infection	HAI
United States	US
Small colony variant	SCV
Wild type	WT
Heparin	HP
Heparan sulfate	HS
Microbial surface components recognising adhesive matrix molecules	MSCRAMMs
Secretable expanded repertoire adhesive molecules	SERAM
Glycosaminoglycan	GAG
Proteoglycan	PG
Deoxyribonucleic acid	DNA
Ribonucleic acid	RNA
Gene ontology	GO
Extracellular deoxyribonucleic acid	eDNA
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Centers for Disease Control and Prevention	CDC
Open reading frame	ORF
Intensive care unit	ICU
Single nucleotide polymorphism	SNP
Post-translational modifications	PTMs
Horizontal gene transfer	HGT
Colony forming units	CFU
Extracellular matrix	ECM
Whole cell lysate	WCL
Excreted cytosolic proteins	ECP
Elongation factor thermo unstable	Ef-Tu
Major histocompatibility complex class II analog protein	MAP
Short linear motif	SLiM
Protein-protein interaction	PPI

### Method-related

Immuno-fluorescence microscopy	IFM
1-Dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis	1D-SDS-PAGE
2-Dimensional polyacrylamide gel electrophoresis	2D-PAGE
Difference in-gel electrophoresis	DIGE
Liquid chromatography	LC



Reversed-phase chromatography	RPLC
High pressure liquid chromatography	HPLC
Isoobaric peptide tags for relative and absolute quantitation	iTRAQ
Isotope-coded affinity tags	ICAT
Stable isotope labels with amino acids in cell culture	SILAC
Hydrophilic interaction liquid chromatography	HILIC
Mass spectrometry	MS
Tandem mass spectrometry	MS/MS
Imaging mass spectrometry	IMS
Matrix assisted laser desorption/ionisation	MALDI
Electrospray ionisation	ESI
Electrospray droplet impact	EDI
Automated direct infusion	ADI
Time-of-flight	TOF
Strong cation exchange	SCX
Polymerase chain reaction	PCR
Semi-quantitative real-time polymerase chain reaction	sqrtPCR
Intelligent data acquisition	IDA

### Solutions

Dithiothreitol	DTT
Acetonitrile	ACN
Trifluoroacetic acid	TFA
Sodium dodecyl sulfate	SDS
Phosphate buffered saline	PBS
Tryptic soy broth	TSB

### Units used

Molar	M
Litre	L
Gram	g
Kilogram	kg
Second	s
Hour	h
Millimetres squared	mm <sup>2</sup>
Nano	n
Milli	m
Micro	μ
Hertz	Hz
Kilo Dalton	kDa
Dalton	Da
Relative centrifugal force	rcf

Degrees celsius	°C
Potential of hydrogen	pH
Weight per volume	w/v
Optical density	OD
Parts per million	ppm
Molecular weight	MW
Amino acid	aa

## **Abstract**

*Staphylococcus aureus* is a Gram-positive pathogen which causes a wide range of afflictions including endocarditis, osteomyelitis, cellulitis, toxic shock syndrome, and necrotising pneumonia. *S. aureus* is a leading cause of hospital-acquired infections and has rapidly acquired resistance to multiple antimicrobials. As such, it was deemed a serious threat by the Centers for Disease Control and Prevention (CDC) in 2013 indicating urgent attention is required to control this pathogen [1]. Currently there are no efficacious vaccines available to treat infections caused by *S. aureus*. With resistance being noted against every class of antibiotic currently available, the development of an alternative therapeutic would not only relieve morbidities, mortalities, and the associated economic burden, but also reduce the selective pressures that drive antibiotic resistance.

To better understand how *S. aureus* interacts with the human host and presents antigens that interact with key host cell receptors, a better understanding of which proteins are displayed on the cell surface is required. This dissertation presents an analysis of the surface proteome of *S. aureus* and describes several potential novel adhesins. Enzymatic cell shaving and surface protein biotinylation were used to catalogue proteins on the cell surface and identify regions within molecules that are surface accessible. Our approaches included methods that maintained protein size context (SDS-PAGE), providing an insight into the extent of surface protein processing. We also characterised heparin-binding proteins in *S. aureus* and interrogated the data in light of our surface proteome studies. This approach enabled us to gain insight into novel binding characteristics used by surface-accessible proteins that could not be predicted using reverse vaccinology and other hypothesis-directed approaches commonly used to develop potential vaccine candidates. Ascertaining the repertoire of heparin-binding proteins was considered important as these proteins are bacterial virulence

factors that facilitate adherence, colonisation, and invasion of target host cells. By coupling these data with other proteomic and bioinformatics techniques, a number of proteins of interest were identified. This includes Elongation Factor Tu, which was found to be surface exposed and highly processed, a finding that has not been seen before in *S. aureus*.

The data presented in the following chapters contributes significantly to the rapidly evolving field of *S. aureus* proteomics. These data will aid in the development of future therapeutic strategies and highlights a number of proteins for further therapeutic investigation.