Phenotypic and Genotypic Investigation of Persistent *Staphylococcus aureus* Bacteraemia Isolates

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Abstract

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Staphylococcus aureus and methicillin-resistant *S. aureus* (MRSA) remain a serious clinical threat and a leading cause of healthcare- and community-associated infections. Blood stream infections or bacteraemia caused by *S. aureus* are further complicated by the phenomenon of bacterial persistence and treatment failure despite confirmed *in vitro* susceptibility of the infecting strain to the administered antibiotics. Moreover, it is unclear how *S. aureus* evades the host immune system for the prolonged duration of a persistent infection. This study aimed to answer these two clinically relevant, biological questions by characterising multiple persistent and resolved MRSA bacteraemia isolates with the view to identifying persistent isolate associated phenotypic and genotypic traits, and novel mechanism(s) for persistence development.

From this work, two distinctly novel *S. aureus* persistence mechanisms are presented. The first involved *in vivo* strain evolution induced by antibiotic exposure (daptomycin) during two independent incidences of persistence (PB1 and PB3), resulting in *mprF* gain-of-function mutations in the persisting bacterial population. This led to daptomycin non-susceptibility and the emergence of novel persistence associated virulence phenotypes, which included a nutrient deprived growth adaption, increased immune evasion, adhesion and stress response associated surface proteins, and attenuated virulence. The second mechanism presented (PB2 and PB5) did not display any defining persistence associated traits; however these bacteraemia were not treated with daptomycin, which further supports the daptomycin induced, MprF mediated persistence mechanism presented for the PB1 and PB3 cases.

This study is the first of its kind to investigate multiple persistent MRSA bacteraemia inclusive of numerous temporally spaced infective isolates, in parallel with

contemporaneous resolved bacteraemia isolates of the same genetic background. The subsequent findings of this study have massive implications for the current clinical regimes implemented during complex *S. aureus* bacteraemia, particularly antibiotic treatment choices, and potentially for other cases of bacterial infection where persistence is frequently observed.

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Finally, there is one really special person I would like to dedicate this to, who is sadly not around to witness this milestone. I love you Dad, I hope this makes you proud.

Contributions

The majority of experimental data presented in this study has been the result of the author's own work. Additionally, all the research, writing and conclusions made within this report are the result of the author's own work.

There were, however, significant contributions made from third parties during certain experimental procedures and subsequent data analysis; these will now be acknowledged.

Firstly, I would like to thank Dr Andrew Bottrill (PNACL, University of Leicester) for conducting in-depth proteomic analysis (iTRAQ LC-MS) as presented in Chapter 3:7:3 and Chapter 5:3:3.

Secondly, I would like to thank Dr Ben Pascoe and Dr Sam Sheppard (University of Swansea) for organising and conducting Illumina whole genome sequence analysis as presented in Chapter 5:4.

Third and finally, I would like to thank Dr Richard Haigh (University of Leicester) for conducting bioinformatic analysis using the genome sequence data as presented in Chapter 5:4.

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Abbreviations

°C	Degrees centigrade
µg / mg / g	microgram / milligram / gram
µl / ml / l	microliter / millilitre / litre
µm / mm / cm	micrometre / millimetre / centimetre
ABC	ATP Binding Cassette
AIP	Auto Inducing Peptide
ATP	Adenosine Triphosphate
BC	Blood Culture
BHI	Brain Heart Infusion
bp / kb / Mbp	base pair / kilobase pair / Megabase pair
BSI	Blood Stream Infection
$C0_2$	Carbon dioxide
CF	Cystic Fibrosis
CFU	Colony Forming Units
CRP	C- Reactive Protein
CRPMI	Chelexed RPMI
CTAB	Cetyl Trimethyl Ammonium Bromide
dH_2O	Distilled water
(c)DNA	(complementary) Deoxyribonucleic Acid
EMRSA	Epidemic Methicillin Resistant *Staphylococcus aureus*
EtBr	Ethidium Bromide
EtOH	Ethanol
FBS	Fetal Bovine Serum
FnBP	Fibronectin Binding Proteins
GoF	Gain of Function
GWAS	Genome Wide Association Study
H_2O_2	Hydrogen Peroxide
HA- / CA-MRSA	Healthcare- / Community-Associated MRSA
HCl	Hydrochloric Acid
HDP / CAMP	Host Defence Peptides / Cationic Antimicrobial Peptides
HGT	Horizontal Gene Transfer
HPLC	High Performance Liquid Chromatography
IE	Infective Endocarditis
IgG	Immunoglobulin G
IPA	Iso-propan-2-ol
IS	Insertion Sequence
iTRAQ	isobaric Tag for Relative Quantification
IV	Intravenous
J-regions	Joining-regions
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MICE / MBD	MIC Evaluator / Micro-Broth Dilution
MLST	Multi-Locus Sequence Typing
MprF	Multi peptide resistance Factor
MPS	Membrane Physical State
MRI	Magnetic Resonance Imaging
(LC-) MS	(Liquid Chromatography-) Mass Spectrometry

MSSA / MRSA	Methicillin Sensitive / Methicillin Resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NGS	Next Generation Sequencing
nM / μM /mM / M	nanoMolar / microMolar / miniMolar / Molar
OD	Optical Density
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
(L-) PG	(Lysyl-) Phosphotidylglycerol
PMP	Platelet Microbicidal Proteins
PNACL	Protein Nucleic Acid Chemistry Laboratory
ppGpp	Guanosine tetraphosphate
PSM	Phenol Soluble Modulins
PVL	Panton Valentine Leukocidin
qPCR / qRT-PCR	quantitative (Reverse Transcription-) PCR
RB	Resolved Bacteraemia
RM-system	Restriction Modification System
(m/r)RNA	(messenger/ribosomal) Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Rotations per minute
SCC(*mec*)	Staphylococcal Cassette Chromosome (*mec*)
SCV	Small Colony Variant
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SNP / INDEL	Single Nucleotide Polymorphism / Insertion-Deletion
ST / CC	Sequence Type / Clonal Complex
TA system	Toxin-antitoxin system
TAE	Tris-Acetate-EDTA (Ethylenediaminetetraacetic acid)
TCR	Two Component Regulator
TSB	Trypticase Soy Broth
TTE / TOE	Transthoracic echocardiogram / Transesophageal echocardiogram
UHL	University Hospitals of Leicester
UOL	University Of Leicester
v/v	Volume by volume
VRSA / (h)VISA	Vancomycin Resistant / (hetero) Vancomycin Intermediate *Staphylococcus aureus*
w/v	Weight by volume

Chapter 1 Introduction

The aim of this study was to genotypically and phenotypically characterise clinical samples originating from multiple *Staphylococcus aureus* bacteraemia for the purpose of identifying putative persistence mechanisms. This chapter will briefly describe *S. aureus*, its genome, virulence regulation and propensity of *S. aureus* to develop antibiotic resistance and its immune evasion capabilities. The phenomenon of bacterial persistence will then be discussed with particular focus on *S. aureus* persistence, current phenotypic associations and suspected persistence mechanisms. The results section will begin with the clinical details of each *S. aureus* infection included in this study and will continue to describe the phenotypic characterisation strategies employed and subsequent data in a further chapter. The third and final results chapter will investigate a potential genetic basis of persistence through mutagenesis and comparative genomic analysis. Finally, this study will speculate on the potential repercussions these findings may have on the clinical management of *S. aureus* associated diseases.

1.1 Microbiology of *Staphylococcus aureus*

Staphylococcus aureus belongs to the genus *Staphylococcus* which can be biochemically recognised from other bacterial genera such as *Enterococci* and *Streptococci* by the expression of catalase (Madigan & Martinko, 2006). *S. aureus* is a Gram-positive, facultative anaerobe; the cells are approximately one micro-meter in size, spherically shaped and form grape-like clusters under the microscope (Madigan & Martinko, 2006). It can be found as a commensal on the skin and mucosal membranes of people colonised; it is estimated that 20 % of humans are persistently and 60 – 80 % intermittently colonised (van Belkum et al., 2009; Garzoni & Kelley, 2009). *S. aureus* is an opportunistic pathogen, with the ability to cause a wide range of human diseases ranging from minor skin infections to pneumonia, meningitis and bacteraemia (Ala'Aldeen & Hiramatsu, 2004), as well as being a leading cause of infective endocarditis (Fowler et al., 2005). *S. aureus* is a highly adaptable pathogen with the ability to accurately assess the environment and respond accordingly via complex gene expression regulation. Additionally, *S. aureus*

displays a repertoire of accessory genes acquired through frequent horizontal gene transfer (HGT) which significantly contributes to the adaptability of *S. aureus*.

1.2 The *Staphylococcus aureus* genome

S. aureus has an approximate genome size of 2.8 Mbp, ~ 33 % GC-content and 2 000 – 2 800 gene coding regions (Lindsay & Holden, 2004). It is estimated that 75 % of the chromosome encodes essential metabolism and maintenance proteins required for critical cellular processes; this comprises the highly conserved, core genome of *S. aureus*. The sequences of seven house-keeping genes within the core genome allows for the separation of individual *S. aureus* strains into different sequence types (ST) within clonal complexes (CC) (Multi-Locus Sequence Typing [MLST]) (Lindsay & Holden, 2004; Enright et al., 2000; Lindsay & Holden, 2006; Feil et al., 2003).

The other 25 % of the genome comprises additional, non-essential genes obtained through HGT. This accessory genome includes bacteriophages, transposons, plasmids, pathogenicity/genomic islands which carry virulence determinants and antibiotic resistance genes (Baba et al., 2008; Lindsay & Holden, 2004; Lindsay & Holden, 2006). Interestingly, there appears to be lineage bias in regards to the presence of certain accessory elements, suggesting difficulty with HGT between different lineages (McCarthy et al., 2012; Lindsay, 2014). This may be attributed to lineage specific restriction-modification (RM) systems which digest "foreign" DNA originating from evolutionary divergent strains (Lindsay & Holden, 2006). Overall, all *S. aureus* strains display a vast array of accessory genetic elements contributing to the high level of genetic diversity experienced in *S. aureus*.

1.2.1 *Staphylococcus aureus* accessory genome

There are multiple pathways for HGT involving various mobile genetic elements (MGE) including plasmids, bacteriophages and Staphylococcal cassette chromosomes (SCC) (Malachowa & DeLeo, 2010).

Plasmids can either be small and multi-copy encoding a single virulence determinant, large and single copy encoding multiple virulence determinants or large multi-factor conjugative plasmids. Perhaps the most currently relevant plasmid is the Tn*1546* plasmid encoding the *vanA* operon (*vanAHXSRYZ*), acquired

by Staphylococcal species from Enterococci presumably during a co-infection. Acquisition and expression of this operon allows for high level vancomycin resistance leading to the emergence of vancomycin resistant *S. aureus* (VRSA) within pre-existing methicillin resistant strains (MRSA) (Chapter 1:4:1). Proteins encoded within this operon initiate the hydrolysis of wild type D-Ala-D-Ala peptidoglycan (VanX and VanY), and synthesise alternative D-Ala-D-Lac precursors (VanA and VanH) which display decreased glycopeptide affinity. The encoded two component regulator system (VanS and VanR) controls operon expression in response to vancomycin exposure (Périchon & Courvalin, 2009; Malachowa & DeLeo, 2010). Another example of plasmid MGE is *erm*(T)-carrying multi-resistance plasmids which contain multi-antibiotic resistance determinants including tetracycline, trimethoprim and kanamycin/neomycin; but also heavy metal resistances such as cadmium (*cadD* and *cadX*) and copper (*copA*) (Gómez-Sanz et al., 2013; Baker et al., 2010).

Bacteriophages are another effective method for genetic transfer between Staphylococcal strains. Bacteriophages are viruses which exclusively infect bacteria, integrate their genetic material into the host genome and synthesise new viral particles using host cell machinery. Newly synthesised viral particles can then be released with minimal damage to the host (temperate phage), through host cell lysis (lytic phage) or can remain within the host genome indefinitely (chronic phage). Prophage associated genetic determinants encode immune modulator proteins (e.g. Scn, Set, Sak), various enterotoxins and the Panton-Valentine leucocidin (PVL) (McCarthy et al., 2012; Malachowa & DeLeo, 2010). PVL is an important virulence factor able to cause apoptosis and necrosis in neutrophils and is associated with abscess formation (Genestier et al., 2005; Malachowa & DeLeo, 2010). Prophages can also have deleterious effect via insertional inactivation; for example the *attB* site for ϕ13 prophage integration is located within the β-haemolysis gene (*hlb*) thereby causing gene inactivation (Coleman et al., 1991).

A third mechanism of HGT involves SCC, e.g. SCC*mec*. Acquisition of this element provides resistance to β-lactams via the expression of *mecA* leading to the designation of methicillin resistance *S. aureus* (MRSA) strains (Baba et al., 2002). The element itself can vary substantially in size (21- to 67 kb) and also in non-

essential gene content; only two gene complexes are essential for methicillin resistance, *mec* and *ccr* (IWG-SCC, 2009; Zhang et al., 2005). Currently there are six types of *mec* gene complex (class A, B, C1, C2, D, E), each containing *mec*A, regulatory encoding genes (*mecI*/*mecR1*) and various insertional sequences (IS) (IWG-SCC; http://www.sccmec.org/Pages/SCC_TypesEN.html; accessed 17-09-2014). The *ccr* gene complex contains cassette chromosome recombinase gene(s) (*ccr*), of which there are current eight recognised types (1 - 8) (Malachowa & DeLeo, 2010; IWG-SCC, 2009; IWG-SCC; http://www.sccmec.org/Pages/SCC_TypesEN.html; accessed 17-09-2014). The different combinations of *mec* and *ccr* gene complexes within SCC*mec* enables the designation of eleven different SCC*mec* types (Figure 1-1 displays SCC*mec* I-VIII) according to the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) (http://www.sccmec.org/Pages/SCC_TypesEN.html; accessed 17-09-2014). Moreover, J regions (joining regions) which contain non-essential elements within SCC*mec* are frequently used as a sub-typing method, adding further distinction within individual SCC*mec* types (IWG-SCC, 2009; Malachowa & DeLeo, 2010).

1.2.2 Virulence determinants and stress response systems

The propensity for pathogenicity between different *S. aureus* strains can vary greatly depending on both the core virulence genes present in individual strains and additional virulence determinants within the accessory genome. These components not only contribute to inter-strain diversity but also gene acquisition can have profound effects on *S. aureus* epidemiology (DeLeo et al., 2010; Otto, 2010).

S. aureus expresses a repertoire of adhesins that are utilised for host factor adhesion and bacterial biofilms; structures which are formed on solid phase structures such as medical devices and/or human tissue. Biofilms have been implicated in numerous chronic diseases such as osteomyelitis, endocarditis, urinary tract infections and cystic fibrosis lung infections (Römling & Balsalobre, 2012; Costerton, 1999). These structures display enhanced resistance to environmental factors such as host defences and antibiotic treatments (Thurlow et al., 2012), which has been attributed to altered metabolism and reduced growth rates exhibited by embedded cells (Fux et al., 2003; Hall-Stoodley & Stoodley, 2009).

Figure 1-1. Diagrammatical representation of the first eight SCC*mec* types (I-VIII) designated by the IWG-SCC. This figure was taken from IWG-SCC (2009); "Classification of Staphylococcal cassette chromosome *mec* (SCC*mec*)", Antimicrob. Agents Chemother. 53(12):4963.

Additionally, dormant cell types (small colony variants [SCV]) which exhibit increased survival capabilities are found within biofilms (Lewis, 2010) (Chapter 1:6:1). It has also been suggested that certain antibiotics may have difficulty diffusing through these structures (Singh et al., 2010), although this suggestion has been disputed (Daddi Oubekka et al., 2012).

There are many different environmental cues and gene regulators which control biofilm formation, the most renowned mechanism involving *icaADBC* (intracellular adhesion operon) (Mack et al., 1996; Mckenney et al., 1998). Ica, which is positively regulated by SarA, controls the expression of key adhesive capsule polysaccharides including PNAG/PIA (poly-N-acetylglucosamine and polysaccharide intercellular adhesion) (Valle et al., 2003; Maira-litrán et al., 2002). These polysaccharides are important for the adherence to cell surfaces and aggregate formation during biofilm development and are frequently induced during osmotic stress in methicillin sensitive *S. aureus* (MSSA) (O'Neill et al., 2007; Maira-litrán et al., 2002).

There are also protein mediated, *ica*-independent biofilm mechanisms which are more favoured by MRSA (O'Neill et al., 2007; Fitzpatrick et al., 2005). Ica independent protein biofilm mediators include Bap (Biofilm-mediated matrix), clumping factors (ClfA and ClfB), autolysin (Atl), extracellular adhesion protein (Eap) and fibronectin binding proteins (FnBP). (Valle et al., 2012; Abraham & Jefferson, 2012; Houston et al., 2011; Melvin et al., 2011; Johnson et al., 2008; O'Neill et al., 2008).

In additional to the involvement of adhesins in biofilm formation, other cell surface associated proteins are known to mediate immune evasion including protein A (SpA) and Sbi (second IgG binding protein). They function by binding the Fc regions of human IgG (immunoglobulins) forming insoluble complexes and subsequently inhibit complement directed phagocytosis (Atkins et al., 2008). Other examples of immune evasion proteins include Eap, which functions as an anti-inflammatory factor by binding host iCAM-1 (intercellular adhesion molecule-1) and inhibiting leukocyte recruitment (Chavakis et al., 2002).

To cause disease, pathogens have to be able to survive the hostile conditions encountered in the host environment including nutrient restriction and exposure to

various toxic agents. Nutrient limitation particularly amino acid starvation can induce a state of dormancy through the activation of the bacterial stringent response. The primary effector of the stringent response, RelA, is capable of inhibiting protein synthesis under austere conditions (Geiger et al., 2012; Geiger et al., 2010). This mechanism allows for minimum survival during hostile conditions until a time when the environment is more favourable for proliferation. Moreover, indiscriminate toxic agents such as hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS) are utilised by the host immune system during phagocytosis; pathogens resists these deleterious agents via induction of the oxidative stress response. Catalase (*katA*) is the main mediator of the oxidative stress response where it metabolises H_2O_2; additional anti-oxidant proteins are also expressed, such as thioredoxin (*trx*), whose function is to "mop-up" ROS (Lu & Holmgren, 2014; Horsburgh et al., 2001; Uziel et al., 2004).

It is clear *S. aureus* has a repertoire of virulence associated genes which span numerous functions, highlighted in Figure 1-2. The next section of this chapter will focus on how the expression of such virulence determinants is controlled.

1.2.3 Gene regulators

There is a vast array of global virulence regulators within the *S. aureus* genome which control the expression of core and accessory genes in response to specific environmental cues. These regulators not only manage their own regulons but also interact with each other to influence gene expression. Gene regulators can either be classified as two component regulators (TCR) or DNA-binding proteins/transcription regulators which directly affect RNA polymerase action. Whole genome sequence analysis of two *S. aureus* strains (N315, Mu50) identified a total of 124 putative transcription regulators inclusive of 17 TCR systems (Kuroda et al., 2001), truly emphasising the highly adaptable nature of S. *aureus* contributing to its success as a global pathogen.

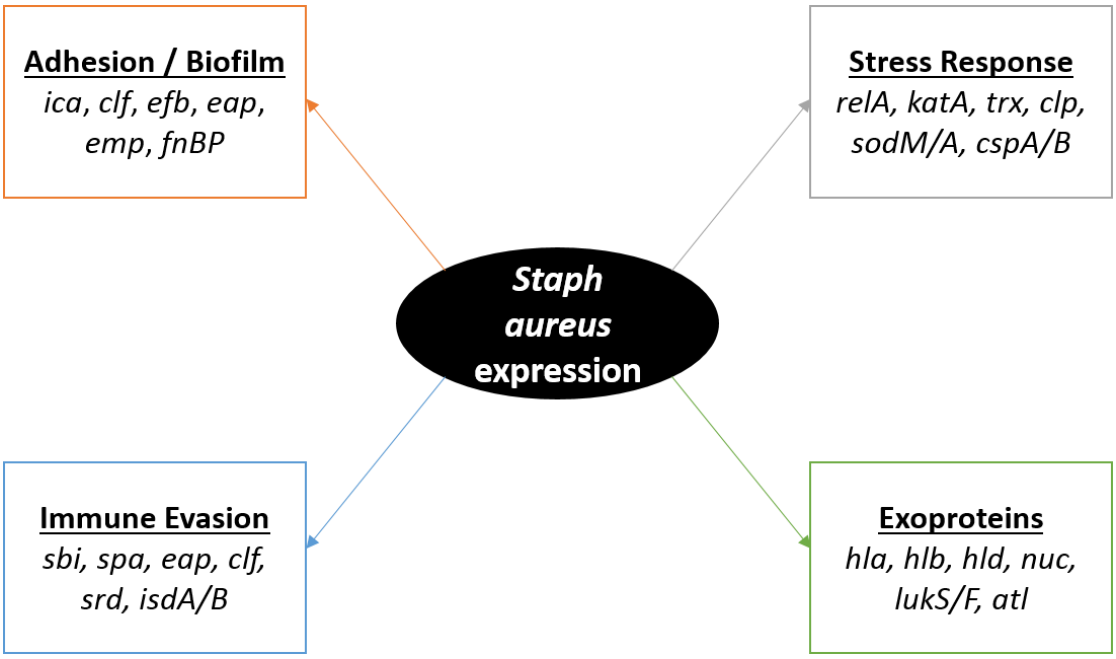

Figure 1-2 A model depicting the functional groups of *S. aureus* virulence determinants. *S. aureus* is able to adapt to a wide range of environments due to apparent expression of a multitude of virulence determinants. Examples of such virulence encoding genes can be found in this figure, loosely grouped according to putative function; namely- adhesion/biofilm, immune evasion, stress response and secreted functions.

Agr/RNAIII

Agr (Accessory Gene Regulator) is a TCR system consisting of two divergently transcribed transcripts (RNAII and RNAIII) controlled by the P2 and P3 promoters respectively. RNAIII is the effector molecule that directly influences virulence expression through RNA - RNA interaction. In contrast RNAII encodes four proteins, AgrACDB; these are collectively responsible for quorum sensing and initiation of RNAIII transcription. RNAIII expression is only initiated once bacterial density has reached a threshold level dictated by the AIP:AgrC ratio (Figure 1-3) (Novick, 2003; Novick et al., 1995; Novick et al., 1993; Ji et al., 1995; Recsei et al., 1986).

Expression of AgrA and subsequently RNAIII leads to the repression of cell surface associated proteins including fibronectin binding proteins (FnBP) and protein A (SpA) but also induces the expression of exoproteins and secreted toxins such as α-toxin (Hla) (Recsei et al., 1986; Somerville & Proctor, 2009). Therefore at low cell densities Agr is inactive, which allows for the expression of adhesins to aid colonisation. As cell density increases expression from the *agr* locus is initiated leading to the secretion of toxins and exoproteins with the aim of scavenging nutrients to maintain the population. RNAIII directed regulation occurs post-transcriptionally, where it functions as an antisense RNA and adheres to the 5' end of target mRNA. This interaction blocks translation initiation and recruits RNA III for targeted mRNA digestion (Huntzinger et al., 2005). RNAIII can also activate expression via the stabilisation and inhibition of secondary structures in target mRNA, allowing for ribosomal entry to the initiation start site (Morfeldt et al., 1995). RNAIII has also been shown to influence other gene regulators specifically the repression of *rot* (repressor of toxins) and *saeRS* expression initiation (Novick & Jiang, 2003; Geisinger et al., 2006). Moreover AgrA not only activates RNAIII transcription but is also known to directly influence the expression of other genes in an RNAIII independent manner. This includes the up-regulation of phenol soluble modulins (PSM) and down-regulation of carbohydrate and amino acid metabolism related genes (Queck et al., 2008).

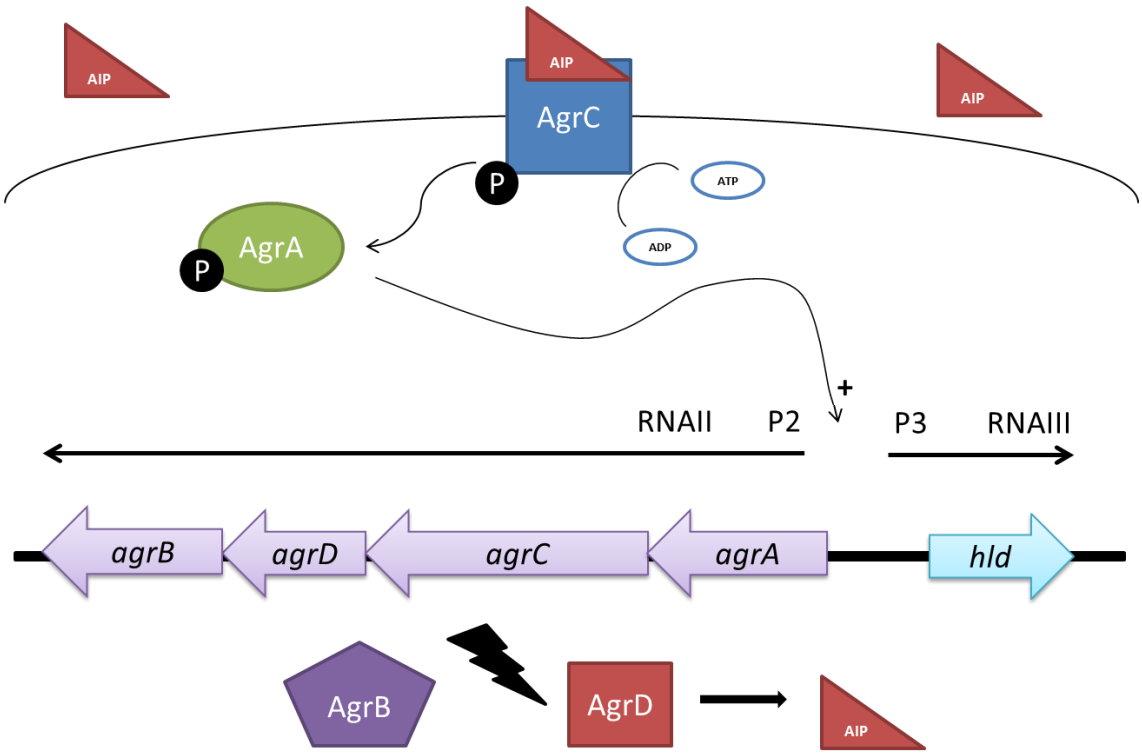

Figure 1-3 Schematic of the Agr TCR system The operon *agrACDB* is transcribed from P2 and translated into four proteins which are responsible for quorum sensing. AgrD is processed by AgrB to form the Auto-Inducing Peptide (AIP) which is exported outside the cell. The concentration of AIP is directly dependent on the density of the bacterial population (quorum sensing) and at a certain threshold AIP can active the transmembrane histidine kinase, AgrC. AgrC can then transfer a phosphate to activate the response regulator, AgrA, which is responsible for the positive regulation of the P2/P3 promoters and RNAIII expression.

SaeRS

The SaeRS system, the second major TCR system in *S. aureus,* consists of four genes (*saePQRS*) where SaeR is the response regulator and SaeS the sensory kinase. SaeP and SaeQ are suggested to be lipo- and membrane proteins respectively which form a complex to activate SaeS's phosphatase activity with the aim to dephosphorylate activated SaeR (Jeong et al., 2013; Giraudo et al., 1994; Adhikari & Novick, 2008). It is proposed that SaeS senses alterations in the cell membrane via its transmembrane domain and begin the phosphorelay (Geiger et al., 2008). However, the exact triggers which active SaeS still remain unclear; but it is plausible to suggest interactions with cell membrane systems may exist. Four individual transcripts have been observed from the *saeRS* locus (Figure 1-4). A and C are transcribed from two promoters, P_A and P_C respectively; whereas it is hypothesised D and B are processed forms of C as there is no known transcriptional start site for B (Adhikari & Novick, 2008; Novick & Jiang, 2003).

Transcription is initiated from P_A after which P_C takes over; this switch is positively regulated by Agr, SarA and SaeRS itself. Environmental signals such as high salt, low pH, high glucose, sub-inhibitory concentrations of clindamycin in addition to the alternative Sigma factor B (SigB) represses SaeRS via P_C whereas sub-inhibitory concentrations of β-lactam antibiotics and H_2O_2 lead to *saeRS* transcription (Novick, 2003; Novick & Jiang, 2003; Adhikari & Novick, 2008; Geiger et al., 2008). SaeRS positively regulates a multitude of secreted and cell surface protein encoding genes (*hla*, *hlb*, *hlg*, *luk, nuc, coa*, *map/eap*, *emp*, *fnb*, *efb*, *sbi*, *spa*) and is thought to work synergistically with Agr to control overall virulence *in vitro* and *in vivo* (Giraudo et al., 1997; Rogasch et al., 2006; Voyich et al., 2009).

There is a multitude of TCR systems in *S. aureus* highlighting its ability to sense the environment and alter virulence expression through the complex regulatory networks of TCR, but also via DNA binding transcription factors.

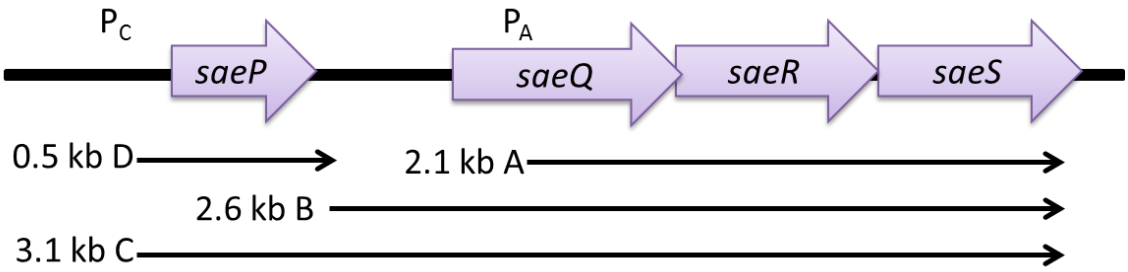

Figure 1-4 Simplistic representation of the *saePQRS* locus. Two individual promoters, P_C and P_A, control the transcription of four overlapping transcripts which encode the four *sae* genes. P_A directs the transcription of A which contains *saeR*/*S* and is thought to begin *saeRS* transcription. There is a subsequent switch from P_A to P_C directed transcription and from this promoter the remaining three transcripts are synthesised. Transcript C contains all four genes, whereas transcripts D and B are thought to be processed forms of transcript C as there is no known transcript B promoter.

SarA protein family and Rot

The SarA (Staphylococcal Accessory gene Regulator A) and the related family consist of either single or double domain, winged helix DNA binding proteins that alter virulence expression by binding the promoters of target genes (Cheung et al., 2008; Cheung et al., 2004; Liu et al., 2006). In general SarA up-regulates adhesins and toxin encoding genes (*fnb*, *efb, hla*, *hlb*, *hld*) and down-regulates protein A (*spa*) and secreted proteases (Cheung et al., 2004). SarA, in combination with SarR, is also known to positively regulate Agr expression (Reyes et al., 2011).

There are multiple SarA homologs, all with similar protein structures but varying influences on virulence expression. Less is known about these homologs compared to SarA but they have been shown to interact forming their own sub-regulatory network (Table 1-1). Rot (Repressor of Toxins) is one such homolog which displays opposing virulence regulation compared to the Agr system i.e. down-regulation of exoproteins and up-regulation of cell surface proteins. However this regulator is ultimately downstream of Agr, where RNAIII has repressive functions against Rot (Said-Salim et al., 2003; Geisinger et al., 2006).

Sigma B

SigB acts as a classic sigma factor guiding RNA polymerase to target virulence gene promoters during late exponential phase (Bischoff et al., 2001). The SigB gene is located within the *rsbUVW-sigB* operon, where the dephosphatase RsbU acts as a positive regulator of SigB, RsbV acts as the anti-anti-SigB factor and RsbW is the anti-SigB factor. SigB expression is induced during heat shock, alkaline shock, $MnCl_2$ and $NaCl_2$ exposure and activation is associated with increased expression of adhesins (*cap*, *atl*, *clfA*, *pho*) and down-regulation of exoproteins (*spl*, *sodM*, *hla*). Additionally, SigB is also known to positively regulate SarA and represses Agr (Bischoff et al., 2001; Bischoff et al., 2004).

Table 1-1 Description of SarA homologs in *S. aureus* N315. Table was adapted from Cheung *et al* (2008), "The SarA protein family of *Staphylococcus aureus*", Int. J. Biochem. Cell Biol. 40(3):358

Gene name	**Putative function and interactions**
SarA	Up-regulates adhesins/toxins: Down-regulates protein A/proteases: Up-regulates Agr (Cheung et al., 2004)
SarR	Manages Agr expression with SarA (Cheung et al., 2004): Down-regulates SarA (Cheung et al., 2004)
SarS	Up-regulates protein A and down-regulates α-toxin (Tegmark et al., 2000)
SarT	Up-regulates SarS (Cheung et al., 2004)
SarU	Up-regulates Agr (Cheung et al., 2004)
Rot	Down-regulates toxins: Opposite effect on virulence expression to Agr (Said-Salim et al., 2003)
SarX	Down-regulates Agr: MgrA-directed activation (Manna & Cheung, 2006)
MgrA	Autolysis and Agr regulator (Truong-bolduc et al., 2003)
SarZ	Up-regulates α-toxin (Kaito et al., 2006)
SarV	Autolysis regulator: Down-regulated by SarA and MgrA (Manna et al., 2004)
SarY	Unknown function

Fur

Ferric uptake regulator (Fur) and related homologs (PerR, Mur and Zur) make up another group of gene regulators which are primarily influenced by the availability of essential metal ions in the environment. Metal ions are essential for normal cellular functions as they are often required for the catalytic site within metalloproteins (Aggett, 1985). However high levels of metals, particular iron, can have adverse effects on the cells such as the production of ROS via Fenton chemistry (Imlay et al., 1988; Repine et al., 1981); therefore intracellular concentrations are tightly controlled. When complexed with iron (II) (Fe^{2+}) Fur is a typical transcription repressor where it is able to recognise and bind specific 19 base pair (bp) DNA sequences called Fur boxes within the promoters of target genes. Through this interaction Fur can block RNA polymerase and inhibit transcription initiation (Troxell & Hassan, 2013). In iron replete conditions Fur down-regulates iron acquisition protein encoding genes, namely siderophores (*fhuC* and *sirA*) thereby controlling intracellular iron concentration (Xiong et al., 2000).

Fur has also shown positive gene regulation in both high and low iron concentrations, examples including catalase (*katA*) and additional gene regulators, *saeRS*, *agrA* and *rot* (Johnson et al., 2011; Horsburgh et al., 2001). Catalase is an essential mediator of the oxidative stress response which is important for immune system survival *in vivo*. Moreover, Fur's influence on downstream gene regulator expression (Agr, SaeRS, Rot) has further implications for the expression of other regulons, highlighting Fur as a major virulence gene regulator (Horsburgh et al., 2001; Johnson et al., 2011). Additionally, Fur's dependency on iron for certain regulatory functions demonstrates the massive influence metal ion concentrations, particularly iron, have on how the bacteria interpret their environment and alter their virulence accordingly. An overview of Fur directed gene regulation is shown in Figure 1-5.

Overall, it is clear *S. aureus* employs a complex network of gene regulators, which not only control their own regulons but also influence the expression and/or activity of other gene regulators. These interactions in turn control the expression of a vast

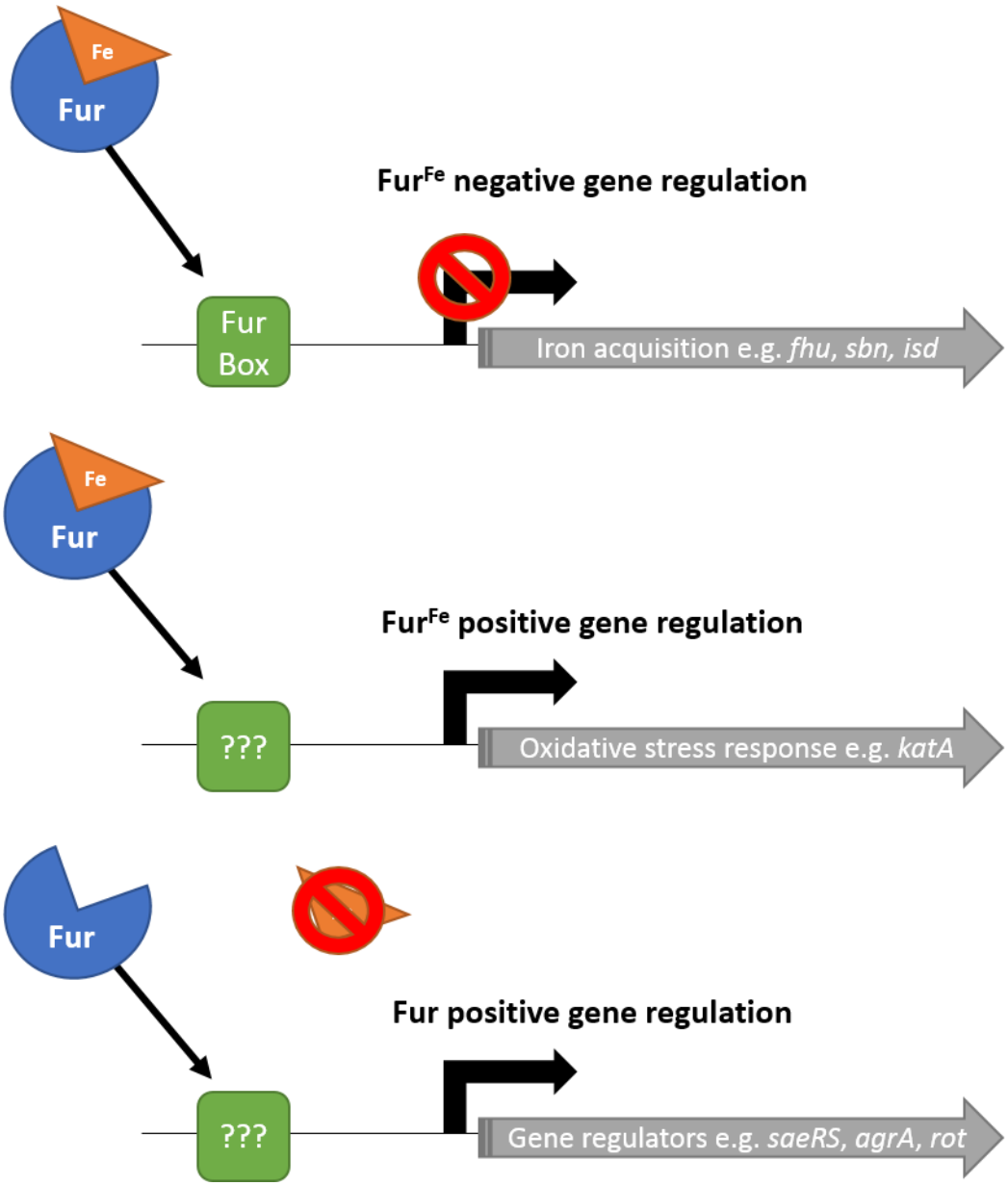

Figure 1-5 Diagrammatical representation of Fur directed gene regulation. In high iron concentration conditions Fur demonstrates classical gene repression by binding Fur boxes within the promoters of target genes. Fur has also demonstrated positive gene regulation in high and low iron concentration conditions, the precise mechanisms for which are unclear at present. Up-regulated Fur regulon genes include *katA*, *saeRS*, *agrA* and *rot*.

array of core and accessory genome associated virulence determinants in response to a multitude of environmental factors. All of these factors contribute to the high level of adaptability and diversity that exists in *S. aureus* strains enabling the species to survive and proliferate in many different niches.

1.3 Methicillin resistant *Staphylococcus aureus* (MRSA)

Methicillin, oxacillin, cephalosporins and other β-lactam derivatives comprise the front-line treatments for Gram-positive pathogens including Staphylococcal species. However, consistent exposure to antibiotics in a clinical setting promotes the development of resistance. One of the major clinical burdens of the last decade has been the expansion of MRSA associated infections. As a consequence alternative antibiotics including vancomycin, linezolid and daptomycin have been used more routinely. However, resistance to these compounds is now being recorded in multi-resistance MRSA clones which highlights our current shortage of appropriate antibiotic choices. The historical evolution of *S. aureus* will be discussed in further detail with particular focus on MSSA and MRSA disease epidemics and current circulating clinical MRSA strains. Resistance to "last-line" antibiotics including vancomycin, linezolid, daptomycin and the molecular mechanisms involved will then be covered.

1.3.1 MRSA and MSSA disease epidemics and statistics

According to the 2014 World Health Organisation (WHO) report on antimicrobial resistance, the proportion of MRSA from the total population of *S. aureus* is estimated to be around 86 % across Africa, the Americas, Europe, South-East Asia, Western Pacific and Eastern Mediterranean regions (WHO, 2014). MRSA infections are associated with severe clinical outcomes including in-hospital and attributed mortality, hospital admission duration and the development of septic shock. Moreover, MRSA infections are responsible for extra cost in a medical setting, associated with increased hospitalisation, antibacterial therapy and additional cost variables (WHO, 2014). The 2010 Euro surveillance report estimated MRSA caused approximately 44 % of 380 000 hospital acquired infections, 22 % of attributed mortalities and 41 % of excess hospital duration; the cost reaching € 380 million per year (Köck et al., 2010; ECDC/EMEA, 2009).

UK surveillance of *S. aureus* bacteraemia have shown MSSA frequencies remained static since formal documentation started in 2011. Moreover, the same suvelliance showed a steep decline in MRSA cases tailing off in recent years (Figure 1-6) (PHE, 2013a; PHE, 2013b; PHE, 2013c). This reduction in MRSA infections may be attributed to the nation-wide effort to reduce healthcare associated infections caused by MRSA and *Clostridium difficile*. This involved enforced hand-washing for all hospital staff and visitors, routine MRSA screening for admitted patients and subsequent mupirocin decolonization for positive individuals. The implementation of these strategies did not however match with the shift in MRSA incidence data; whereas a nation-wide reduction in ciprofloxacin prescriptions correlated well. Ciprofloxacin resistance is universally observed in hospital MRSA clones, due to the high level of use experienced historically. Therefore it is hypothesised a reduction in ciprofloxacin prescription means the loss of selective pressure for MRSA infections and a consequent decline for MRSA infection rates (Knight et al., 2012). Although this suggestion has been disputed recently as carriage of large MGE was not found to affect bacterial fitness (Knight et al., 2013).

1.3.2 Healthcare associated- (HA-) and community acquired- (CA-) MRSA

Historically MRSA infections occurred only after or during hospital exposure particularly in immunocompromised individuals, leading to the designation of healthcare associated (HA-) MRSA. HA-MRSA strains typically display the larger SCC*mec* elements (SCC*mec* type I, II and III) and encode additional antibiotic resistance components leading to the multi antibiotic resistance profiles classically exhibited by these strains (Diederen & Kluytmans, 2006).

In recent year's hyper-virulent MRSA strains have emerged capable of causing disease in otherwise healthy individuals in the community, leading to the designation community acquired (CA-) MRSA. These strains contain the smaller SCC*mec* elements (SCC*mec* types IV and V) often including extra virulence determinants such as PVL (Diederen & Kluytmans, 2006). CA-MRSA infections are highly prevalent in the USA, due to USA300, which is associated with significantly higher recorded incidences compared to HA-MRSA attributed infections (Liu et al., 2008).

Figure 1-6 Voluntary surveillance of MRSA, MSSA and total *S. aureus* bacteraemia in England, Wales and Northern Ireland. This figure was taken from Public Health England (2012), "Voluntary *Staphylococcus aureus* bacteraemia report"; 2012:4.

There has been a major shift in recent years in regards to circulating MRSA strains found in the healthcare setting. Specifically in the UK, the main infective MRSA strain currently is epidemic MRSA-15 (EMRSA-15; CC22 [ST22], SCC*mec* IV) which took over from epidemic MRSA-16 (EMRSA-16; CC30 [ST36], SCC*mec* II) (Figure 1-7) (Wyllie et al., 2011; Knight et al., 2012; Ellington et al., 2010; Rao et al., 2011). EMRSA-15 has been shown to be the fitter clone of the two displaying increased desiccation tolerance, competitive and non-competitive *in vitro* growth, biofilm capabilities, host cell invasion and intracellular persistence, virulence and prolonged duration during murine bacteraemia models (Knight et al., 2012; Baldan et al., 2012). It has been shown that carriage of the larger SCC*mec* type II elements in EMRSA-16 clones entails a significant fitness burden, whereas there is no fitness effect from SCC*mec* IV carriage in EMRSA-15 strains (Knight et al., 2013; Collins et al., 2010). Moreover, constitutively high PBP2a expression from SCC*mec* II has been shown to negatively effects Agr quorum sensing; this is in contrast SCC*mec* IV/V which display inducible PBP2a expression resulting in uninterrupted virulence expression via Agr (Rudkin et al., 2014; Rudkin et al., 2012). Despite these findings, SCC*mec* type and carriage of additional antibiotic resistance determinants on MGE do not provide sufficient evidence to explain the change in MRSA epidemiology witnessed in recent years, indicating additional lineage specific factors are responsible for the shift from CC30 to CC22 epidemic clones (Knight et al., 2013).

1.4 Resistance to non β-lactam antibiotics

In recent years, the incidence of multi-resistant MRSA strains has been increasing, this has also been documented for other bacterial species such as *E. coli* and *Klebsiella* (carbapenemase-producing Enterobacteriaceae). The current antibiotic shortage crisis has led to a global initiative to identify new antimicrobial compounds. β-lactam alternatives for MRSA include vancomycin, linezolid and daptomycin; but in recent years resistance and/or reduced susceptibility to these agents in circulating MRSA strains have been recorded (Boyle-Vavra et al., 2011; Chang et al., 2003; Cui et al., 2010; Gao et al., 2010; Hentschke et al., 2008; Hiramatsu et al., 1997; Long et al., 2006; Lowy, 2003; van Hal et al., 2011; Zhu et al., 2007).

Figure 1-7 Proportion of different *S. aureus* lineages during random sampling by Knight *et al* (2012) in the St Georges Healthcare Trust. This figure was taken from Knight *et al*. (2012), "Shift in dominant hospital associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones over time", Journal of Antimicrobial Chemotherapy 67(10):2516.

1.4.1 Vancomycin resistance *Staphylococcus aureus* (VRSA/VISA)

Traditionally, high level vancomycin resistance, designated vancomycin resistant *S. aureus* (VRSA) is associated with the acquisition of the *vanA* operon (Chapter 1:2:1), which results in altered peptidoglycan precursors and reduced glycopeptide affinity in the bacterial cell wall. There are additional mechanisms for reduced vancomycin susceptibility and/or intermediate resistance (VISA), which do involve acquisition of accessory genetic elements. One of the first reported cases of a VISA phenotype, Mu50, involved up-regulation of cell wall biosynthesis and PBP2a expression, resulting in a vancomycin minimum inhibitory concentration (MIC) of 8 μg/ml (Hiramatsu et al., 1997). The phenomenon of hetero-resistance has also been observed (hVISA) where sub-populations exhibits a VISA phenotype within a majority background of vancomycin susceptible cells (Lowy, 2003).

1.4.2 Daptomycin and linezolid reduced susceptibility

Daptomycin is a lipopeptide antibiotic capable of forming pores in the bacterial cell membrane leading to an efflux of potassium ions, loss of cell membrane potential and subsequent inhibition of protein, DNA and RNA synthesis (Silverman et al., 2003). Reduced daptomycin susceptibility is often observed in conjunction with a VISA phenotype as a thickened cell wall negatively impacts the pore forming ability of daptomycin (van Hal et al., 2011). There are numerous alternative mechanisms for the development of daptomycin non-susceptibility often involving genetic mutations within genes involved in cell wall and/or membrane biosynthesis. Genes frequently associated with this phenotype include *mprF*, *cls2*, *pgsA*, *yycG*/*walK*, which encode membrane modifications enzymes (Patel et al., 2011; Peleg et al., 2012). Alterations in such enzymatic functions have been shown to alter cell membrane charge and/or lipid content resulting in repulsion of cationic agents including daptomycin coupled with calcium (Boyle-Vavra et al., 2011). Additionally, mutations within a RNA polymerase subunit (*rpoB*) also shows a similar phenotype due to its influence on *dlt* expression (Cui et al., 2010), an operon encoding a cell wall modification enzyme (DltB) frequently associated with cell wall thickening (Bayer et al., 2013).

Linezolid, an oxazolidinone, inhibits translation initiation by targeting 23S rRNA of the 50S ribosomal subunit (Kohanski et al., 2010; Hentschke et al., 2008). Mutations within the 23S rRNA alter the binding site of linezolid and inhibit its action (Hentschke et al., 2008; Zhu et al., 2007). An alternative mechanism of linezolid resistance is mediated by ribosomal methyltransferases, including those encoded within the genome e.g. *rlmN*, and accessory genome encoded methytransferase genes e.g. *cfr* (Gao et al., 2010; Long et al., 2006).

What has been discussed so far has focused upon the acquisition of MBE which genotypically confers antibiotic resistance leading to treatment failure *in vivo*. However, treatment failure is also witnessed in infecting populations which do not genotypically exhibit typical antibiotic resistance determinants. Potential factors for "antibiotic tolerance" include the accumulation of mutations in seemingly unrelated genes indirectly leading to reduced antibiotic susceptibility (Jones et al., 2008; Boyle-Vavra et al., 2011). Additionally, a state of dormancy is well regarded as an universal antibiotic tolerance mechanism due to the decreased cellular metabolism exhibited by such affected cells (Lewis, 2007; Fauvart et al., 2011). A final option involves physical localisation and reduced exposure to deleterious environmental agents including antibiotics (von Eiff et al., 2001; Löffler et al., 2013).

In addition to *S. aureus*'s ability to resist and tolerate prescribed chemotherapeutic agents, it also exhibits a wide variety of immune evasive strategies. Such strategies will now be discussed in relation to the specific immune response elicited by the human body when challenged with *S. aureus*.

1.5 The human immune response against *Staphylococcus aureus*

The next section will discuss the human immune response against an invasive infection, with particular focus on *S. aureus* infections and specific immune evasion strategies employed by *S. aureus*.

1.5.1 Pathogen recognition receptors

The innate immune system provides the first line of defence against pathogens and is a universal strategy independent of antigenic recognition. In replace of the specificity antigens provide, the innate immune system utilises pathogen

recognition receptors (PRR) (Medzhitov & Janeway 1997). PRRs function by recognising structures conserved across different bacterial species, named pathogen-associated molecular patterns (PAMPs) (Takeuchi & Akira 2010; Medzhitov & Janeway 1997). Examples include the LPS (lipopolysaccharide) of Gram-negative bacteria, lipoteichoic acids of Gram-positive bacteria, peptidoglycan and other cell-wall associated components (Janssens & Beyaert 2003; Akira et al. 2006). Toll-like receptors (TLRs), the most characterised group of PRRs, can be found on various immune cells including dendritic cells and macrophages, but are also expressed by nonprofessional immune cells such as keratinocytes, endothelial and epithelial cells (Akira et al. 2006; Takeuchi & Akira 2010). TLR2 and NOD2, an intracellular PRR, play important roles in *S. aureus* recognition, specifically via peptidoglycan stimulation (Müller-Anstett et al. 2010). Once activated PRRs initiate a signalling cascade via interleukin- (IL-) 1R and MyD88 pathways, resulting in the expression and secretion of various antimicrobial and proinflammatory components including host defence peptides (HDPs), cytokines and chemokines (Tosi 2005; Medzhitov 2001; Krishna & Miller 2011).

1.5.2 Host defence peptides

HDPs, otherwise known as cationic antimicrobial peptides (CAMP) constitute a major part of the innate immune response. The most prominent HDPs in humans are α- (human neutrophil peptides; HNP) and β-defensins (human beta defensins; HBD) which are primarily expressed from neutrophils and epithelial cells respectively (Ganz 2003). They can either be constitutively expressed, as is the case for neutrophil dependent α-defensins (e.g. HNP1-3) and certain β-defensins (e.g. HBD1); or the expression can be inducible, for example HBD2 expression from epithelial cells is dependent on cytokine stimulation (IL-1 and nuclear factor-κB [NF-κB]) (Tsutsumi-Ishii & Nagaoka 2003; Ganz 2003). HDPs are indiscriminately bactericidal in that they adhere to the microbial cell surface through electrostatic interactions and exert their antimicrobial action by distorting and/or forming pores within the pathogen's cell membrane resulting in cell lysis (Ganz 2003; Wimley et al. 1994).

Bacterial species have evolved various strategies to combat the effects of HDPs such as the expression of HDP specific efflux systems (e.g. *mtr* in *Neisseria gonorrhoeae*)

and HDP targeting membrane proteases (e.g. *Salmonella typhimurium*) (Guina et al. 2000; Hafer & Aring 1998). In *S. aureus* specifically, multiple HDP evasion mechanisms exist including the production of staphylokinase and aureolysin which function to inactivate α-defensin and human cathelicidin, another human HDP, respectively (Jin et al. 2004; Sieprawska-lupa et al. 2004). Moreover, *S. aureus* is able to modify its cell surface thereby negatively affecting the ability of HDPs to adhere and/or enter the cell. MprF and Dlt function by modifying the cell membrane and cell wall respectively resulting in an altered surface charge which reduces HDP affinity; additionally, IsdA expression leads to decreased cellular hydrophobicity thereby increasing resistance to HDP membrane penetration (Peschel et al. 2001; Peschel et al. 1999; Clarke et al. 2007).

1.5.3 Cytokines, chemokines and leukocyte migration

A second consequence of PRR activation is the secretion of cytokines (e.g. IL-1, IL-6 and TNF-α) and chemokines (e.g. CXCL1/Gro-α and CXCL8/IL-8), soluble cell signalling proteins which modulate the immune response and act as ligands for G protein-coupled transmembrane receptors used in the chemotactic recruitment of leukocytes (Tosi 2005; Ley et al. 2007; Krishna & Miller 2011). Stimulation by cytokines induces the expression of adhesion molecules on the surface of endothelial and immune cells which are required for neutrophil migration down the chemokine gradient (Ley et al. 2007). Integrins such as L-selectin and lymphocyte function-associated antigen 1 (LFA1) on the neutrophil cell-surface interact with immunoglobulin-like adhesion molecules (P-/E-selectin and iCAM-1 [intercellular adhesion molecule-1]) and extracellular matrix proteins on the endothelium causing neutrophils to "roll" and enter infected tissues ready to initiate phagocytosis (Ley et al. 2007; Krishna & Miller 2011).

S. aureus exhibits multiple strategies which interfere with neutrophil migration. One such example is the expression of CHIPS: *CH*emotaxis *I*nhibitory *P*roteins of *S*taphylococci, which block the formyl peptide (FPR) and C5a receptors on leukocytes from binding of their complementary agonists which subsequently inhibits chemotactic migration. Additionally, Eap (extracellular adhesion protein) of *S. aureus* is able to bind iCAM-1 blocking any interaction with LFA-1, thereby inhibiting neutrophil adhesion and migration across the endothelium.

1.5.4 Complement mediated opsonisation

One of the ways phagocytosis can be activated is by complement mediated opsonisation. There are three pathways which result in complement fixation, the classical pathway, the lectin pathway and the alternative pathway; all of which result in the cleavage of C3 producing C3a and C3b, via the formation of the C3 convertase (Tosi 2005). The lectin and alternative pathways are classified under the innate immune system whereas the classical pathway is mediated by adaptive immunity i.e. mediated through antigenic recognition by antibodies. The lectin pathway involves the MBL (mannan-binding lectin) protein and its associated proteases (MASP) binding the microbial cell surface via mannose-containing carbohydrates. C4b and C2a are then recruited causing the formation of the C3 convertase, C4b2a, and subsequent cleavage of C3 (Suzan H M Rooijakkers, van Kessel, et al. 2005). The alternative pathway involves the consistent hydrolysis of C3 at low levels (C3(H_20)), the product of which is bound by factor B and cleaved by factor D producing the alternative C3 convertase, C3bBb. The subsequent cleavage of C3 results in a low level of circulating C3b which is able to adhere to microbial cell surfaces (Suzan H M Rooijakkers, van Kessel, et al. 2005). In contrast, the classical pathway involves formation of the C1 complex (C1q, C1r and C1s) and subsequent binding to antibodies which have recognised antigens on the bacterial cell surface. The C1 complex is then able to cleave C4 and C2 thereby forming the same C3 convertase as in the lectin pathway, C4bC2a (Tosi 2005; Suzan H M Rooijakkers, van Kessel, et al. 2005). Once C3 is cleaved through either of the three pathways described, C3a is released which functions as an additional chemoattractant for further immune cells; whereas C3b remains on the microbial cell surface to bind phagocytic complement receptors CR1 (CD35) and CR3 (CD11b/CD18) located on neutrophils and macrophages thereby triggering phagocytosis (Tosi 2005).

Predictably, *S. aureus* also employs various mechanisms designed to interfere with the complement system thereby aiding to evade the immune system. For example, extracellular fibrinogen-binding protein (Efb) and *Staphylococcus* complement inhibitor (SCIN) are able to bind C3 and both forms of the C3 convertase respectively, thereby inhibiting C3 cleavage and subsequent C3b deposition on the microbial cell surface (Lee, Ho, et al. 2004; Lee, Liang, et al. 2004; Suzan H M

Rooijakkers, Ruyken, et al. 2005). Moreover, *S. aureus* can activate host plasminogen via the action of staphylokinase; activated host plasmin is then able remove C3b and IgG which has already bound to the cell surface therefore reversing opsonisation (S H M Rooijakkers et al. 2005).

1.5.5 Phagocytosis

Once at the site of infection, neutrophils recognise opsonised pathogens either through complement recognition receptors (CR1/CR3) or IgG Fc specific receptors, leading to engulfment and capture of the microbe inside the phagosome. Lysosomal compartments then merge with the phagosome and release lethal agents. Such antimicrobial agents include reactive oxygen species (ROS), proteases (e.g. cathepsin G, elastase, gelatinase) and acid hydrolases, HDPs including cathelicidin, lysozyme and α-defensins, and metal ion chelators (e.g. lactoferrin and calprotectin) (Krishna & Miller 2011; Segal 2005).

Assuming the inflammatory cascade has continued thus far despite the numerous evasion strategies already employed, *S. aureus* has a variety of mechanisms which inhibit opsonisation, resist phagocytosis, cause damage to immune cells and also tolerate the antimicrobial agents within the phagosome.

Arguably the most characterised immune evasion protein of *S. aureus* is protein A (SpA), which functions by binding and coating the bacterial cell in IgG. Importantly this interaction causes the Fc portion to be in the wrong orientation for neutrophil Fc receptor recognition thereby inhibiting phagocytosis (Foster 2005; Suzan H M Rooijakkers, van Kessel, et al. 2005; Krishna & Miller 2011). Clumping factor A (ClfA) and fibrinogen-binding protein (FnBPs) are also hypothesised to bind and subsequently coat the bacterial cell in host proteins, i.e. fibrinogen, and in doing so block opsonin adhesion (Foster 2005; Krishna & Miller 2011). It has also been shown the presence of a bacterial capsule can provide some form of protection against opsonisation and therefore making encapsulated strains more resistant to phagocytosis (Thakker et al. 1998; Foster 2005).

However, if these strategies are unsuccessful and *S. aureus* is localised within the phagosome, this bacteria is also known to secrete numerous cytolytic toxins which cause neutrophil lysis. Examples include α-lysin, leukotoxins (e.g. Panton-Valentine

leucocidin; PVL) and phenol soluble modulins (PSM) (Krishna & Miller 2011; Foster 2005; Wang et al. 2007; Suzan H M Rooijakkers, van Kessel, et al. 2005). Moreover, *S. aureus* has been shown to be relatively tolerant of the hostile phagocytic environment, especially during ROS exposure due to the expression of antioxidants (e.g. carotenoid pigment) and superoxide dismutases (e.g. SodM) (Liu et al. 2005; Karavolos 2003).

1.5.6 T-cell activation and function

The adaptive immune response works in conjunction with innate immunity by providing specificity against infective agents. Activation of the adaptive immune response is dependent on antigen presenting cells (APCs) which include both professional (e.g. macrophages and dendritic cells) and non-professional (e.g. epithelial and endothelial cells) cell types. During PAMP directed stimulation of PRR, pathogenic antigens are processed and presented on co-stimulatory (CD08/86) and major histocompatibility class II (MHC-II) molecules to naïve T-cells causing maturation into T-helper effector cells (Medzhitov 2001; Banchereau & Steinman 1998). Upon activation T-cells can differentiate into either T helper-1, -2 and -17 (T_H1/ T_H2/ T_H17) cells all of which produce specific cytokines that either promote the cell-mediated response (T_H1 via IFN-γ), humoral response (T_H2 via IL-4 and IL-13), or abscess formation (T_H17 via IL-17, IL-21, IL-22 and L-26) (Girardi 2007; Cells et al. 2010).

Interestingly, the recruitment of neutrophils through the T_H1 dependent response (i.e. IFN-γ) was shown to be effective for *S. aureus* related infections (McLoughlin et al. 2008), whereas a T_H2 response leads to exasperation of infective symptoms (Cho et al. 2001). It is hypothesised that during the T_H2 response, IL-4 promotes the expression of host fibronectin and fibrinogen, which actually aids *S. aureus* colonisation through Efb and FnBP adhesion (Cho et al. 2001). The T_H17 pathway appears to be the most effective strategy against *S. aureus* infections particularly of the skin, as shown by patients with immunodeficiency disorders (Krishna & Miller 2011). T_H17/IL-17 promotes the chemotactic migration of neutrophils by secreting various chemokines (e.g. CXCL1, 2, 5 and 8) and induces HDP secretion from keratinocytes (Peric et al. n.d.; Korn et al. 2009), which appears to be the most effective T-cell dependent strategy against *S. aureus* skin infections.

1.5.7 B-cell differentiation and antibody action

In addition to T-cell specific responses to infections, T_H cells also activate resting B-cells and initiate the differentiation into immunoglobulin producing plasma cells. A large repertoire of antibodies can be found after an invasive *S. aureus* infection, specifically targeted against secreted toxins, cell surface and cell wall components, and other extracellular virulence factors expressed by *S. aureus* (Holtfreter et al. 2010). *S. aureus*, however, is able to combat these effects via the expression of immunoglobulin binding proteins, such as the previously described protein A but also Sbi (staphylococcal binder of immunoglobulin). Sbi is thought to employ a slightly different mechanism compared to protein A whereby it binds to and subsequently sequesters antibodies away from the bacterial cell (Bröker et al. 2014). Moreover, *S. aureus* expresses "superantigens" (e.g. enterotoxins A/B and toxic shock syndrome toxin-1 [TSST-1]) which have the ability to bind MHC-II on APCs and T-cell receptors (TCRs) independent of antigenic specificity. This not only exasperates the inflammatory response leading to host tissue damage but also depletes the *S. aureus* specific B-cell pool, due to the short half-lives of antibody secreting cells, and initiates T- and B-cell suppression (Leung et al. 1998; Parcina et al. 2013; Bröker et al. 2014). Overall, this data may help to explain why the development of a *S. aureus*/MRSA specific vaccine has yet to be clinically successful.

Overall, it is clear *S. aureus* has a large repertoire of strategies designed to enable the evasion of the human immune system, an overview of what has been discussed is diagrammatically represented in Figure 1-8. Combined with its obvious propensity to develop antimicrobial resistance, as discussed in the previous section, it is clear *S. aureus* makes for a reputable pathogen. Successful implementation of these two mentioned pathogenic traits leads to serious disease complications, such as "bacterial persistence". *S. aureus* persistence, a phenomenon of great clinical concern, will be discussed in further detail in the next section.

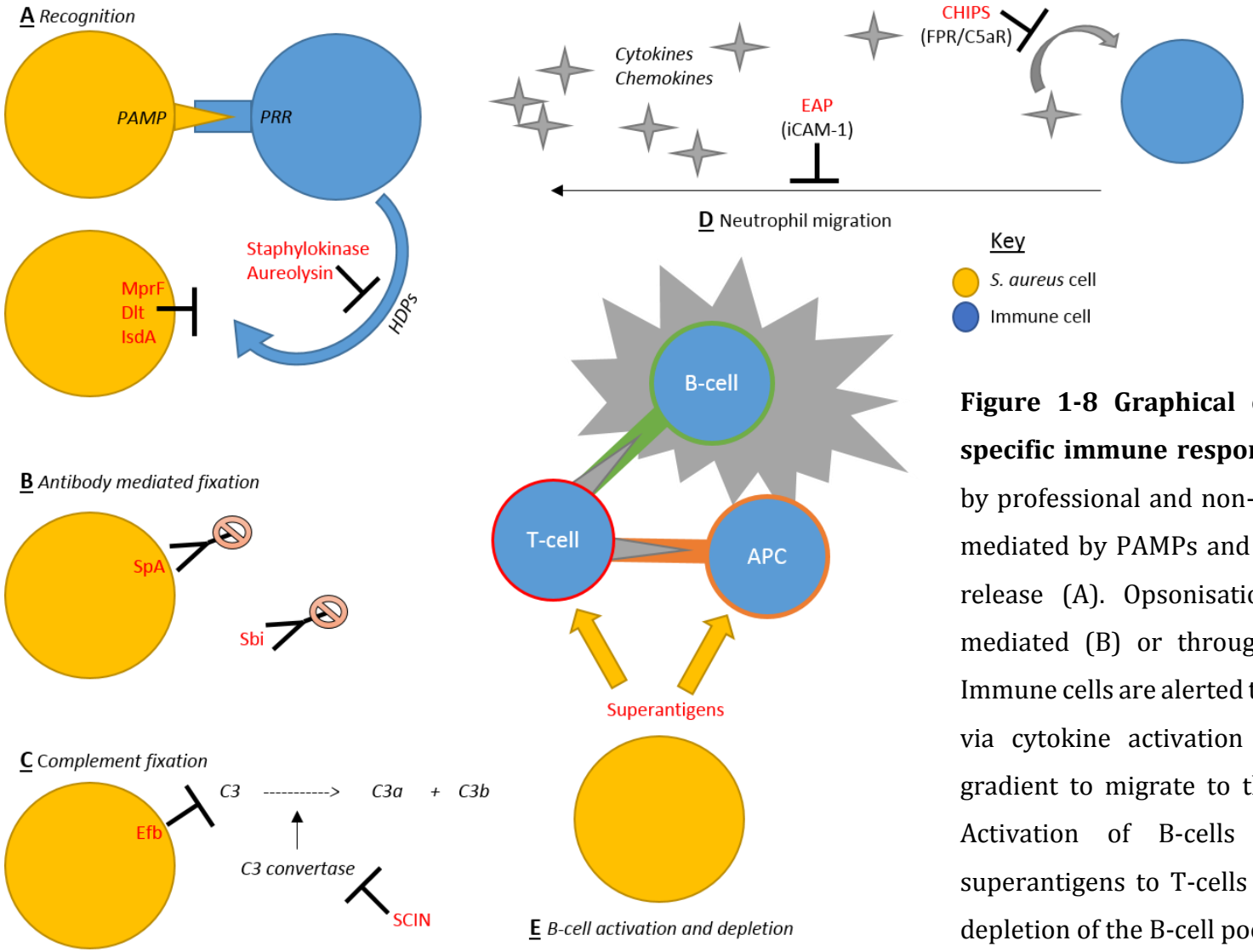

Figure 1-8 Graphical overview of an *S. aureus* specific immune response. Recognition of *S. aureus* by professional and non-professional immune cells is mediated by PAMPs and PRRs which stimulates HDP release (A). Opsonisation can either be antibody mediated (B) or through complement fixation (C). Immune cells are alerted to the presence of an infection via cytokine activation and utilise the chemokine gradient to migrate to the site of inflammation (D). Activation of B-cells via the presentation of superantigens to T-cells and other APCs can lead to depletion of the B-cell pool (E).

1.6 *Staphylococcus aureus* bacteraemia and persistence

1.6.1 Clinical diagnosis of a bacteraemia

Incidences of *S. aureus* infections, specifically cases of bacteraemia and endocarditis are often associated with patients which have experienced prolonged hospitalisation in combination with antibiotic treatment and/or surgical procedures (Mitchell & Howden, 2005). *S. aureus* bacteraemia also correlates with a local extravascular source of infection such as an infected wound, osteomyelitis and pneumonia or an intravascular source including medical devices and intravenous drug use (Mitchell & Howden, 2005). In any of these situations, the presence of any additional infective symptoms implicates a bacteraemic episode.

In general, sepsis can be associated with SIRS, Systemic Inflammatory Response Syndrome, of which approximately one fifth of sepsis cases are defined as bacteraemia (PHE, 2014). SIRS include the presentation of the following clinical features- increased heart rate, hyperventilation, increased white blood cell counts and abnormal body temperatures (PHE, 2014). If sepsis/bacteraemia is suspected in a patient, the Department of Health guidelines recommend the collection of two blood cultures (20 – 30 ml each) from different sites prior to the start of antimicrobial therapy if possible. Blood samples are then diluted in nutrient broth and incubated for 5 – 7 days using automated blood culture instruments which will document the presence of microbiological growth (Murray & Masur, 2012; PHE, 2014). Alternative molecular techniques that are quicker and demonstrate increased sensitivity also exist such as Matric Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF) mass spectroscopy and these should be used when available. All positive blood cultures are then sub-cultured onto recommended agar plates, depending on the clinical details observed, in combination with microscopy (plus Gram staining) and antimicrobial susceptibility testing (PHE, 2014). Empirical therapy is prescribed to the patient until accurate microbiological identification is reported to the clinician and appropriate treatment is initiated (PHE, 2014).

1.6.2 *Staphylococcus aureus* persistent bacteraemia

A persistent *S. aureus* bacteraemia can be defined as the "continuation or recurrence of a documented blood stream infection over a period in excess of seven days during which time the patient in question has received appropriate antibiotic therapy(ies) to which the infecting isolate has been deemed susceptible to by conventional methods" (Fowler et al., 2004; Xiong et al., 2009; Park et al., 2012; Seidl et al., 2011). This is in contrast to a "resolved bacteraemia" where the patient's infective symptoms diminish and/or a sterile blood culture is documented prior to the seven-day infection cut-off. Despite variations in epidemiological designs it is clear persistent infections make up a significant proportion of reported MRSA infections (~ 6 – 38 %) (Khatib et al., 2009; Khatib et al., 2006; Chong et al., 2013). These infections are associated with poorer patient outcomes as demonstrated by increased duration of hospitalisation, incidence of metastatic infections, high levels of attributed and in-hospital mortality (Chong et al., 2013; Lin et al., 2010; Khatib et al., 2006; López et al., 2012).

1.6.3 Clinical and microbiological traits of *Staphylococcus aureus* persistence

Epidemiological studies focused upon *S. aureus* bacteraemia have presented various clinical features which are over-represented in persistent cases. These include the presence of metastasis and infective foci, such as infective endocarditis (IE), indwelling devices such as catheters and clinically recognised co-morbidities such as diabetes and septic shock (López et al., 2012; Chong et al., 2013; Lin et al., 2010; Neuner et al., 2010; Khatib et al., 2006; Khatib et al., 2009; Yoon et al., 2010). Other persistence associated traits have included the presence of methicillin resistance and VISA phenotypes (Chong et al., 2013; Khatib et al., 2009; Lin et al., 2010; Yoon et al., 2010; Neuner et al., 2010).

A hand full of studies have retrospectively investigated terminal persistent *S. aureus* isolates in parallel with resolved bacteraemic isolates, with the view to assess differences in basic phenotypic attributes. Improved resistance to host defence cationic peptides (HDP; aka. cationic antimicrobial peptides- CAMP) is frequently associated with persistent isolates, specifically human neutrophil peptide 1 (hNP-1) and thrombin induced platelet microbicidal proteins (tPMP) (Fowler et al. 2004;

Khatib et al. 2006; Seidl et al. 2011; Xiong et al. 2010). There is experimental evidence to suggest PMP comprise a major defensive strategy against endovascular infections. Moreover *in vitro* determined PMP resistance levels were found to be proportional to endovascular disease severity (Kupferwasser, 2002), which truly highlights the relevance of increased HDP resistance in persistent isolates. A positive correlation was also found between persistence duration and the combination of HDP resistance and biofilm capabilities (Seidl et al., 2011).

Another suggested persistence associated trait includes improved fibrinogen, fibronectin and endothelial cell adhesion (Xiong et al., 2009), which would theoretically aid bacterial colonisation and infection foci formation, however this particularly trait was disputed by Seidl *et al*. (2011) who found no significant link with persistent isolates. Interestingly, reduced activity of δ-lysin has also been linked with persistent *S. aureus* isolates on multiple occasions (Sakoulas et al., 2005; Schweizer et al., 2011; Tsuji et al., 2011; Fowler et al., 2004). A decrease in δ-lysin could indicate *agr* dysfunction and subsequent reduced toxin activity in persistent isolates.

Over-representation of specific genotypes in persistent isolates has also been recorded including SCC*mec* type II, *agr* type II/III and ST30 (Park et al., 2012; Xiong et al., 2009; Fowler et al., 2004; Seidl et al., 2011), which may suggest there is certain strain bias in regards to the ability to form a persistent infection. However findings are often contradictory between individual studies (Neuner et al., 2010; Chong et al., 2013), suggesting over-representation of one particular genotype may be due to circulating strain bias specific to the individual studies.

Overall it is clear no one feature of *S. aureus* persistent isolates or persistent infections so far presented has been recognised across all epidemiological studies conducted. This truly highlights the heterogeneous nature of bacterial persistence and that multiple mechanisms are likely employed, each with its own associated phenotypic traits.

1.7 *Staphylococcus aureus* persistence mechanisms

There are two main obstructions for an infecting organism to overcome to develop a successful persistent infection. These include tolerance of antibiotic therapies and evasion of host immune system detection and defensive strategies. These two factors need to be sustained for the prolonged duration of a persistent infection. There have been many presented mechanisms as to how these aims are achievable, which will now be discussed.

1.7.1 Small colony variants (SCV)

A specific cell type called small colony variants (SCV) are typically slow-growing, non-pigmented, non-haemolytic cells which can be identified by their small colony sizes (approx. one tenth the size of phenotypically wild type cells) (Proctor et al., 1995; Balwit et al., 1994; Sendi & Proctor, 2009). Importantly these cells display elevated antibiotic resistance levels despite genotypic susceptibility, therefore they have often been associated with clinical treatment failure and cases of bacterial persistence (von Eiff et al., 2001; Proctor et al., 1998; von Eiff, Bettin, et al., 1997). The formation of these cell types is a cost effective, reversible alternative to traditional antibiotic resistance development which can have a significant fitness cost attributed to the entire bacterial population. SCV formation reduces bacterial proliferation for only a subset of cells for a limited time period with the added gain of improved survival capabilities (Massey et al., 2001). SCV have been frequently isolated from clinical samples documented as far back as 1978 originating from varied, chronic diseases including osteomyelitis, Darier's disease, cystic fibrosis associated lung infections, abscesses and bacteraemia (von Eiff, Bettin, et al., 1997; Musher et al., 1979; von Eiff et al., 2001; Wolter et al., 2013; Yagci et al., 2011; Seifert et al., 1999; Gao et al., 2010; Proctor et al., 1995; Acar et al., 1978). This further highlights their clinical relevance and association with *S. aureus* persistence.

In most cases these cells display auxotrophism for compounds including haemin and menadione as a result of mutations within the corresponding biosynthetic encoding genes (*hemB*, *menD*). Limitations of these compounds negatively impacts the electron transport system leading to a reduced electrochemical gradient across the cell membrane and limited production of ATP (adenosine triphosphate) (von Eiff,

Heilmann, et al., 1997; von Eiff et al., 2006; Proctor et al., 2006; Sendi & Proctor, 2009). It is this reduction in membrane potential which is thought to enable antibiotic tolerance; particularly antibiotics that require a charge to enter the cell e.g. aminoglycosides. Moreover, SCV are slow growing meaning they exhibit a global reduction in cellular activities; therefore antibiotics which target processes such as cell wall metabolism (β-lactams etc.) prove to be less effective against SCV populations (Proctor & von Humboldt, 1998; Proctor et al., 1998; Proctor et al., 2006; von Eiff et al., 2006; Sendi & Proctor, 2009; Peschel, 2002). Diagnosis and isolation of SCV have been historically difficult as they are often overlooked, especially in mixed cultures, as wild type *S. aureus* can easily out grow them (Sendi & Proctor, 2009; Proctor et al., 2006). Additionally, SCV can rapidly revert back to the wild type phenotype through sub-culturing, which severely impacts research capabilities (Tuchscherr et al., 2011; Sendi & Proctor, 2009).

The term "persister cell" is often used to refer to SCV-like cells exhibited by other bacterial species during chronic infections. These cell types have been extensively investigated, particularly in regards to *E. coli* and are frequently observed in a multitude of chronic bacterial infections including cystic fibrosis (CF) and non-CF related bronchiectasis (*Pseudomonas aeruginosa, Haemophilus influenza, Streptococcus pneumoniae*), latent tuberculosis (TB [*Mycobacterium tuberculosis*]), oral thrush (*Candida albicans*), Lyme disease (*Borrelia burgdorferi*) and chronic gastritis (*Helicobacter pylori*) (Lafleur et al., 2010; Mulcahy et al., 2010; Stewart et al., 2003; Gao et al., 2010; Dimitriou et al., 2011; Berndtson, 2013; Salama et al., 2013; Chalmers & Hill, 2012). Potential dormancy mechanisms involved in persister cell formation include accumulation of ppGpp (guanosine tetraphosphate) (Maisonneuve et al., 2011; Maisonneuve et al., 2013), induction of toxin-antitoxin (TA) modules (Pandey & Gerdes, 2005; Lewis, 2010; Gerdes & Maisonneuve, 2012), induction of the bacterial stringent response (RelA/SpoT) and SOS/oxidative stress response activity (Godfrey et al., 2002; Chatterji & Ojha, 2001; Fraud & Poole, 2011; Balaban et al., 2013). Due to the phenotypic similarities observed between SCV and persister cells, it is likely they are one and the same; therefore conclusions drawn in regards to persister cells could potentially apply to SCV.

1.7.2 Invasion and intracellular persistence

Host cell invasion and intracellular localisation is another suspected persistence mechanism commonly found in combination with the presence of SCV (von Eiff et al., 2001; Sendi & Proctor, 2009; Vesga et al., 1996). This mechanism would provide its own advantages for promoting a prolonged/persistent infection in addition to those already provided by SCV. Extracellular antibiotic therapies, especially those which have reduced membrane penetration capabilities, display decreased effectiveness against intracellular bacteria (Clement et al., 2005; Garzoni & Kelley, 2009). This subsequently enables the bacteria to survive antibiotics to which they display susceptibility *in vitro*. Secondly, this localisation would also help avoid detection by the host immune system and diminish the effects of any previously mounted immune system defence (Tuchscherr et al., 2011; Garzoni & Kelley, 2011). Third and finally, this localisation could be used to establish a source of pseudo-protected bacterial propagation from which chronic infections could disseminate from (Garzoni & Kelley, 2011; Prajsnar et al., 2012)

Mycobacterium tuberculosis, responsible for TB is an effective facultative intracellular pathogen, able to survive within macrophages post phagocytosis allowing for infection (Guirado et al., 2013). *Yesinia pestis*, the causative agent of the bubonic plague, is also able to actively or passively invade and survive within macrophages and non-professional phagocytic host cells such as epithelial cells (Ke et al., 2013). These findings emphasises the role of invasion and intracellular localisation in the progression of chronic diseases by other bacterial species; consequently this mechanism should be considered a real possibility for *S. aureus* persistence.

Not previously regarded as an intracellular pathogen, *S. aureus* is now the focus of intracellular research especially in regards to chronic infections and SCV development. *S. aureus* is known to survive phagocytosis by neutrophils and macrophages and actively proliferate in the cytoplasm prior to host cell lysis (Kubica et al., 2008). Whereas, invasion and intracellular persistence in non-professional phagocytic host cells such as epithelial and endothelial cells are primarily associated with chronic infections (Park et al., 2007; Tuchscherr et al., 2011).

The process of invasion can involve FnBP, fibronectin and host cell $\alpha_5\beta_1$ integrins; To a lesser extent Eap may also be involved, particularly in regards to fibroblast cell types and if FnBP is unavailable (Haggar et al., 2003; Hussain et al., 2002; Sinha & Herrmann, 2005; Sinha & Fraunholz, 2010). Additionally, the entire process is thought to be mediated by ClpC (caseinolytic protein C), the ATPase subunit of the ClpP protease, possibly through FnBP regulation (Nair et al., 2000; Capestany et al., 2008). ClpC has also been the focus of more general links to *S. aureus* persistence, specifically involving metabolism regulation (Chatterjee et al., 2009).

Garzoni *et al* (2007) found persistently internalised cells (< 2 weeks) exhibited immediate down-regulation of metabolism implying a selective pressure to move into a dormant state. Additionally there was rapid repression of toxin expression, particularly *hla* (α-lysin) presumably to limit potential damage to the host and keep its niche viable (Garzoni et al., 2007). Moreover levels of host chemokines and iCAM-1 (intercellular adhesion molecule 1; aka CD54) were found to return to basal levels only fourteen days post-inoculation (Tuchscherr et al., 2011). These data provide evidence that the mechanism of dormancy whilst internalised successfully enables host immune system avoidance and promotes a successful chronic infection.

An overview of what is currently known and suggested regarding *S. aureus* persistence is presented in Figure 1-9.

1.7.3 Genetic basis of persistence (Gao et al., 2013, 2010)

A third potential theory for the development of *S. aureus* persistence involves targeted genome evolution *in vivo*. This aspect relies on the hypothesis that a subset of gene (networks) are responsible for bacterial persistence and that persistence specific alleles exist for these genes. Whole genome comparisons between persistent and non-persistent isolates would be able to identify such candidate genes (networks) related to persistence.

Gao *et al* (2010:2013) conducted one such study investigating a single persistent MRSA bacteraemia where the initial infecting isolate was compared to the subsequent clinical isolate collected during infection progression. A SCV phenotype was exhibited by the terminal clinical isolate, however it did not display traditional auxotrophy for haemin or menadione.

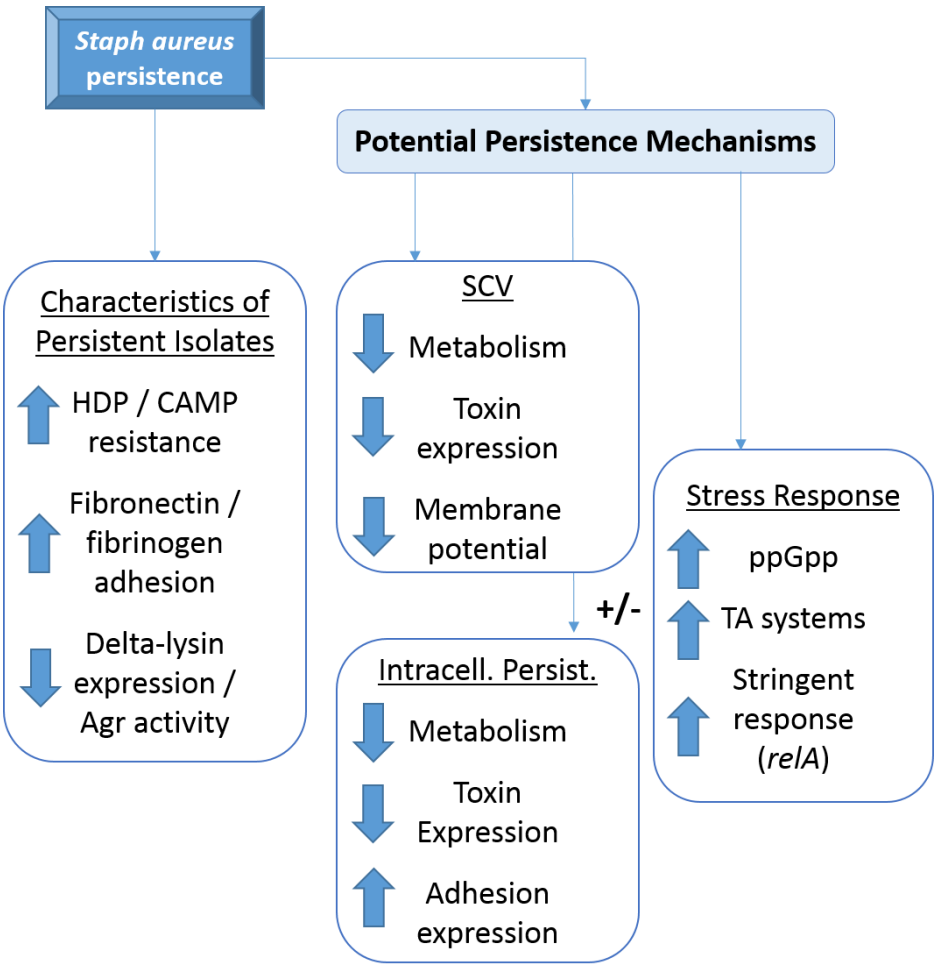

Figure 1-9 Brief overview of current *S. aureus* persistence associated traits and suggested mechanisms. Several characteristics have been previously associated with persistent *S. aureus* isolates, listed on the left. Moreover, suggested mechanisms of *S. aureus* persistence are also listed including the defining traits of each mechanism.

Genome sequence analysis revealed four functional mutations between initial and SCV; three of which were associated with the development of antibiotic resistance for rifampicin, ciprofloxacin and linezolid. Further investigations revealed the *rpoB* rifampicin resistance mutation also led to increased capsule biosynthesis and increased *agr* transcription. The fourth mutation caused an amino acid substitution within the RelA hydrolase domain, leading to the accumulation of ppGpp resulting in a permanently activated stringent response. The development of persistence in this infection was subsequently attributed to stringent response activity and capsule up-regulation via *rpoB* variation (Gao et al., 2010; Gao et al., 2013). Despite the novel findings this study presents it must be taken into account such conclusions are based exclusively on a single case of persistent MRSA bacteraemia. Therefore it is unwise to apply these findings to any subsequent cases of *S. aureus* infections without further investigations involving multiple incidences of persistence.

1.8 Aims and objectives

This chapter has attempted to describe the importance of *S. aureus* in regards to its pathogenicity and subsequent clinical impact. The success of *S. aureus* as a pathogen can be attributed to its complex gene regulatory systems, vast repertoire of virulence determinants and its ability to respond and evolve to changing environments. Further research is paramount due to the increasing risk this pathogen poses within the community, its continuous antibiotic resistance development and also the phenomenon of persistence.

The main aim of this study was to answer two biologically relevant questions in regards to *S. aureus* persistent infections- how do persistent *S. aureus* isolates evade the host immune system, and how do they tolerate appropriate antibiotic therapies for prolonged periods of time? To adequately answer these questions, clinical cases of persistent *S. aureus* bacteraemia were identified and all available archived isolates relating to these multiple cases were collected for *in vitro* characterisation. Additionally, several cases of resolved *S. aureus* bacteraemia were identified, the relevant archived isolate collected and characterised in parallel with persistent samples.

Broad-spectrum phenotypic assays involved microbiological and biochemical assessment using a range of conditions including nutrient replete and nutrient/metal ion restricted environments, which are more reflective of the *in vivo* situation. Specifically phenotypic assessment covered antibiotic susceptibilities, growth characteristics, exoprotein activity and protein profiling using 1D SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis). Further investigation involved iTRAQ LC-MS (Isobaric tag for relative and absolute quantitation, liquid chromatography–mass spectrometry) which aimed to identify proteomic alterations during infection progression. The overall virulence potentials and intracellular persistence capabilities were assessed via a *Galleria mellonella* based model of infection and a tissue culture based model of invasion. Furthermore, potential *in vivo* genome evolution within each individual case of persistence was investigated by comparative genomics using data derived from Illumina whole genome next generation sequencing (NGS). The resulting genetic data including potential SNP (single nucleotide polymorphism) and INDEL (insertions/deletions) detected between initial and persistent isolates was related back to the previously recorded persistence associated phenotypic traits with the aim to identify potential cause/effect (genotype to phenotype) connections.

This strategy of investigating *S. aureus* persistence was unique because multiple incidences of *S. aureus* persistent bacteraemia were analysed, inclusive of the initial and all available sequential persistent isolate(s). Through this approach phenotypic and genotypic evolutionary changes which occurred during each case of persistence were identifiable. Additionally, evolutionary changes which were experienced in more than one infection will suggest a common pathway for persistence development exhibited by *S. aureus*. Resolved bacteraemia incidences involving the same circulating *S. aureus* isolate were analysed in parallel with persistent infection isolates. This additional comparison allowed for the distinction between general infection associated phenotypes (i.e. those observed in both resolved and persistent isolates) from phenotypes arising as a direct consequence of persistence development.

Overall, this study aims to identify a common mechanism(s) of *S. aureus* persistence and discuss the potential impact these finding will have on *S. aureus* infection treatment and clinical management.

Chapter 2 Materials and Methods

2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 2-1. The pIMAY vector and recombinant plasmid maps are shown in Figure 5:2. Bacterial frozen cultures were stored in Trypticase soy broth (TSB) with 20 % (v/v) glycerol at - 80 °C. For standard analysis such as antibiotic investigations bacterial strains were plated onto Luria-Bertani (LB) agar plates and incubated overnight at 37 °C, followed by inoculating a single colony into LB broth and incubated shaking at 200 rpm at 37 °C. For nutrient replete conditions, TSB was inoculated with a single colony from LB plates and incubated shaking at 37 °C. For nutrient restricted, metal ion depleted conditions 6 % horse blood agar and CRPMI (chelexed RPMI-1640) medium with 10 % (v/v) RPMI-1640 was used and cultures grown statically at 37 ˚C in 5 % CO_2.

2.2 Growth media and supplements

All media was prepared using dH_20 and sterilised either by autoclaving (heated to 120 ˚C at 15 pSI for 15 minutes) or filter sterilisation. Stericup filtration systems (Millipore) with a 0.22 µm pore size were used for amounts more than 50 ml. Alternatively 0.2 µm Acrodisc membranes (Pall) attached to plastic syringes were used for amounts less than 50 ml.

2.2.1 Antibiotics and additional supplements

When required, the following antibiotics were added to the media at the following concentrations- anhydrotetracycline (ATc) 1 µg/ml; tetracycline (Tet) and chloramphenicol (Cm) 10 µg/ml; gentamicin 200 µg/ml. Additionally supplements including 1 % glucose (w/v), 2 - 4 % NaCl (w/v) and 5 % human serum (v/v) were added to media after initial sterilisation followed by filter sterilisation.

Table 2-1 Bacterial strains and plasmids used in this study

Strain	Genotype	Reference
DC10B	High-efficiency *E. coli* cloning strain	(Monk et al. 2012)
RN4220	Restriction deficient 8325 derivative	Laboratory stock
Newman	Lab MSSA isolate	(Duthie & Lorenz 1952)
Newman*Δfur*	Made via transduction from 8325-4 *Δfur::tet*	(Johnson et al. 2005; Horsburgh et al. 2001)
PM25	Epidemic MRSA-15 isolate	(Moore & Lindsay 2002)
PM64	Epidemic MRSA-16 isolate	(Moore & Lindsay 2002)
PM64*Δfur*	Made via transduction from Newman *Δfur::tet*	Dr. Jon Baker and (Horsburgh et al. 2001)
N315	Clinical MRSA isolate	(Okonogi et al. 1989)
N315*Δfur**	Deletion of *fur* via pIMAY-*Δfur** transformation	This study and (Monk et al. 2012)
PB1-1	Clinical MRSA isolate	University hospital of Leicester
PB1-15-1	Clinical MRSA isolate	University hospital of Leicester
PB1-15-2	Clinical MRSA isolate	University hospital of Leicester
PB2-1	Clinical MRSA isolate	University hospital of Leicester
PB2-35	Clinical MRSA isolate	University hospital of Leicester
PB3-1	Clinical MRSA isolate	University hospital of Leicester
PB3-1*Δfur**	Deletion of *fur* via pIMAY-*Δfur** transformation	This study and (Monk et al. 2012)
PB3-29	Clinical MRSA isolate	University hospital of Leicester
PB3-32-1	Clinical MRSA isolate	University hospital of Leicester
PB3-32-2	Clinical MRSA isolate	University hospital of Leicester
PB3-72	Clinical MRSA isolate	University hospital of Leicester
PB3-74	Clinical MRSA isolate	University hospital of Leicester
PB5-1	Clinical MRSA isolate	University hospital of Leicester

PB5-30	Clinical MRSA isolate	University hospital of Leicester
PB5-41	Clinical MRSA isolate	University hospital of Leicester
RB1	Clinical MRSA isolate	University hospital of Leicester
RB4	Clinical MRSA isolate	University hospital of Leicester
RB4*Δfur*	Made via transduction from Newman *Δfur::tet*	This study and (Horsburgh et al. 2001)
RB5	Clinical MRSA isolate	University hospital of Leicester

Plasmids	**Description**	**Reference**
pIMAY	Vector (pWV01ts replicon, *cat*-Cm resistance counter selection-*secY* antisense RNA)	(Monk et al. 2012)
pIMAY-*Δfur**	Recombinant pIMAY vector containing *S. aureus Δfur** deletion	This study and (Monk et al. 2012)

2.2.2 Blood agar

Blood agar was prepared with 4 % Blood agar base (Oxoid) and autoclaved to sterilise. The agar was cooled to 60 °C before the addition of either 6 % horse, sheep or rabbit blood.

2.2.3 Brain Heart Infusion (BHI) broth

BHI broth was prepared with 3.7 % (w/v) Brain Heart Infusion broth powder (Oxoid) and autoclaved.

2.2.4 Chelexed RPMI (CRPMI)

CRPMI was prepared by stirring 6 % Chelex 100 (Sigma Ltd) in RPMI-1640 (Sigma Ltd) overnight at 4 °C. This was filter sterilised to remove the Chelex and the pH was altered to either pH 7.0, pH 8.8 or pH 10.0. The medium was filter sterilised for a second time before 10 % (v/v) RPMI-1640 was added to allow for minimal growth. CRPMI was stored at 4 °C. In some circumstances 1 % (v/v) casamino acids was added to CRPMI cultures from an autoclaved 10 % (w/v) stock to improve growth when a large amount of culture was required for analysis.

2.2.5 Dulbecco's Modified Eagle Medium (DMEM) supplemented with Fetal Bovine Serum (FBS)

DMEM high glucose, glutaMAX plus pyruvate (Fisher Scientific) was supplemented with 10 % Fetal Bovine Serum (FNS; PAA Laboratories) and stored at 4 °C.

2.2.6 DNase agar

DNase agar was prepared with 4.2 % DNase agar powder (Fluka BioChemika) and autoclaved.

2.2.7 Luria-Bertani (LB) medium

LB broth was prepared with 1 % (w/v) Tryptone (Oxoid), 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl and the pH adjusted to 7.5. For LB agar (LA), 1.5 % Bioagar was added. Both were autoclaved to sterilise.

2.2.8 LK broth and agar

LK media was prepared according to the instructions for LB and LA but 0.7 % KCl replaced NaCl. Both were autoclaved to sterilize.

2.2.9 Mueller Hinton agar (MHA) and broth (MHB)

MHA was prepared with 3.8 % (w/v) Mueller Hinton agar powder (Oxoid). MHB was prepared with 2.1 % (w/v) Mueller Hinton broth powder (Oxoid). Both were autoclaved to sterilise. For cation adjusted MHB (CAMHB) 10 mg/ml of Mg^{2+} and Ca^{2+} were added.

2.2.10 Trypticase soy broth (TSB)

TSB was prepared with 3 % BBL Trypticase Soy Broth (BD Diagnostic Systems) and autoclaved.

2.3 DNA preparations and manipulations

2.3.1 *S. aureus* genomic DNA extraction (Ausubel et al., 1995)

1.5 ml of a 5 ml LB overnight liquid culture was centrifuged at 13000 rpm for 3 minutes to pellet the cells, the supernatant was discarded. The cell pellet was resuspended in 250 µl of P1 buffer (Qiagen) containing 100 µg/ml lysostaphin and incubated at 37 °C for 15 minutes. 2 µl of 25 mg/ml proteinase K was added, followed by 27 µl of 10 % (w/v) SDS, the solution inverted to mix after each new addition. Mixtures were incubated at 37 °C for 20 - 30 minutes. 97 µl 5 M NaCl was added, followed by 81 µl of CTAB (cetyl trimethyl ammonium bromide), which was pre-warmed to 65 ˚C. Mixtures was incubated at 65 °C for 20 minutes. An equal volume of 24:1 chlorofom:isoamylalcohol was added and the tube centrifuged at 13000 rpm for 10 minutes. The upper layer was transferred to a fresh eppendorf and an equal volume of iso-propan-2-ol (IPA) was added. The tubes were centrifuged again at 13000 rpm for 10 minutes and the supernatant discarded. The resulting DNA pellet was resuspended in 100 µl dH_2O and stored at - 20 °C.

2.3.2 *S. aureus* genomic DNA extraction for Illumina genome sequencing

1.5 ml of a 5 ml LB overnight liquid culture was centrifuged at 13000 rpm for 3 minutes to pellet the cells, the supernatant was discarded. The pellet was resuspended in 100 µl 50 mM EDTA containing 200 µg/ml lysostaphin and incubated at 37 °C for 30 minutes. From this step onwards the Wizard Genomic DNA Purification kit (Promega) was used and the manufacturer's instructions were followed. Further steps briefly involved the addition of 600 µl nuclei lysis buffer to the samples which was mixed thoroughly and incubation at 80 °C for 1 hour. Samples were allowed to cool before adding 3 µl RNase A solution and incubated at 37 °C for 1 hour. 200 µl of protein precipitate solution was added and the samples were vortexed for 20 seconds and incubated on ice for 5 minutes. Samples were centrifuged at max speed for 3 minutes. The supernatant was transferred to a fresh eppendorf containing 600 µl IPA and samples were inverted to precipitate the DNA. These were then centrifuged for 2 minutes and the pellet washed with 70 % EtOH before a final spin to pellet the purified DNA. DNA was resuspended in 100 µl pre-heated dH_2O and incubated at 65 °C for 1 hour to aid resuspension. A further clean-up step was completed using Genomic DNA clean and concentrator columns (Cambridge Bioscience) according to manufacturer's instructions. Concentrations and purification ratios were determined using a Thermo-Scientific nano-drop and sent to the University of Swansea (Dr Sam Sheppard) for collaborative Illumina genome sequencing which was conducted at the Wellcome Trust Centre, Oxford.

2.3.3 Plasmid DNA preparations

1.5 ml of a 5 ml LB overnight liquid culture was used for plasmid extraction with the E.Z.N.A Plasmid Mini Prep Kit I (Omega, Bio-tek). The manufacturer's instructions were followed with an additional lysis step for *S. aureus* which involved the addition of 100 µg/ml lysostaphin to Solution I and incubation at 37 °C for 15 minutes.

2.3.4 Polymerase chain reaction (PCR)

Each 10 µl reaction contained the following- 2.5 µl DNA (from chromosomal or plasmid prep), 5 µl FailSafe PCR 2x Premix D (Epicentre, Biotechnologies), 1 µl of each forward and reverse primer from a 10 µM stock (Table 2-2) and 0.5 µl Kapa *Taq* DNA polymerase (ABgene). Kapa *Taq* DNA polymerase was replaced with Bio-

X-act DNA polymerase (Bioline) for cloning. Reactions were performed in a G-storm GS1 thermal cycler. A typical thermal cycler reaction consisted of 98 °C for 2 minutes, 30 cycles of 98 °C for 30 seconds, Tm °C (depending on the specific primer set used) for 30 seconds, 72 °C for 1 minute and a final extension at 72 °C for 10 minutes. For transcripts longer than 1 kb, the elongation step was increased by 30 seconds for every 1 kb increase. For colony PCR, 2.5 µl dH_2O containing a single *E. coli* colony replaced the 2.5 µl DNA; an additional step of 10 minutes at 97 °C prior to PCR cycling was included to lyse the cells.

2.3.5 Agarose gel electrophoresis

1.5 % (w/v) Agarose (Lonza SeaKem) was dissolved in 1x TAE (Tris-Acetate-EDTA) buffer by boiling. Once cooled to 60 ˚C, 25 µg/ml ethidium bromide (EtBr) was added and stored at 60 °C for future use. 5 µl Hyperladder I (Bioline) was used for size markers and 2 – 5 µl of each sample was mixed with 5x TAE loading buffer (12.5 % (w/v) Ficoll, 0.1 % (w/v) Bromophenol blue) prior to loading the gel. Gels were run at 100 V and bands were visualised using a UV transilluminator (Gene Genius Bio Imaging System, Syngene).

2.3.6 Gel extraction

DNA samples were separated by electrophoresis followed by excision and purification using the Zymogen Gel Extraction kit (Zymo Research Company). The manufacturer's instructions were followed with the additional final spin step to ensure adequate removal of alcohol. This method was used to purify transcripts for cloning.

2.3.7 PCR clean up and Sanger DNA sequencing

Resulting PCR reactions were purified using the Cycle Pure kit (Omega, Bio-Tek) according to the manufacturer's instructions to remove any contaminating oligo-nucleotides before being used as a template for DNA sequencing. Each 8 µl sequencing reaction contained 3 – 15 ng of template DNA (depending on the size of target to be sequenced), 2 µl forward or reverse primer (from 10 µM stock) and 2 µl

Table 2-2 Primers used in this study

Primer Name	Sequence 5'-3'	Tm˚C	Application	Reference
AGR1F	ATCGCAGCTTATAGTACTTGT	63.7	*agr* Typing- Type I	(Campbell et al. 2008)
AGR1R	CTTGATTACGTTTATATTTCATC			
AGR2F	AACGCTTGCAGCAGTTTATTT	62.7	*agr* Typing- Type II	(Campbell et al. 2008)
AGR2R	CGACATTATAAGTATTACAACA			
AGR3F	TATATAAATTGTGATTTTTTATTG	58.0	*agr* Typing- Type III	(Campbell et al. 2008)
AGR3R	TTCTTTAAGAGTAAATTGAGAA			
AGR4F	GTTGCTTCTTATAGTACATGTT	57.0	*agr* Typing- Type IV	(Campbell et al. 2008)
AGR4R	CTTAAAAATATAGTGATTCCAATA			
β	ATTGCCTTGATAATAGCCYTCT	55.0	SCC*mec* Typing Multiplex	(Boye et al. 2007)
α3	TAAAGGCATCAATGCACAAACACT			
*ccrC*F	CGTCTATTACAAGATGTTAAGGATAAT	55.0	SCC*mec* Typing Multiplex	(Boye et al. 2007)
*ccrC*R	CCTTTATAGACTGGATTATTCAAAATAT			
1272F1	GCCACTCATAACATATGGAA	55.0	SCC*mec* Typing Multiplex	(Boye et al. 2007)
1272R1	CATCCGAGTGAAACCCAAA			
5R*mecA*	TATACCAAACCCGACAACTAC	55.0	SCC*mec* Typing Multiplex	(Boye et al. 2007)
5R431	CGGCTACAGTGATAACATCC			

ccrB2 F	CGAACGTAATAACATTGTCG	48.0	SCC*mec* Type IV Subtyping Multiplex	(Milheiriço et al. 2007)
ccrB2 R	TTGGCWATTTTACGATAGCC			
IVA F	ATAAGAGATCGAACAGAAGC	48.0	SCC*mec* Type IV Subtyping Multiplex	(Milheiriço et al. 2007)
IVA R	TGAAGAAATCATGCCTATCG			
IVB F	TTGCTCATTTCAGTCTTACC	48.0	SCC*mec* Type IV Subtyping Multiplex	(Milheiriço et al. 2007)
IVB R	TTACTTCAGCTGCATTAAGC			
IVC F	CCATTGCAAATTTCTCTTCC	48.0	SCC*mec* Type IV Subtyping Multiplex	(Milheiriço et al. 2007)
IVC R	ATAGATTCTACTGCAAGTCC			
IVD F	TCTCGACTGTTTGCAATAGG	48.0	SCC*mec* Type IV Subtyping Multiplex	(Milheiriço et al. 2007)
IVD R	CAATCATCTAGTTGGATACG			
IVGF	TGATAGTCAAAGTATGGTGG	48.0	SCC*mec* Type IV Subtyping Multiplex	(Milheiriço et al. 2007)
IVGR	GAATAATGCAAAGTGGAACG			
IVHF	TTCCTCGTTTTTTCTGAACG	48.0	SCC*mec* Type IV Subtyping Multiplex	(Milheiriço et al. 2007)
IVHR	CAAACACTGATATTGTGTCG			
arc-Up	TTGATTCACCAGCGCGTATTGTC	55.0	MLST	(Enright et al. 2000)
arc-Dn	AGGTATCTGCTTCAATCAGCG			
aroE-Up	ATCGGAAATCCTATTTCACATTC	55.0	MLST	(Enright et al. 2000)
aroE-Dn	GGTGTTGTATTAATAACGATATC			

glpF-Up	CTAGGAACTGCAATCTTAATCC	55.0	MLST	(Enright et al. 2000)
glpF-Dn	TGGTAAAATCGCATGTCCAATTC			
gmk-Up	ATCGTTTTATCGGGACCATC	55.0	MLST	(Enright et al. 2000)
gmk-Dn	TCATTAACTACAACGTAATCGTA			
pta-Up	GTTAAAATCGTATTACCTGAAGG	55.0	MLST	(Enright et al. 2000)
pta-Dn	GACCCTTTTGTTGAAAAGCTTAA			
tpi-Up	TCGTTCATTCTGAACGTCGTGAA	55.0	MLST	(Enright et al. 2000)
tpi-Dn	TTTGCACCTTCTAACAATTGTAC			
yqiL-Up	CAGCATACAGGACACCTATTGGC	55.0	MLST	(Enright et al. 2000)
yqiL-Dn	CGTTGAGGAATCGATACTGGAAC			
spa F	AGACGATCCTTCGGTGAGC	60.0	*spa* typing	(Shopsin et al. 1999)
spa R	GCTTTTGCAATGTCATTTACTG			
fur F	CTATCATTGCATTGCAAGAC	55.0	Targets *S.aureus fur*	Dr Julie Morrissey
fur R	CTGCGTCGCCTAATGGTAC			
16S RT F*	ACGGTCTTGCTGTCACTTATT	60.0	qRT-PCR	(Wada et al. 2010)
16S RT R*	TACACATATGTTCTTCCCTAATAA			
gyr RT_F*	GACTGATGCCGATGTGGA	60.0	qRT-PCR	(Harraghy et al. 2005)
gyr RT_R*	AACGGTGGCTGTGCAATA			

IM151 IM152	TACATGTCAAGAATAAACTGCCAAAGC AATACCTGTGACGGAAGATCACTTCG	60.0	Flanks pIMAY insert site	(Monk et al. 2012)
3'*fur*FLAP-pIMAYF **5'*fur*FLAP**-pIMAYR	***AAGTGTTGGCTTTATTAGA***GGTACCCAGCTTTTG TTCCCTTTAGTGAGG ***CTACTGGTTTACGATACTGTTT***GAGCTCCAATTC GCCCTATAGTGAGTCG	65.0	Recombinant pIMAY-*fur** amplification	(Monk et al. 2012) and this study
***kpn1*FLAP**-3'*fur*F **5'*fur*FLAP**-3'*fur*R	***TAAAGGGAACAAAAGCTGGGTACC***TCTAATAAA GCCAACACTTCGTCAAC ***GATTAAATCGCGTTAAGCAA***GTTGAAAATGAGT TCAATTTTAAAA	55.0	Recombinant pIMAY-*fur** amplification	(Monk et al. 2012) and this study
3'*fur*FLAP-5'*fur*F ***sacI*FLAP**-5'*fur*R	***TTTTAAAATTGAACTCATTTTCAAC***TTGCTTAACG CGATTTAATCG ***CGACTCACTATAGGGCGAATTGGAGCTC***AAACA GTATCGTAAACCAGTAG	55.0	Recombinant pIMAY-*fur** amplification	(Monk et al. 2012) and this study
IN*fur* F IN*fur* R	TTGCTGTTTGTGCATTGAC GACTGCGTCGCCTAATG	55.0	Flanks *fur* gene. Confirms *fur* deletion	This study
mco F *mco* R	GGTTCCTAATGATACTTTTGCA CGTTTCAATTTCCCAAGTTT	55.0	*mco* detection	Dr Miranda Johnson

*used designated qRT-PCR cycle program

diluted Big Dye; containing Big Dye v3.1 (Applied Biosystems) diluted 1 in 8 with 5x DNA sequencing buffer. Reactions were subjected to 30 cycles of 96 °C for 20 seconds, 50 °C for 20 seconds and 60 °C for 4 minutes using a G-storm GS1 thermal cycler. 2 µl of 2.2 % (w/v) SDS and 8 µl dH_2O were added to each reaction and heated to 98 °C for 8 minutes. Unincorporated dyes and excess primers were removed using Performa Gel Filtration Cartridges (Edge Biosystems). Analysis was conducted on a BioSystems 3730 sequencer by PNACL (Protein Nucleic Acid Chemistry Laboratory, University of Leicester).

2.3.8 One-step isothermal DNA recombination (Gibson et al., 2009)

Desired DNA fragments were amplified with a high fidelity DNA polymerase (Bio-X-act, Bioline) using specific primers (**Table 2-2**) which included overhangs complementary to the adjacent transcript. Amplified plasmid DNA (pIMAY) was treated twice with DpnI (NEB) to digest template DNA and all PCR products were purified via gel extraction. Fragments were mixed together in equimolar amounts totalling 5 µl, added to 15 µl of isothermal DNA assembly reaction mixture and incubated at 50 °C for 1 hour. The isothermal DNA assembly reaction mixture consisted of 4.5 µl *Taq* DNA ligase, 0.9 µl T5 exonuclease (0.2 U/µl), 0.56 µl Phusion DNA polymerase (2 U/µl) and 9 µl 5x IRB (Isothermal reaction buffer). 5x IRB was made up with 25 % PEG-8000, 500 mM Tris HCl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT and 1 mM of each dNTP.

2.4 Pulse field gel electrophoresis (PFGE) (Mulvey et al., 2001)

2.4.1 Plug preparation and SmaI digestion

3 ml BHI was inoculated with a single *S. aureus* colony and incubated shaking at 37 °C overnight. Cells were recovered from a 150 µl aliquot by centrifugation and resuspended in 150 µl cell suspension buffer (10 mM Tris HCl pH 7.2, 20 mM NaCl, 50 mM EDTA) containing 200 µg/ml lysostaphin. This was mixed with 150 µl pre-melted 2 % low melting point agarose (Sigma Aldrich) and specialised PFGE plug moulds were filled and allowed to set for 15 minutes at room temperature. Each plug was incubated in 2 ml lysis buffer (10 mM Tris HCl pH 7.2, 50 mM NaCl, 50 mM EDTA, 0.2 % (v/w) deoxycholate, 0.5 % (v/w) sarkosyl) at 37 °C for 1 hour. Lysis

buffer was replaced with 2 ml PK buffer (250 mM EDTA pH 9.0, 1 % (v/w) sarkosyl) containing 100 µg/ml proteinase K and incubated at 50 °C for 30 - 60 minutes. Plugs were washed three times in 10 ml wash buffer (10 mM Tris HCl pH 7.6, 0.1 mM EDTA), each wash lasted 30 minutes. Plugs were then stored at 4 °C in 2 ml wash buffer. Approximately one third of each plug was equilibrated by incubation in 300 µl of 1x restriction enzyme buffer 4 (NEB) for 1 hour. This was replaced with 150 µl of fresh 1x restriction enzyme buffer 4 containing 25 U SmaI (NEB) and incubated at room temperature overnight.

2.4.2 PFGE protocol

A CHEF DR-II Bio-Rad electrophoresis tank was prepared by allowing dH_2O to run through for 1 hour, followed by two refills of 0.5x TBE (10x TBE: 0.89 M Tris HCl pH 8.4, 0.89 M boric acid, 0.02 M EDTA) each for 1 hour. During the second passage of 0.5x TBE the tank was allowed to cool to 14 °C. The gel was made from 1 % pulse field certified agarose (BioRad) in 0.5x TBE. The digested plug slices were arranged on the comb and placed inside the gel whilst it was setting, together with a plug slice of CHEF DNA size standard lambda ladder (BioRad). Once set, the comb was removed and gaps filled with melted agarose. The gel was run with switch times 5.3 to 34.9 at 6.0 V/cm, 14 °C for 20 hours. The gel was stained using 0.5x TBE containing 0.5 µg/ml EtBr for 30 minutes followed by three washes in dH_2O. Bands were visualised with UV transilluminator (Gene Genius Bio Imaging System, Syngene).

2.5 Transformations and transductions

2.5.1 Electrocompetent *E. coli* (DC10B) and transformation

5 ml LB was inoculated with a single DC10B *E. coli* colony and incubated shaking at 37 °C overnight. 1 ml was diluted into 100 ml pre-warmed LB and incubated shaking at 37 °C until the culture reached OD_{600nm} 0.6. The cells were rapidly chilled in an ice slurry and harvested via centrifugation at 4000 rpm for 10 minutes at 4 °C. Pellets were washed three times with decreasing amounts of ice cold 10 % (v/v) glycerol (1/1; 1/2; 1/4), and stored at - 80 °C. Plasmid DNA was dialysed for 30 - 60 minutes using a 0.02 µm 13 mm nitrocellulose disc (Millpore) prior to electroporation.

Competent DC10B cells were defrosted on ice for 10 minutes and 50 µl was mixed with 10 µl dialysed plasmid DNA. This was transferred to a pre-chilled 0.2 cm electroporation cuvette (Geneflow) and electroporated using a Biorad gene pulser at 1.8 kV, 100 Ω, and 25 µF. 1 ml LB was immediately added and the culture incubated at 37 °C for 1 hour. 200 µl of the culture was plated in triplicate onto LA containing the required antibiotics and incubated overnight at 37 °C.

2.5.2 Electrocompetent *S. aureus* and transformations (Löfblom et al., 2007)

20 ml TSB/BHI was inoculated with a single *S. aureus* colony and incubated shaking overnight at 37 °C. The culture was pelleted, resuspended in residual culture and added drop wise to 100 ml pre-warmed TSB/BHI to OD_{578nm} 0.5. This was incubated shaking at 37 ˚C until the culture reached OD_{578nm} 0.6. The cells were rapidly chilled on an ice slurry and pelleted via centrifugation at 4000 rpm for 10 minutes at 4 °C. Cells were then washed twice in an equal volume of ice cold dH_2O. This was followed by three washes in decreasing amounts of 10 % (v/v) glycerol (1/10; 1/25; 1/200) and the cells stored at - 80 ˚C in 50 µl aliquots. Highly concentrated plasmid DNA was dialysed for 30 - 60 minutes using a 0.02 µm 13 mm nitrocellulose disc (Millipore) prior to electroporation. Competent *S. aureus* cells were defrosted on ice for 5 minutes, then at room temperature for 5 minutes. Cells were pelleted and resuspended in 50 µl 500 mM sucrose + 10 % (v/v) glycerol. 5 µg of dialysed plasmid DNA was added and the mix was transferred to a 0.1 cm electroporation cuvette (Geneflow) and electroporated using a Biorad gene pulser at 2.1 kV, 100 Ω, and 25 µF. 1 ml TSB + 500 mM sucrose was immediately added and the culture incubated at 28 ˚C for 1 hour. 200 µl of the culture was plated in triplicate onto LA containing the required antibiotics and incubated overnight at 28 °C.

2.5.3 *S. aureus* phage lysate production and transductions

5 ml LB was inoculated with the donor strain of *S. aureus* and incubated shaking overnight at 37 °C. This was diluted to OD_{600nm} 0.05 in 25 ml LB + 10 mM $CaCl_2$ + 10 mM $MgSO_4$ and incubated shaking at 37 ˚C until OD_{600nm} 0.2. 10 ml was subcultured into fresh 25 ml LB + 10 mM $CaCl_2$ + 10 mM $MgSO_4$ with 1 ml phage ϕ11 and incubated shaking at 37 °C for a further 4 hours or until cells have lysed. The culture was centrifuged at 4000 rpm for 10 minutes, the supernatant filter sterilised and

stored at 4 ˚C for future use. The recipient strain was inoculated into 20 ml LK broth and incubated shaking at 37 ˚C overnight. The cells were harvested and resuspended in 1 ml LK broth. Duplicate tubes (test and negative control) were set up containing 500 µl cells and either 500 µl donor phage lysate (in the test sample) or 500 µl LK broth, both were made up to 2 ml using LK + 10 mM $CaCl_2$. Cultures were incubated static at 37 ˚C for 25 minutes followed by shaking at 37 ˚C for a further 15 minutes. 1 ml ice cold 2 mM Na citrate was added and the culture centrifuged at 4000 rpm for 10 minutes at 4 °C. The cell pellets were resuspended in 1 ml ice cold 2 mM Na citrate and incubated on ice for 2 hours minimum. 100 µl were plated in triplicate onto LK agar plates + 0.05 % (w/v) Na citrate containing required antibiotics and incubated for 24 - 48 hours at 37 °C.

2.6 Antibiotic sensitivity testing

2.6.1 Micro-broth dilution (Cockerill et al., 2011; Wikler et al., 2007)

5 ml MHB overnight cultures were centrifuged at 4000 rpm for 5 minutes to pellet the cells and the concentrated cell pellet was added drop-wise into 10 ml fresh CAMHB to 0.1 OD_{600nm}. This was further diluted one log_{10} in fresh CAMHB. In a 96 well round bottomed plate (Nunc), 50 µl of CAMHB was pipetted into each well apart from the third column. 100 µl of CAMHB + 64 µg/ml vancomycin or CAMHB + 256 µg/ml oxacillin was added to the third column and a one log_2 serial dilution of the antibiotic was made in subsequent columns from left to right. 50 µl of the CAMHB + antibiotic was also added to the first column to serve as the negative control (antibiotic only, no inoculum). To columns two to twelve, 50 µl inoculum was added, making the second column the positive control (inoculum only, no antibiotic) and subsequent columns containing decreasing concentrations of antibiotic. The plate was then incubated at 37 °C for 24 - 48 hours and the MIC was determined by the minimum concentration of antibiotic that inhibited growth. CAMHB + 2 % (w/v) NaCl was used for oxacillin susceptibility testing.

2.6.2 MIC Evaluator test strips and MAST rings (Cockerill et al., 2011; Wikler et al., 2007)

Sterile cotton swabs were saturated with bacteria by submerging in 5 ml LB overnight cultures and were used to streak MHA plates in multiple directions resulting in confluent growth. Once dry, the MIC Evaluator test strips (Oxoid) were applied to the plates using sterile tweezers with the MIC scale facing upwards and the plates incubated at 37 °C for 24 - 48 hours. The MIC was recorded where the inhibition of growth crosses the MICE test strip. If the growth continued throughout the strip, the MIC was recorded as more than the maximum concentration. For oxacillin MIC testing, MHA + 2 % (w/v) NaCl was used. For antibiogram analysis, M13 and M43 systemic gram positive MAST rings (MAST group) were applied to inoculated MHA plates and incubated as above. Zones of inhibition were recorded and translated into an arbitrary scale of low, intermediate and high tolerances.

2.7 Growth analysis

2.7.1 24 hour growth curves

For nutrient replete conditions TSB was used. 5 ml TSB static overnight at 37 ˚C in 5 % CO_2 was sub-cultured into 20 ml fresh TSB to OD_{600nm} 0.05. Cultures were incubated static at 37 ˚C in 5 % CO_2. For nutrient restricted conditions CRPMI was used. 10 ml CRPMI static overnight at 37 ˚C in 5 % CO_2 was harvested and the concentrated cell pellet was added drop-wise into 20 ml fresh CRPMI to OD_{600nm} 0.1. Cultures were incubated static at 37 ˚C in 5 % CO_2. To investigate the effect of oxidative stress, 10 ml TSB shaking overnight at 37 ˚C was sub-cultured into 40 ml fresh TSB to OD_{600nm} 0.05. Cultures were split in two 20 ml where 2 µl of 30 % H_2O_2 was added to one set. Cultures were incubated shaking at 37 °C. 1 ml aliquots were taken every hour for the first 7 hours and OD_{600nm} was recorded using a spectophometer, followed by a final reading 24 hours post inoculation. The means were calculated from three independent experiments and plotted using GraphPad Prism 6 software.

2.7.2 96-well plate growth analysis

10 ml CRPMI static overnight at 37 ˚C in 5 % CO_2 was harvested and the concentrated cell pellet was added drop-wise into 20 ml fresh CRPMI to OD_{600nm} 0.1. Cultures were separated into aliquots where increasing concentrations of specific stressors (0, 50, 100, 200 µM $FeSO_4$ or $CuCl_2$) were added to the cultures and used to fill triplicate wells of a 96 well flat bottom plate. Plates were incubated at 37 ˚C in 5 % CO_2 and OD_{600nm} recorded after 24 hours using a BMG Labtech FLUOstar Omega plate reader. Means were calculated from three independent experiments and plotted +/- one standard deviation from the mean. Statistical significance, as determined by student T-tests, was indicated by asterisks (*) using the following scale: ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

2.8 Exoprotein analysis

2.8.1 Quantitative haemolysis activity assay

10 ml LB overnight liquid cultures were prepared and the OD_{600nm} were measured to check growth was comparable between strains. Cultures were centrifuged at 4000 rpm for 5 minutes and the supernatants sterilized and concentrated using Amicon Ultra Centrifugal Concentrators (Millipore) by centrifugation at 4000 rpm for 10 minutes. 2 ml of defibrinated sheep or rabbit blood (Oxoid) was centrifuged at 6000 rpm for 1 minute and 400 µl of the bottom layer was resuspended in 10 ml PBS. 50 µl of PBS was added to all wells of a 96-well round bottomed plate (Nunc) except the first column. 50 µl of the concentrated supernatant was added to the PBS in the third column and mixed thoroughly by pipetting and a log_2 serial dilution made in subsequent wells from left to right. 50 µl of 4 % sheep/rabbit blood was added to all wells and the plate incubated at 37 °C for 30 minutes. The terminal dilution where complete haemolysis still occurred was recorded for each sample.

2.8.2 DNase and haemolysis plate activity assay

5 ml LB overnight liquid cultures were prepared and the OD_{600nm} were measured to check growth was comparable between strains. 10 µl of each culture was spotted onto DNase, sheep blood and rabbit blood agar plates and allowed to dry. The plates were incubated at 37 °C for 24 hours (in 5 % CO_2 for blood agar plates). To measure

DNase activity, DNase plates were flooded with 1 M HCl to precipitate polymerised DNA which produced clear halos where DNA was hydrolysed. The radiuses of the clear halos around bacterial growth indicating haemolysis or DNase activity were measured and recorded.

2.8.3 *agr* dysfunction (Marks and Vaughan, 1952; Sakoulas et al., 2002)

A single colony of RN4220, a δ-lysin defective strain, was streaked in a single line on a sheep blood agar plate. Single colonies of test isolates were streaked perpendicular to RN4220 leaving a 2 mm gap approximately. Plates were incubated for 24 hours at 37 ˚C in 5 % CO_2 and analysed for complete clearing between the test isolate and RN4220 indicating δ-lysin activity and a functional *agr* system.

2.9 Staphylococcus aureus protein extraction

10 ml CRPMI overnight liquid cultures were centrifuged at 4000 rpm for 5 minutes to collect the cell pellet. The supernatant was removed and kept for further analysis. The cell pellet was weighed and used for normalisation calculations.

2.9.1 Secreted protein fraction

The supernatant was sterilized and concentrated using Amicon Ultra Centrifugal Concentrators (Millipore) leaving approximately 200 µl of concentrated supernatant. Samples were normalised relative to 10 mg of cell pellet weight.

2.9.2 Non-covalently bound protein fraction

10 µl (per mg of pellet) of 125 mM Tris HCl pH 7.0 + 2 % (w/v) SDS was used to resuspend the cells and the solution boiled for 3 minutes. Samples were centrifuged at 13000 rpm for 3 minutes and the supernatant retained. For iTRAQ protein preparations 125 mM Tris HCl pH 7.0 + 2 % (w/v) SDS was replaced with 125 mM MOPS pH 7.0 + 2 % (w/v) SDS and samples sent to PNACL (University of Leicester) for iTRAQ labelling and LC-MS analysis.

2.9.3 Cell wall protein fraction

25 µl (per mg of pellet) of 400 µg/ml lysostaphin in 1 mg/ml benzamidine in PBS + 30 % (w/v) raffinose was used to resuspend the cells and incubated at 37 °C for 20

minutes. Samples were centrifuged at 13000 rpm for 5 minutes and the supernatant retained.

2.10 SDS-PAGE protein gels

2.10.1 Protein loading preparation

40 µl of the non-covalently bound protein fraction and 60 µl of the secreted and cell wall protein fractions were mixed with equal volume of 2x Laemmli sample loading buffer (20 % (v/v) Glycerol, 4 % (w/v) SDS, 100 mM Tris HCl (pH 7.0), 200 mM DTT (Dithiothreitol), 0.2 % (w/v) Bromophenol Blue). These were boiled for 3 minutes and briefly centrifuged. The prepared protein samples were loaded into the gel along with 20 µl of PageRuler prestained protein ladder (Fermentas).

2.10.2 1D SDS PAGE

The proteins were separated on a SDS-PolyAcrylamide Gel by Electrophoresis (SDS-PAGE) using a 10 % separating gel and a 5 % stacking gel. Prepared gels were run at 60 mA with maximum voltage in 1x SDS-PAGE running buffer (3.2 g Tris, 14.4 g Glycine, 1 g SDS in 1 l dH_20). Protein bands were visualised by staining the gel with Coomassie blue overnight (22.5 % (v/v) methanol, 10 % (v/v) glacial acetic acid, 0.25 % Coomasie brilliant blue R250 [Sigma]) followed by destaining (Coomassie stain excluding the dye) to remove the excess dye. Protein bands of interest were identified by PNACL (Protein and Nucleic Acid Chemistry Laboratory) within the University of Leicester using MALDI-ToF (Matrix Assisted Laser Desorption/Ionization- Time of Flight) mass spectrometry.

2.10.3 10% separating gel (25ml)

12.3 ml buffer A (45.4 g Tris, 1 g SDS in 500 ml dH_2O, pH 8.8), 8.3 ml acrylamide, 3.5 ml dH_2O, 864 µl 1 % APS and 68.2 µl Temed (tetramethylethylenediamine).

2.10.4 5 % stacking gel (7 ml)

3.5 ml buffer B (15.1 g Tris, 1 g SDS in 500 ml dH_2O, pH 6.8), 1155 µl acrylamide, 2156 µl dH_2O, 175 µl 1 % (v/w) APS and 14 µl Temed (tetramethylethylenediamine).

2.11 *Staphylococcus aureus* biofilms (Johnson et al., 2008)

Quadruplicate wells of a 96 well flat bottomed microtiter plate (Nunc) were inoculated with 200 µl OD_{600nm} 0.05 bacterial inoculum and incubated statically for 24 hours at 37 °C. OD_{600nm} was read at time 0 and 24 h to record potential growth differences on a BMG Labtech FLUOstar Omega plate reader. The wells were gently washed three times with sterile phosphate buffered saline (PBS) and heat fixed at 60 C for 30 minutes. The wells were stained with 200 µl of 1 % safranine for 30 minutes and washed twice with deionised water. Biofilms values were measured at OD_{490nm}. Means were calculated from three independent experiments and plotted +/- one standard error from the mean. Statistical significance was indicated by asterisks (*) using the following scale: ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

2.12 Fibrinogen and fibronectin binding assay

Fibrinogen or fibronectin coated plates were prepared by adding 100µl of either 10 µg/ml fibrinogen or fibronectin (Sigma Aldrich Ltd) suspended in 0.02 % sodium carbonate (pH 9.6) to 96 well flat bottomed plates and kept at 4 ˚C overnight. Plates were washed three times with sterile PBS and 100 µl 3 % bovine serum albumin (BSA: Sigma Aldrich Ltd) was added to stop non-specific binding. Plates were incubated for 2 hours at 37 ˚C and washed as before with sterile PBS. 100 µl of the bacterial inoculum (log_{10} dilution of BHI + 1 % glucose overnight culture into fresh media) was added to quadruplicate wells of coated plates and incubated for 2 hours at 37 °C. Plates were washed as before in PBS and the adhered cells fix at 55 ˚C for 30 minutes. Plates were stained with 100 µl 0.5 % crystal violet (Sigma Aldrich Ltd) for 30 minutes followed by three washes with dH_2O and drying at 55 ˚C for 15-30 minutes. Fibrinogen and fibronectin binding capabilities were assessed by recording optical densities at 570nm. Means were calculated from three independent experiments and plotted +/- one standard error from the mean.

2.13 Gentamicin persister assay

10 ml LB overnight cultures were subcultured into 20 ml fresh media to OD_{600nm} 0.1 and incubated shaking at 37 ˚C for 1 hour. At this point 1 ml aliquots were taken to

be used for CFU plating. Cultures were split into two and 10x MIC of gentamicin was added to one set. Cultures were re-incubated for a further 6 hours. 1 ml aliquots were taken, serially diluted and plated out onto LA and LA + 10x gentamicin MIC.

2.14 Eukaryotic cell invasion assay

2.14.1 Maintenance of H9C2 cell line

An adhesive cell line, H9C2, was seeded and maintained in DMEM glutamax (Gibco) + 10 % fetal bovine serum (FBS; PAA laborarories). The cells were allowed to reach 70 % confluency before they were treated with 1x trypsin EDTA (PAA Laboratories) to release them from the surface of the culture flask; at this point cells were diluted into fresh media and split into new flasks for incubation. In preparation for invasion experiments, cultures were split into 12-well tissue culture plates (Nunc) and grown to 100 % confluency.

2.14.2 Adhesion, invasion and intracellular persistence assay

The previous media of DMEM glutamax + 10 % FBS was aspirated from the 12-well plates and replaced with DMEM glutamax + 1 % FBS 30 minutes before the start of the experiment to allow the cells to acclimatise. For the bacterial inoculum, 10 ml overnight cultures were harvested, the bacterial pellets washed three times in sterile PBS and resuspended in PBS to OD_{600nm} 1.0 (approximately 10^7 CFU per 10 µl). Inoculum CFU were confirmed during each experiment via serial dilutions and plating. 10 µl of each culture was used to inoculate triplicate wells in three separate 12 well plates containing confluent H9C2 cells, and plates incubated at 37 °C in 5 % CO_2 for 2 hours. For two of the three plates, media was replaced with DMEM glutamax + 1 % FBS plus 200 µg/ml gentamicin (Sigma-Aldrich) and incubated for a further 3 hours (for invasion) or 72 hours (for intracellular persistence). The final plate was used immediately without further media replacement or incubations (adhesion). Media was aspirated and wells washed twice with PBS. The H9C2 cells were lysed in 1 ml 1 % triton-X-100 (Sigma-Aldrich) in PBS for 10 minutes at room temperature, serial dilutions were made and plated onto non-selective media to calculate bacterial CFU. In normal circumstances LB broth was used for bacterial overnight cultures and CFU were plated onto LA. It was found that Δ*fur* mutants

struggled to grow in LB and on LA plates, therefore overnight cultures were prepared using CRPMI + 1 % casamino acids and CFU plated onto horse blood agar plates. Means were calculated from three independent experiments and plotted +/- one standard error from the mean. Statistical significance was indicated by asterisks (*) using the following scale: ns P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

2.15 *Galleria mellonella* larva infection model

2.15.1 Virulence assay

Galleria mellonella larvae were purchased from Live Foods UK Ltd (www.livefoods.co.uk) and were stored at 4 °C for up to one week. Before each experiment, *Galleria* larvae were left overnight at room temperature. Overnight cultures in 5 ml LB were centrifuged, pellets washed three times in sterile PBS and resuspended in fresh sterile PBS to OD_{600nm} 0.1 (approximately 10^6 CFU per 20 µl). Groups of 10 *Galleria* larvae were used for each infecting isolate. Larva were injected with 20 µl of bacterial inoculum via their last proleg using micro-fine 1 mL insulin syringes (BD) with a stepper repetitive pipette (Tridak), incubated at 37 °C and fatalities recorded every 24 hours for 96 hours. Additionally, groups of 10 larvae were either injected with 20 µl sterile PBS and not injected at all and were used as negative controls; if a total of two larvae died from any one or a combination of the two groups, the experiment was discarded. Comparable inoculums between different strains were confirmed via CFU plating. Data from three independent experiments were plotted on Kaplan-Meier survival graphs and statistical significance indicated by asterisks (*) using the following scale: ns P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

2.15.2 48 hour bacterial burden assessment

Relative bacterial burdens were investigated 48 hours post *Galleria* larvae inoculation and additional larvae were inoculated in parallel to each virulence experiment for this purpose. 48 hours post inoculation two viable larvae per infecting strain per experiment were homogenised in 2 ml sterile PBS using 4 x 2.5 mm zirconia/silica beads (Thistle Scientific, UK) in a mini bead beater (Biospec

products, Inc) on high for 30 s. These homogenates were centrifuged at 2500 rpm for 5 minutes at 4 °C and the liquid phase retained and kept on ice. Serial dilutions were made, plated onto *Staphylococcus* selective media (mannitol salt agar; Oxoid) and CFU were calculated after a minimum of 48 hours incubation at 37 °C.

2.16 RNA preparation and qRT-PCR

2.16.1 RNA preparation using TRIreagent

The remaining liquid phase was centrifuged at full speed for 15 minutes, pellets resuspended in 100 µl TE^{LL} (TE buffer containing 3 mg/ml lysozyme; Sigma-Aldrich, and 40 µg/ml lysostaphin; Ambi products LLC, USA) and incubated at 37 °C for 15 minutes. 900 µl TRI-reagent (Sigma-Aldrich) was added and samples incubated for a further 5 minutes at room temperature. 200 µl chloroform was added and vortexed for 15 s followed by 15 minutes incubation at room temperature. Samples were centrifuged for 15 minutes at full speed at 4 °C, the upper aqueous phase transferred to a fresh tube for RNA isolation. To the upper aqueous phase, 0.5 ml IPA was added, inverted and incubated at room temperature for 10 minutes. Samples were centrifuged at full speed for 8 minutes at 4 °C. The RNA pellet was washed with 1 ml 75 % ethanol and centrifuged at 10500 rpm for 5 minutes at 4 °C. The pellet was briefly air dried before re-suspension in 50 µl DEPC treated water and storage at - 80 °C.

2.16.2 qRT-PCR

RNA samples were DNase treated using Turbo DNase (Ambion) according to manufacturers' instructions, the resulting samples quantified using a Thermo Scientific 2000c nano-drop and normalised so that 12 µl contained 2 µg total RNA. Samples were stored at - 80 °C. Omniscript reverse transcription kits (Qiagen) were used to convert RNA to cDNA, using 12 µl (2 µg) of DNase treated RNA; RNase inhibitor at 10 U/µl (Ambion) and random hexamers at 10 µM (Applied Biosystems) were used as recommended but not supplied within the kit; samples stored at - 20 °C for up to one week. qPCR was conducted using fast SYBR green technology on an ABI 7500 machine. Set up briefly involved 20 µl reactions containing 10 µl fast SYBR green master mix (Applied Biosystems), 0.5 µl of each forward and reverse 16S

rRNA primers (10 µM stock) and template cDNA. The following cycle conditions were used- 94 °C for 20 s and 40 cycles of 95 °C for 3 s, 60 °C for 30 s, ending in a disassociation curve to confirm accurate primer binding and disassociation. Two technical repeats were completed per RNA sample. The resulting Ct values were inverted and multiplied by 1000 to make them more manageable for comparisons.

2.17 Illumina genome sequencing and bioinformatic analysis

2.17.1 Illumina genome sequencing

Conducted in collaboration with Dr Sam Sheppard and Dr Ben Pascoe (University of Swansea, 2013/2014).

Genomic DNA was prepared as described in section 2.3.2. A minimum of 4 µg purified DNA of minimum concentration 40 ng/µl resuspended in dH_2O was sent to Wellcome Trust Centre, Oxford. Samples were subjected to Illumina genome sequencing workflow as described in Sheppard et al. (2013). This briefly involved shearing 2 µg genomic DNA using a Covaris E210 sonicator followed by enrichment of 200 bp fragments. Fragments were end repaired, bead purified (Ampure paramagnetic beads; Beckman Coulter Inc) and a-tailed. Adaptors were ligated and samples were subjected to primer extension via PCR to introduce Illumina specific sequencing indexes. Indexed DNA samples were purified a final time before quantification and equimolar pooling. Pooled samples were subjected to paired-end high-throughput genome sequencing performed at the Wellcome Trust Centre for Human Genetics in Oxford, UK using an Illumina HiSeq 2500 machine. The resulting raw sequencing reads were demultiplexed and delivered to University of Leicester for bioinformatic analysis.

2.17.2 Bioinformatic analysis of genomic sequencing data

Conducted by Dr Richard Haigh (University of Leicester, 2013/2014).

All bioinformatic analysis was conducted on SPECTRE (Special Computational Teaching and Research Environment), a High Performance Cluster hosted by the University of Leicester. Firstly, raw paired-end sequencing reads were quality checked using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/),

specifically fastq_quality_trimmer and fastq_quality_filter programs were utilised. These programs filtered out poor quality reads and end-trimmed the remaining reads to remove poor quality bases and barcodes. Trimmed reads were then aligned to a reference genome, HO 5096 0412 member of the EMRSA15 lineage (Holden et al. 2013). Alignments were constructed using a combination of the following programs- Picard (http://picard.sourceforge.net.) to create a dictionary for the reference genome, BWA-MEM (Li 2013 [http://bio-bwa.sourceforge.net/]) to create a SAM file of mapped reads and SAMtools view, sort and index programs (Li et al. 2009 [http://samtools.sourceforge.net/]) to convert the SAM file to a sorted, indexed BAM file which could be viewed using Tablet viewer (Milne et al. 2013 [http://bioinf.hutton.ac.uk/tablet]). Genetic variations when compared to the reference genome and between individual isolate sequence data such as initial and persistent isolate comparisons were identified using VarScan.v2.3.6 (Koboldt et al. 2012 [http://bioinf.hutton.ac.uk/tablet]) where a variant threshold of > 80 % was used. Each identified SNP/INDEL was mapped back to the reference genome where the biological consequence could be determined, i.e. position in regards to known open-reading frames and whether they were synonymous or non-synonymous.

Chapter 3 Characterisation of clinical *Staphylococcus aureus* isolates

3.1 Introduction

The primary aim of this study is to identify putative genotypic and phenotypic traits associated with persistent *S. aureus* isolates. So far no universal "persistence indicator(s)" has been agreed to by all investigators. This is likely due to the epidemiological design of the majority of persistence studies, where the focus was the size of the cohort collection as opposed to in-depth analysis of individual persistent infections. This may have meant that subtle persistence traits could have been overlooked. The design of this investigation took into account the suggested limitations of previous studies and consequently it was decided that an in-depth analysis should be conducted into multiple persistent infections; and where possible all clinical isolates relating to each individual infection should be included in analysis. Additionally, a selection of isolates originating from resolved bacteraemia cases were investigated so data could be compared between the two groups. All clinical isolates were collected from a single location within a single time period thereby limiting any bias resulting from different circulating *S. aureus* strains.

3.2 Clinical details of *S. aureus* bacteraemia isolates

The University Hospitals of Leicester (UHL) archive was studied for *S. aureus* samples originating from bacteraemia patients from March 2010 to April 2012. Persistent *S. aureus* bacteraemia were defined as blood stream infections (BSI) lasting more than seven days during which appropriate antibiotics were administered. In contrast, resolved *S. aureus* bacteraemia were BSI successfully treated using clinically relevant antibiotics; confirmed by diminished infective symptoms and/or negative blood culture (BC) within seven days of the first positive BC. Additional information regarding the infection and patient details remained anonymous until after lab research was conducted to avoid adding unnecessary bias to the cohort collection. Individual infection timelines are displayed in Figure 3-1 to Figure 3-4 and a brief overview can be seen in Table 3-1. Microbiological samples were taken from the UHL's frozen bacterial beads made from single colonies

originating from the primary BC plates and additional frozen cultures made for the purpose of lab analysis which were stored at the University of Leicester (UOL).

3.2.1 Persistent bacteraemia 1 (PB1)

A 76 year old female was admitted to the UHL with chest pain and suspected sepsis (day 0). On admission, BC were taken and broad spectrum antibiotics, co-amoxiclav, clarithromycin and vancomycin were immediately prescribed (Figure 3-1). On day 3 post admission MRSA was identified as the infecting isolate and treatment was subsequently changed to linezolid. The archived infecting isolate is referred to as PB1-1. On day 6 a transthoracic echo (TTE) indicated mitral valve IE, consequently daptomycin replaced linezolid on day 7. A follow up BC on day 7 was positive for MRSA, but not archived therefore not available for analysis. The patient's symptoms persisted and two surveillance BC taken on day 15 were positive for MRSA (PB1-15-1, PB1-15-2). The patient's health continued to deteriorate until they passed away on day 18.

3.2.2 Persistent bacteraemia 2 (PB2)

A 70 year old female was already under the care of the UHL for the management of pustular psoriasis prior to diagnosis of *S. aureus* bacteraemia. Eleven days post admission left arm pain and congestion of the capillaries was reported. BC were taken and flucloxacillin immediately prescribed which was later replaced with a 14 day prescription of vancomycin when MRSA was confirmed (Figure 3-2). The culture was archived and available for analysis (PB2-1). Symptoms appeared to diminish until the patient's CRP (C-reactive protein) levels rose unexpectedly 35 days after the initial BC was taken. This coincided with sacral wound deterioration and a BC confirmed MRSA infection (PB2-35). Vancomycin was re-commenced but terminated after two days as the patient appeared systemically well and the positive BC was considered possible contamination. Eight days later the wound looked increasingly sore and had increased in size, therefore vancomycin treatment was re-commenced. A four week prescription of daptomycin replaced vancomycin treatment shortly after due to difficulty in maintaining vancomycin levels. The sacral wound showed improvement and infective symptoms did not reappear.

3.2.3 Persistent bacteraemia 3 (PB3)

A 45 year old man was admitted to the UHL with fever and an infected left foot ulcer (day 0), BC were taken on admission and intravenous (IV) flucloxacillin was started confirmed (Figure 3-3). Vancomycin was prescribed on day 3 when MRSA was identified but treatment was changed to daptomycin on day 4 due a vancomycin induced rash. The initial bacterial sample was archived and consequently collected for lab analysis (PB3-1). Negative BC were documented on day 7 and 13 but due to the severity of the infection the left foot was amputated on day 20. On day 27 daptomycin treatment was terminated, but after two days the patient spiked a temperature and a positive BC was acknowledged (PB3-29). A 4 week course of linezolid was prescribed but surveillance BC taken on day 32 (PB9-32-1/PB9-32-2) remained positive, despite a negative TTE for heart valves vegetations. Surveillance BC on days 36, 37, 40, 42, 44, 47, 50, 57 and 61 were negative and the patient was discharged on day 46. On day 72 an additional BC (reason not documented) was positive for MRSA (PB3-72) and a subsequent BC was positive (PB3-74). On day 76 the patient was re-admitted and commenced IV tigecycline and rifampicin for a further 10 weeks. A MRI (magnetic resonance imagining) showed signs of infection within the disc space between the V2/V3 vertebra, a paraspinal abscess and right foot osteomyelitis; but a transoesophageal echocardiogram (TOE) showed no evidence of IE. The patient was discharged well and afebrile on day 146 with a 3 month course of oral doxycycline. BC taken on day of discharge were negative.

3.2.4 Persistent bacteraemia 5 (PB5)

A 78 year old male was admitted with right side renal pain and was known to have renal stones. A permacath line was inserted for haemodyaliasis and the following day the patient spiked a temperature. BC were taken and vancomycin and co-amoxiclav was immediately administered (Figure 3-4). MRSA was confirmed 3 days later; the infecting isolate was archived and therefore available for analysis (PB5-1). As a result treatment was changed to a 14 day course of linezolid and the permacath line removed. After 4 days of treatment a chest wall abscess was drained and patient

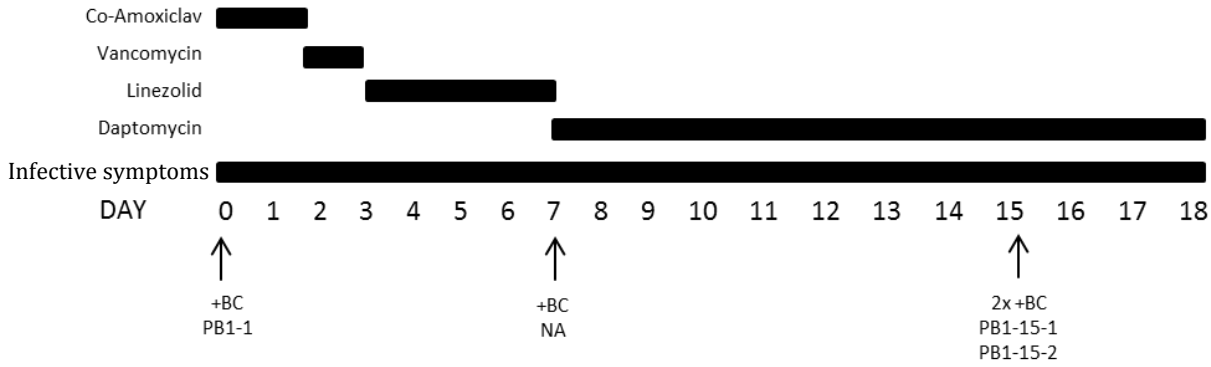

Figure 3-1 PB1 bacteraemia timeline. The patient was admitted on day 0 and BC taken (PB1-1). Co-amoxiclav was prescribed followed by vancomycin, linezolid and daptomycin. A positive BC was taken on day 7 (not archived) and two independent positive BC on day 15 (PB1-15-1 and PB1-15-2). The patient passed away on day 18. Timings for the bacteraemia are relative to the collection of the initial BC. The periods of infective symptoms are estimated from clinical documentation.

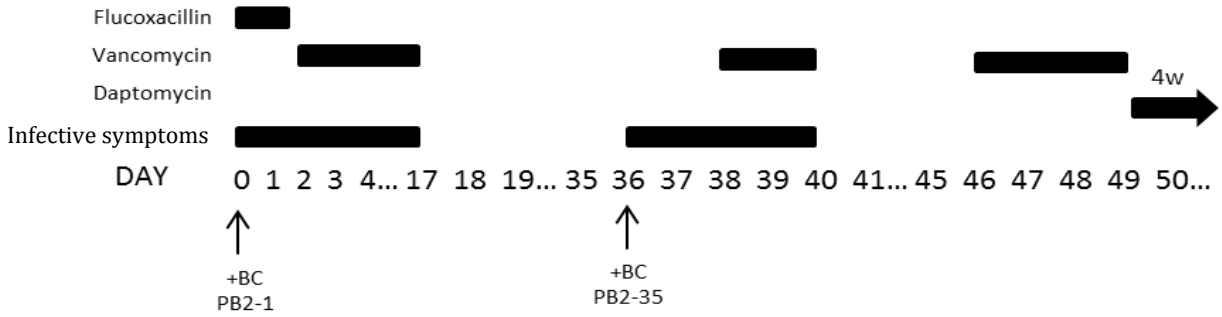

Figure 3-2 PB2 bacteraemia timeline. On day 0 the initial positive BC (PB2-1) was taken. Another positive BC was taken on day 36 (PB2-35). Antibiotics included flucoxacillin, three occasions of vancomycin, followed by a four week prescription of daptomycin. Symptoms appeared diminished after daptomycin therapy. Timings for the bacteraemia are relative to the collection of the initial BC. The periods of infective symptoms are estimated from clinical documentation.

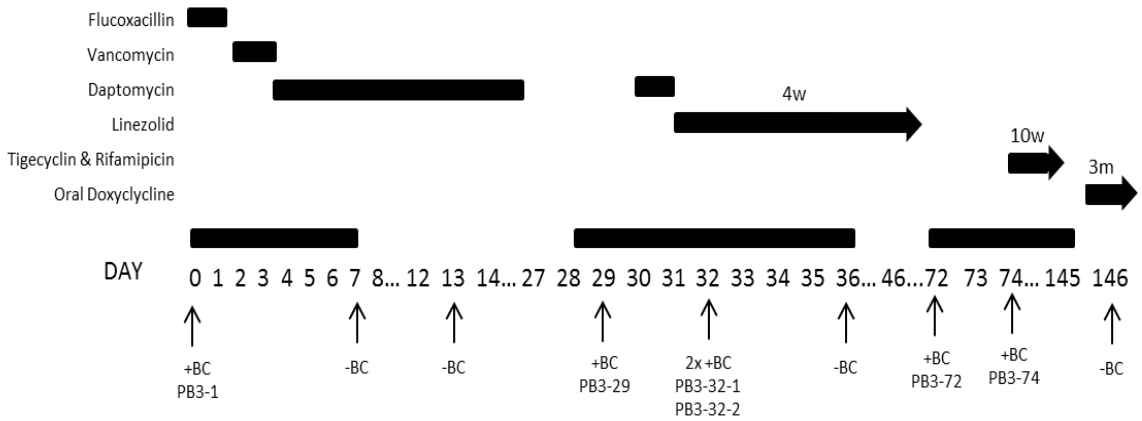

Figure 3-3 PB3 bacteraemia timeline. On day 0 a positive BC was collected (PB3-1). Antibiotic treatment initially involved flucoxacillin, vancomycin then daptomycin. Two negative BC were recorded and treatment ended on day 27. Surveillance BC on day 29 and 32 were positive (PB3-29, PB3-32-1/2) so daptomycin was prescribed followed by four weeks of linezolid. Negative BC were recorded and the patient discharged on day 46. On 72 and 74 BC were positive therefore 10 weeks of tigecyclin and rifamipicin were administered. The patient was discharged again on day 146 with 3 months of oral doxyclycline. Timings for the bacteraemia are relative to the collection of the initial BC. The periods of infective symptoms are estimated from clinical documentation.

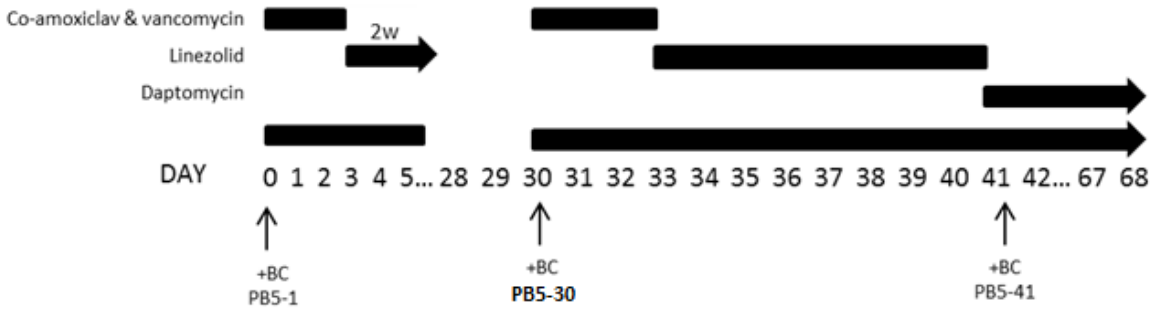

Figure 3-4 PB5 bacteraemia timeline. The initial BC (PB5-1) was taken on day 0. A positive surveillance BC was taken on day 30 (PB5-30). Antibiotics included co-amoxiclav, vancomycin, linezolid and daptomycin. Disease severity continued until the patient passed away approx. one month later. Timings for the bacteraemia are relative to the collection of the initial BC. The periods of infective symptoms are estimated from clinical documentation.

Table 3-1 Overview of persistent and resolved *S. aureus* bacteraemia clinical information. The bacteraemia reference is given for each bacteraemia case (PB- Persistent; RB- resolved bacteraemia), antibiotics specific to the MRSA bacteraemia are stated and day which positive and negative BC were acknowledged (D- day number relative to the initial isolate- D0). Details of the potential infective focus and final patient outcome are also given

Case ref	Classification	Antibiotics	Positive BC	Negative BC	Potential/ suspected foci	Final patient diagnosis
PB1	Persistent	Vancomycin, linezolid, daptomycin	D0- PB1-1 D7- not archived D15- PB1-15-1 D15- PB1-15-2	None	Mitral valve endocarditis	Fatal, patient passed away on day 18
PB2	Persistent	Vancomycin, daptomycin	D0- PB2-1 D36- PB2-35	None	Sacral wound	Resolved and discharged
PB3	Persistent	Vancomycin, daptomycin, linezolid, tigecycline/ rifampicin, doxycycline	D0- PB3-1 D29- PB3-29 D32- PB3-32-1 D32- PB3-32-2 D72- PB3-72 D74- PB3-74	D7, 13, 36, 37, 40, 42, 44, 47, 50, 57, 61, 146	Left foot ulcer Paraspinal abscess & right foot osteomyelitis	Resolved and discharged on day 146

Continued from previous page…

Case ref	Classification	Antibiotics	Positive BC	Negative BC	Potential/ suspected foci	Final patient diagnosis
PB5	Persistent	Linezolid, vancomycin, daptomycin	D0- PB5-1 D30- PB5-30 D41- PB5-41	None	Permacath line Chest wall abscess Disc space inflammation	Deemed unsuitable for surgical intervention, patient passed away approx. 1 month after last BC
RB1	Resolved	Linezolid	D0- RB1	None	Infected hemithorax	Resolved and discharged
RB4	Resolved	Vancomycin	D0- RB4	D3	Catheter an JJ stents	Resolved and discharged
RB5	Resolved	Vancomycin	D0- RB5	None	Infected prosthetic hip joint	Resolved and discharged

was diagnosed with multiple myeloma with bony metastases throughout the body. The patient's developed infective symptoms once again 10 days after the linezolid treatment finished so BC were taken and co-amoxiclav prescribed. MRSA was confirmed (PB5-30) and linezolid treatment was re-administered. Eleven days later surveillance BC (PB5-41) still tested positive for MRSA therefore daptomycin replaced treatment. An MRI showed inflammation of vertebrae and disc space with a paraspinal abscess but the patient was deemed unsuitable for surgical intervention. The patient's health continued to deteriorate until they passed away a month later.

3.2.5 Resolved bacteraemia- RB1, RB4 and RB5

RB1 involved a 64 year old male admitted to the UHL with mesothelioma of the lung and elected for removal of the cancerous membrane via thoracotomy decortication surgery; a chest drain remained. Seven days later, the patient spiked a temperature and co-amoxiclav was prescribed. After a further seven days, raised white blood cell counts and CRP levels were noted, therefore BCs were taken and treatment was changed to tazocin and vancomycin. The patient was taken back to theatre for re-thoractomy; pus was drained for the right hemithorax and the infected diaphragmatic patch was removed. Two days later MRSA was confirmed in the sputum, pleural fluid and BCs, resulting in linezolid replacing vancomycin for treatment. Fourteen days after linezolid was initiated, the patient was discharged with no infective symptoms remaining present.

A 41 year old female with recurrent pyelonephritis, Crohn's disease, a long term catheter and ureteral stents was admitted the Leicester General Hospital with loin pain and haematuria. Imepenem was administered due to an allergy to penicillin and initial BCs were negative for bacterial culture. Fifteen days later, the patient developed abdominal pain and exhibited raised white blood cell counts and CRP levels, therefore Imepenem was re-administered. Two days later, BCs confirmed MRSA and a 14 day course of vancomycin was initiated. Repeat BCs taken the next day were negative, with no re-emergence of infective symptoms.

RB5 originated from an 80 year old female who had undergone a total right hip replacement six months previously, and was admitted to UHL with five day hip pain

Clinical observation noted sepsis with fever, raised white blood cell counts and CRP levels, therefore flucloxacillin was prescribed. BCs taken on admission confirmed MRSA and vancomycin replaced flucloxacillin of day 3. O day 10 the patient was taken to theatre for right hip washout and debridement; samples later confirmed the suspected MRSA infective focus. After completing the 48 day course of vancomycin, the patient was discharged.

3.3 Molecular typing & antibiogram profiles

A series of published molecular and phenotypic typing methods were used to determine the isolates' genetic backgrounds including ST and CC. Additionally, these techniques were used to confirm all isolates collected from an independent infection originated from one persisting *S. aureus* strain as opposed to multiple cases of different infecting isolates.

3.3.1 SCC*mec*, J-region and *agr* typing

All *S. aureus* isolates chosen for this study were methicillin resistant as proven by the UHL. Multiplex PCR were used to determine each isolates SCC*mec* type (Boye et al 2007) and J-region sub-type (Milheiriço et al., 2007). The multiplex SCC*mec* PCR used four primer pairs to target different areas of the SCC*mec* element producing distinct DNA fragment banding patterns corresponding to one of the first five SCC*mec* types (I to V). Two representatives of the epidemic MRSA clones in the UK were included as typing controls (PM64, EMRSA-16, SCC*mec* II & PM25, EMRSA-15, SCC*mec* type IV) (Moore & Lindsay, 2002). All clinical isolates produced two bands with estimated sizes of 400 bp and 950 bp relating to the IS*1272* and *ccrA2-B* loci (Figure 3-5A). This combination depicts SCC*mec* type IV and this was confirmed by identical bands in the PM25 lane. The PM64 test sample displayed the ~ 950 bp band only, confirming SCC*mec* II.

The isolates' SCC*mec* elements were further classified by J-region typing specific to SCC*mec* type IV (Milheiriço et al., 2007). In this multiplex PCR six primer pair sets were used, each targeting one or two SCC*mec* IV subtypes (IVa-IVh); the relevant sub-type was identified from the size of the PCR product. Again PM25 and PM64 were included as SCC*mec* IVh positive control and SCC*mec* type II negative control

respectively. All clinical isolates displayed bands of approximately 650 bp indicating SCC*mec* IVh, which was verified with PM25 (Figure 3-5B).

Another variable locus in the *S. aureus* genome is the global virulence regulator gene *agr* of which there are four types (I to IV). The isolates *agr* types were investigated by running four individual PCR specific to each *agr* type (Campbell et al., 2008). PM25 (*agr* type I) and PM64 (*agr* type III) were included as typing controls. Positive bands for the clinical isolates were observed in the *agr* type I PCR, as was the case for PM25, indicating *agr* type I (Figure 3-5C). PM64 displayed a positive PCR product for the *agr* type III PCR, as expected (data not shown).

3.3.2 Multi-locus sequence typing (MLST) and *spa* typing

ST and CC were determined by Multi Locus Sequence Typing (MLST) (Enright et al., 2000). This method involved sequencing seven housekeeping genes (*arc*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL)* and the resulting allele combination matched to a ST (CC). All isolates from the PB1 and PB2 infections plus RB4 were used for MLST analysis. The trimmed DNA trace files were used to query the *S. aureus* MLST database (http://saureus.mlst.net/) and designated a ST (CC). All isolates analysed were ST22 (CC22); the same clonal complex as EMRSA-15 (PM25). Isolates from the PB3, PB5, RB1 and RB5 infections were not used for MLST analysis as it was decided all clinical samples would undergo whole genome sequencing; therefore determining the ST without the need for independent MLST analysis (Chapter 5:4).

Due to the lack of differentiation between isolates in the analysis so far, molecular typing was investigated further via *spa* typing (Shopsin et al., 1999; Harmsen et al., 2003). There are multiple *spa* types for each CC, therefore it was assumed this method could be used to differentiate between different infecting strains. Trimmed DNA trace files were used to query *spa* type reference sequences associated with ST22 strains and the specific *spa* type was determined via the Ridom *Spa* Server. Results showed all PB1 and PB2 isolates were *t032*, which is the same *spa* type as EMRSA-15. Isolates from the PB3, PB5, RB1, RB4 and RB5 infections were not used for *spa* analysis due to imminent genome sequencing data.

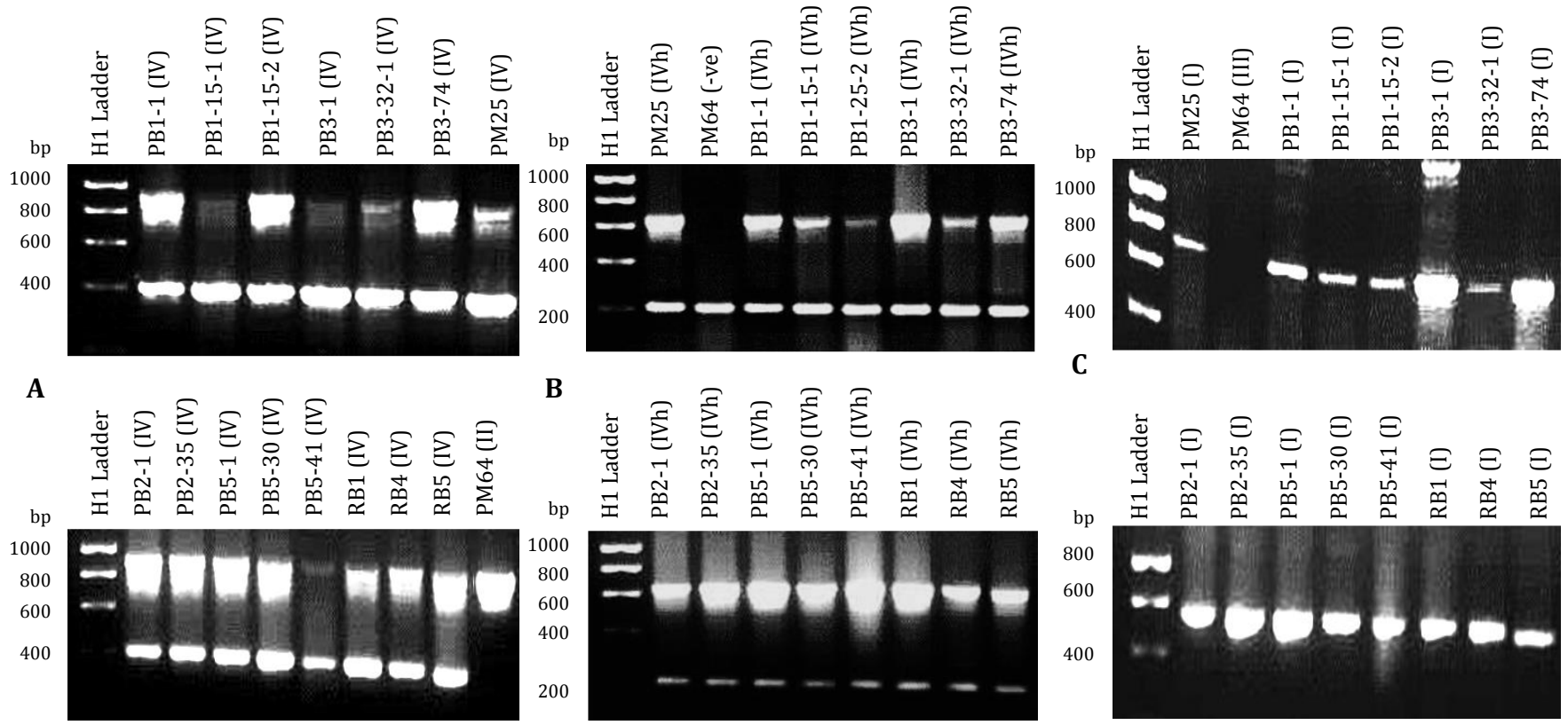

Figure 3-5 SCC*mec*, J-region and *agr* typing agarose gels. SCC*mec* typing was conducted as described (A), SCC*mec* type IV profiles are indicated by two bands ~ 400 bp and ~ 950 bp; a single band ~ 950 bp indicates SCC*mec* type II. SCC*mec* IV J-region subtyping was conducted (B) where ~ 650 bp band relates to SCC*mec* IVh; a band ~ 200 bp relates to the *ccrB2* internal control confirming successful PCR. Agr typing involved four individual PCR targeting each *agr* type; *agr* type I primers were used (C) and a 550 bp band confirms *agr* type I. PM25 (SCC*mec* type IVh, *agr* I) and PM64 (SCC*mec* type II, *agr* III) were included as controls.

Overall, the combination of SCC*mec*, *agr*, MLST and *spa* typing methodologies suggests all clinical isolates to be descendants from EMRSA-15. But these results based upon core genome typing do not help to differentiate between isolates from different infections; consequently, further typing methods were employed.

3.3.3 Antibiogram profiles and pulse field gel electrophoresis (PFGE)

Two types of MASTRING-S™ systemic Gram-positive antibiotic rings were used (Chapter 2:6.2) to determine the isolates' antibiogram profiles (Table 3-2). All isolates originating from the same infection showed identical antibiogram profiles, but strains isolated from different infections demonstrated variable antibiotic profiles. For example, PB5 isolates displayed a high level of erythromycin tolerance, and intermediate tolerance to trimethroprim, sulphamethoxazole, chloramphenicol and streptomycin were exhibited by PB1 and PB2 isolates and in some instances the resolved isolates. PB3 is the only collection of isolates to display low levels of tolerance to all antibiotics except β-lactam antibiotics, which was a trait shared by all isolates. Overall, this data indicates accessory genome differences between isolates from different infections. Moreover, there are no observable differences between isolates within individual infections supporting the assumption that all isolates collected from individual infections originated from the same infecting strain.

To further confirm all isolates collected from each persistent bacteraemia originated from the same infecting strain pulse field gel electrophoresis (PFGE; Chapter 2:4) was used (Mulvey et al., 2001). PFGE banding patterns are identical for isolates within the same infection but isolates from separate infections show slightly different patterns as indicated by asterisks in Figure 3-6. These results, in combination with the antibiogram profiles, provides evidence to suggest all isolates originating from one independent infection evolved from the same infecting isolate. Subsequently each infection involved true persistence of one infecting isolate; not multiple cases of different, infecting strains.

Table 3-2 Antibiogram profiles. MASTRING-S™ antibiotic rings were used to record the isolates tolerances to a series of antibiotics. The inhibition radiuses were converted to an arbitrary scale of H for high (> 4 mm), I for intermediate (2 – 4 mm) and L for low (< 2 mm). Experiments were done in duplicate to confirm reproducibility.

	Penicillin G (1 unit)	Clindamycin (2µg)	Gentamicin (10µg)	Fusidic acid (10µg)	Erythromycin (5µg)	Trimethroprim (1.25µg)	Sulphamethoxazole (25µg)	Chloramphenicol (25µg)	Oxacillin (10µg)	Novobiocin (5µg)	Streptomycin (10µg)	Tetracycline (25µg)
PB1-1	H	L	L	L	L	I	I	I	H	L	I	L
PB1-15-1	H	L	L	L	L	I	I	I	H	L	I	L
PB1-15-2	H	L	L	L	L	I	I	I	H	L	I	L
PB2-1	H	L	L	L	L	I	I	I	H	L	I	L
PB2-35	H	L	L	L	L	I	I	I	H	L	I	L
PB3-1	H	L	L	L	L	L	L	L	H	L	L	L
PB3-32	H	L	L	L	L	L	L	L	H	L	L	L
PB3-74	H	L	L	L	L	L	L	L	H	L	L	L
PB5-1	H	L	L	L	H	L	I	L	H	L	L	L
PB5-30	H	L	L	L	H	L	I	L	H	L	L	L
PB5-41	H	L	L	L	H	L	I	L	H	L	L	L
RB1	H	L	I	L	L	L	I	L	H	L	I	L
RB4	H	L	L	L	L	H	I	L	H	L	L	L
RB5	H	L	L	I	L	L	L	L	H	L	I	L

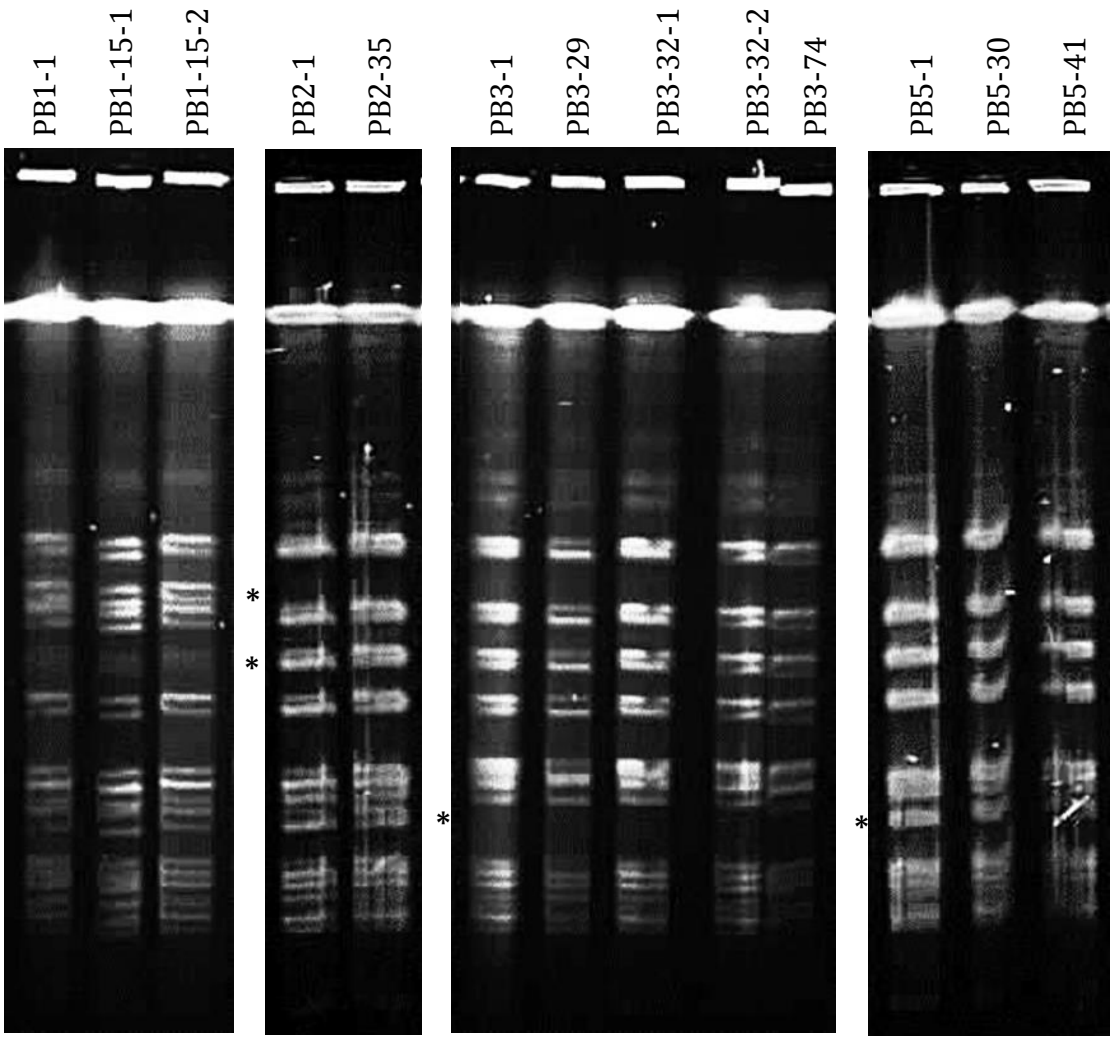

Figure 3-6 PFGE banding pattern for PB isolates. Chromosomal DNA was prepared for isolates originating from the persistent bacteraemia and subject to PFGE. DNA banding patterns were identical between isolates within individual persistent infections. DNA banding patterns of isolates from different infections show several differences indicated by *. Restriction digestions and PFGE gels were run twice to confirm reproducibility.

3.4 Antibiotic sensitivities to treatment antibiotics

The aim of this section was to investigate whether any of the persistent, infecting isolates developed tolerances to the treatment antibiotics. The *S. aureus* isolates' antibiotic sensitivities were assessed using two CLSI recommended methods (Table 3-3), M.I.C. Evaluator™ (MICE) test strips (Oxoid) and micro-broth dilution (MBD) (Chapter 2:6). To classify whether or not a particular MIC is resistant or sensitive, MIC breakpoints supplied by BSAC (British Society of Antibiotic Chemotherapy) in combination with EUCAST (European Committee on Antibiotic Susceptibility Testing) were used.

Oxacillin was used to assess the isolates' MIC in regards to methicillin as the former is currently used in hospital treatment regimes. As expected all isolates displayed a resistant MIC of > 2 μg/ml, in agreement with SCC*mec* typing results (Chapter 3.3.1). Traditionally, EMRSA-15 isolates exhibit a relatively low level of oxacillin resistance as shown by PM25 (32 μg/ml), in contrast to a high level exhibited by EMRSA-16 isolates such as PM64 (> 128 μg/ml). All clinical isolates in this study exhibited high MIC more indicative of EMRSA-16, suggesting an alteration in *mecA* expression has occurred during the EMRSA-15 lineage evolution.

All clinical isolates showed susceptible MBD determined vancomycin MIC (0.5 - 2 μg/ml). However the MICE method designated PB1 and PB2 isolates as vancomycin resistant (3 - 4 μg/ml). This data may suggest an intermediate susceptibility to vancomycin in these isolates.

All clinical isolates displayed a susceptible linezolid MIC and the majority of isolates showed daptomycin susceptibility. Interestingly, persistent isolates from the PB1 and PB3 infections (PB1-15-1/2 and PB3-32-1) exhibited resistant daptomycin MIC (2 - 4 μg/ml); whereas their corresponding initial isolates (PB1-1 and PB3-1) showed susceptibility (1 μg/ml). These data may suggest daptomycin tolerance was developed during the course of both the PB1 and PB3 infections.

Table 3-3 Antibiotic sensitivities of clinical isolates against treatment antibiotics. The antibiotic sensitivities of the clinical isolates, displayed as MIC in μg/ml, are shown for the four main antibiotics prescribed. Where available data relating to two different methods are shown, MICE and MBD. MIC which are classified as resistant are in **bold**. Experiments were independently repeated three times to confirm reproducibility. A $\log_2$ dilution factor is displayed where appropriate.

	Oxacillin		**Vancomycin**		**Linezolid**	**Daptomycin**
	MICE	**MBD**	**MICE**	**MBD**	**MICE**	**MICE**
PB1-1	**128**	**64-128**	**3-4**	0.5-2	2	1
PB1-15-1	**128**	**64-128**	**4**	1-2	2	**2-4**
PB1-15-2	**128**	**64-128**	**4**	1-2	2	**2-4**
PB2-1	**> 256**	**64-128**	**3-4**	0.5-2	2	0.5
PB2-35	**> 256**	**64-128**	**3-4**	0.5-2	2	0.5
PB3-1	**64**	**64**	1-2	0.5-1	1-2	1
PB3-32-1	**128**	**64**	2	1-2	1-2	**2**
PB3-74	**64**	**64**	2	0.5-2	1-2	1
PB5-1	**> 256**	**> 128**	2	2	1-2	1
PB5-30	**> 256**	**> 128**	2	2	1-2	1
PB5-41	**> 256**	**> 128**	2	2	1	1
RB1	**64**	**32-64**	2	1-2	2	0.5
RB4	**> 256**	**64-128**	2	1-2	2-4	0.5
RB5	**64-128**	**32-64**	2	1-2	1-2	0.5
PM25 (EMRSA-15)	**32**	**32**	0.5-1	0.25-0.5	1-2	0.5
PM64 (EMRSA-16)	**> 256**	**> 128**	0.5-1	0.5-1	1	0.25-0.5

3.5 Growth characteristics of clinical isolates

3.5.1 None of the clinical isolates display a small colony variant (SCV) phenotype

SCV are proposed to play an important role in the development of *S. aureus* persistence (von Eiff et al., 2006; Proctor et al., 2006; Gao et al., 2010; Proctor et al., 1995). As a result the appearance of the clinical isolates on solid phase media was scrutinised for the presence of SCV (Figure 3-7). There was no indication of SCV on horse blood agar or any other media tested for any of the clinical isolates. The colony morphology and colour of all the clinical isolates were typical for wild type *S. aureus*. Additionally, there were no observable differences between isolates taken at different time points within an infection or between isolates from different infections. These results confirm all isolates, including initial, persistent and resolved isolates, do not exhibit SCV phenotypes.

3.5.2 PB1 & PB3 persistent isolates exhibit improved growth after 24 hours in nutrient deprived, metal ion restricted media (CRPMI)

To fully assess the possible involvement of dormancy in these infections the clinical isolates' growth in liquid phase media was investigated. Two different media were used; a nutrient replete media (TSB) and nutrient restricted tissue culture medium, RPMI-1640, which had been chelexed to remove the metal ions (CRPMI). Overnight cultures were diluted into fresh media, incubated and optical density (OD_{600nm}) readings were taken every hour for 7 hours and 24 hours post inoculation.

In nutrient replete (TSB) medium there were no obvious differences between any of the isolates within the same infection from different time-points or between isolates from resolved and persistent infections, when comparing the 0 - 7 hours growth (data not shown) or at the 24 hour time-point (Figure 3-8A). In CRPMI, no substantial differences were seen in the first 7 hours of growth between isolates within infections or between different infecting strains (data not shown).

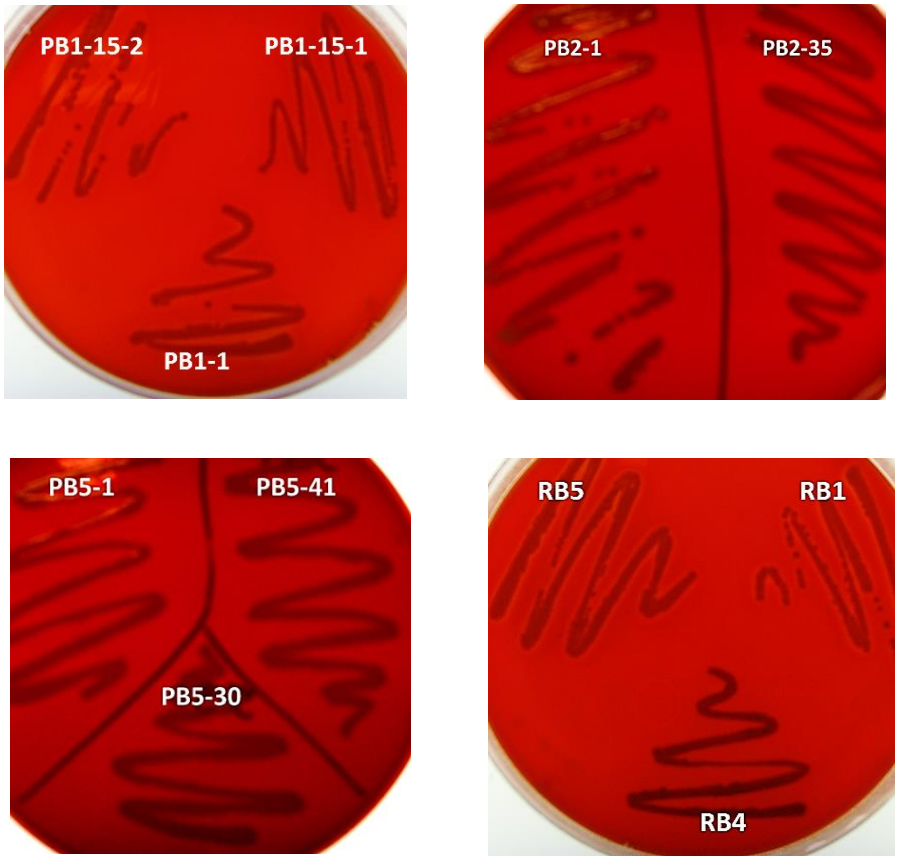

Figure 3-7 Colony morphology of clinical isolates on horse blood agar. There was no indication of SCV in any of the isolates. Additionally, the colony morphology had not changed during the course of any infection and no differences were observed between isolates from persistent or resolved infections.

At the 24 hour time-point the PB1 and PB3 persistent isolates (PB1-15-1/2 and PB3-32-1) showed significantly higher growth values compared to their respective initial isolates (PB1-1 and PB3-1) (Figure 3-8B $P < 0.05$ and $P < 0.01$ respectively). Additionally, the PB1 persistent isolates (PB1-15-1/2) were significant higher compared to the resolved isolates (RB1, RB4 and RB5) ($P < 0.05$). Interestingly, PB3-74 exhibited similar growth characteristics compared to the PB3 initial isolate (PB3-1) as it also had a significantly lower growth value at 24 hours than PB3-32-1 ($P < 0.01$). This may indicate PB3-74 isn't a true persistent isolate and it was in-fact re-emergence of the initial isolate (PB3-1) from an infective focus.

Overall, there was no indication of dormancy in any of the isolates tested in TSB or CRPMI ruling out the possible involvement of SCV in these persistent infections. In fact, data indicates the PB1 and PB3 persistent isolates exhibit a nutrient deprived specific growth advantage, suggesting the PB1 and PB3 infecting strains underwent a growth adaption to *in vivo* conditions.

3.5.3 PB1 & PB3 growth advantage is neutral pH specific

Shortly after the growth analysis was conducted a change in the isolates growth profiles was observed. The pH of the CRPMI media was found to have a slightly lower pH than usual (from pH 8.8 to pH 7.0). Therefore the effect of slight alterations in pH on the isolates' growth behaviours was further investigated.

Experiments were conducted as before using CRPMI pH 7.0 but only 24 hour readings were recorded as no differences were observed in the exponential (0 - 7 hours) growth phase previously. Additionally, only isolates from the PB1 and PB3 infections (plus the resolved isolates for comparison purposes) were used for further analysis as these were the only isolates to respond to a change in pH (Figure 3-8).

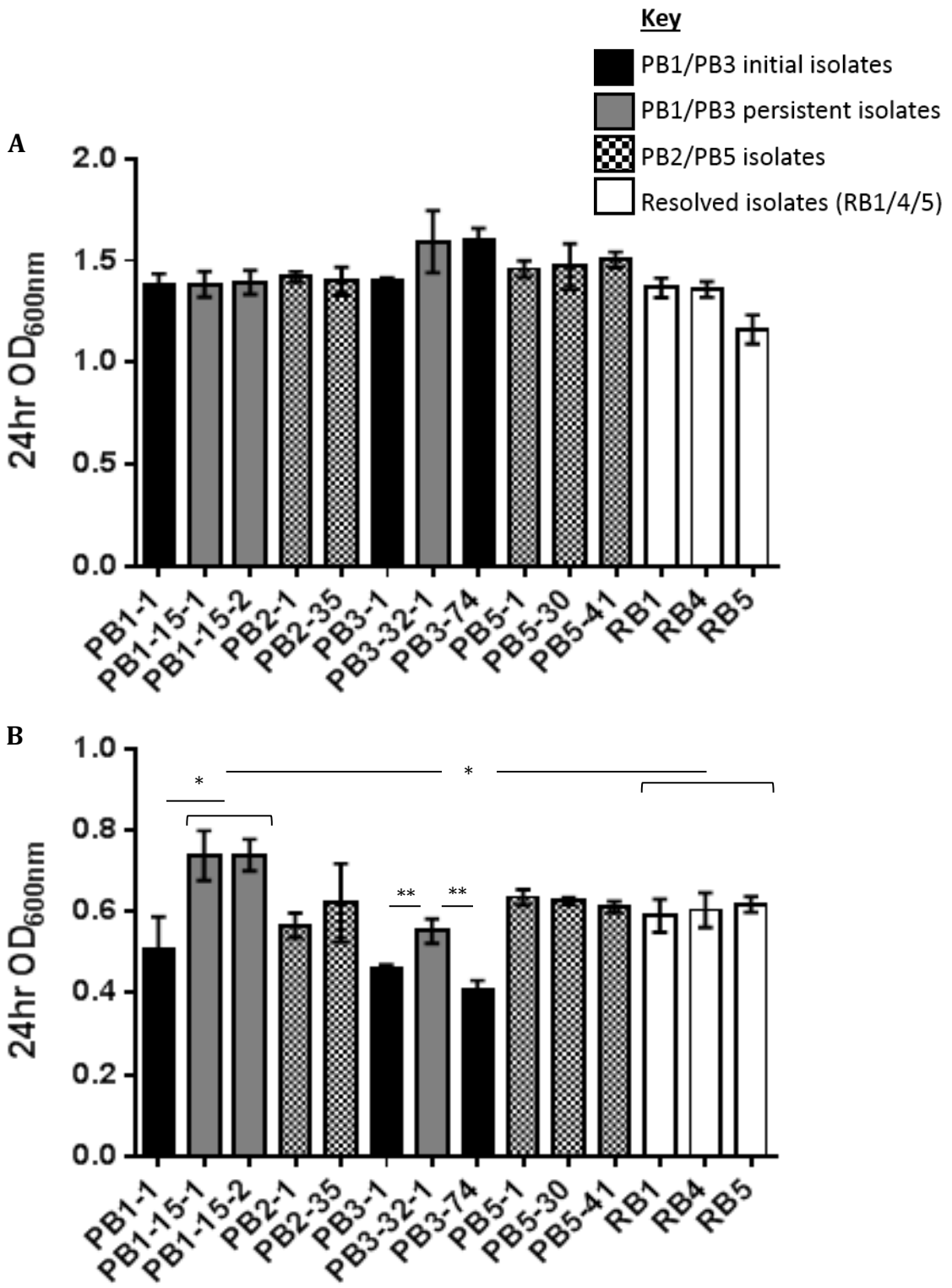

Figure 3-8 24 hour growth analysis in TSB and CRPMI. The mean 24 hour OD_{600nm} from three independent experiments +/- one standard deviation were plotted for TSB (A) and CRPMI (B). Statistical analysis was conducted using the student T-test within the GraphPad Prism 6 software and significant differences are indicated by asterisks (*).

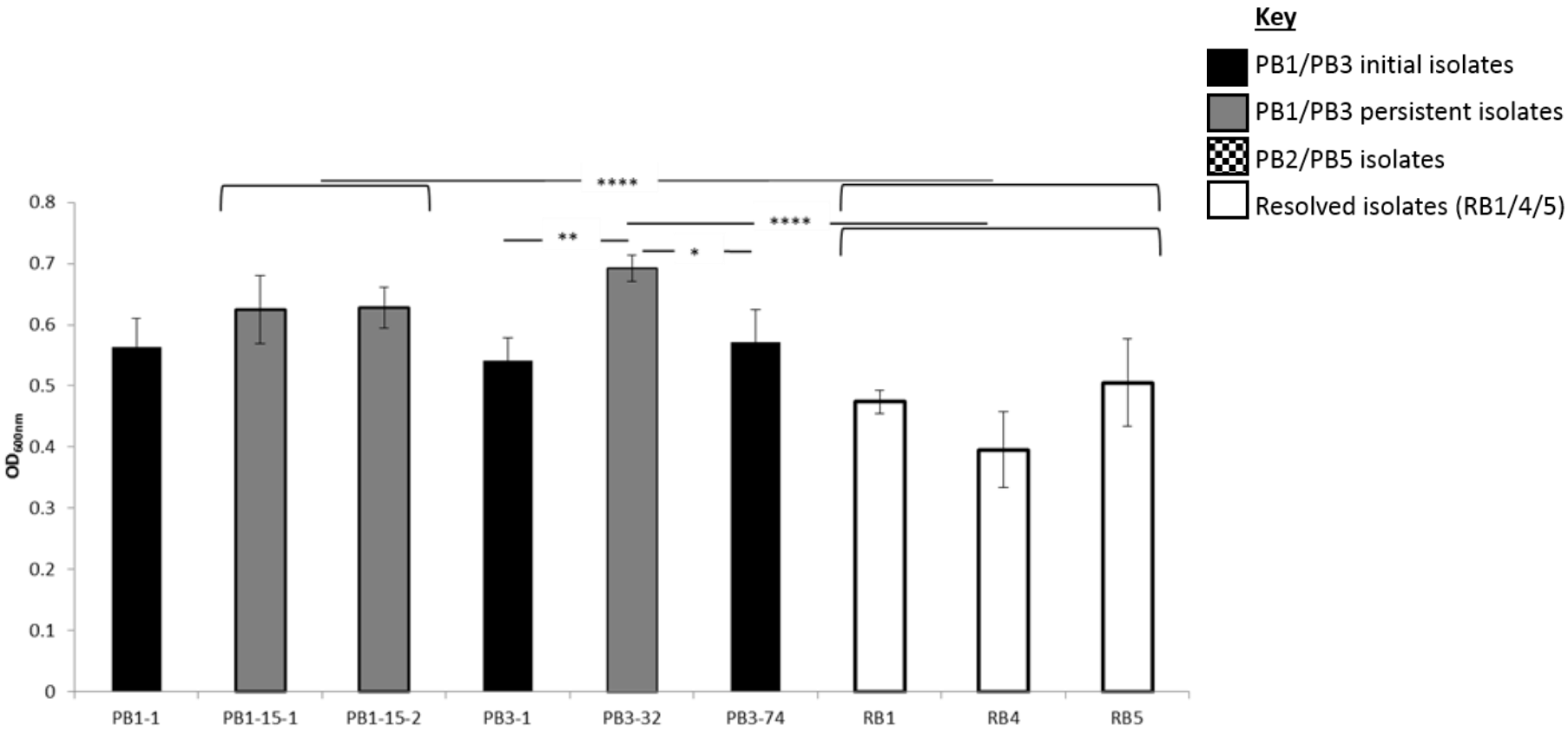

Figure 3-8 CRPMI pH 7.0 24 hour growth analysis. The mean 24 hour OD_{600nm} from three independent experiments after incubation in CRPMI pH 7.0 were plotted +/- one standard deviation. Student T-tests were used for statistical analysis and significance is indicated by asterisks (*).

Numerous differences were observed between the PB1 and PB3 clinical isolates after 24 hours incubation in CRPMI pH 7.0. Specifically, persistent isolates in both the PB1 and PB3 infections (PB1-15-1/2 and PB3-32-1) exhibited higher 24 hour growth values in a pH 7.0 environment compared to their initial isolates (PB1-1 and PB3-1), but the difference was only significant between the PB3 isolates' ($P < 0.01$). Additionally, the PB1 and PB3 persistent isolates exhibited significantly greater 24 hour growth values in comparison to the resolved isolates average ($P < 0.0001$) and to a lesser extent, so did the PB1 and PB3 initial isolates ($P < 0.05$).

Overall, this data not only indicates that the PB1 and PB3 infecting strains have developed a growth advantage in nutrient and metal ion restricted environments; but also that this growth advantage is accentuated at near neutral pH's.

3.6 Exoprotein activities

3.6.1 Possible *agr* dysfunction in RB4 but not in PB isolates

Previous studies found a possible association between *agr* dysfunction and persistent isolates (Park et al., 2012; Fowler et al., 2004); therefore this potential persistence trait was investigated by assessing the activity of δ-haemolysin, an indicator of *agr* activity (Figure 3-9), protocol details can be found in Chapter 2:8.3. All isolates from the PB1, PB2, PB3 and PB5 infections and RB1 and RB5 showed additional red blood cell clearing where there was an overlap of haemolytic activity with RN4220. These data indicate isolates have operational *agr* systems, which is in contrast to the previously suggested association of *agr* dysfunction with persistent isolates. RB4 is the only isolate which may exhibit *agr* dysfunction due to the absence of additional haemolytic activity

3.6.2 No association between DNase, α- or β-haemolysis with persistent isolates

Investigation of δ-haemolysin activity indicated that the clinical isolates may exhibit different levels of haemolytic activity therefore the activities of two additional haemolysins (α-, β-) and one exoprotein (DNase) were investigated (Chapter 2:8).

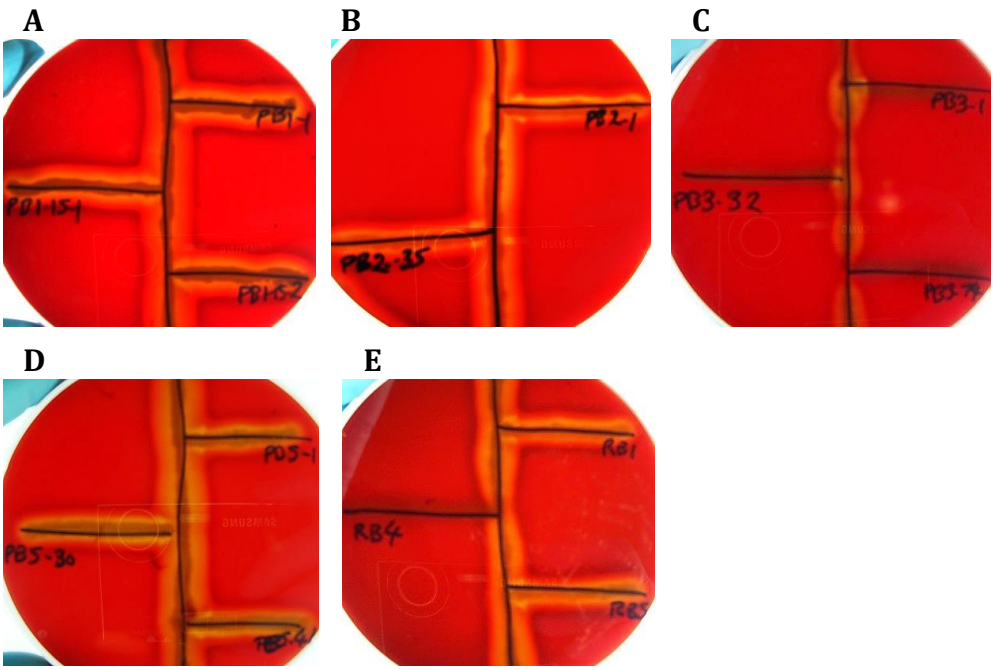

Figure 3-9 δ-haemolytic activity on sheep blood agar plates. RN4220, a δ-lysin deficient strain was used to assess the clinical isolates delta-lysin activity. Experiments were completed in duplicate to confirm reproducibility. RB4 was the only isolate to not exhibit additional haemolytic activity when in close proximity to the RN4220 strain suggesting *agr* dysfunction.

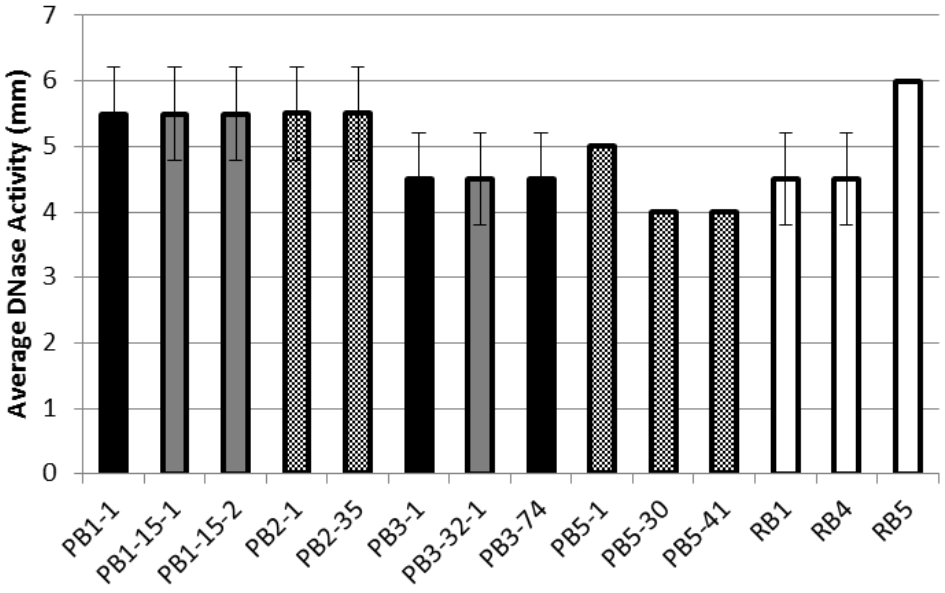

Figure 3-10 DNase activity. DNase activity is given by the size of halo (mm) around the test isolates indicating hydrolysed DNA. Means were calculated and plotted from three independent experiments +/- one standard deviation. No significant differences were seen between any of the persistent or resolved isolates.

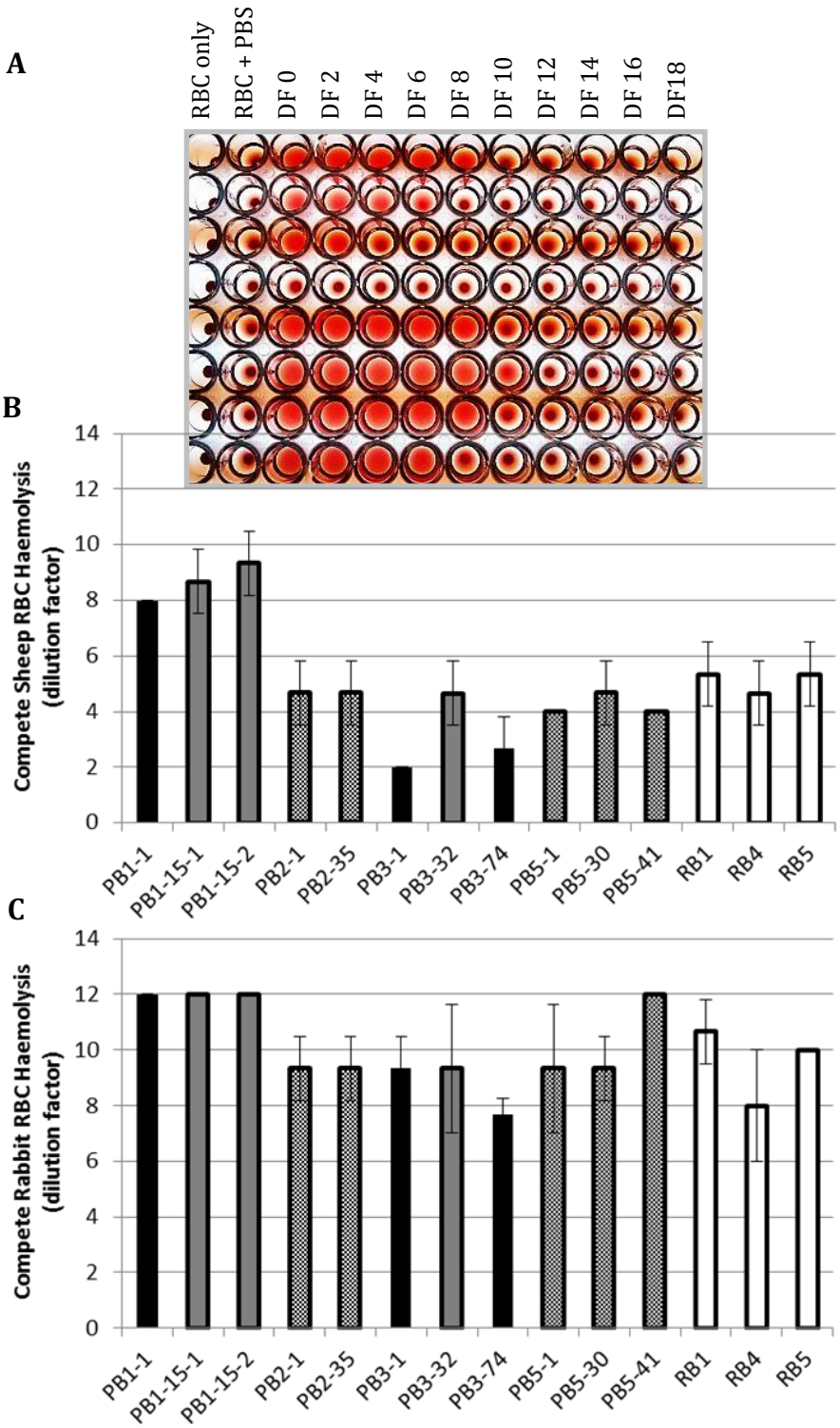

Figure 3-11 α-and β-haemolysin activities. Haemolytic activities are given as the final dilution (DF- dilution factor) of concentrated bacterial supernatant where complete haemolysis still occurred, an example of a test plate can be seen in A. Sheep (B) and rabbit (C) red blood cells were both used as they are substrates for different haemolysins. Dilution factor means were calculated and plotted from three independent experiments +/- one standard deviation.

No differences in DNase activity were seen between isolates within the PB1, PB2 or PB3 infections, but there was a slight decrease from PB5-1 to PB5-30/PB5-41 (Figure 3-10). There were varied results for the resolved isolates where RB1 and RB4 showed slightly reduced activity, whereas RB5 exhibited the highest DNase activity. Overall though, no association of DNase activity can be observed between persistent and resolved isolates.

The activities of two additional haemolysins (α-, β-) were also investigated. It has been demonstrated that the use of different types of red blood cells can distinguish between the activities of individual haemolysins (Dinges et al., 2000). Specifically, α-haemolysin is active against rabbit red blood cells whilst β-haemolysin is active against sheep. No differences were seen between isolates in the PB1, PB2 and PB5 infections in regards to α- or β-haemolysin activity; but the persistent PB3-32-1 isolate displays increased β-haemolysin activity compared to the initial isolate PB3-1 and to a lesser extent, PB3-74 (Figure 3-11). Overall, there is no association between the expression of exoproteins tested here and the PB isolates characterised in this study.

3.7 Protein composition on the bacterial cell surface

The aim of this part of the project was to investigate potential proteomic differences in the clinical isolates' bacterial cell surfaces. The bacterial cell surface is particularly important to investigate as it comprises the interactive interface between the infecting cell and its environment, specifically host factors; and is therefore is under immense selective pressures *in vivo*. Protein fractions were prepared from the clinical isolates after incubation in CRPMI. CRPMI is more indicative of the host environment and therefore would give a more realistic impression of isolates' behaviours *in vivo*.

3.7.1 Multiple differences were observed between PB1 and PB3 initial and persistent isolates within the non-covalently bound cell surface protein fraction

No obvious differences were found in the secreted or the cell wall protein fraction between isolates within persistent infections (Figure 3-12). Additionally, there are

no common differences between the three resolved isolates and isolates from persistent infections in these two fractions; suggesting no association with persistence in this study. Interestingly, there are multiple differences observed in the non-covalently bound, cell surface protein fraction (Figure 3-13). The most prominent change being an increase in a 70 kDa protein band in the PB1 persistent isolates, which is also visualised to a lesser extent, in PB3-32-1. Additionally, PB1 and PB3 persistent isolates showed similar or increased levels of this protein compared to the resolved isolates. In contrast, the same protein is shown to decrease between PB2-1 and PB2-35 and be expressed at relatively low levels in PB5 isolates. This band was identified by mass spectrometry (mass spec) as Extracellular Adhesion Protein (Eap) otherwise known as Map1, a known immune evasion protein (Lee et al., 2002).

Analysis of the other differentially expressed proteins bands by MS was unfortunately unsuccessful due to insufficient amounts of protein within individual bands or because the band consisted of multiple proteins.

One general feature of the cell surface associated protein fraction was that PB1, PB2 and PB3 persistent isolates showed an overall decrease in the number and intensity of background protein bands. This may suggest a global decrease of cell surface associated proteins could be associated with persistence.

3.7.2 CRPMI pH 7.0 specific increase of cell surface associated Eap in PB1 and PB3 persistent isolates

Following the discovery of a pH dependent growth phenotype (Chapter 3.5.3), the potential effect of a pH change on the cell surface protein fraction was investigated. The same method was used but the isolates were incubated in CRPMI pH 7.0. PB1 and PB3 isolates were chosen for further investigation as they were affected by the pH change during growth analysis; resolved isolates were also included for comparison purposes.

The previously observed increase in Eap (~ 70 kDa) from initial to persistent isolate in the PB1 and PB3 infections was more pronounced in CRPMI pH 7.0. Additionally, a number of other bands displayed changes in intensity between isolates within individual infections suggesting these protein changes are neutral pH specific and

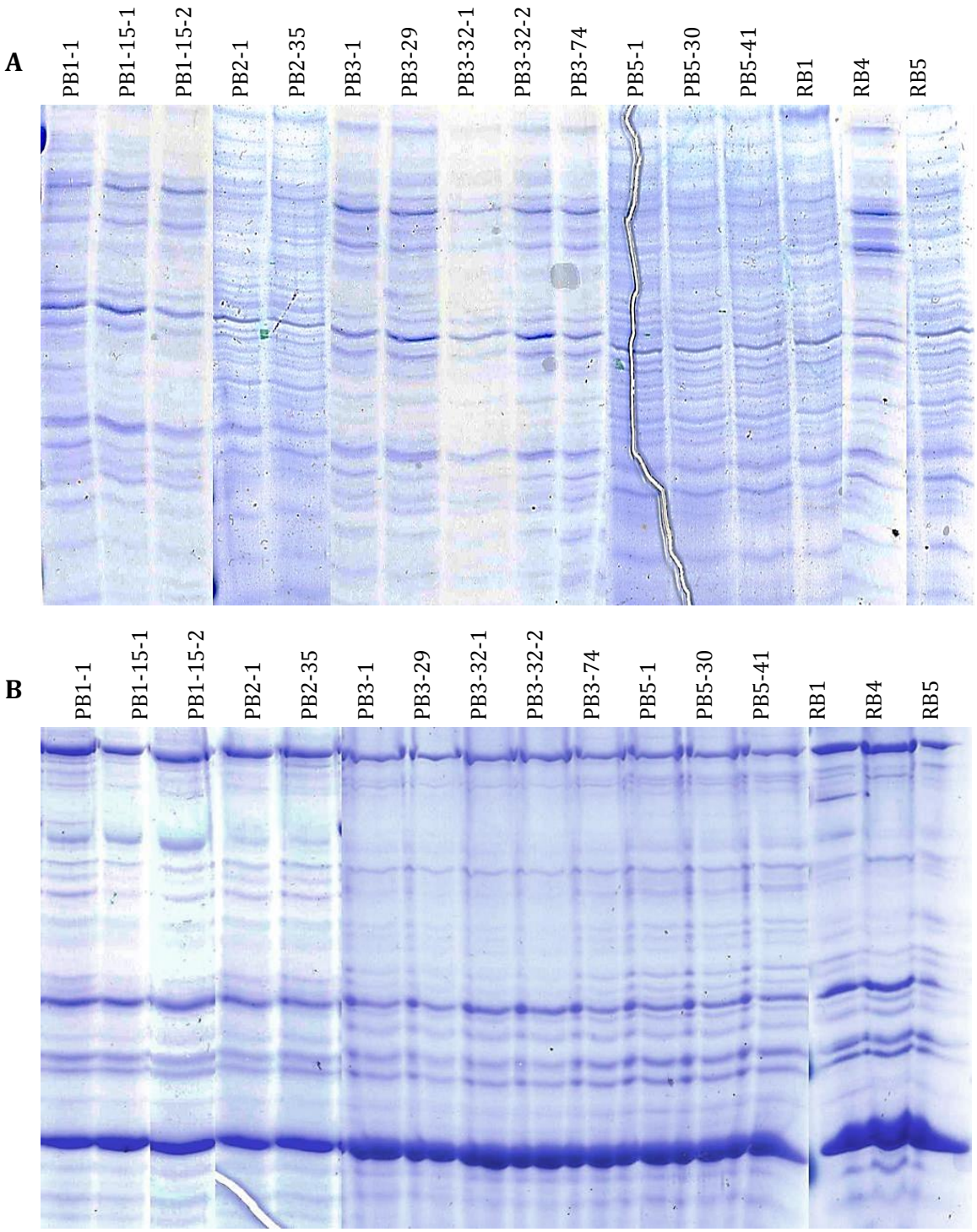

Figure 3-12 Secreted and cell wall protein fractions displayed on 1D SDS-PAGE. The secreted (A) and cell wall (B) bound protein fractions displayed on 1D SDS-PAGE after 16 hours incubation in CRPMI pH 8.8 at 37 °C in 5 % CO_2. Experiments were repeated independently three times to confirm reproducibility

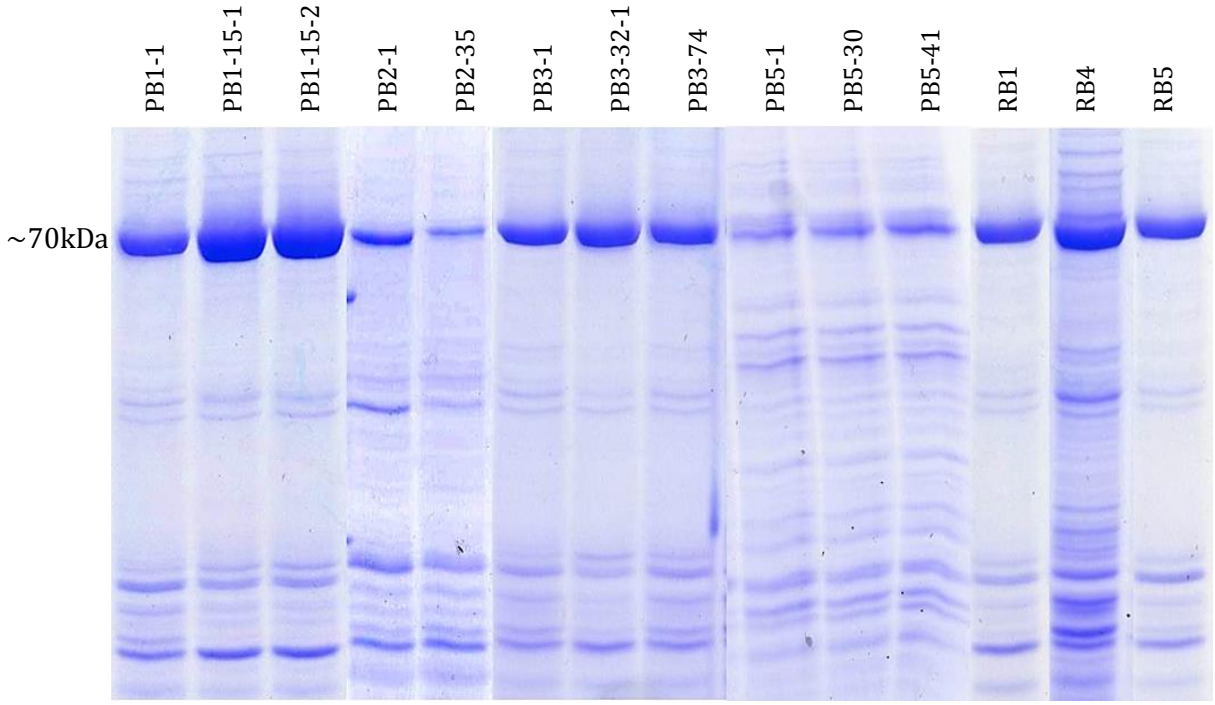

Figure 3-13 Non-covalently bound cell surface associated protein fraction displayed on 1D SDS-PAGE. The cell surface associated protein fraction displayed on 1D SDS-PAGE after 16 hours incubation in CRPMI pH 8.8 at 37 °C in 5 % CO_2. Experiments were repeated independently three times to confirm reproducibility. Individual bands were analysed by mass spectrometry to identify the particular proteins.

could show an association with persistence (Figure 3-14). Due to the large number of protein bands which exhibited different intensities an alternative high sensitivity method was employed, namely iTRAQ (isobaric tag for relative and absolute quantification) in combination with LC-MS (liquid chromatography-mass spectrometry) conducted by PNACL at the UOL.

3.7.3 Further investigation of cell surface proteins by iTRAQ LC-MS

Cell surface associated protein fractions were prepared as before from cultures grown in CRPMI pH 7.0 and normalised according to pellet weight. The concentrations of the protein samples were then assessed by nano-drop and normalised again ensuring the submission of equal protein masses for all of the isolates included in this analysis. Samples were then submitted to PNACL (UOL) where iTRAQ and LC-MS was conducted. This process firstly involves trypsin digestion of each protein sample resulting in a collection of proteolytic peptides. These peptides are uniquely labelled using the isobaric iTRAQ reagents consisting of N-methyl piperazine reporter groups with various masses (113, 114, 115, 116, 117, 118, 119, 121), a balance group and an N-hydroxy succinimide ester group; this group functions by reacting with the primary amines of the peptides effectively labelling them. All digested and labelled samples are then pooled into one sample mixture and subjected to LC-MS where individual peptides are identified by their mass-to-charge ratios and quantified relative to the reporter ion signals also included in the MS scan. The collection of peptides originating from individual samples can be distinguished by the different reporter groups used for iTRAQ labelling, thereby enabling direct quantitative comparisons between samples using Scaffold4 software. RB4 was analysed separately from the rest of the isolates (Chapter 5:3:3) as a maximum of eight samples can be labelled and analysed at any one time by this method. Individual protein levels for the persistent and resolved isolates were calibrated independently to the PB1 and PB3 initial isolates and proteins which showed > 0.5 $\log_2$ fold difference are listed in Table 3-4.

A total of 214 and 178 proteins were identified in the cell surface extract during the first and second biological repeats respectively demonstrating the high resolution of this method. Proteins which exhibited > 0.5 $\log_2$ difference between initial, persistent and/or resolved isolates included known adhesion and immune evasion

proteins, stress response proteins, metabolism and cell maintenance proteins, general virulence and antibiotic resistance proteins.

Proteins which differed from initial to persistent isolate in both the PB1 and PB3 infections included extracellular adhesion protein (Eap), penicillin binding protein 2a (PBP2a), acyl carrier protein, putative DNA directed RNA polymerase subunit E (RpoE) and thioredoxin (Trx). Enolase (Eno) only showed a substantial increase within the PB1 infection; whereas extracellular matrix binding protein (Emp), bi-functional autolysin (Atl), immunoglobulin binding protein (Sbi) and cold shock protein (CspA) only showed increases within the PB3 infection. A number of proteins showed a decrease in the resolved isolates compared to the PB1 initial isolate including Eap, Emp, Atl, Sbi, CspA, PBPa and acyl carrier protein. Eno was the only protein to show a significant decrease in resolved isolates compared to the PB3 initial isolate (PB3-1) compared to the resolved isolates. Again PB3-74 generally displayed values mirroring the PB3 initial isolate (PB3-1) providing more evidence to suggest PB3-74 emerged from a source of the initial isolate.

Overall, this data suggests the PB1 and PB3 infecting strains evolved in parallel during both infections leading to similar changes in their cell surface protein profile; additionally these attributes were not observed in resolved isolates, indicating an association with persistence development.

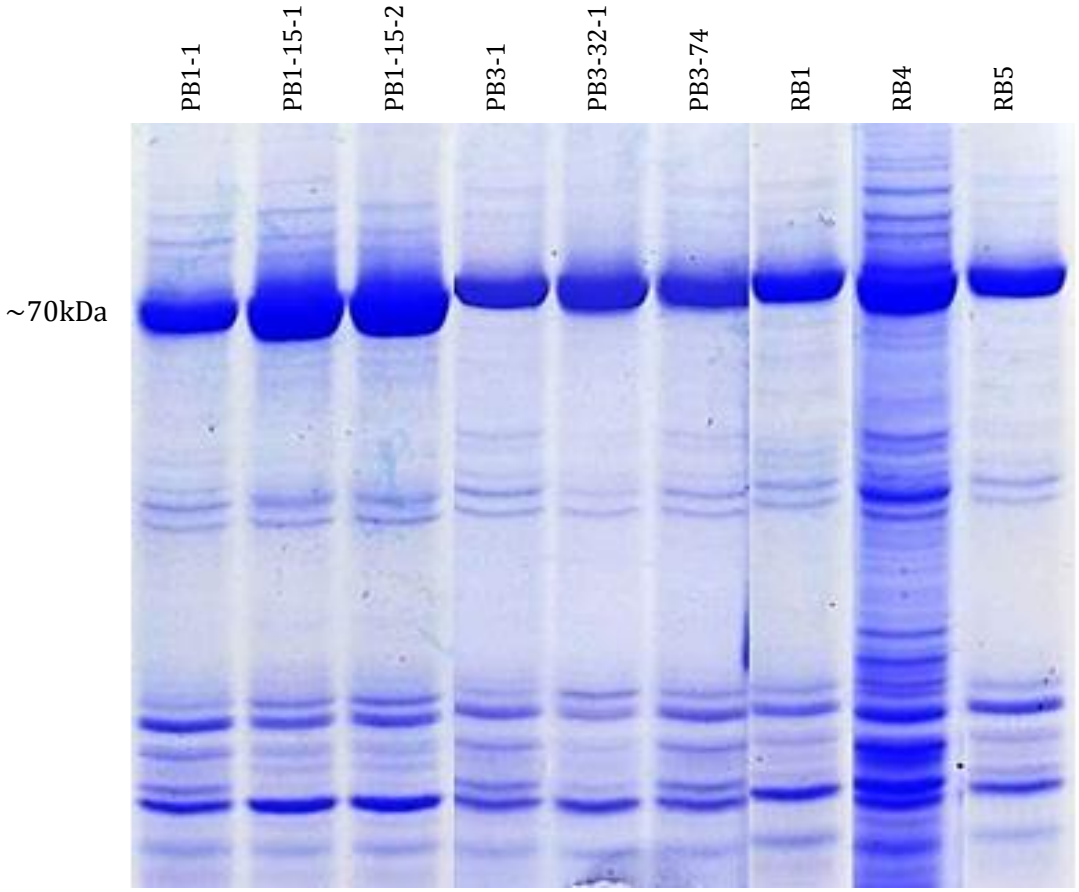

Figure 3-14 Non-covalently bound cell surface associated protein fraction prepared from CRPMI pH 7.0. The cell surface associated protein fraction displayed on 1D SDS-PAGE after 16 hours incubation in CRPMI pH 7.0 at 37 °C in 5 % CO_2. Experiments were repeated independently three times to confirm reproducibility.

Table 3-4 Relative quantities of cell surface associated proteins as determined by iTRAQ LC-MS. The proteins which displayed > 0.5 log_2 fold difference between initial, persistent and/or resolved isolates are listed below, including the related accession numbers and putative protein function. The relative protein amounts are averages of two biological repeats (each biological repeat inclusive of two technical repeats) and are expressed as log_2 ratios relative to either PB1-1 or PB3-1 as indicated. Log_2 ratios are colour coded to indicate relative increases (red) and decreases (blue), with increasing intensity of colour to signify higher values.

			Protein conc relative to PB1-1 (log_2)					**Protein conc relative to PB3-1 (log_2)**				
Accession Number	**Protein Names**	**Protein Function**	**PB1-1**	PB1-15-1	PB1-15-2	RB1	RB5	**PB3-1**	PB3-32-1	PB3-74	RB1	RB5
D2N8U8; Q8NVR4; Q2YU86; Q5HEI2	Cluster of Map (Eap) proteins	Low-iron biofilms, adhesion to host proteins, disrupts T-cell responses	**0.0**	0.85	0.65	-0.75	-0.95	**0.0**	1.1	-0.2	0.05	-0.2
Q2FIK4; Q99VJ2; D2N5J8; D9RFH0	Cluster of extracellular matrix binding proteins (Emp)	Low iron biofilms, adhesion to host proteins	**0.0**	0.2	0.45	-0.6	-1.15	**0.0**	1.35	-0.05	-0.1	-0.7
D2N627; E5QQT8; Q6GI31	Bifunctional autolysin (Atl)	Agr regulated virulence determinant	**0.0**	0.45	0.35	-0.5	-1.15	**0.0**	0.55	0.2	0.3	-0.3

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			Protein conc relative to PB1-1 (log_2)					Protein conc relative to PB3-1 (log_2)				
Accession Number	**Protein Names**	**Protein Function**	**PB1-1**	PB1-15-1	PB1-15-2	RB1	RB5	**PB3-1**	PB3-32-1	PB3-74	RB1	RB5
Q2YVZ4; D2NA02	Cluster of immunoglobulin binding proteins (Sbi)	Immune evasion factor: binds IgG to prevent opsonisation	**0.0**	0.0	0.25	-0.6	-1.25	**0.0**	0.7	0.05	0.6	-0.2
Q2FH36	Cold shock protein (CspA)	Involved in the cold shock	**-0.05**	0.45	0.3	-0.75	-1.0	**0.0**	0.8	0.15	-0.3	-0.55
Q5HJW3	Penicillin-binding protein 2' (PBP2')	Involve in *β*-lactam antibiotic resistance	**0.0**	0.75	0.85	-0.55	-1.6	**-0.05**	1.05	0.05	-0.05	-1.1
A7WZT2; D2N5I4; D9RFF8	Enolase (Eno)	Phosphopyruvate hydratase, converts 2-PG to PEP	**0.05**	1.7	1.7	0.0	0.15	**-0.05**	0.05	-0.05	-0.7	-0.5
A7X1J8	Acyl carrier protein	Cell maintenance	**0.0**	0.5	0.3	-0.55	-0.8	**0.0**	0.8	-0.05	-0.05	-0.25
D8HDA2; D9RBT9; Q2YUM5	Probable DNA-directed RNA polymerase (RpoE)	Cell maintenance	**0.0**	3.15	2.7	0.85	1.1	**0.0**	0.95	0.35	0.1	0.35
Q2FHT6	Thioredoxin	Antioxidant protein	**-0.05**	2.15	1.15	-0.1	-0.1	**0.0**	1.05	0.2	-0.35	-0.3

3.8 Discussion

The aim of the chapter was to identify and characterise four independent cases of persistent *S. aureus* bacteraemia (PB1, PB2, PB3, PB5) in parallel with three separate incidences of resolved bacteraemia (RB1, RB4, RB5). Uncharacterised persistence phenotypes were observed in this chapter without the association of previously published *S. aureus* persistence characteristics, such as SCV, which suggests novel persistence mechanisms were employed during these infections. Additionally, such persistence associated characteristics were observed in isolates collected late during the persistent bacteraemia (persistent isolates) which suggests the infecting strains adapted during infection progression demonstrating *in vivo* strain evolution.

3.8.1 Experimental design and cohort collection

All isolates from both persistent and resolved infection types were shown to display the same genetic background (SCC*mec* IVh, *agr* type I) and were therefore deemed to have comparable genetic backgrounds. Selected isolates were found to be ST22 and t*032* suggesting the clinical isolates are descendants from EMRSA-15, the main circulating MRSA strain in the UK today (Ellington et al., 2010; Johnson et al., 2001; Rollason et al., 2008). This study analysed isolates from two types of infections (resolved and persistent) but also temporal variations within persistent infections, i.e. samples collected on day zero (initial isolate) compared to samples collected further on during infection progression (persistent isolates). The cohort collection was designed to enable three distinct comparisons, diagrammatically represented in Figure 3-15.

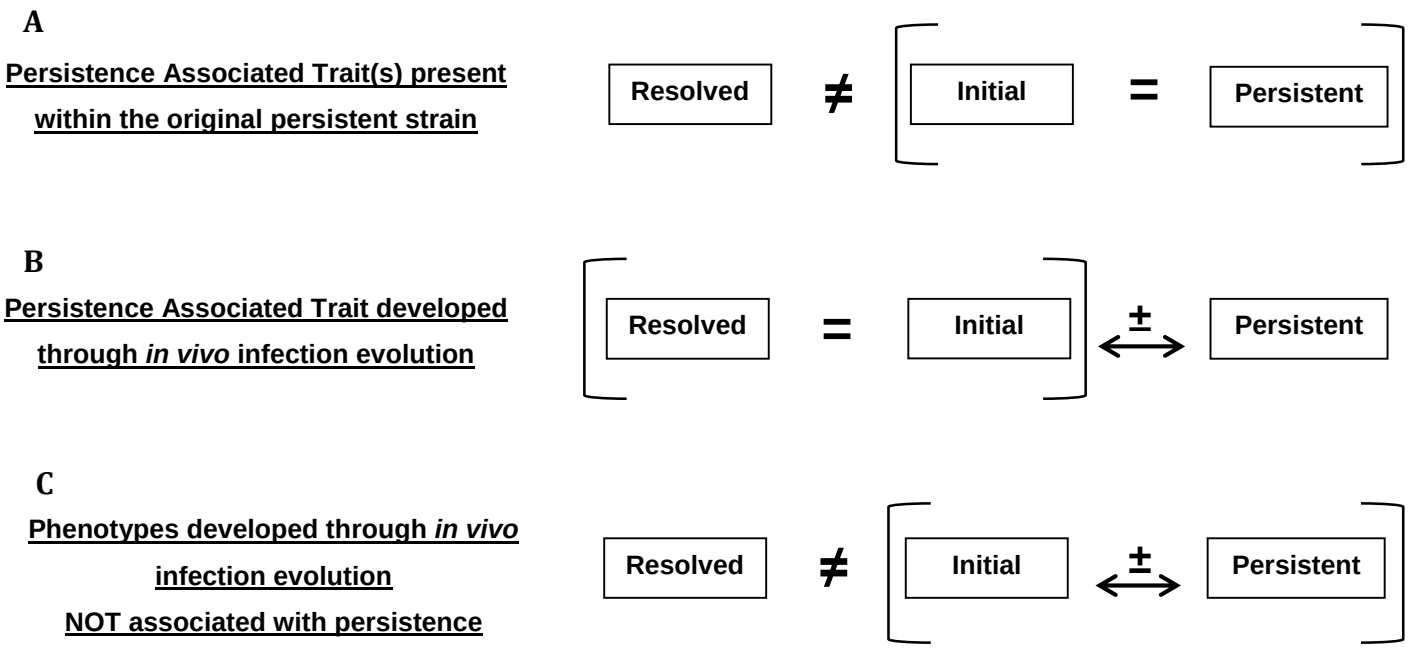


Figure 3-15 Diagrammatical representation of comparisons made with initial, persistent and resolved isolates and the subsequent conclusions. When the same phenotype is observed between the initial and persistent isolate but distinct from resolved isolates (A), then this is referred to as a pre-infection persistence associated trait. If the initial and resolved isolates display the same phenotype which is distinct from the persistent isolates (B), then this is a persistence associated trait developed through *in vivo* strain evolution. If there is a development from initial to persistent isolate but there is no related pattern to resolved isolates (C) then these phenotypes are a consequence of *in vivo* evolution but not associated to persistence. This highlights the importance of all three isolate types being included in persistence investigations.

Firstly, comparisons between the persistent and resolved strains should identify so called "predisposing" persistence traits; with the assumption that not all strains are equally capable at developing a persistent infection. Such traits could be used to predict persistence at initial infection diagnosis by the identification of high risk strains. A second comparison would involve differences between the initial and subsequent/persistent isolates within an infection, where the persistent isolates are phenotypically distinct from resolved isolates. Such traits would be developed during infection progression otherwise referred to as *in vivo* strain evolution; these would be defined as "persistence-associated" as they are not observed in the resolved isolates. This comparison assumes all strains are equally likely to produce a persistent infection, but development of such traits is dependent on specific evolutionary events occurring during the infection. Such traits could be used practically during infection progression to diagnose persistence by confirming the presence of these characteristics. The third and final comparison would also involve *in vivo* strain evolution between the initial and persistent isolates, but the resolved isolates would not be phenotypically disguisable. These traits would have been developed during the infection but they would not directly associate with persistence due to the influence of different strain backgrounds.

Previous studies either involved the comparison between resolved isolates and the initial isolates from persistent infections, likely overlooking persistence associated *in vivo* evolved traits (Abdelhady et al., 2014; Chong et al., 2013; Fowler et al., 2004; Hawkins et al., 2007; Neuner et al., 2010; Park et al., 2012; Seidl et al., 2011; Xiong et al., 2009; Yoon et al., 2010). Other publications included all isolates originating from one or more persistent infections but did not include isolates from resolved infections (Gao et al., 2013; Gao et al., 2010; Howden et al., 2008; Howden et al., 2006; Proctor et al., 1995; Khosrovaneh et al., 2004). This experimental design may lead to false positives where not all *in vivo* evolved traits are truly persistence associated and are in fact infection artefacts. The inclusion of all three isolate types enables the identification of persistence specific phenotypes resulting from *in vivo* strain evolution, by eliminating general infection-associated traits previously thought to be persistence-associated phenotypes.

3.8.2 Clinical differences between persistent infections including suspected infective foci and different treatment schemes

Infection foci and metastatic infections are commonly suggested as predictors for persistence development and mortality (Kim et al. 2003; Chong et al. 2013; Khatib et al. 2009; Khatib et al. 2006; Lin et al. 2010). PB1, PB2 and PB5 experienced clinically distinct infection foci during the time of persistent isolate collection, specifically mitral valve IE, sacral wound and paraspinal abscess. In contrast, the PB3 primary focus (foot ulcer) was eradicated prior to persistent isolate collection. A paraspinal abscess was later identified but the specific timing of foci formation is unknown therefore its presence during sample collection cannot be ruled out. Despite displaying clinically different types of infection foci, phenotypic analysis indicated there were considerable similarities between the PB1 and PB3 infections, and also for PB2 and PB5. This suggests there was limited, if any, overall influence by the individual infection foci present on the behaviours of each infecting strain and during persistence development.

An additional difference between persistent infections was antibiotic treatment schemes. The three main antibiotics used for treatment included vancomycin, linezolid and daptomycin, but the timing and order of these prescriptions varied between infections. In particular, treatment for the PB1 and PB3 infections included daptomycin prior to persistent isolate collection; whereas daptomycin was administered after final sample collection in the PB2 and PB5 infections. As a consequence of treatment PB1 and PB3 persistent isolates developed reduced daptomycin susceptibility; whereas the daptomycin MIC for PB2 and PB5 persistent isolates remained the same.

Increasing daptomycin MIC during persistent infections in response to treatment has been observed previously (32 - 60 %) and such cases are associated with significant therapeutic failure rates (19 - 41 %) (Gasch et al., 2014; Fowler et al., 2006; Sharma et al., 2008; Boyle-Vavra et al., 2011). This is also observed for vancomycin treatment where the development of the (h)VISA phenotype is frequently observed (Howden et al., 2006; van Hal et al., 2011; Khosrovaneh et al., 2004; Lin et al., 2010). Additionally a VISA phenotype is suggested as a risk factor for persistent infections (Lin et al., 2010; Yoon et al., 2010; Neuner et al., 2010). This

is particularly relevant as both PB1 and PB2 isolates displayed VISA MIC using the MICE susceptibility method. Previous research also presents a link between VISA development and reduced daptomycin susceptibility, usually because of a thickened cell wall (Patel et al., 2006; van Hal et al., 2011; Cui et al., 2010; Cui et al., 2006). Therefore the prescription of vancomycin prior to daptomycin treatment in the PB1 and PB3 infections may have contributed to the development of reduced daptomycin susceptibility in these strains.

As previously stated, PB1 and PB3 infecting strains are phenotypically distinct from PB2 and PB5 strains; this cannot be explained by differences in infection foci, but may be potentially linked with differences in antibiotic treatment, particularly daptomycin. Evidence suggests daptomycin and vancomycin treatment can induce phenotypic changes within bacterial populations *in vitro* and *in vivo*. Exposure to vancomycin and development of a VISA phenotype has been associated with increases in biofilm formation and fibronectin adhesion (Hsu et al., 2011; Abdelhady et al., 2014). Additional, vancomycin (in combination with *agr* dysfunction) and daptomycin have been shown to induce reduced susceptibility to HDP/CAMP (Sakoulas et al., 2005; Mishra et al., 2011). Therefore it is possible the phenotypic similarities seen for PB1 and PB3 isolates may be a result of daptomycin treatment *in vivo*, as these specific traits aren't observed in persistent infections without daptomycin exposure (PB2 and PB5).

3.8.3 Two distinct persistence mechanisms are observed neither of which involved SCV or *agr* dysfunction

PB2 and PB5 infecting strains did not exhibit any distinguishable traits in relation to the basic assessment used in this chapter either between initial and persistent isolates or in comparison with resolved isolates. This is in stark contrast to the observations for PB1 and PB3 cases which displayed signs of *in vivo* strain evolution contributing to persistence development. Importantly, none of these four infections involved SCV, a common mechanism of persistence (von Eiff et al., 2006; Proctor et al., 2006; Gao et al., 2010; Proctor et al., 1995). Neither did they exhibit *agr* dysfunction, another associated persistence trait (Park et al., 2012; Fowler et al., 2004) and no differences were observed in activity of three secreted exoproteins (α-, β-haemolysin, DNase). This suggests two diverse mechanisms of persistence were

employed during these four infections; but also these mechanisms are distinct from previous publications involving SCV and reduced toxin expression through *agr* dysfunction, highlighting the novelty of this study's data.

3.8.4 Two persistent infections (PB1 and PB3) display novel *in vivo* evolved persistence associated traits

As stated there was no indication of SCV in any of the isolates tested, neither was there any differences in growth using nutrient replete media supporting the fact that dormancy was not involved in these infections. In fact, PB1 and PB3 infecting strains displayed a growth adaptation to a nutrient deprived, metal ion restricted media at a near neutral pH. Moreover, this phenotype was lost when the media was slightly basic, e.g. pH 10.0 (data not shown). This is the first time to our knowledge a persistence associated **growth adaption** has been presented in regards to *S. aureus* persistence or indeed for any other bacterial species. The use of nutrient deprived, metal ion restricted media at pH 7.0 is crucial as it mimics the persistent environment i.e. within the host niche the availability of nutrients is kept low, freely available metal ions are sequestered and the blood in particular is kept at pH 7.4. Therefore the use of this media more accurately assesses how the bacteria behaved under these particular stress conditions whilst promoting an infection. Overall, these data suggests the PB1 and PB3 infecting strains adapted to the specific environment they persisted in; it also emphasises the need to assess bacteria under *in vivo*-like conditions otherwise important phenotypes could be overlooked.

PB1 and PB3 persistent isolates also displayed significant increases in cell surface associated proteins specifically after incubation in the nutrient deprived, metal ion restricted media. Such proteins are involved in adhesion, immune system evasion, cytotoxicity and stress response systems. An overview of all differentially expressed proteins is displayed in Figure 3-16. Several of these proteins exhibited substantial increases from initial to persistent isolate in one or both of the infecting strains signifying an *in vivo* evolved trait. Other proteins showed comparable values between initial and persistent isolates but displayed a lower level of expression in resolved isolates. Subsequently, this study proposes persistence development required a threshold level of these proteins to be present; if levels were below this

value the PB1/PB3 strains evolved to up-regulate expression leading to a comparable protein profile exhibited by PB1 and PB3 persistent isolates.

An additional feature observed in this chapter was that the phenotypes of PB3-74 mirrored the initial isolate (PB3-1); such phenotypes included daptomycin MIC, growth analysis and proteomic profiles. Due to the stability of the persistence phenotypes observed, it is doubtful that PB3-74 reverted back to the initial isolate's phenotypes after undergoing *in vivo* persistence evolution. It is more likely that PB3-74 originated directly from an unidentified infection focus which was formed prior to *in vivo* adaption and remained present until day 74.

Differentially expressed proteins listed in Table 3-4 and Figure 3-16 cover a wide variety of functions including adhesion, immune system evasion and stress response. Emp and Eap are both mediators of low iron biofilms and adhesion to a range of host proteins including fibronectin and fibrinogen (Johnson et al., 2008; Hussain et al., 2001). Also, Eap displays immune evasion properties such as inhibiting leukocyte recruitment via iCAM-1 (intercellular adhesion molecule 1), interrupting neutrophil binding to endothelial cells and reducing T-cell proliferation (Chavakis et al., 2002; Haggar et al., 2004; Lee et al., 2002). Other immune system evasion proteins include Sbi, which binds IgG and β_2-glycoprotein but has also been shown to inhibit neutrophil directed opsonophagocytosis and complement by binding host factors H/C3b/C3d (Zhang et al., 1998; Zhang et al., 1999; Haupt et al., 2008; Smith et al., 2011). Eap is also thought to be essential for keratinocytes internalisation (Bur et al., 2013) in addition to Atl which has been shown to interact with Hsc70 during endothelial cell invasion and also plays a role in FnBP mediated biofilms (Hirschhausen et al., 2010; Houston et al., 2011). Two additional proteins which are implicated in this chapter are Trx and Csp, which mediate two major bacterial stress response systems, namely oxidative stress and cold-shock (Uziel et al., 2004; Katzif et al., 2003; Lu & Holmgren, 2014). The functions of other differentially expressed proteins include antibiotic resistance (PBP2a), glycolysis and laminin adhesion (Eno) and generalised cell maintenance (RpoE, Acyl carrier). Due to the putative functions of the identified proteins, it would be fair to hypothesis the persistent isolates exhibit significant phenotypic traits. These would include biofilm formation, adhesion and eukaryotic cell invasion and adhesion to other host

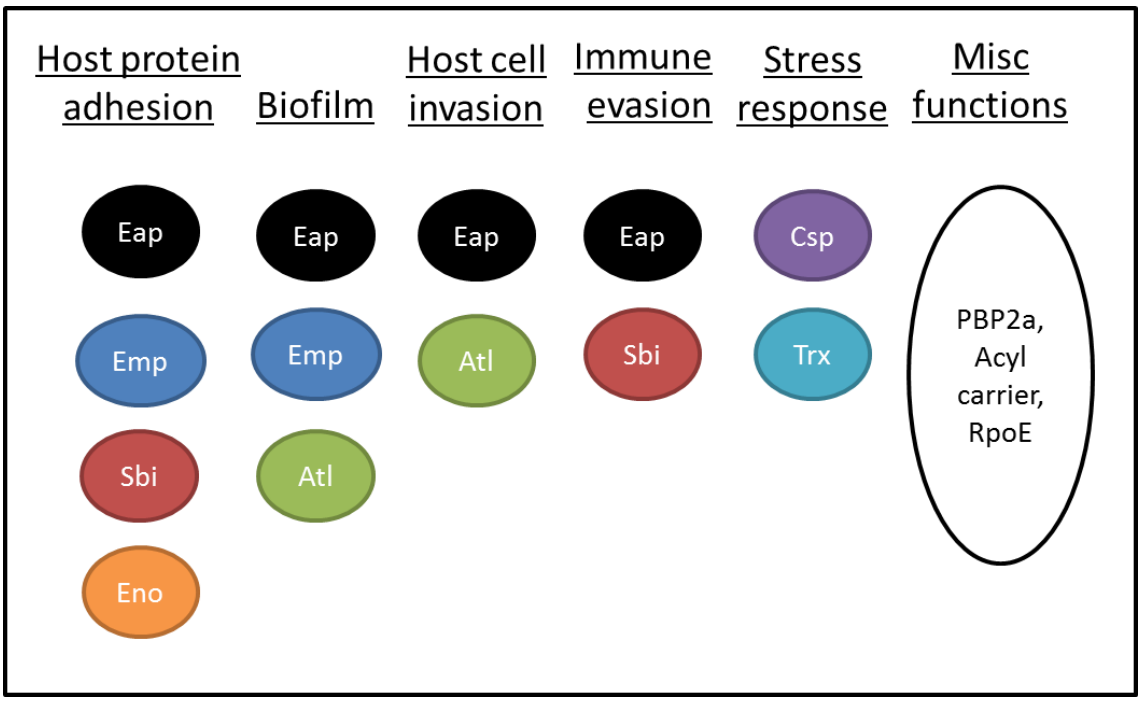


Figure 3-16 Overview of cell surface associated proteins up-regulated in PB1 and PB3 persistent isolates. All cell surface associated proteins which displayed > 0.5 $\log_2$ increases in persistent isolates identified by iTRAQ LC-MS, are listed. Each protein is colour-coded and categorised according to function; proteins which display multiple functions are included multiple times. The miscellaneous functions category includes proteins whose function does not fit into any of the functional groups listed, such as antibiotic resistance, general cell maintenance and biosynthesis.

factors such as fibronectin/fibrinogen, immune system evasion and survival in hostile conditions such as defence against ROS.

Overall, many of these proteins are predicted to have a major influence *in vivo*. In particular, immune system evasion is one of the hurdles an infecting organism needs to overcome to sustain a prolonged infection. Additionally, the infecting bacteria will encounter many stressors *in vivo* including oxidative stress, therefore up-regulation of stress response proteins would aid this purpose. Finally, many of these proteins are known to adhere to a range of host factors and mediate bacterial biofilms. Therefore one could hypothesise these phenotypes are altered according to the proteomic changes witnessed here, such phenotypes will be investigated in the next section of this study.

3.8.5 Implication of global regulators, SaeRS and Fur, in regards to the PB1 and PB3 proteomic changes

Several of the differentially expressed proteins associated with persistence are included in the SaeRS regulon. This particular TCR system senses cell membrane alterations and works synergistically with Agr to up-regulate key secreted and cell surface proteins including haemolysins, nuclease, coagulase, a multitude of adhesins (Eap/Map1, Emp, FnBP, Efb) and immune evasion proteins (Sbi, Spa) (Geiger et al., 2008; Giraudo et al., 1997; Rogasch et al., 2006; Voyich et al., 2009). In this case, SaeRS is known to positively regulate Eap, Emp and Sbi, which is in agreement with these data. However, rather conversely to these proteomic results SaeRS down-regulates PBP2a and Trx (Kuroda et al., 2007; Rogasch et al., 2006; Nygaard et al., 2011; Voyich et al., 2009). This possibly indicates the involvement of multiple regulators in addition to SaeRS. To investigate the possible involvement of SaeRS, the *sae* promoter, *saeR*, *saeS* and *eap* promoter were sequenced in the PB1 initial and persistent isolates in addition to PM25 which was used as a closely related strain comparison (data not shown). These loci were identical in sequence between initial, persistent and PM25 reference sequence. Therefore a mutation in the *saeRS* locus is not the cause of these proteomic changes.

Another implicated gene regulator is Fur. Fur is known to positively regulate Eap, Sbi and other gene regulators such as SaeRS, Agr and Rot (Repressor Of Toxins),

however Fur also down-regulates Atl (Johnson et al., 2011), which is again in contrast to the observed proteomic changes. It is becoming more likely multiple factors are involved, possibly other gene regulators, to achieve the proteomic changes seen in this study as SaeRS/Fur regulation does not fully correlate with the observed results.

3.8.6 Conclusions

During this chapter, two distinct sets of defining traits have been observed in the four infections investigated, none of which included previously regarded persistence traits such as SCV or *agr* dysfunction. An overview of this chapter's results is shown in Figure 3-17. It can be immediately concluded that different infective foci did not influence persistence development in this study as each infection displayed substantially different clinical attributes. However, differences in antibiotic treatment, particularly daptomycin prescription may be a defining cause for the different persistence traits observed between PB1/PB3 and PB2/PB5. The mechanism employed by PB2 and PB5 has yet to display any phenotypic attributes associated with persistence in response to the general characterisation assays used during this chapter. This is in contrast to PB1 and PB3, where despite differences in their clinical details, both infecting strains displayed similar defining traits developed though infection progression. Persistent isolates from both infections exhibited growth adaptations and increases in cell surface associated proteins involved in adhesion, immune evasion and stress response. These changes were experienced exclusively in a nutrient deprived, metal ion restricted environment at a near neutral pH, reflective of the host niche in which they persisted. Following on from this, the next chapter will focus upon the assessment of specific virulence determinants linking with the proteomic phenotypes witnessed and aim to identify virulence signatures associated with persistence development.

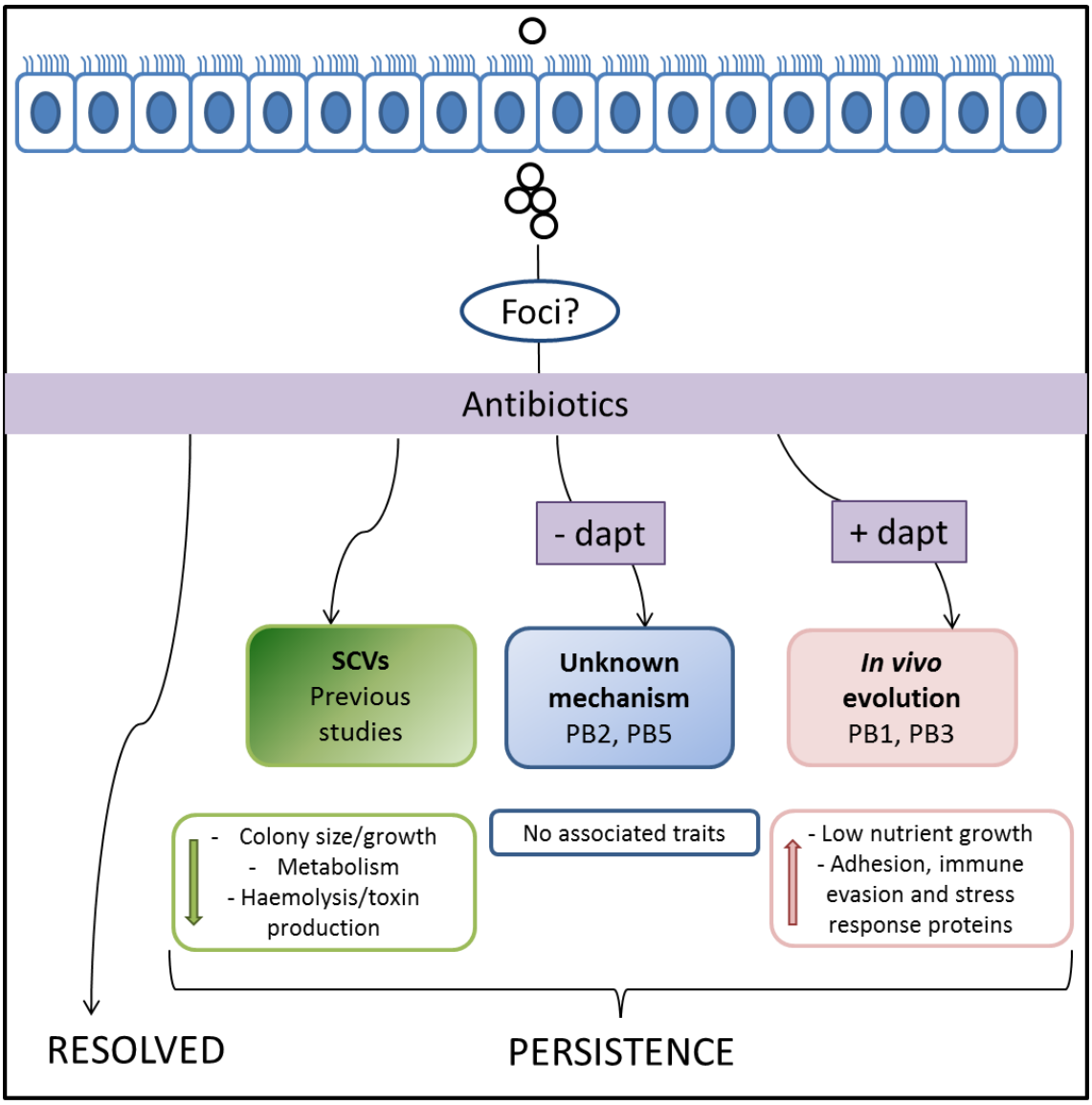


Figure 3-17 Current persistence model presented after Chapter 3's data. This diagram highlights the main pathways of *S. aureus* bacteraemia known currently including resolved and persistent outcomes. Persistence mechanisms include the previously regarded SCV pathway and its associated traits. Also displayed is the PB2/PB5 mechanism investigated in this study, which has not exhibited any defining traits so far. Finally a third mechanism is presented which involves *in vivo* strain evolution and the development of specific persistence associated traits, experienced in PB1 and PB3

Chapter 4 Persistence Associated Virulence Determinants

4.1 Introduction

Chapter 3 focussed upon general persistence associated phenotypic traits. Such "persistence traits" could be used as indicators of persistence during infections and allude to the physical properties of the persisting bacteria. However, a series of associated bacterial characteristics does not fully explain the persistence mechanism and the associated virulence determinants employed during the infections. Investigations need to consider the possibility of continuous bacterial adaptation and behavioural changes occurring *in vivo* and investigate the potential influence of environmental factors (e.g. immune system defences, antibiotic therapies).

For this part of the study, the aim was to investigate a series of virulence determinants for an association with persistence; additionally, the effect of external factors on the clinical isolates was analysed. The previously characterised *S. aureus* strains were assessed for their ability to form biofilms and persist intracellularly within nonprofessional phagocytic eukaryotic host cells. Both of these defensive strategies are effective at providing protection to indwelling bacterial cells from external elements such as host immune system defences and administered antibiotic therapies (Clement et al., 2005; Garzoni & Kelley, 2009; Dimitriou et al., 2011; Seidl et al., 2011). The isolates' general virulence capabilities were investigated using a *Galleria mellonella* larva infection model and the involvement of bacterial stress response systems was analysed via exposure to bactericidal antibiotic concentrations, toxic levels of metals and H_2O_2.

4.2 Biofilm capabilities of clinical *Staphylococcus aureus* isolates

The ability to form biofilms is a major bacterial virulence factor and is known to be a risk factor in patients with indwelling devices such as urinary and central venous catheters and prosthetic heart valves (Agarwal et al., 2010). Biofilms are a serious clinical issue as they are notoriously difficult to treat, which subsequently makes them an ideal persistence mechanism.

4.2.1 PB1 and PB3 persistent isolates show improved glucose mediated biofilms in BHI

There are multiple pathways by which biofilms can be formed depending on the adhesion factors involved, the expression of which are influenced by the environment (O'Neill et al., 2007; O'Neill et al., 2008; Thompson et al., 2010). Therefore the clinical isolates' abilities to form biofilms were assessed in BHI and in the presence of different supplements (NaCl, human serum, glucose) known to induce the expression of certain biofilm mediators (Chapter 2:11).

Clinical isolates failed to form biofilms in BHI, with or without the addition of NaCl or human serum supplements, however all isolates were able to form glucose mediated biofilms (Figure 4-1). Interestingly, significant differences were observed between initial and persistent isolates within the PB1 and PB3 infections. The PB1 persistent isolates (PB1-15-1/2) exhibited a significantly higher level of biofilm formation than PB1-1 ($P = 0.0012$ & $P < 0.0001$), similarly the PB3 persistent isolate (PB3-32-1) was also significantly higher than PB3-1 and PB3-74 ($P < 0.0001$). Additionally, PB3-32-1 exhibited a significantly higher level of biofilm formation than the resolved isolates ($P < 0.0001$), possibly suggesting an association between glucose mediated biofilm and persistence during the PB3 infection. Overall, this data suggests *in vivo* strain evolution during the PB1 and PB3 infections resulted in increased glucose induced biofilm capabilities, but this was not seen during the PB2 or PB5 infections.

4.2.2 Differential biofilm values are not related to variations in fibronectin or fibrinogen binding capabilities

It has been previously published that mildly acidic conditions resulting from glucose metabolism induces the expression of known biofilm mediators, fibrinogen and fibronectin binding proteins (FnBP, Efb) (O'Neill et al., 2008). To investigate whether these adhesins are involved in this study the isolates abilities to bind fibrinogen and fibronectin coated surfaces was assessed (Chapter 2:12). No differences were observed between any of the isolates in regards to fibrinogen or fibronectin binding capabilities (Figure 4-2). These data indicates the observed biofilm phenotype was not a direct result of changes in fibronectin or fibrinogen

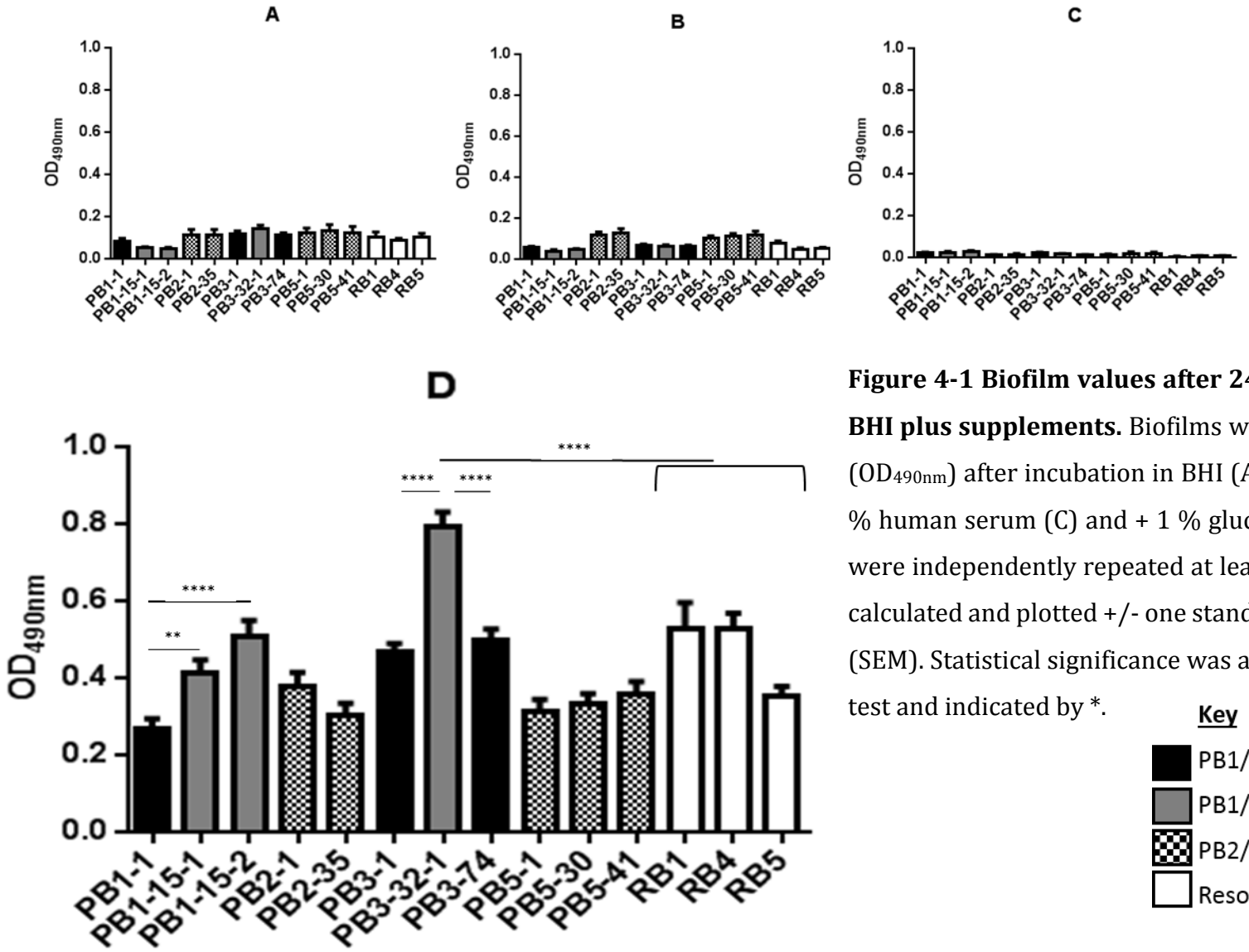


Figure 4-1 Biofilm values after 24 hour incubation in BHI plus supplements. Biofilms were optically assessed (OD_{490nm}) after incubation in BHI (A), + 4 % NaCl (B), + 5 % human serum (C) and + 1 % glucose (D). Experiments were independently repeated at least three times, means calculated and plotted +/- one standard error of the mean (SEM). Statistical significance was analysed by student T-test and indicated by *.

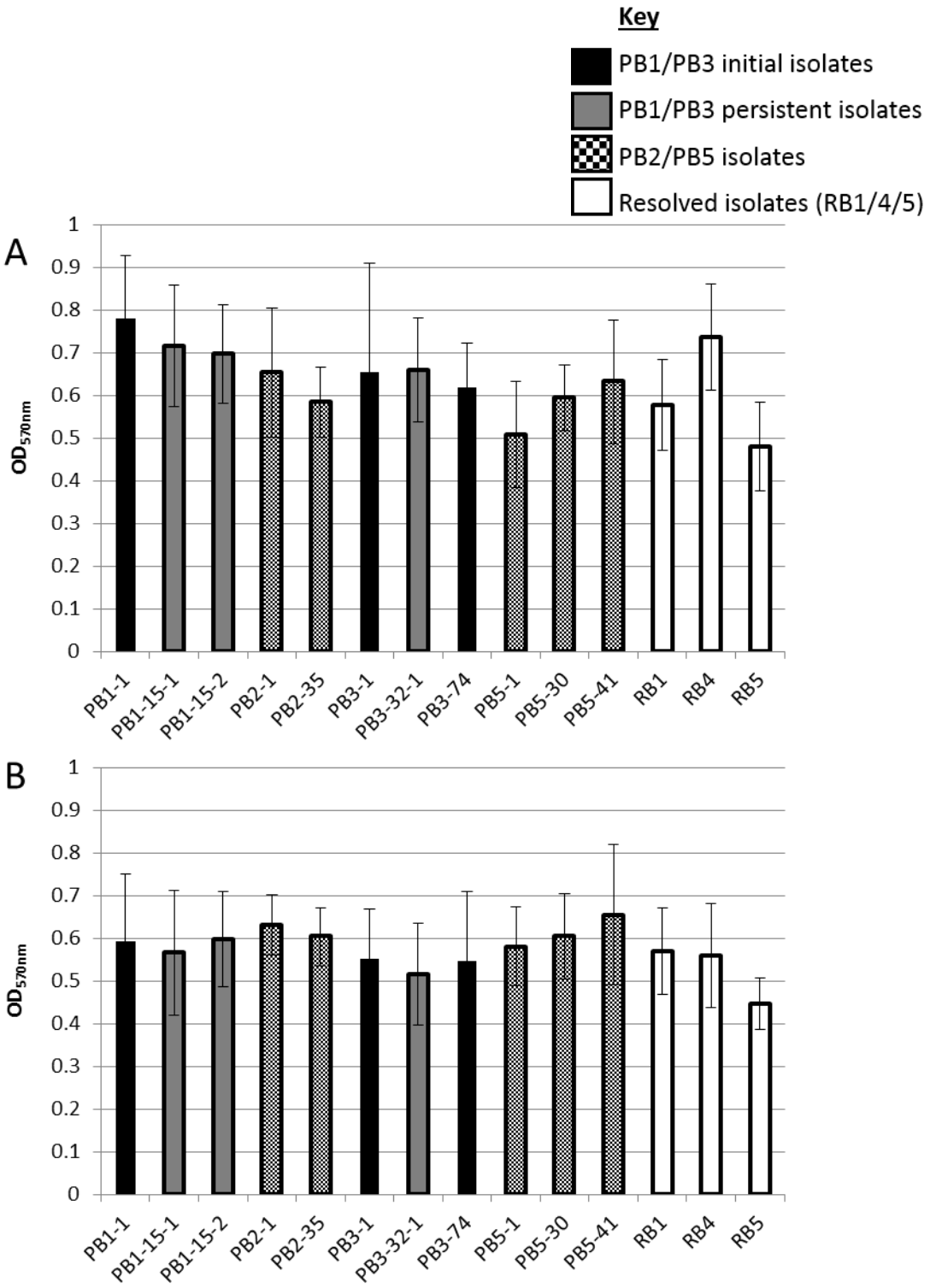
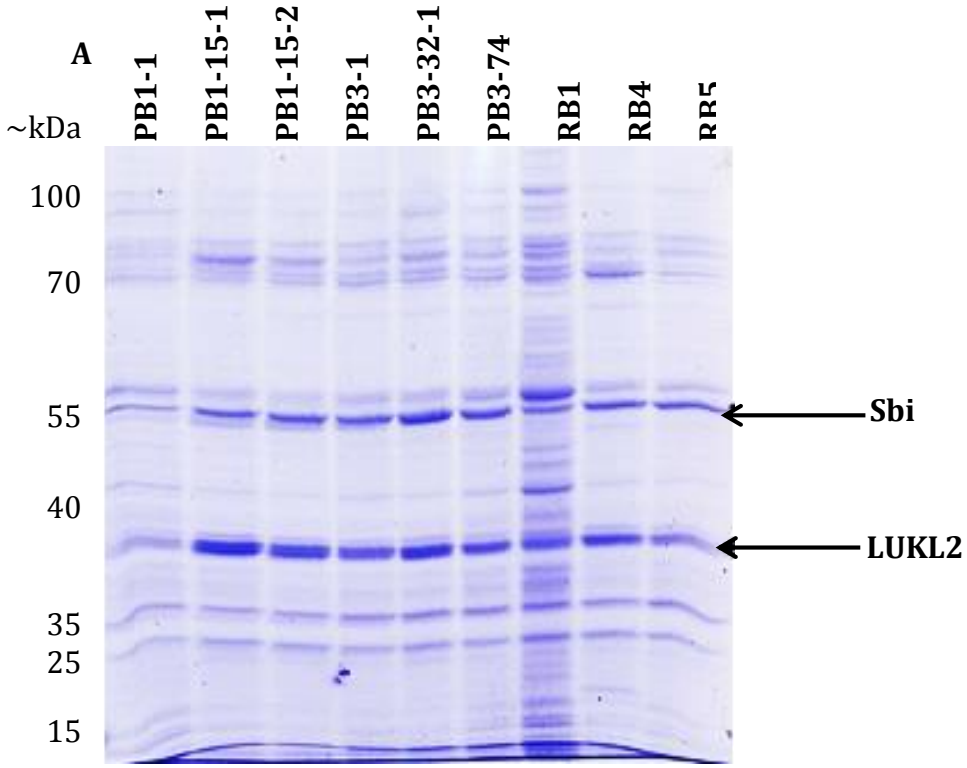


Figure 4-2 Fibrinogen (A) and fibronectin (B) binding capabilities. The isolates' abilities to bind fibrinogen or fibronectin coated surfaces at a set inoculum was optically assessed (OD_{570nm}) after 2 hours incubation in BHI + 1 % glucose. Experiments were independently repeated three times, means calculated and plotted +/- one standard deviation. No differences were seen between any isolates in the ability to bind fibrinogen or fibronectin.

A



B

		Area	Percent	Relative Density
SBI	PB1-1	7806.083	9.106	100.00%
	PB1-15-1	9787.134	11.417	125.38%
	PB1-15-2	9704.305	11.32	124.31%
	PB3-1	10031.38	11.702	100.00%
	PB3-32-1	10795.38	12.593	107.61%
	PB3-74	9836.912	11.475	98.06%
LUKL2	PB1-1	9328.861	14.096	100.00%
	PB1-15-1	12676.52	19.154	135.88%
	PB1-15-2	11410.81	17.241	122.31%
	PB3-1	10690.4	16.153	100.00%
	PB3-32-1	11540.81	17.438	107.96%
	PB3-74	10535.23	15.918	98.55%

Figure 4-3 Cell surface associated protein fraction after 24 hour incubation in BHI + glucose. The non-covalently bound cell surface protein fraction was prepared as previously described after 24 hours incubation in BHI + 1 % glucose and visualised via 1D SDS-PAGE (A). Two individual bands which displayed differential abundances between PB1 and PB3 isolates were identified by MS as SBI and LUKL2. ImageJ was used to quantify the SBI and LUKL2 protein bands in the persistent isolates relative to their respective initial isolates (B).

adhesion capabilities. Therefore it is reasonable to hypothesise additional factor(s) are involved in glucose mediated biofilms which are as yet undetermined.

4.2.3 PB1 and PB3 persistent isolate display increased cell surface associated Sbi and leukocidin-like protein 2 in response to glucose

The cell wall and cell surface associated protein composition after incubation in BHI + glucose was assessed using 1D SDS-PAGE to further investigate other possible glucose induced biofilm mediators. No differences were seen in the cell wall protein fraction after incubation in BHI + glucose (data not shown); but multiple differences were seen in the cell surface associated protein fraction between PB1 and PB3 initial and persistent isolates (Figure 4-3A). Two protein bands of interest were identified by MS as Sbi (second immunoglobulin binding protein of *S. aureus*) and LUKL2 (uncharacterised leukocidin-like protein 2). These proteins show PB1 and PB3 persistent isolate specific increases compared to their relative initial isolates, calculated using ImageJ densitometry analysis (Figure 4-3B), which correlates with glucose mediated biofilm data (Chapter 4.2.1). These data may indicate that one or both of these proteins may responsible for the glucose mediated biofilm phenotype observed in the previous section.

4.3 Eukaryotic cell invasion assay

The isolates abilities to adhere, invade and persist intracellular within a rat myocardium cell line, H9C2, was investigated as a potential mechanism of persistence. This particular cell line was chosen as myocardium cells would be a cell type often encountered by the bacteria during a BSI.

4.3.1 PB1 and PB3 persistent isolates show decreased intracellular persistence capabilities compared to their respective initial isolates

Confluent H9C2 cells were inoculated with standardised bacterial counts and incubated in the presence or absence of gentamicin. Gentamicin is traditionally used in this assay as it is unable to penetrate eukaryotic cell membranes; therefore internalised bacteria are protected. At set times points, infected H9C2 cells were lysed and homogenates plated out to give an estimate of bacterial CFU correlating with adhesion, invasion or intracellular persistence numbers (Chapter 2:14).

Bacterial inoculum CFU were also analysed by one way Anova to confirm they were sufficiently comparable between different strains and experimental repeats. Plotted mean CFU for adhesion, invasion and intracellular persistence data from three independent experiments can be viewed in Figure 4-4.

No significant differences in adhesion or invasion numbers were observed between any of the initial, persistent or resolved isolates. Despite intracellular survival being thought as an important persistence trait, both PB1 and PB3 persistent isolates actually show decreased intracellular CFU compared to their relative initial isolates (Figure 4-4C*). There was also considerable variation in data between individual infecting strains indicating a strong effect due to the different strain backgrounds. For example, both the persistent PB3 and resolved RB4 infecting strains showed significantly higher intracellular persistence as compared to all other isolates.

In conclusion, there appears to be no association between adhesion, invasion or intracellular persistence capabilities with the persistent *S. aureus* bacteraemia cases characterised; therefore it is unlikely to be a potential persistence mechanism in this study.

4.4 *Galleria mellonella* larva virulence model

Decreased virulence has been previously implicated in persistence, usually involving SCV (Fowler et al., 2004; Park et al., 2012; Gao et al., 2010; Gao et al., 2013). What is unclear is if attenuated virulence is still involved in persistence with phenotypically wild type persistent isolates. Therefore, the general virulence capabilities of the clinical bacteraemia isolates needed to be assessed using an assay/model indicative of the human environment.

G. mellonella larva are frequently used to assess the virulence characteristics of human pathogens including *S. aureus*; specifically the ability to cause tissue damage, propensity for immune system evasion and the effectivity of antimicrobial agents can be and, has been investigated accurately using this model (Peleg et al., 2009; Desbois & Coote, 2011; Purves et al., 2010; Gao et al., 2010). The main reason they are effective models is that they demonstrate a complex innate immune response similar to a human's; for example, they have the ability to phagocytose infecting

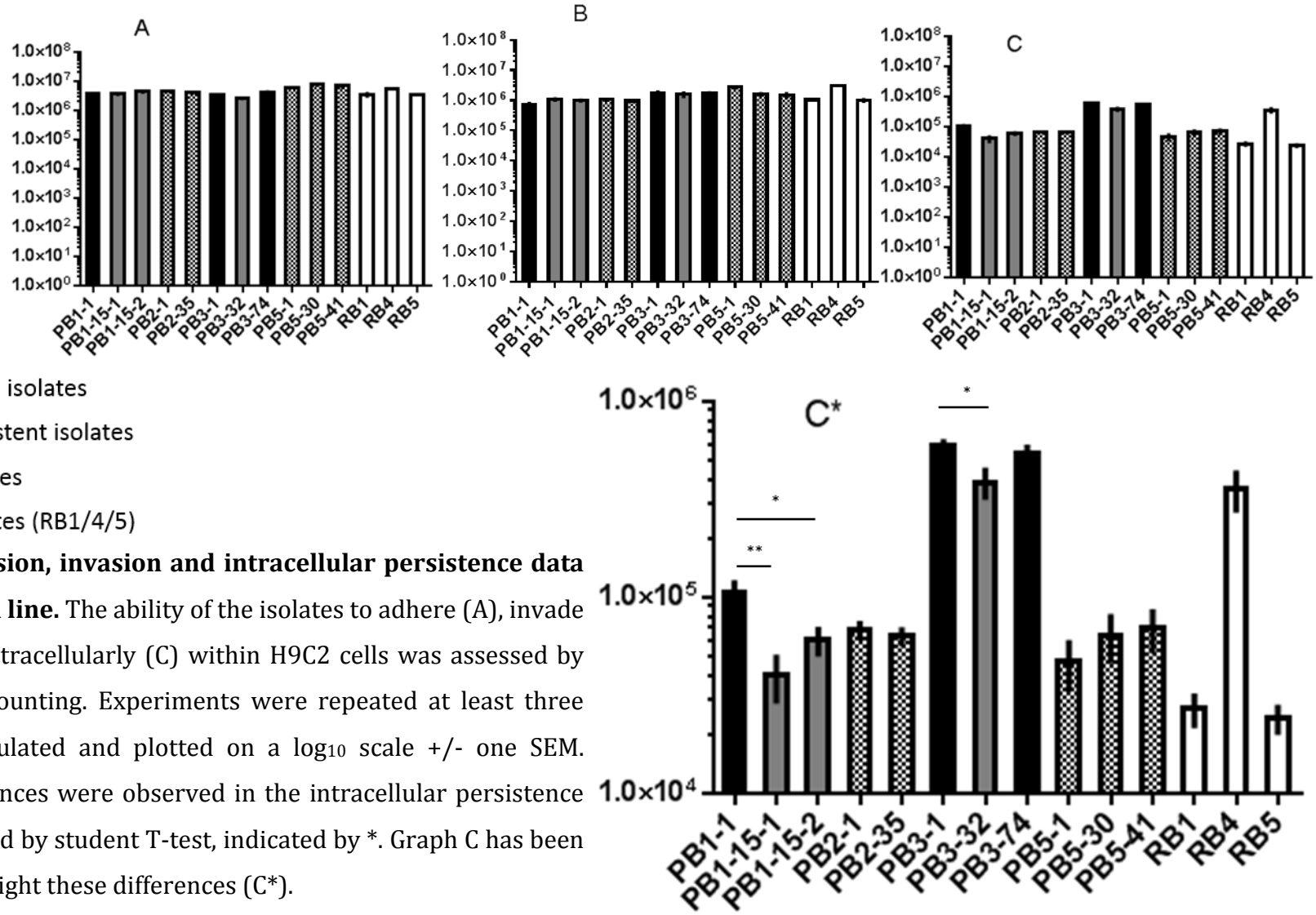


Figure 4-4 Adhesion, invasion and intracellular persistence data using a H9C2 cell line. The ability of the isolates to adhere (A), invade (B) and persist intracellularly (C) within H9C2 cells was assessed by traditional CFU counting. Experiments were repeated at least three times, mean calculated and plotted on a $\log_{10}$ scale +/- one SEM. Significant differences were observed in the intracellular persistence data as determined by student T-test, indicated by *. Graph C has been expanded to highlight these differences (C*).

pathogens and initiate lysosomal digestion via oxidative stress pathways and also trigger the release of various HDPs (Cook & McArthur, 2013; Ramarao et al., 2012). Moreover, there are certain similarities between the epithelial and digestive tissues between *Galleria* larva and humans, subsequently the ability of pathogens to adhere and colonise the host can be reliably assessed (Cook & McArthur, 2013; Ramarao et al., 2012). The main drawback to using this model is the length of time the larva remain viable for once infected, i.e. 5 days max. Consequently this model could be not used as "persistence" model as pathogenic incubation within the larva host would need to be sustained post 7 days. However, the possibility of differential virulence potentials between the initial, persistent and resolved isolates could be accurately assessed using this model.

4.4.1 *Galleria mellonella* larva infected with PB1 and PB3 persistent isolates show improved survival rates

Figure 4-5 shows the survival plots of infected *G. mellonella* larva (Chapter 2:15). High mortality rates were experienced for larva infected with PB1 and PB3 initial isolates, PB2 and PB5 initial or persistent isolates and all three resolved isolates. In contrast, *G. mellonella* larva infected with PB1 or PB3 persistent isolates showed significantly lower fatalities compared to all other isolates including their respective initial isolates and resolved; specifically PB1-15-1/2 versus PB1-1 $P < 0.0001$; PB3-32-1 versus PB3-1 $P < 0.01$, versus PB3-74 $P < 0.05$.

Importantly, the observed attenuated virulence exhibited by the PB1 and PB3 persistent isolates has been found without the association of SCV development. This suggests attenuated virulence is a true determinant of persistence, not only in infections involving SCV but also with phenotypically wild type persistent isolates.

4.4.1 Infected *G. mellonella* larva had similar bacterial burdens when inoculated with PB1 and PB3 initial and persistent isolates as shown by traditional CFU counting and quantitative molecular techniques

The larvae bacterial burdens were assessed to confirm the observed persistence associated attenuated virulence was not caused by differences in bacterial loads between different infecting isolates.

For this assessment, additional larva were inoculated in parallel with the virulence experiments and after 48 hours were homogenised, plated out using Staphylococcal selective media and CFU counted (Figure 4-6). Unfortunately, data retrieved from this method were extremely variable, with CFU differing substantially not only between individual repeats but also between larvae infected with the same inoculum within the same experiment. Additionally, it was noted that the time taken for isolates to recover using this selective media varied. In particular, persistent isolates from the PB1 and PB3 infections took a minimum of 48 hours to recover on Mannitol salt agar compared to 24 - 48 hours for their respective initial isolates. Interestingly, this prolonged recovery time on Mannitol salt agar was not experienced by *in vitro* cultures (data not shown). Comparable recovery times were observed when homogenates were plated onto non-selective media. Unfortunately, it was impractical to use non-selective media to assess *S. aureus* specific CFU due to contaminating species. This finding may indicate increased sensitivity to high salt stress exhibited by persistent isolates post *in vivo* larvae incubation, which would subsequently influence bacterial recovery and affect accurate assessment of bacterial burdens. As a result, traditional CFU counting from selective media may not display a true representation of bacterial burden; therefore a combination of qPCR and qRT-PCR was employed as a molecular measurement of bacterial burden.

A series of control experiments were initially conducted to optimise the protocol. Set numbers of bacterial cells encompassing the range which is usually retrieved 48 hours post infection (10^4-10^9) were injected into individual larva. CFU were plated as before, in addition to total DNA and RNA extraction from individual homogenates. A PCR targeting the *S. aureus fur* gene confirmed *S. aureus* specific DNA was in proportion to the size of the inoculum used for inoculation (Figure 4-7A). The 16S rRNA gene/transcript was chosen as the target for quantification as this was the only primer set to produce a single band from the heterogeneous DNA samples; indicating *S. aureus* specificity (Figure 4-7B-E). Both DNA and RNA control samples were then used for qPCR and qRT-PCR to assess the most accurate template type to use for assessment (Figure 4-8). Quantitative assessment of DNA was unable to distinguish below 10^6 CFU; likely due to the low ratio of *S. aureus* DNA to contaminating host DNA. In contrast, all inoculum sizes were distinguishable using RNA; therefore this template type was used in analysis.

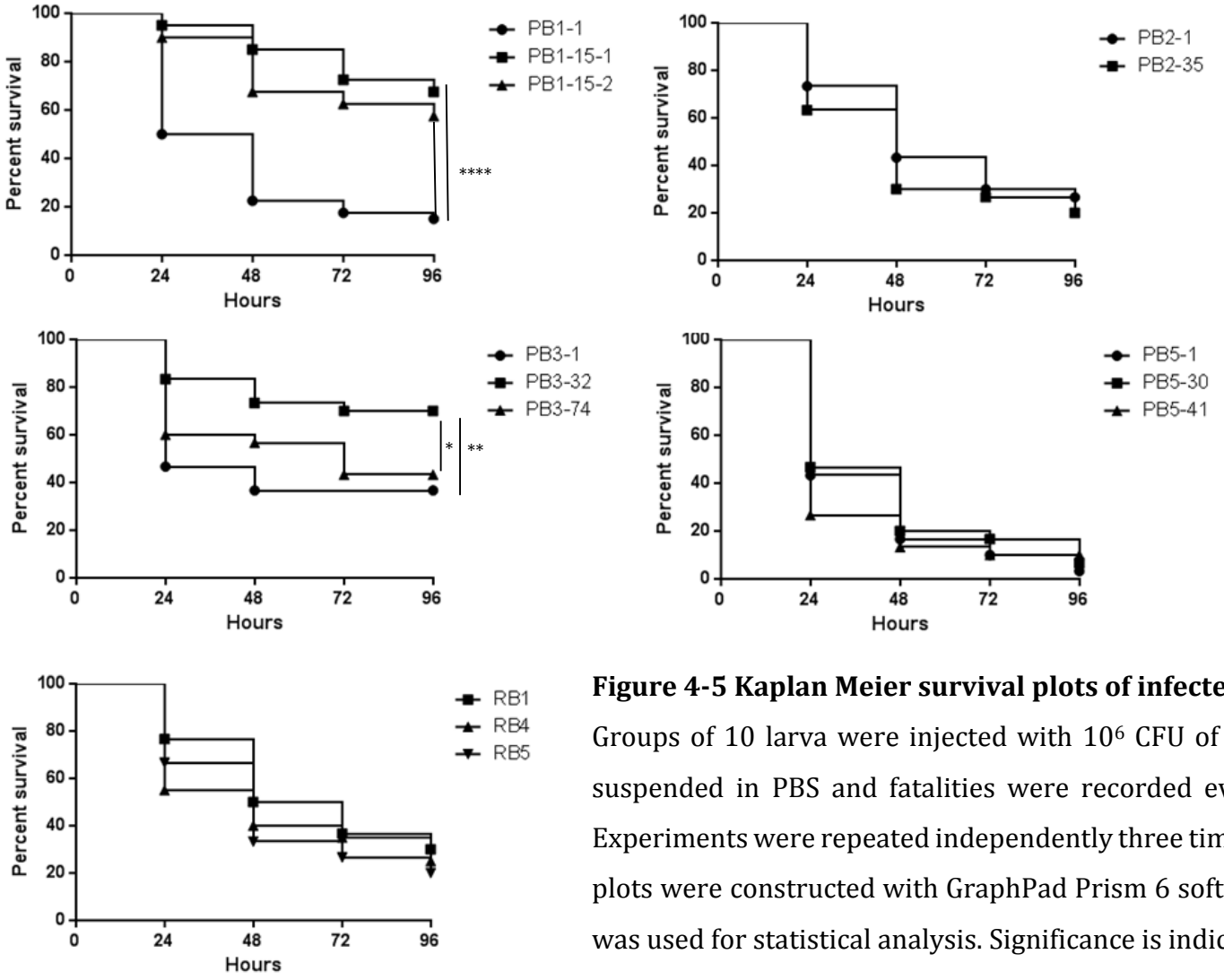


Figure 4-5 Kaplan Meier survival plots of infected *Galleria mellonella* larva. Groups of 10 larva were injected with 10^6 CFU of individual bacterial cultures suspended in PBS and fatalities were recorded every 24 hours for 96 hours. Experiments were repeated independently three times and Kaplan Meier survival plots were constructed with GraphPad Prism 6 software. Log-rank (Mantel-Cox) was used for statistical analysis. Significance is indicated with *.

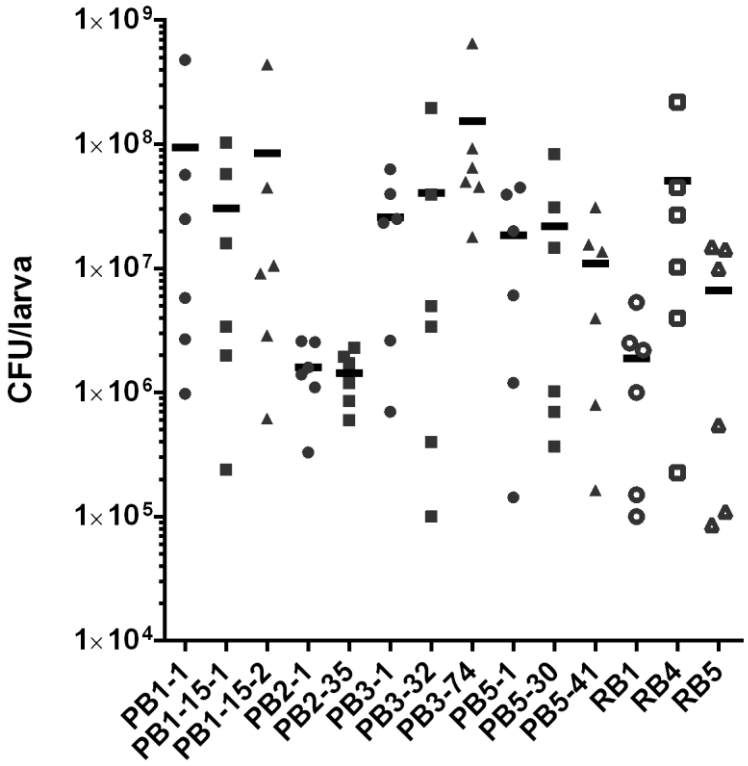


Figure 4-6 48 hour bacterial burdens as calculated from traditional CFU plating onto selective media. Two viable larva were homogenised 48 hours post infection in parallel with each virulence experiment, serially diluted and plated onto Staphylococcal selective media (Mannitol salt agar). CFU were counted and plotted on a scatter graph using a $\log_{10}$ scale (dots) including an indication of the mean (line).

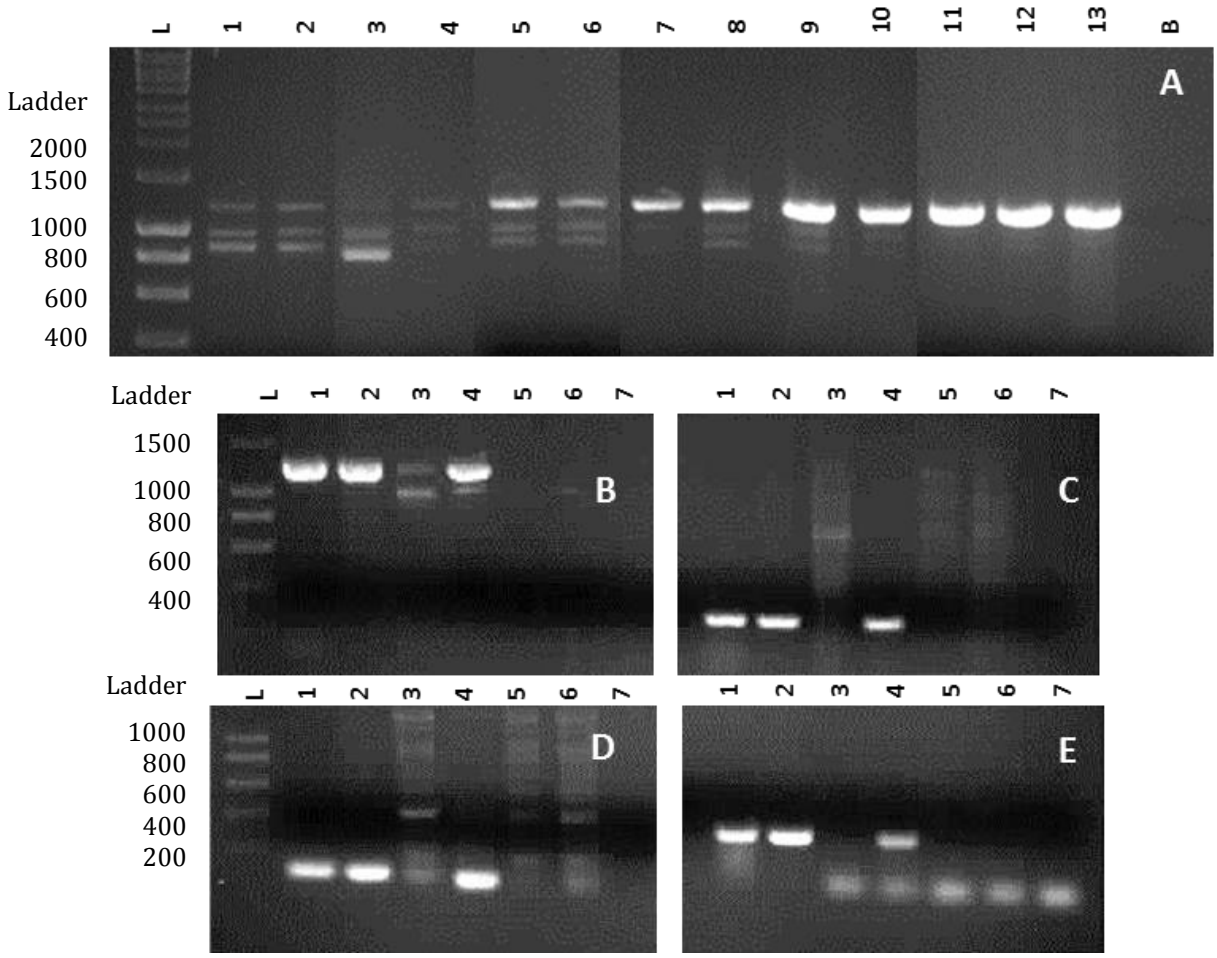


Figure 4-7 Molecular quantification of DNA and RNA isolated from infected larva. DNA isolated from individual larva infected with specified bacterial inoculum sizes were used as templates in a *fur* specific PCR (A). Lanes contained H1 DNA ladder (L), duplicate larva infected with 10^4 bacteria (1+2), 10^5 bacteria (3+4), 10^6 bacteria (5+6), 10^7 bacteria (7+8), 10^8 bacteria (9+10) and 10^9 (11+12). *S. aureus* chromosomal DNA (13) and water (B) were included as positive and negative controls respectively. Larva isolated DNA was also used to assess the specificity of four primer pairs; *fur* (B), *nuc* (C), *gyr* (D) and 16S (E). Lanes contained H1 ladder (L), chromosomal DNA (1+2; positive controls), larva infected with 10^4 bacteria (3), 10^9 bacteria (4), PBS injected duplicate larva (5+6; negative control) and water (7; negative control). Non-specific binding was observed in *fur*, *nuc* and *gyr* PCRs due to the presence of contaminating larva DNA and were unsuitable for use in qPCR analysis. A single band is observed with the 16S rRNA primer set and was therefore used in further analysis by qPCR.

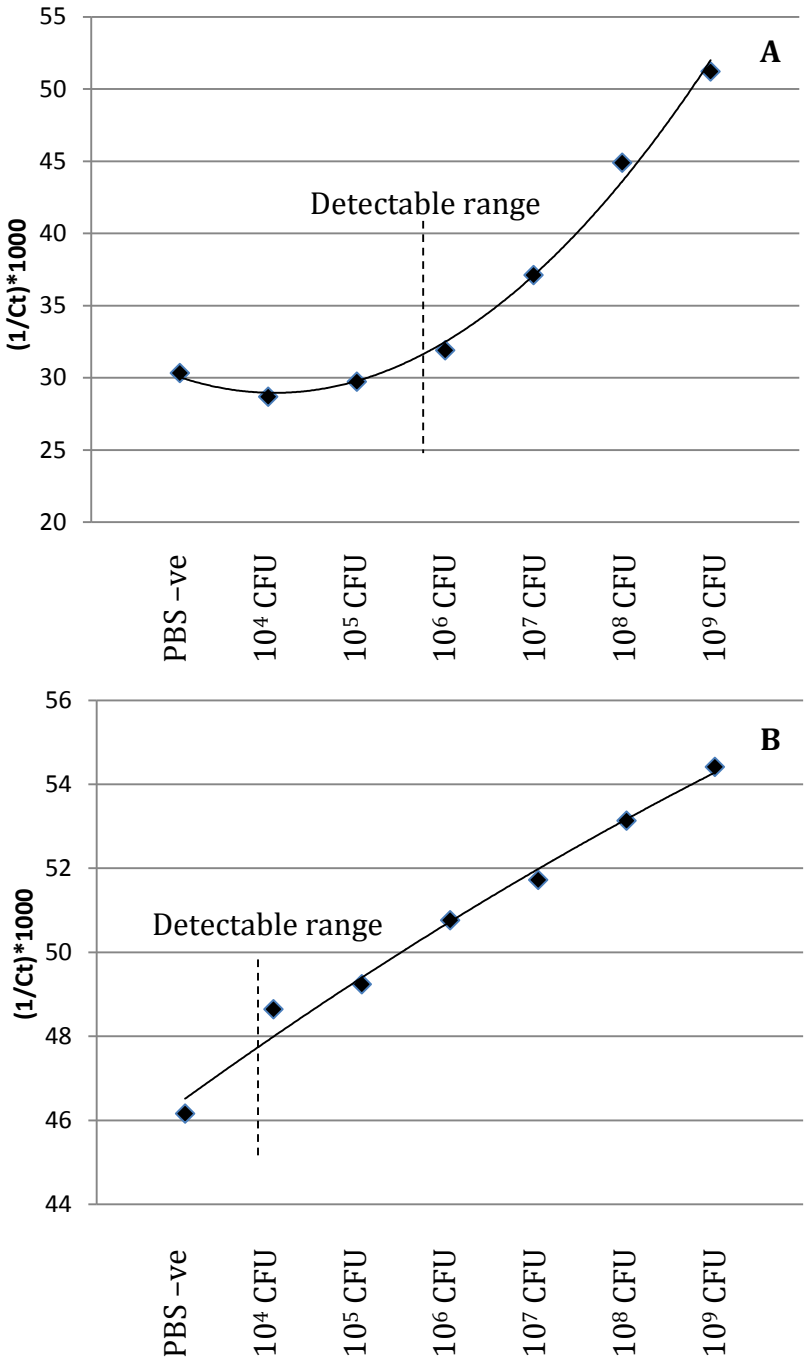


Figure 4-8 Assessment of qPCR and qRT-PCR using DNA (A) and RNA (B) isolated from *S. aureus* infected *G. mellonella* larva. DNA (A) and RNA (B) template types were assessed for suitability in estimating bacterial burden via 16S rRNA quantification. Mean Ct values were calculated from three independent experiments with duplicate larva. These were inverted and multiplied by 1000 to give a manageable value for comparisons. Data is plotted on a scatter graph with a closest fit curve.

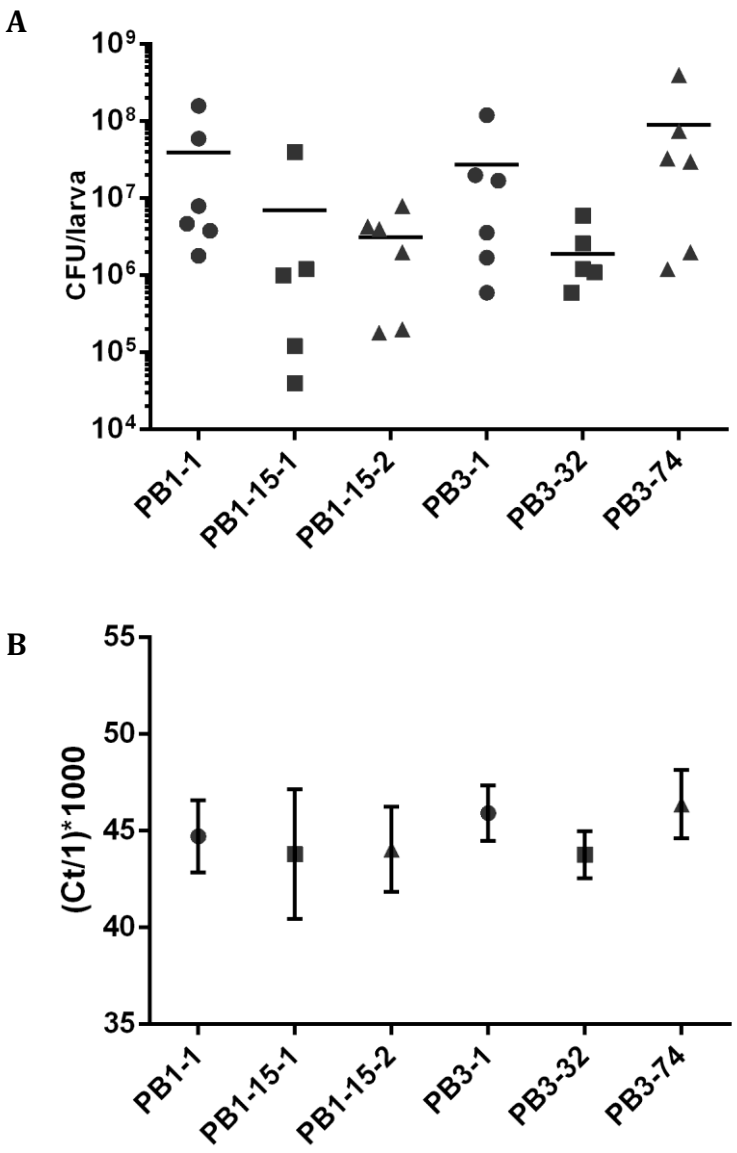


Figure 4-9 Bacterial burdens 48 hours post infection- a comparison of methodologies. Bacterial CFU were calculated from individual, homogenised larva, plotted (dots) on a scatter plot using a $\log_{10}$ scale, means are indicated with lines (A). Total RNA was also extracted from these homogenised larva and used for qRT-PCR as an alternative method to estimate relative bacterial burdens via quantification of 16s rRNA. Ct values were inverted and multiplied by 1000 to give a manageable value for comparisons; means plotted +/- one standard deviation (B). Duplicate larva were analysed for each of the three independent repeats and two technical qRT-PCR repeats were completed for each RNA sample. No significant differences, as calculated by one way Anova, were found between PB1 and PB3 initial and persistent isolates in regards to CFU or qRT-PCR analysis.

PB1 and PB3 isolates were the only isolates investigated further as they exhibited differences in virulence. RNA was isolated from larva 48 hours post inoculation and used for qRT-PCR, in parallel to traditional CFU plating onto selective media (Figure 4-9). One way Anova analysis showed there were no significant differences between the bacterial burdens of isolates within the PB1 or PB3 infections; either using traditional CFU counting or through qRT-PCR analysis.

Overall, the PB1 and PB3 persistent isolates exhibit attenuated virulence which was not explained by differences in bacterial burdens, as shown by two different methodologies. Therefore this trait can be presented as a true persistence-associated determinant in the PB1 and PB3 infections.

4.5 Gentamicin induced persister assay

It has been hypothesised antibiotic exposure can induce a phenotypic switch within individual *S. aureus* cells leading to a "persister phenotype" (Johnson & Levin, 2013). These cell types appear to be indistinguishable from the SCV phenotype where an accumulation of genetic errors leads to arrested cell cycles and dormancy during antibiotic treatment. Once treatment ceases these cell types return to wild type behaviours. Due to the implication of "phenotypic switching" within persistent infections, the effect of antibiotic exposure was investigated. Gentamicin was chosen as the test antibiotic because all clinical isolates were shown to be gentamicin sensitive.

4.5.1 No association between the ability to tolerate gentamicin therapy and persistence

To assess gentamicin survival duplicate bacterial cultures were incubated with or without a lethal dose of gentamicin (10x MIC) and CFU were plated after 6 hours exposure. CFU values with gentamicin were divided by CFU values without gentamicin and expressed as per cent mile values (pcm; 1 part in 100 000). No pattern was observed between any of the isolates in their ability to survive lethal doses of gentamicin (Figure 4-10). In fact, significant variation can be seen between isolates and also between repeats for the same isolate. This demonstrates the highly

variable responses exhibited by sensitive bacterial populations to antibiotic exposure.

4.5.2 Exposure to lethal levels of gentamicin leads to three distinct colony types in surviving bacterial sub-populations

In addition to counting CFU from non-selective agar plates, the effect of gentamicin exposure on colony appearance was assessed. Exposure to gentamicin induced two colony forms in all isolates, a large, pale colony which is typical of the wild type appearance, and a pin-prick sized, colourless colony type, which is likely a type of SCV which has been experienced in previous studies (Massey et al., 2001). An additional colony type was seen for all isolates except PB5, which was bright yellow/orange; this may indicate a mutation in SigB which is responsible for pigment production i.e. staphyloxanthin expression (Giachino et al., 2001). Pictures of example plates can be seen in Figure 4-11 and all different colony forms were confirmed as *S. aureus* via *S. aureus* specific PCR (*nuc*) and 16S rRNA targeted DNA sequencing (data not shown).

All three colony types were found to be transiently tolerant of gentamicin as no colony tested was able to survive sub-culturing onto gentamicin containing plates. Additionally, overnight incubation in non-selective broth was sufficient for each colony type to revert back to wild type form, signifying instability. Overall, gentamicin exposure is shown to induce multiple transient changes in bacterial populations that correlate with changes in colony morphology, potentially allowing for antibiotic tolerance.

To assess the effect of solid phase exposure to gentamycin, *S. aureus* cultures grown with and without gentamicin were plated onto gentamicin containing plates. Only the PB1 (PB1-15-1/2) and PB3 (PB3-32-1) persistent isolates were shown to grow on agar plates containing gentamicin. Additionally, there were significantly lower survival numbers on gentamicin containing plates after liquid phase pre-exposure to gentamicin compared to unexposed controls (Figure 4-12). The three different colony forms previously described were again observed in this assay, but surviving colonies remained pseudo-resistant to gentamicin after sub-culturing onto gentamicin containing plates. These colonies types were also tested for their

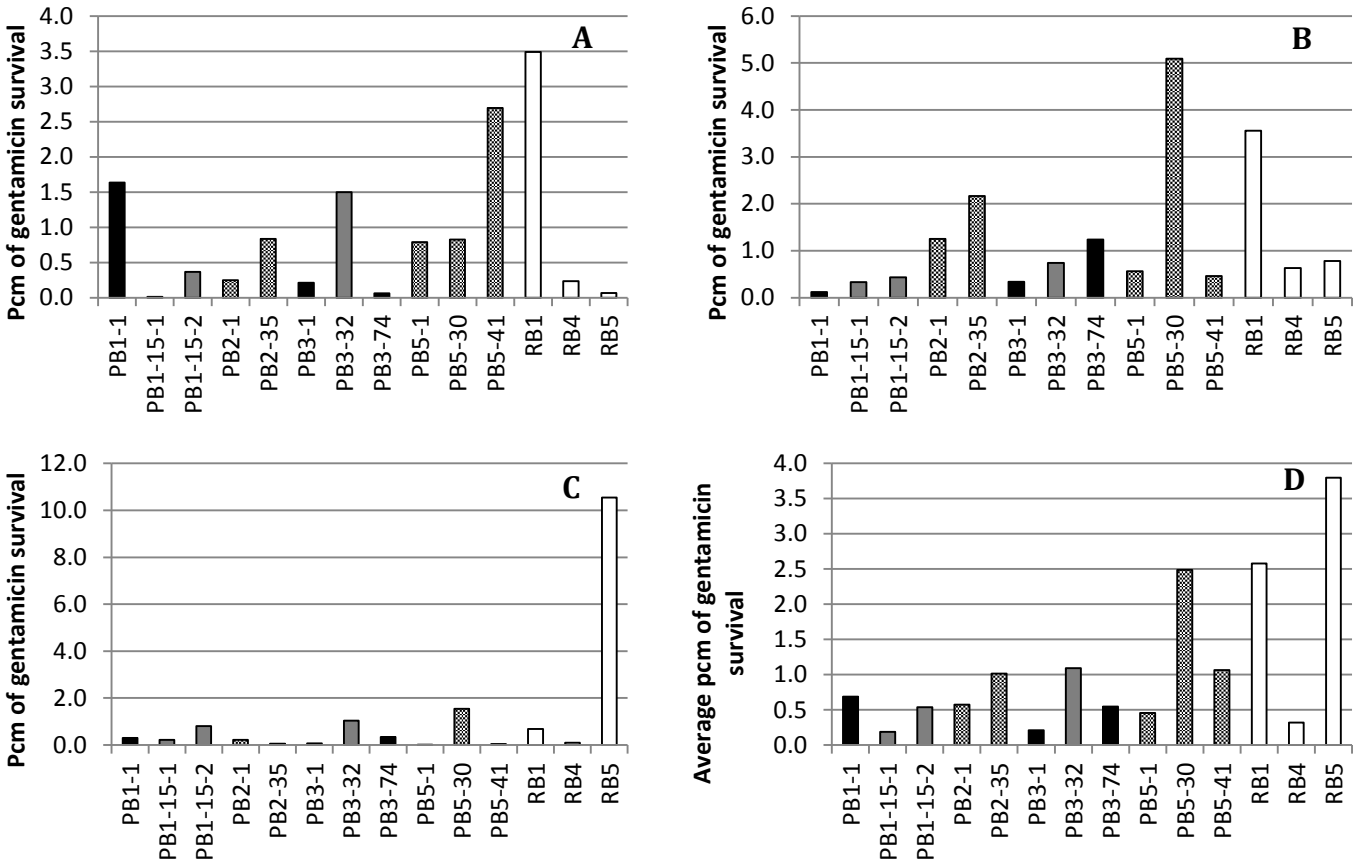


Figure 4-10 Pcm (per cent mile) survival after 6 hours exposure to 10x gentamicin MIC. Duplicate cultures of exponential phase bacteria were incubated for 6 hours shaking at 37 °C, one set with TSB (positive control), the other set with TSB + 10x of gentamicin MIC (10 µg/ml). CFU were counted from non-selective media and numbers from TSB + gentamicin cultures were divided by TSB control cultures. Per cent mile (pcm) figures are plotted for each of the three repeats (A, B and C) and the calculated means of three repeats (D).

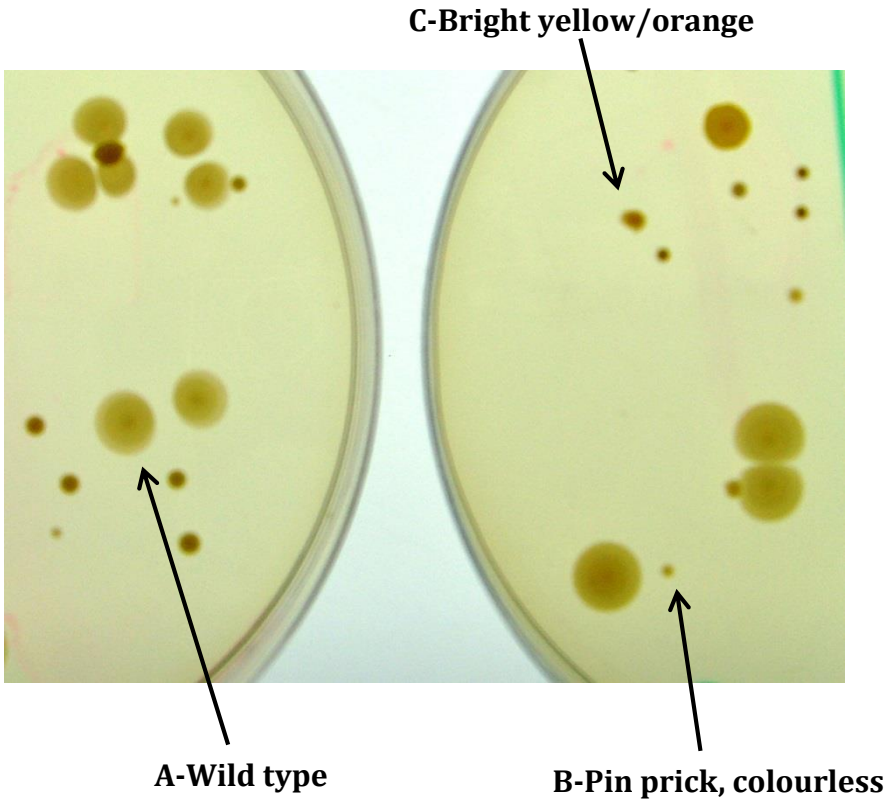


Figure 4-11 Example plate containing different colony types post gentamicin exposure. Exponential phase bacterial cultures were exposed to 10x gentamicin MIC in TSB for 6 hours, serially diluted and plated onto non-selective media (LA). The colony appearances were recorded and pictures taken. Experiments were repeated independently three times to confirm reproducibility. Up to three colony types were observed, wild type colonies (A- large and pale), pin prick colourless colonies (B) and bright yellow/orange colonies (C).

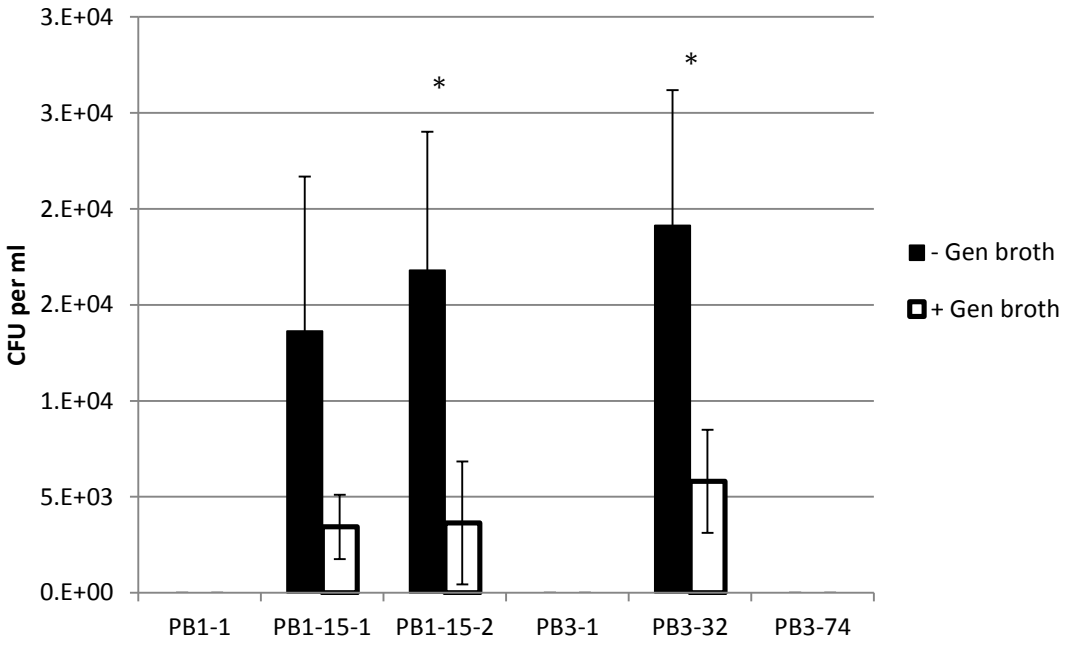


Figure 4-12 A comparison of CFU values from gentamicin containing plates after 6 hours incubation in TSB or TSB + 10x gentamicin MIC. 6 hour bacterial cultures with and without 10x gentamicin MIC were plated onto gentamicin containing plates of the same concentration. Mean CFU from three independent experiments were plotted +/- one standard deviation. T- Tests were used for statistical analysis and indicated by *.

ability to revert back to wild type behaviours after overnight incubation in non-selective broth. Isolates were shown to revert but the rate was not 100 %; in fact rates were so variable that this process appeared to be due to chance in the absence of any selective pressure (data not shown).

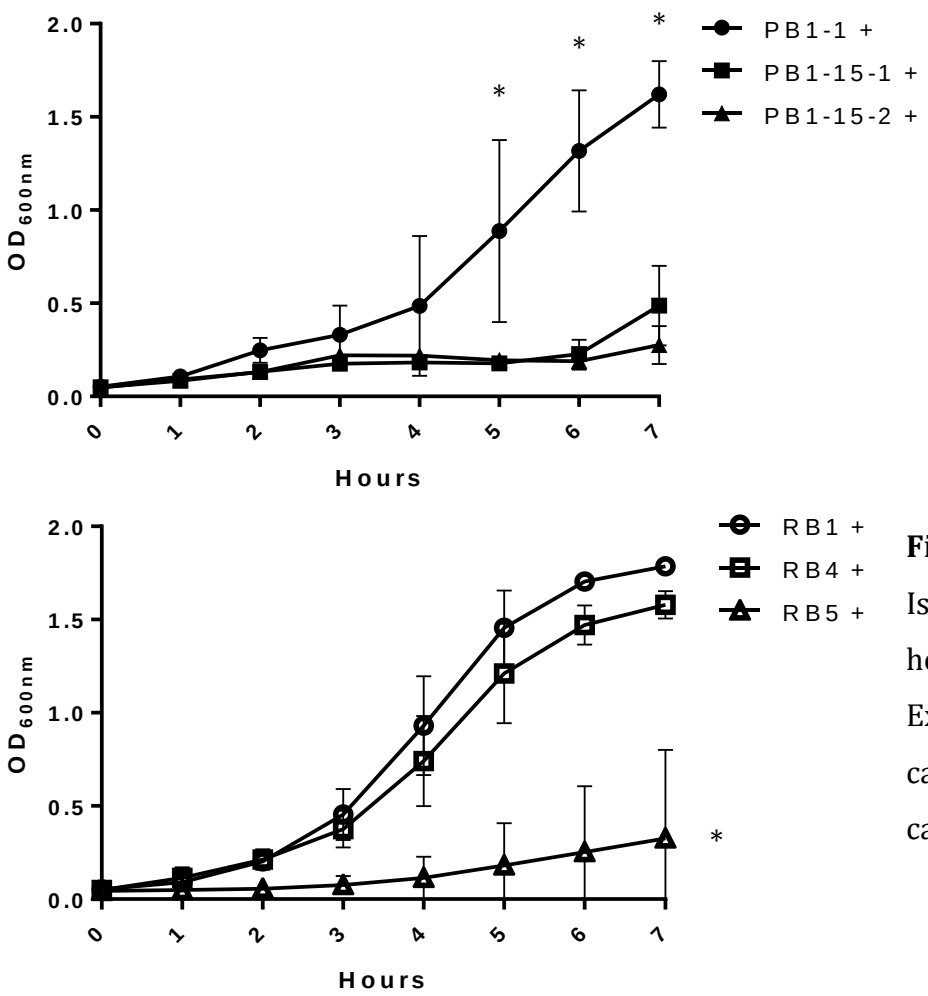
These results suggest that there is a background bias within the PB1 and PB3 persistent isolates for the formation of these different colony types in response to solid phase gentamicin exposure, possibly indicating a pseudo-mutator phenotype within these isolates. Therefore it can hypothesised genetic predisposition exists within PB1/PB3 persistent isolates which is associated with persistence development.

4.6 Exposure to environmental stressors

The host environment is a very toxic place for infective species and as a consequence pathogenic bacteria have developed their own ways of tolerating stressors encountered whilst within the human body and out. Here, the effect of exposure to additional external stimuli was assessed to investigate a potential involvement of bacterial stress response systems during persistence. Specifically, the effect of oxidative stress and toxic metal concentrations on the isolates' growth behaviours was analysed. Only PB1 and PB3 isolates were used in further analysis as these strains displayed differential phenotypes when exposed to solid phase gentamicin in the previous section.

4.6.1 PB1 persistent isolates and RB5 showed increased sensitivity to hydrogen peroxide

S. aureus isolates were incubated in TSB with or without the presence of H_2O_2 and OD_{600nm} were recording hourly to assess the effect of oxidative stress on bacterial growth. Growth without H_2O_2 was identical between all isolates tested (data not shown) as stated previously (Chapter 3:5:2). Interestingly, the PB1 persistent isolates (PB1-15-1/2) experienced significant growth defects in the presence of H_2O_2 (Figure 4-13) compared to their respective initial isolate (PB1-1) at 5, 6 and 7 hours ($P < 0.0001$), but recovered by 24 hours post inoculation (data not shown).



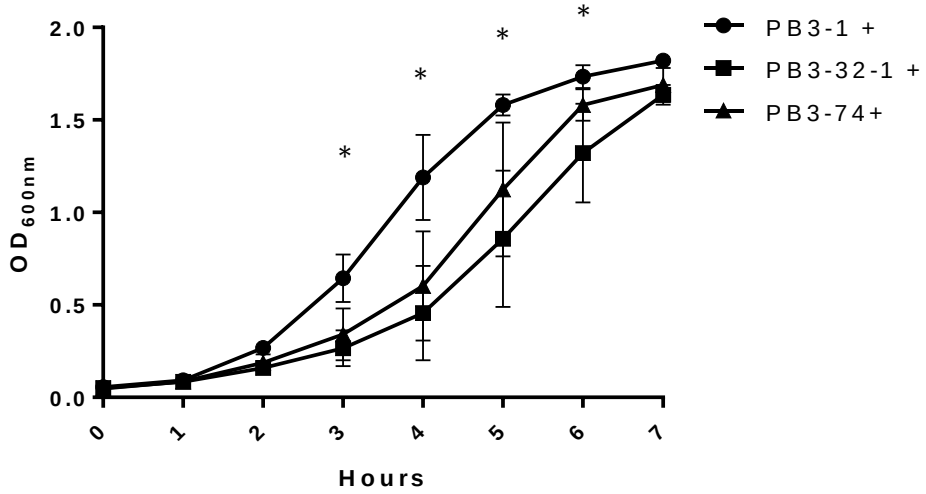


Figure 4-13 Growth analysis in TSB in the presence of H_2O_2. Isolates were incubated shaking in TSB + 0.003 % H_2O_2 at 37 °C for 24 hours where OD_{600nm} was recorded every hour for 7 hours. Experiments were repeated independently three times, means calculated and plotted +/- one standard deviation. Significance was calculated using multiple student T-Tests and indicated by *.

To a lesser extent, the PB3 persistent isolate (PB3-32-1) was found to have significantly lower values compared to the PB3 initial isolate (PB3-1) at 3 ($P < 0.01$), 4 ($P < 0.0001$), 5 ($P < 0.0001$) and 6 ($P < 0.01$) hours, but displayed similar values post 7 hours. This data suggests persistent isolates from the PB1 and PB3 infections take longer to adapt to oxidative stress compared to their initial isolates, but do eventually recover after 7 hours. RB5 also experiences a growth deficit in the presence of H_2O_2, whereas RB1 and RB4 have growth curves comparable to the PB1 and PB3 initial isolates.

4.6.2 PB1 and PB3 persistent isolates show improved tolerance to toxic levels of iron

To further analyse the potential involvement of bacterial stress response in the development of persistence, the isolates' growth characteristics were optically assessed after 24 hours incubation with and without toxic levels of iron and copper (Figure 4-14). CRPMI was chosen as the incubation media as it is more reflective of the host environment in which isolates persisted.

Firstly, the persistent isolate specific 24 hour growth advantage in CRPMI is clearly observed in this data, as mentioned previously (see Chapter3:5:2). In regards to the effect of toxic copper concentrations, initial and persistent isolates both exhibited significant growth reductions resulting in overall comparable values. Therefore copper tolerance does not show a persistence association.

In contrast to all other isolates, RB1 shows a significant increase in growth when exposed to high copper levels. Previous protein analysis (Chapter 3:7:3) found an unusually high amount of cell surface associated multi-copper oxidase (Mco) in RB1 (Figure 4-15A). The encoding gene (*mco*) is found on plasmids in certain *S. aureus* strains either integrated into the genome or freely replicating (Gómez-Sanz et al., 2013). Genetic analysis of chromosomal and plasmid DNA preparations (Figure 4-15B) showed all test isolates except RB5 contain the *mco* gene; but interestingly RB1's *mco* copy is located on a freely replicating plasmid and is likely multi-copy. This would explain the increased amount of Mco on the bacterial cell surface and the improved copper tolerance displayed by RB1.

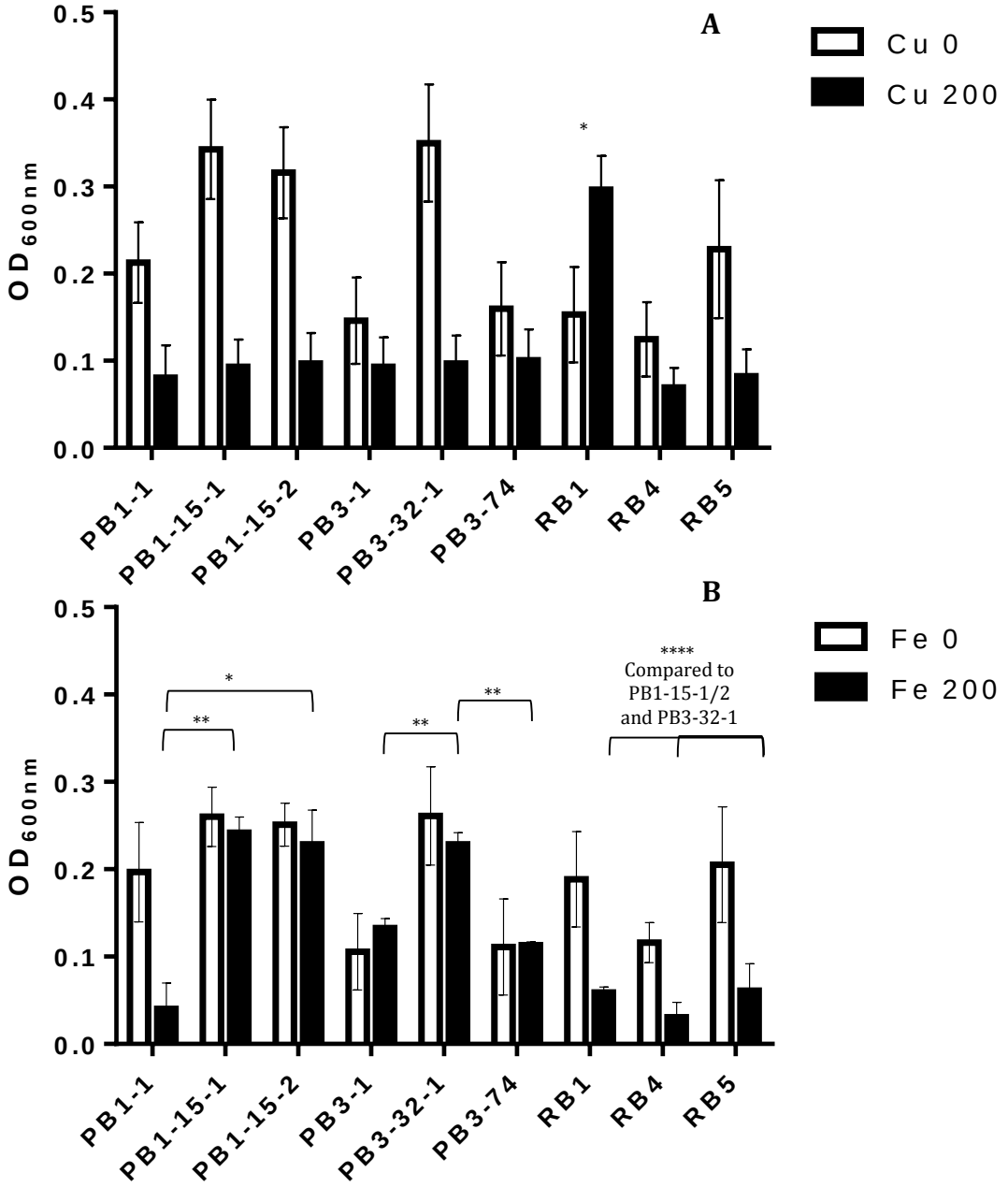


Figure 4-14 24 hour growth values after incubation in CRPMI + $CuCl_2$ or $FeSO_4$. Isolates were incubated in CRPMI for 24 hours at 37 °C in 5 % CO_2 with or without 200 µM $CuCl_2$ (A) or 200 µM $FeSO_4$ (B). OD_{600nm} were recorded, means calculated from three independent experiments and plotted +/- one standard deviation. Statistical analysis was conducted using the student T-test and significance is indicated by *.

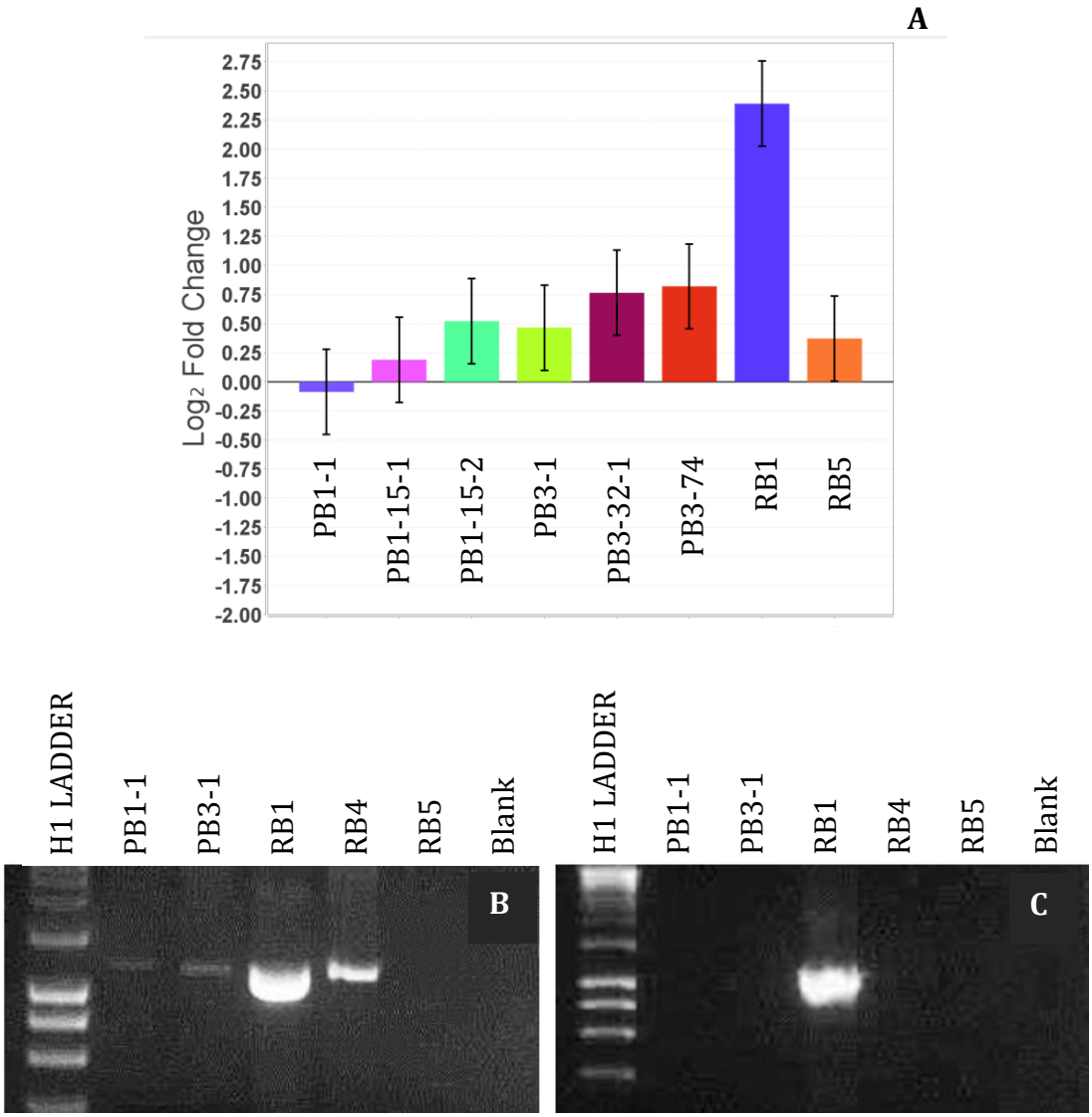


Figure 4-15 Protein and DNA quantification of multi-copper oxidase between isolates. Protein preparation and iTRAQ LC-MS analysis was conducted as stated elsewhere (Chapter 2:9:2). Log_2 fold changes calibrated to PB1-1 for Mco were plotted with 95 % confidence intervals using Scaffold 4 software (A). Genomic DNA (B) and plasmid DNA (C) preparations were used as templates for *mco* targeted PCR. PB1-1, PB3-1, RB1 and RB4 display *mco* positive PCR bands from gDNA preps. Only RB1 displayed a plasmid specific *mco* band indicating *mco* is located on a freely replicating plasmid and therefore likely to be multi-copy.

In regards to the effect of toxic iron concentrations, it appears the persistent isolates from both the PB1 and PB3 infections display increased iron tolerance compared to their respective initial isolates. Statistical analysis of the 200 μM iron growth values showed PB1-1 was significantly lower than PB1-15-1 ($P < 0.01$) and PB1-15-2 ($P < 0.05$) and PB3-1/74 were significantly lower than PB3-32-1 ($P < 0.01$). Additionally, resolved isolate data at 200 μM iron was significantly lower than the PB1 and PB3 persistent isolates ($P < 0.0001$).

Overall, these data suggests both the PB1 and PB3 infecting strains developed improved iron tolerance which is observed in PB1 and PB3 persistent isolates. This is the first time metal tolerance has been investigated in regards to persistent isolates, highlighting the novelty of this finding.

4.7 Discussion

This chapter aimed to further investigate *S. aureus* persistence by assessing specific candidate virulence determinants and potential persistence mechanisms for an association with these persistent infections. Consistent with the previous chapter, isolates from the PB1/PB3 infections displayed clear signs of *in vivo* adaptation; whereas PB2/PB5 isolates did not exhibit any defining characteristics and therefore will not be discussed in detail in this section. In addition to the PB1/PB3 persistence traits presented in Chapter 3, several virulence phenotypes were discovered to be persistent isolate associated, including increased biofilm formation, decreased virulence in a *G. mellonella* model, increased H_2O_2 sensitivity and improved iron tolerance. Several traits were not experienced in resolved isolates demonstrating *in vivo* adaptation specifically in response to persistence development. Others did not exhibit clear phenotypic patterns between persistent and resolved infections and were therefore deemed to be evolved *in vivo* but not associated with persistence due to substantial strain variation observed. The stability of these persistence associated phenotypes provides evidence to suggest a genetic basis of persistence, particularly for the PB1 and PB3 infections where similar phenotypic patterns are observed.

4.7.1 Biofilm formation and intracellular eukaryotic cell persistence are not involved in *S. aureus* persistence cases characterised in this study

Biofilm formation cannot be associated with persistence in this study due to the high level of strain variability observed. This is in contrast to previous studies presenting an association between biofilm formation and persistence in *S. aureus* in combination with HDP/CAMP resistance (Seidl et al., 2011). However, both the PB1 and PB3 infecting strains exhibited improved glucose mediated biofilms; consequently this trait can be described as a general infection characteristic, but not directly related to persistence development. Previous research showed exposure to vancomycin improved biofilm formation and fibronectin adhesion in persistent *S. aureus* isolates via SarA regulation (Abdelhady et al., 2014). Therefore it is possible the biofilm phenotype observed in this study is a result of antibiotic prescription during the infections, in particular exposure to vancomycin and/or daptomycin.

Fibrinogen and fibronectin binding proteins (FnBP, Efb) are known mediators of glucose induced biofilm formation (O'Neill et al., 2008) and were therefore investigated for a potential role in the glucose mediated biofilm phenotype. Despite this, the observed increase in biofilm formation was not explained by a parallel increase in fibronectin or fibrinogen adhesion. These data suggests there are additional biofilm mediators induced in response to glucose in addition to fibronectin/fibrinogen binding proteins. Proteomic analysis after glucose incubation implicated two further cell surface associated proteins for a role in glucose induced biofilm formation. Sbi and leukocidin-like proteins were over represented on the cell surface in persistent isolates in response to glucose as shown by 1D SDS-PAGE analysis (Chapter 4.2.3), of which Sbi also exhibited persistent isolate specific increases after nutrient restricted growth (Chapter 3:7:3). As previously stated Sbi is a well characterised Staphylococcal IgG binding protein involved in immune system evasion via adhesion to multiple host factors and subsequent interruption of complement (Zhang et al., 1998; Zhang et al., 1999; Haupt et al., 2008; Smith et al., 2011). Leukocidin proteins mediate lysis of eukaryotic host cells, particularly polymorphonuclear leukocytes (PMN) via the formation of pores on the cell membrane (Kaneko & Kamio, 2004; Yanai et al., 2014). These two cell surface associated proteins have not been previously linked to

biofilm formation, potentially suggesting a novel mechanism of biofilm formation in *S. aureus*.

Likewise, invading and persisting intracellularly within eukaryotic host cells is not associated with persistent infections in this study. In fact, PB1 and PB3 persistent isolates displayed decreased cell numbers able to persist intracellularly within a rat myocardium cell line compared to their initial isolates. These data conflicts with the previous suggestion that intracellular localisation is a major persistence mechanism (Garzoni & Kelley, 2009; Clement et al., 2005); it is likely this mechanism is observed more frequently in cases involving SCV, which are adapted cell types for this process (Sendi & Proctor, 2009; Tuchscherr et al., 2010; von Eiff et al., 1997). The persistent isolate associated reduction in intracellular numbers may suggest an alteration in bacterial fitness, possibly affecting their responses to environmental stressors.

In response to antibiotic pressure on solid media specifically, PB1 and PB3 persistent isolates exclusively formed various mutated colony types that remained transiently tolerant during subsequent sub-culturing. Furthermore, persistent isolates displayed increased sensitivity to H_2O_2 and decreased colony growth specifically on high salt media after larvae *in vivo* incubation. Importantly, colony sizes were identical between initial, persistent and resolved isolates when larva homogenates were plated onto non-selective media such as horse blood agar, thereby excluding the possibility of SCV involvement. This data suggests the persistent isolates developed increased sensitivity to environmental factors, such as antibiotic exposure, oxidative stress and osmotic pressures, indicating a possible fitness alteration. The two component regulator, KdpDE, has been proposed to be involved in both oxidative and osmotic pressures in additional to its primary role in potassium ion homeostasis (Freeman et al., 2013). *S. aureus kdpD/kdpE* knock-outs show attenuated survival in human blood and macrophages (Zhao et al., 2010). Moreover, research using *Salmonella typhimurium* showed mutations in *kdpD/kdpE* led to diminished infection capabilities in a *Caenorhabditis elegans* model and also decreased survival during macrophage assays (Alegado et al., 2011). Interestingly, this two component system is positively regulated by Fur in high iron conditions (Johnson et al., 2011), which was implicated in the previous chapter for an involvement in persistence development. Moreover, Fur is a known oxidative stress

modulator in *S. aureus* via the up-regulation of catalase and control of iron uptake, providing more evidence for the involvement of Fur in this study (Horsburgh, Ingham, et al., 2001).

4.7.2 Novel *in vivo* evolved persistence phenotypes include attenuated virulence and improved tolerance to toxic iron concentrations

Both the PB1 and PB3 persistent isolates displayed true persistence associated attenuated virulence using a *Galleria mellonella* larvae based model of infection, which is supported by comparable bacterial burdens between all isolates. *G. mellonella* larva (Figure 4-16) are proven to be effective models when assessing human pathogens such as *Campylobacter*, *Listeria*, *Pseudomonas* and *Candida* (Senior et al., 2011; Lionakis, 2011; Joyce & Gahan, 2010; Lorè et al., 2012). They are ideal to use in these situations as they possess similar humoral and cellular immune response systems to humans, they can be maintained at 37°C and do not carry the ethical restraints that are associated with vertebrates (Cook & McArthur, 2013; Hoffmann, 1995; Lionakis, 2011). Interestingly, *in vitro* assessment of virulence expression, specifically Agr activity, haemolysin and nuclease secretion (Chapter 3:6) did not indicate a difference in virulence between the isolates. It is possible additional virulence determinants, which were not assessed during this study, exhibit differential activity *in vivo* contributing to the observed attenuated virulence exhibited by persistent isolates.

Reduced larvae mortality has been experienced previously in *S. aureus* persistence cases involving SCV (Gao et al., 2010), persistent cystic fibrosis *Pseudomonas aeruginosa* isolates (Lorè et al., 2012) and also in parallel with increasing vancomycin MIC in *S. aureus* isolates (Peleg et al., 2009). This is the first time however attenuated virulence, in regards to *S. aureus* persistence, has been observed in phenotypically wild type strains, implying that it is an exclusive trait commonly found among persistent *S. aureus* isolates. Logically, reducing virulence would limit the potential impact exoprotein secretion would have on host viability and also avoid alerting the host immune system to the presence of an infection (Chalmers & Hill, 2012), thereby enabling a long-term

Figure 4-16 *Galleria mellonella* larva image. The image depicts two *G. mellonella* larvae. On the top left is an infected, deceased larva as shown by the dark discolouration and flatten appearance; on the bottom right is a healthy, viable larva with light colouration. A size scale of 5 mm is indicated.

infection. Inactivation of Fur in *S. aureus* has shown to attenuate virulence in murine models, although this may be in part due to decreased bacterial burdens in the *fur* mutants (Horsburgh, Ingham, et al., 2001; Torres et al., 2010). However, the effect of *fur* inactivation in *Galleria* models has not been studied previously and therefore would need to be investigated for any definite conclusions to be made.

As previously stated the persistent isolates had difficulty recovering on high salt media post *in vivo* larva incubation. This phenotype was not observed on non-selective media suggesting a fitness disadvantage when faced with this type of stress, rather than formation of SCV *in vivo*. Subsequent sub-cultures using the same high salt media showed persistent isolates eventually recovered (~3 sub cultures; data not shown); additionally persistent isolates did not display this phenomenon when plated from *in vitro* cultures onto high salt media. This data indicates the salt sensitivity observed is at least partially transient and developed as a result of *in vivo* adaption.

The second persistence associated phenotype found during analysis included improved survival against toxic iron levels compared to both initial and resolved isolates. This is the first time iron sensitivity has been investigated in regards to *S. aureus* persistence, or indeed in other bacterial species, and could indicate an important trait of persistent isolates. Iron is an essential component of many cellular processes due to its role as an electron carrier, but this attribute can also make it extremely toxic to cells via the formation of superoxide anions and hydroxyl radicals (Schaible & Kaufmann, 2004). Therefore free iron is tightly regulated in the human body by sequestering it into protein complexes; specifically, plasma located iron is transferrin bound which can then be utilised by erythrocytes to form haemoglobin (Schaible & Kaufmann, 2004). Macrophages are responsible for the digestion of old erythrocytes for the purpose of recycling iron; consequently within these cells is the highest concentration of free iron that can be found in the human body (De Domenico et al., 2008). Increased iron tolerance would theorectically enable better survival in a macrophage environment, however this study has shown persistent isolates are more sensitive to oxidative stress, a trait which would negate this theoretical adaptation to the macrophage niche. Elsewhere in the host the availability of free iron is kept low and meaning that the infecting organisms did not

encounter high levels of iron often during each of the infections. Therefore it is unlikely this improved iron tolerance is a direct adaption trait, but it could be an indication that the regulators involved in iron homeostasis and oxidative stress are altered in these isolates.

4.7.3 Potential involvement of Fur in persistence associated virulence phenotypes

In high iron conditions, Fur represses iron import into the cell through down-regulation of siderophores and iron uptake transporters; but it also positively regulates catalase expression (*katA*) thereby influencing the oxidative stress response (Horsburgh, Ingham, et al., 2001). PerR is another metal ion (manganese) dependent repressor, which specifically down-regulates the expression of iron storage proteins (ferritin etc), oxidative stress proteins (*katA*, *trxB*) and Fur itself (Horsburgh, Clements, et al., 2001). De-repression of the PerR regulon is observed in the presence of peroxides identifying PerR as a peroxide sensor (Horsburgh, Clements, et al., 2001). Figure 4-17shows a simplistic diagram of this regulation.

Throughout this chapter, certain persistence phenotypes show a link with the Fur regulon such as oxidative stress sensitivity, iron tolerance and attenuated virulence. Additionally in Chapter 3, proteomic data included many Fur regulated proteins; however the expression pattern observed for these proteins did not fully fit with Fur directly gene regulation. Consequently, this study presents an alternative Fur/PerR regulation model involving *fur* dysfunction in the persistent isolates (Figure 4-18). Inactivation of *fur* would lead to de-repression of the PerR regulon via increased intracellular iron levels and peroxides. Improved iron storage would allow the persistent isolates to survive at higher iron concentrations but as there would be no Fur directed up-regulation of *katA*, these isolates would be more sensitive to oxidative stress.

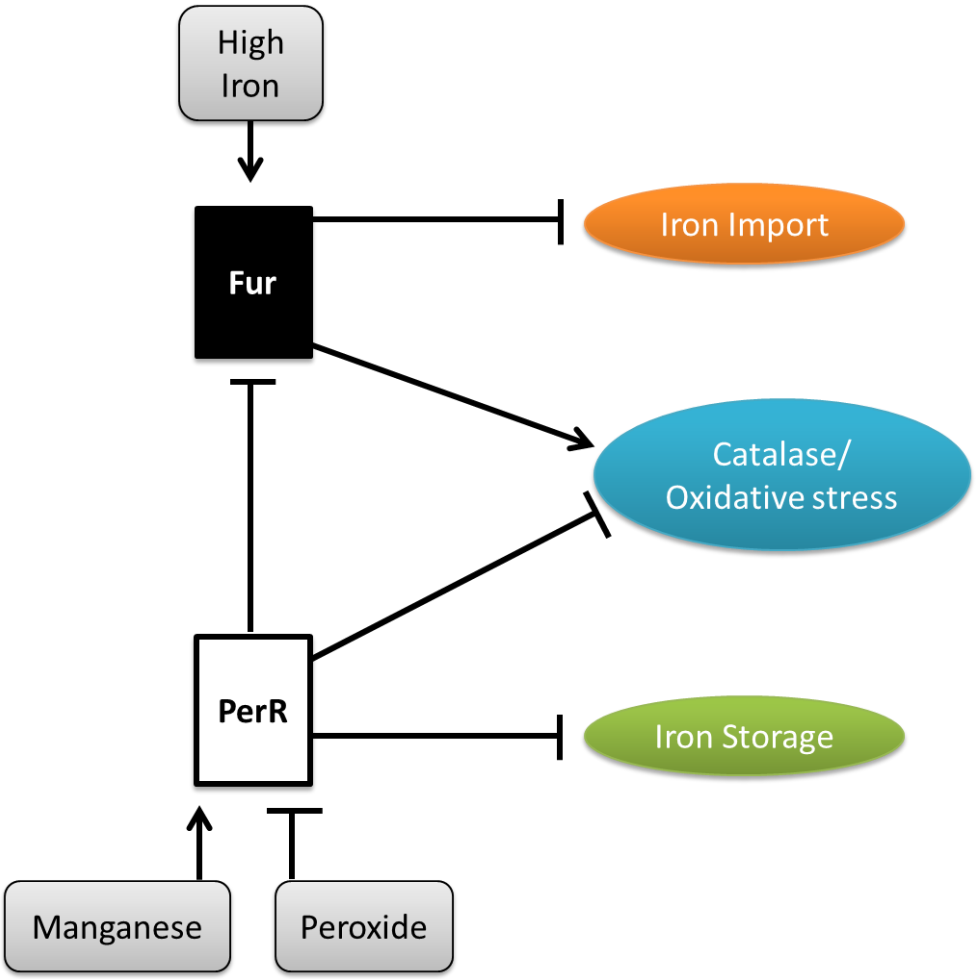


Figure 4-17 Simplistic interactions between Fur and PerR in regards to oxidative stress and iron homeostasis. Under high iron conditions Fur acts as a transcriptional repressor down-regulating iron uptake among other processes. It also up-regulates catalase (*katA*) expression. PerR represses the expression of iron storage systems such as ferritin and also oxidative stress genes (*katA*, *txrB*) in a manganese dependent manor. De-repression of the PerR regulon is observed in the presence of peroxides, suggesting PerR also acts as a peroxide sensor.

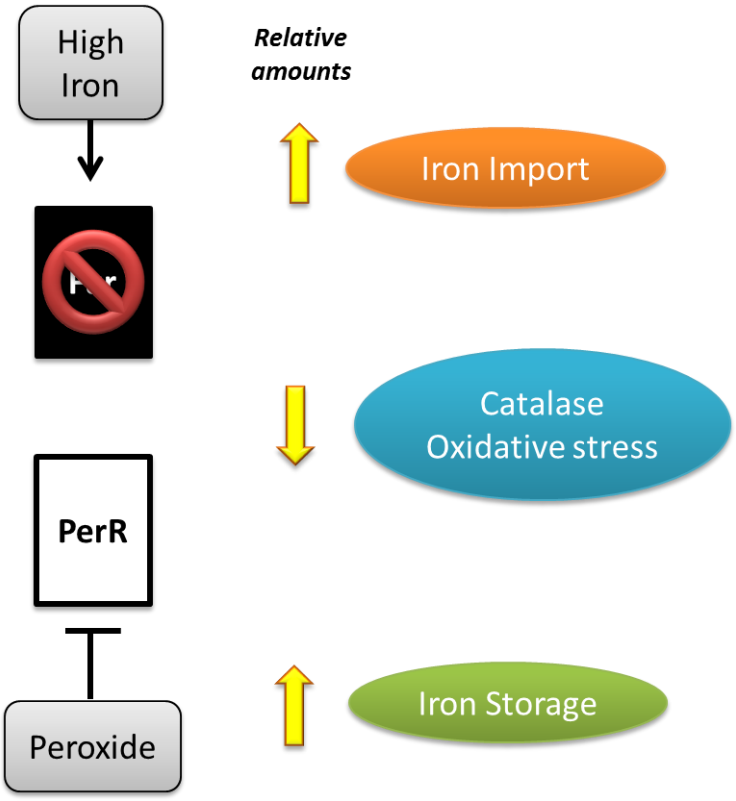


Figure 4-18 Hypothesised simplistic interactions between Fur and PerR gene regulators and subsequent consequences on oxidative stress and iron homeostasis in PB1 and PB3 persistent isolates. It is hypothesised PB1 and PB3 persistent isolates exhibit *fur* dysfunction; consequently there is no Fur directed repression on iron import. This leads to an increase in peroxide within the cell, thereby causing de-repression of the PerR regulon. Excess intracellular iron would be sequestered by the increase in iron storage thereby improving iron tolerance. Additionally, without the Fur directed up-regulation of *katA*, the persistent isolates are more sensitive to oxidative stress.

4.7.4 Conclusions

During this chapter four virulence specific traits were shown to be developed *in vivo* during both the PB1 and PB3 infections. Two of which were incidences of general infection evolution, specifically increased glucose media biofilm and increased H_2O_2 sensitivity. The remaining two traits namely attenuated virulence in an insect model of infection and increased tolerance to toxic iron levels, associated exclusively with persistent isolates (Figure 4-19). A genetic basis for persistence in this study is strongly implicated firstly due to the stability of the observed phenotypes exhibited within the PB1 and PB3 strains; but also because two independent infecting strains exhibit parallel evolutionary patterns, suggesting similar mutations have occurred in each instance. It has been hypothesised Fur may be involved but the proposed models are purely speculative and to fully investigate the potential involvement of Fur, knock-out *fur* mutations were attempted which are discussed in the next chapter. Additionally, it is possible other gene regulators or cellular processes could be responsible for the observed phenotypes, independent of *fur*, therefore whole genome sequencing was conducted on all strains. This is especially important for the PB2 and PB5 infections where no phenotypic differences have been acknowledged thus far; it would be interesting to see if any causative mutations for persistence could be identified in these cases.

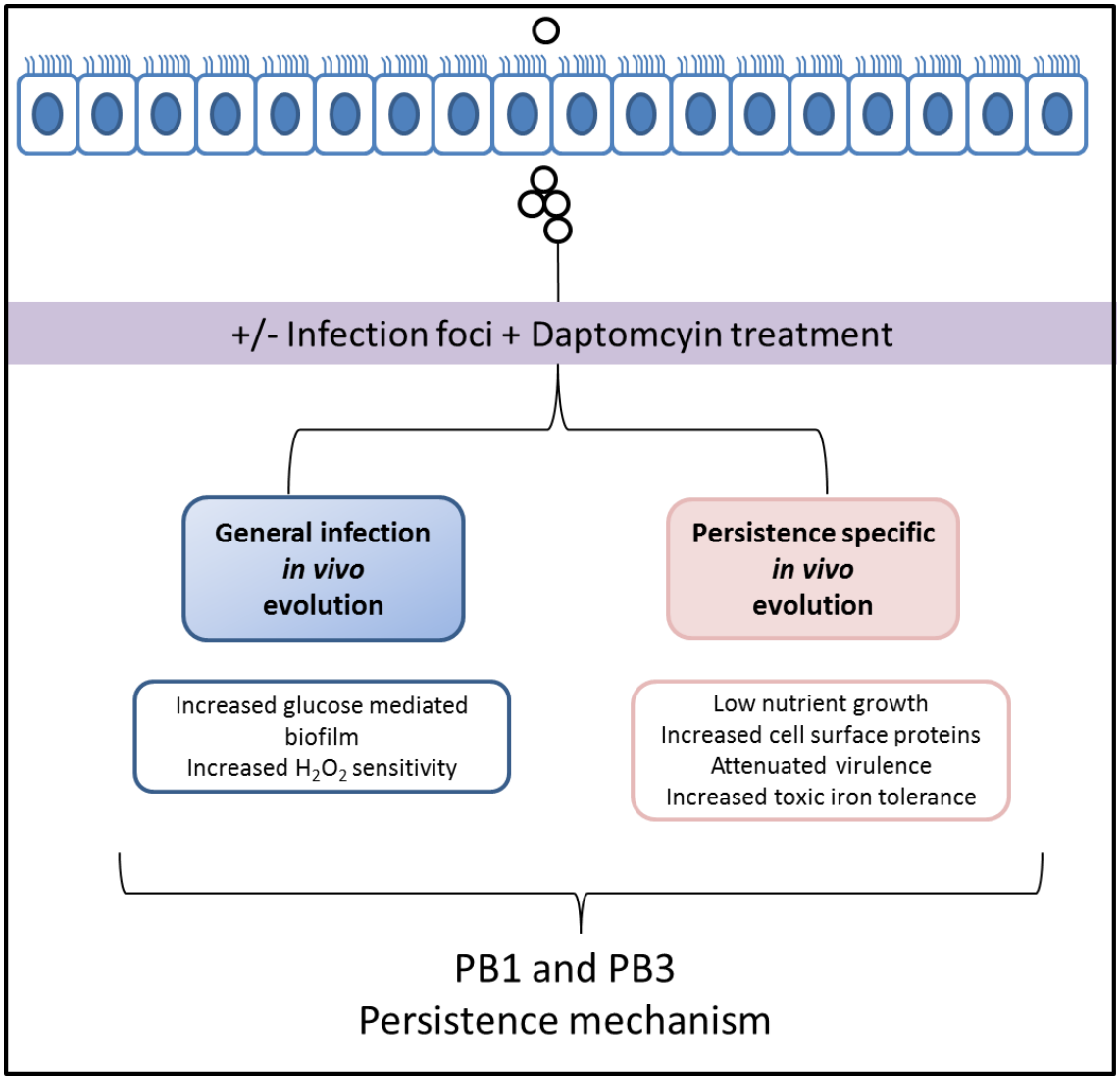


Figure 4-19 Current PB1 and PB3 persistence model after Chapter 4's data. This diagram highlights the two types of *in vivo* evolved traits, general infection evolution and persistence specific. Increased glucose mediated biofilm formation and increased H_2O_2 sensitivity were designated general infection traits as there was no clear pattern compared to resolved isolates. Attenuated virulence in a larva model of infection and increased iron tolerance are persistence specific traits as they are not observed in resolved isolates. These traits are in addition to the low nutrient growth adaptation and increased concentrations of certain cell surface associated proteins as shown in Chapter 3.

Chapter 5 Genetic Basis of Persistence- Mutant Construction and Comparative Genomics

5.1 Introduction

This study has so far determined several phenotypic traits and virulence determinants which exclusively associate with persistent *S. aureus* bacteraemia isolates compared to those from resolved bacteraemia. These traits were shown to be reproducibly stable through multiple repeat analyses suggesting the cause has a genetic basis and has arisen through genome evolution *in vivo*. Additionally, similar phenotypes are observed in two independent cases of persistence (PB1 and PB3), possibly indicating the same genetic network was affected in both persisting strains and this particular adaptation mechanism is a common pathway for persistence.

Genetic mutations have been previously implicated in persistence, mostly in reference to SCV development, including mutations within ribosomal proteins (*rpoB*), stress response systems (*relA*) and other biosynthetic pathways (*hemB*, *menD* etc.) (Gao et al., 2013, 2010; Proctor et al., 1995; von Eiff et al., 1997). It is currently unclear whether SCV are essential for persistence development, i.e. the causative mutation leading to the SCV phenotype is just circumstantial; or if that particular mutation is specifically required for persistence and the formation of SCV is an unavoidable side-effect.

A possible genetic basis for persistence was chosen to be investigated in this study by Illumina whole genome sequencing which aimed to identify persistence specific markers(s) within these persistent infections compared to resolved bacteraemia cases. Furthermore, candidate gene regulators implicated in persistence development during previous chapters were targeted for knockout mutations whilst the genome sequencing data was being processed. Previous analyses were repeated using these mutants to investigate possible associations with persistence.

5.2 Genetic manipulations of CC22 clinical isolates

The regulons of two global gene regulators exhibited significant overlap with data observed in previous chapters, namely the two component regulator system SaeRS and the global virulence regulator Fur (Chapter 3:8:5 & Chapter 4:7:3). Whilst Illumina genome sequencing was being processed (Chapter 5:4), knockout mutations within these regulator genes were attempted in several initial, persistent and resolved isolates characterised in this study. To our knowledge, mutagenesis has not been previously published in current MRSA CC22 strains, additionally the effect of *fur* or *saeRS* inactivation has not been previously assessed within MRSA isolates.

5.2.1 Low transduction efficiencies for CC22 clinical isolates

Donor *S. aureus* strains containing the *Δsae*::Tn*917* and *Δfur*::*tet* constructs (Horsburgh et al., 2001; Johnson et al., 2005) were used for bacteriophage lysate production and transductions into recipient clinical *S. aureus* isolates. (Chapter 2:5:3). Unfortunately, transductions involving either construct were largely unsuccessful. Through multiple efforts only one successful *Δfur*::*tet* mutant was constructed and confirmed by a *fur* specific PCR (data not shown) named RB4*Δfur*.

Recent studies have implicated novel restriction-modification (RM) systems as the reason for unsuccessful genetic manipulation within new clinical MRSA strains (Corvaglia et al., 2010; Veiga and Pinho, 2009; Waldron and Lindsay, 2006). A subsequent study developed a way of circumventing this problem using an *E. coli dcm* methylation mutant (DC10B) (Monk and Foster, 2012; Monk et al., 2012). DNA isolated from this particular cloning strain is deficient in methylated cytosine residues which improves transformation efficiencies. This new transformation system was therefore utilised for all further genetic manipulation into clinical MRSA CC22 strains.

5.2.2 Transformation and allelic replacement using pIMAY-*Δfur** (Monk *et al* 2012)

Our current *Δfur* construct (*Δfur::tet*) had to be reconstructed for use in this new manipulation technique; the new construct will henceforth be referred to as *Δfur** whereas the previous *fur* mutant construct containing a tetracycline cassette created by Horsburgh *et al* (2001) will retain the reference *Δfur or Δfur::tet.* The recommended vector, pIMAY, was chosen for the *Δfur** reconstruction as its temperature sensitive system allows integrant selection at 37 °C. This is opposed to integrant selection at 43 °C, used by most vectors, which has been shown to induce secondary mutations in the *saeRS* locus (Sun et al., 2010). Diagrams of the *fur* locus, pIMAY vector and recombinant *Δfur** plasmid can be seen in Figure 5-1 and Figure 5-2.

The 450 bp *S. aureus fur* gene sequence was scrutinised for presumptive promotor and terminator sequences using the Softberry BPROM server. This was to avoid interruption of any potential regulatory sequences utilised by adjacent genes contained within the *fur* gene. From this analysis 330 bp (encoding 110 amino acids) of the *fur* gene was designated for deletion. Primers were designed to amplify two 500 bp regions located either side of the designated *fur* deletion region (including *xerD* and SAR*1575* gene sequences). All primers used for construction, including pIMAY vector amplification primers, contained additional "primer flaps" on their 3' ends. These "flaps" were designed to be homologous to the adjacent DNA fragment in the recombinant pIMAY *Δfur** plasmid construction, therefore enabling recombinant plasmid assembly using the one step isothermal DNA assembly method by Gibson et al (2009). This resulted in a recombinant pIMAY plasmid containing a 1 kb insertion; which itself contained 120 bp of *fur* (450 bp wild type gene minus 330 bp designated for deletion) flanked by partial *xerD* and SAR*1575* coding sequence.

Once assembled the recombinant plasmid was transformed into *E. coli* DC10B and selected by chloramphenicol resistance. Successful recombinant plasmid transformants were confirmed by PCR using primers (IM151/2) which flanked the *Δfur** gene sequence contained within the plasmid (expected product length ~ 1200 bp). Plasmid DNA was isolated from confirmed DC10B recombinant clones and

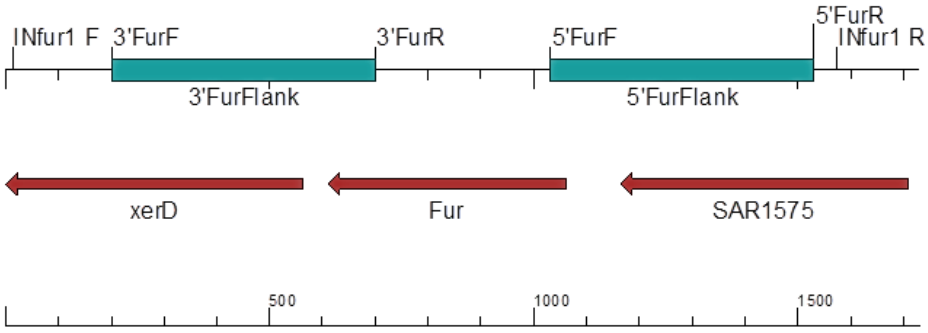


Figure 5-1 Diagrammatical representation of the *S. aureus fur* locus; areas of PCR amplification and primers used are indicated. The entire 450 bp *fur* locus was analysed for presumptive promoter/terminator sequences using the Softberry BPROM server to avoid introducing polar effects during mutagenesis. Two 500 bp *fur* gene fragments were designated and PCR amplified separately. The first fragment (3'FurFlank) contained 90 bp from the downstream *fur* gene region with an additional 410 bp into *xerD* (3'FurF/3'FurR). The second fragment (5'FurFlank) contained 30 bp from the upstream end of *fur* and 470 bp into SAR*1575* (5'FurF/5'FurR). In total, 330 bp of the *fur* gene was excluded from the wild type length of 450 bp. The locations of final integration/mutant confirmation genome specific primers are also indicated (INfur1F/R).

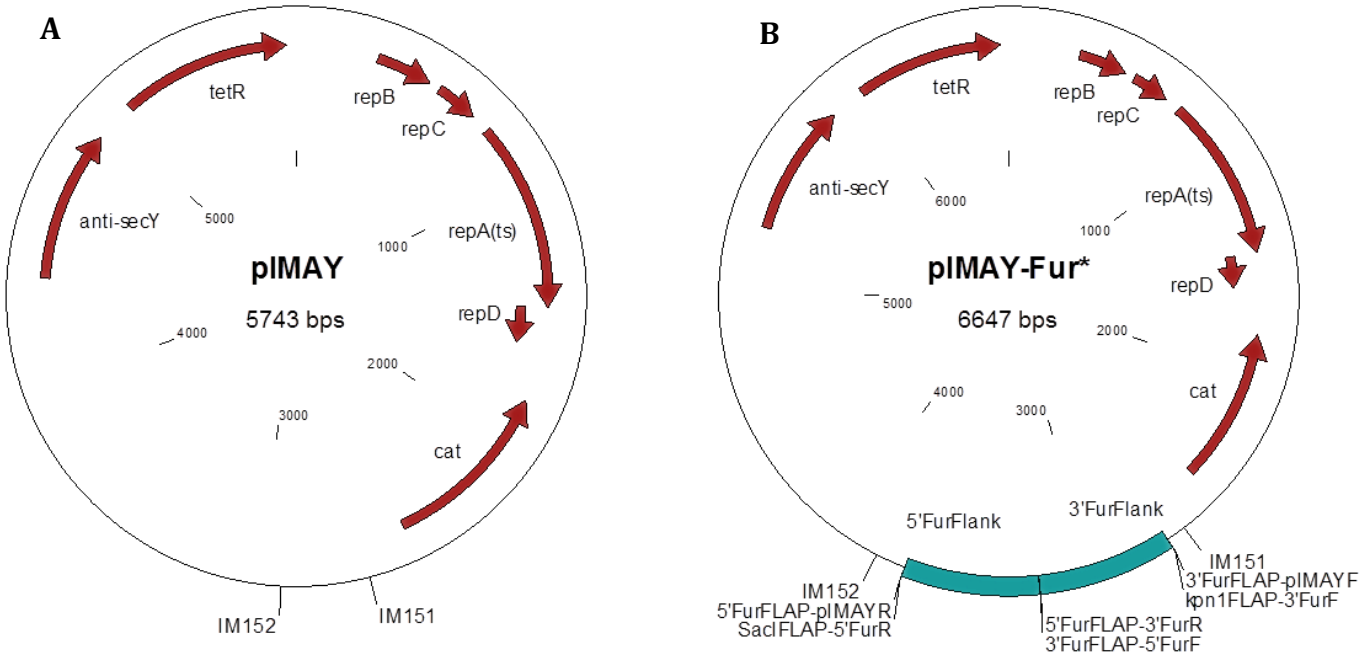


Figure 5-2 Construction of the recombinant pIMAY-*Δfur plasmid.** The pIMAY vector (A) and recombinant pIMAY-*Δfur** (B) are shown. All genes used for selection/counter-selection are labelled and all primers (plus homologous flaps) used are indicated. The pIMAY vector was PCR amplified (pIMAYF/pIMAYR) along with the separate *fur* fragments (Figure 5-1) and the recombinant plasmid assembled using the one step isothermal DNA assembly method (Gibson et al., 2009). Briefly, additional DNA sequences were artificially included in each of the primer pairs (primer flaps); these sequences were designed to be homologous to the adjacent DNA fragment in the recombinant plasmid, which enabled recombinant plasmid assembly. Specific primer sequences including homologous flaps and full protocol details can be seen in Chapter 2:3:8 and Table 2:2.

transformed directly into competent *S. aureus* cells at 28 °C, the permissive temperature for free plasmid replication. A temperature shift to 37 °C, a non-permissive temperature for plasmid replication, allows for the selection of clones containing genome integrated plasmid. Successful integrants were confirmed using a combination of plasmid specific and genome specific PCR primers (IM151/INfur1R and IM152/INfur1F). Rolling circle plasmid replication was initiated by a third temperature shift back to 28 °C and clones negative for plasmid DNA (integrated or freely replicating) were counter-selected for using anhydrotetracycline. Subsequent *S. aureus* clones were then either left with the wild type *fur* locus or the deletion (*Δfur**).

The genotypes of candidate mutant colonies were investigated by PCR using genome specific primers (INfur1F/R). These primers were designed to amplify the region encompassing the designated *fur* deletion, therefore the sizes of the amplified products depicted either a wild type *fur* gene sequence, i.e. 1600 bp, or a smaller PCR product of ~ 1250 bp, demonstrating the *Δfur** mutation.

Initially, *Δfur** mutagenesis was attempted using only the clinical MRSA isolates characterised in this study. However, preliminary antibiotic sensitivity data using the RB4*Δfur* transduction mutant suggested a novel association between *fur* and oxacillin resistance (see Chapter 5.3.1). Therefore, additional MRSA strains exhibiting various SCC*mec* types were included in mutagenesis. PM64*Δfur* (CC30, SCC*mec* type II) containing the *Δfur::tet* transduction mutation (Dr Jon Baker) was included, in additional to the RB4*Δfur* mutant transduced during this study. Moreover, two *Δfur** mutants were constructed including the initial PB3 isolate, PB3-1*Δ fur** (CC22, SCC*mec* type IV), and N315 (CC5, SCC*mec* type II), a well characterised MRSA lab isolate (N315*Δfur**).

5.3 Characterisation of Δ*fur* in a CC22 MRSA genetic background

The previously listed *Δfur* mutants, either containing *Δfur::tet* or *Δfur** constructs were analysed in parallel with their isogenic wild type isolates. Subsequent assays included previous persistence experiments used in Chapter 3 and 4 to investigate the potential involvement of *fur* in persistence.

5.3.1 SCC*mec* type dependent involvement of *fur* in oxacillin resistance

Preliminary data using RB4*Δfur* suggested a novel interaction between *fur* and oxacillin resistance. Therefore the effect of *Δfur* on the isolates' antibiotic resistances to oxacillin, vancomycin, linezolid and daptomycin was assessed using M.I.C. Evaluator™ test strips (Chapter 2:6:2). No differences were observed between wild type and *fur* mutant MIC for vancomycin, linezolid or daptomycin (data not shown). In contrast, there were several incidences where *fur* mutants displayed the phenomenon of heteroresistance against oxacillin compared to the high level of homogenous resistance exhibited by their isogenic wild type strains. Unfortunately, pictures did not adequately demonstrate this phenotype and have been omitted from this study. However, this data suggests a novel association between Fur function and the mechanism of oxacillin resistance. It would be interesting to investigate if this phenotype is observed during exposure to other antibiotics and also if it is a common trait in all MRSA strains

5.3.2 PM64*Δfur* and RB4*Δfur* exhibit TSB specific exponential growth reductions but no effect in CRPMI

To assess whether Fur had any involvement in the nutrient deprived specific growth advantage observed for persistent isolates (Chapter 3:5:2) 24 hour growth curves were performed as previously described (Chapter 2:7:1) using two different mediums, TSB and CRPMI pH 7.0. Overnight cultures were diluted to a set inoculum in fresh media (either TSB or CRPMI pH 7.0) and incubated for 24 hours at 37 °C in 5 % CO_2. Optical density readings (OD_{600nm}) were taken every hour for the first 7 hours to assess their exponential growth phases and 24 hours after inoculation (Figure 5-3 [TSB]; Figure 5-4 [CRPMI]).

Significant growth defects are observed in PM64*Δfur* and RB4*Δfur* compared to their isogenic wild type isolates in TSB. Specifically, PM64*Δfur* is found to be significantly lower than PM64 at 2 ($P < 0.01$), 3, 4 ($P < 0.001$), 5, 6 and 7 ($P < 0.01$) hours; and RB4*Δfur* lower than RB4 at 3, 4, 5 ($P < 0.001$), 6 and 7 ($P < 0.01$) hours as shown by multiple T-tests using Graphpad Prism 6 software. Interestingly, there were no differences between the wild type and *Δfur** within the PB3-1 or N315 strains during the first 7 hours growth in TSB. At the 24 hour time point PM64*Δfur* ($P < 0.05$),

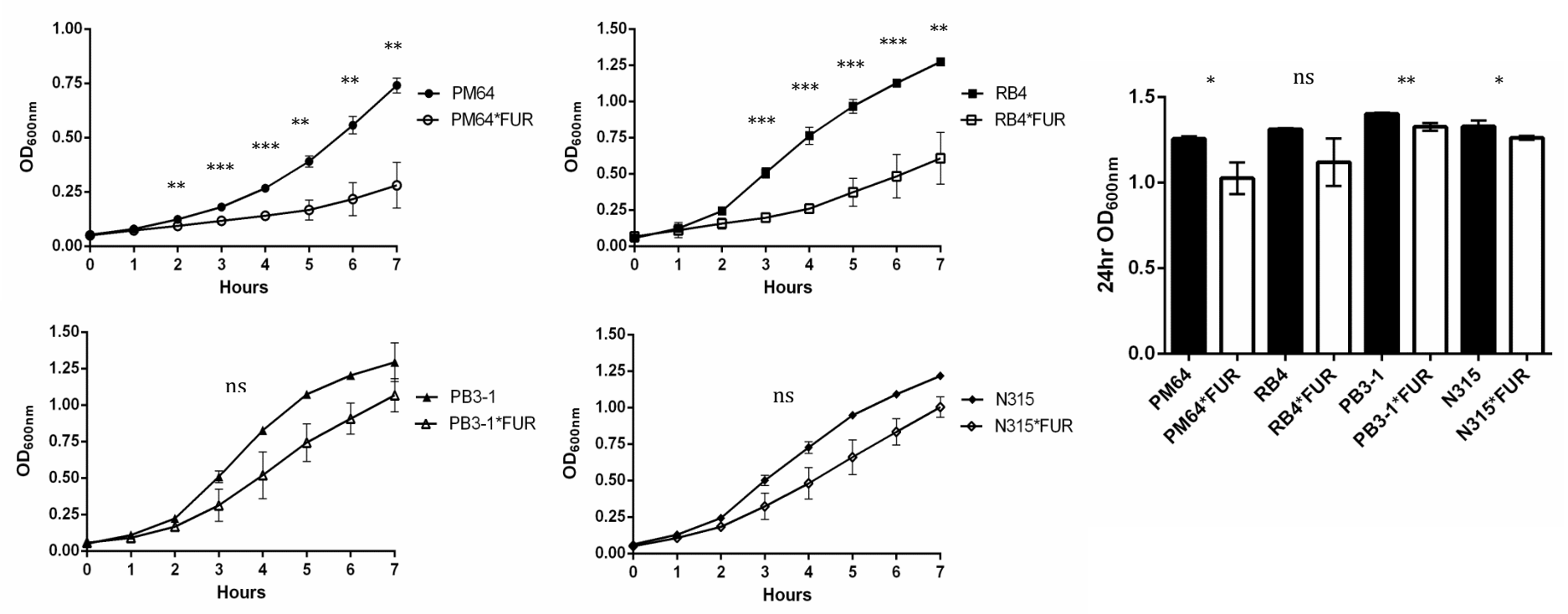


Figure 5-3 Growth analysis of MRSA *Δfur* mutants in TSB, 0 - 7 hours and 24 hour time points. All isolates were incubated static at 37 °C in 5 % CO_2 for 24 hours in TSB. OD_{600nm} were recorded every hour for 7 hours (A) and 24 hours (B) post inoculation. Experiments were repeated independently three times, means calculated and plotted +/- one standard deviation. Multiple T-tests for each individual time point were used to calculate statistical significance and indicated on graphs with * or ns.

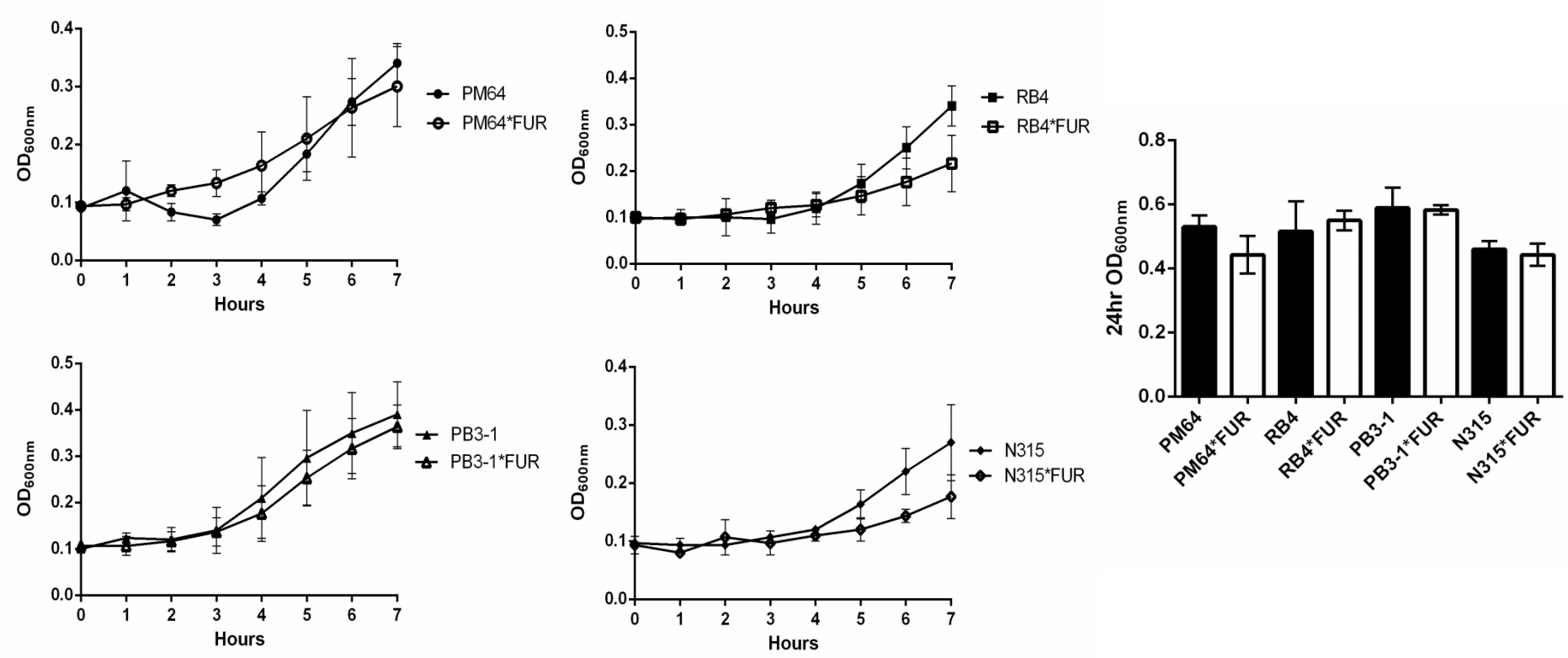


Figure 5-4 Growth analysis of MRSA *Δfur* mutants in CRPMI pH 7.0, 0 - 7 hours and 24 hour time points. All isolates were incubated static at 37 °C in 5 % CO_2 for 24 hours in CRPMI pH 7.0. OD_{600nm} were recorded every hour for 7 hours (A) and 24 hours (B) post inoculation. Experiments were repeated independently three times, means calculated and plotted +/- one standard deviation.

PB3-1*Δfur** ($P < 0.01$) and N315*Δfur** ($P < 0.05$) all showed significantly lower values compared to their respective wild type isolates; RB4*Δfur*'s significance just fell short of the 0.05 threshold ($P = 0.073$). Comparisons made between the isolates' growth characteristics in CRPMI showed no differences between any wild type or *fur* mutant combination in regards to either the first 7 hours growth or after 24 hours incubation.

Overall, this data suggests the two *fur* mutants containing the tetracycline construct suffer growth impediments particularly after 24 hours growth in rich media (TSB) but not in nutrient restricted environments (CRPMI). It is interest however that this phenotype is not exhibited by the alternative *fur** mutants, not containing a tetracycline cassette. This suggests presence and/or expression of the tetracycline cassette has a detrimental effect on growth in TSB, independent of Fur function. One possible explanation is that there is a fitness cost placed on the mutant clone for the expression of tetracycline, therefore *fur* mutants (*Δfur**) lacking this fitness burden do not exhibit the observed growth defect. A second explanation could be that the presence of the tetracycline cassette has unidentified polar effects on neighbouring gene expression such as *xerD*. XerD is responsible for the resolution of chromosomal multimers during segregation and has been shown to be essential for viability in *S. aureus* (Chalker et al., 2000). Further work would need to be conducted to ascertain which of these suggestions, if any, are correct.

Overall though, there appears to be no evidence to suggest *fur* was involved in the persistence associated nutrient deprived growth phenotype witnessed previously. The inactivation of *fur* in a persistent isolate background would confirm this but genetic manipulation in persistent isolates was unsuccessful during this time.

5.3.3 MRSA *Δfur* isolates display decreased adhesion protein and increased virulence factor expression

Persistent *S. aureus* isolates were shown by proteomic analysis to display increased concentrations of cell surface associated proteins involved in adhesion, immune evasion, cytotoxicity and stress response including Eap, Emp, Sbi, Atl and Trx (Chapter 3:7:3). Many of these proteins such as Eap, Emp and Sbi are known to be under SaeRS control (Kuroda et al. 2007; Rogasch et al. 2006; Nygaard et al. 2011;

Voyich et al. 2009), which is subsequently under Fur positive regulation (Johnson et al., 2011). To determine if Fur plays a role in the differential protein regulation found in persistent *S. aureus* clinical isolates the cell surface protein fractions were scrutinised after 16 hour incubation in CRPMI (Figure 5-5, protocol details Chapter 2:9:2).

RB4*Δfur* and PM64*Δfur* exhibited decreased cell surface associated Eap (~70 kDa), compared to their respective wild type isolates. However Eap levels appear unchanged in PB3-1*Δfur** compared to its isogenic wild type isolate. There was no difference in cell surface associated Eap between N315/N315*Δfur** either, this fact is unclear Figure 5-5 due to protein loading differences but has been demonstrated in previous gel analysis (data not shown). These data may be indicative of background strain differences within different MRSA strains or could suggest presence of the tetracycline cassette is affecting several phenotypes in addition to the differential exponential growth phenotype witnessed previously. To investigate these phenotypic discrepancies further, the new construct (*Δfur**) could be used to transform a lab strain such as Newman which has been well characterised using the previous *Δfur::tet* construct. Phenotypic differences between Newman*Δfur* and Newman*Δfur** such as those witnessed here would suggest secondary effects originated from the tetracycline cassette. Without this data, such phenotypic discrepancies cannot be attributed to differences in *Δfur* constructs or to differences in strain backgrounds.

Prior to making PB3-1*Δfur** and N315*Δfur**, the cell surface associated protein extracts of RB4 and RB4*Δfur* were further analysed by iTRAQ LC-MS in parallel with previous persistence analysis (Chapter 3:7:3). This was to further investigate the potential involvement of Fur in regards to the persistence associated proteomic data, using a more sensitive technique. A table containing the full list of differentially expressed cell surface proteins in RB4*Δfur* can be found in the appendix (Table A) but an overview is given in Table 5-1. Using the previous limits, i.e. > 0.5 $\log_2$ fold difference, a total of 40 cell surface associated proteins were down-regulated in the *fur* mutant and 58 up-regulated. Included in this list were numerous persistence associated proteins described previously.

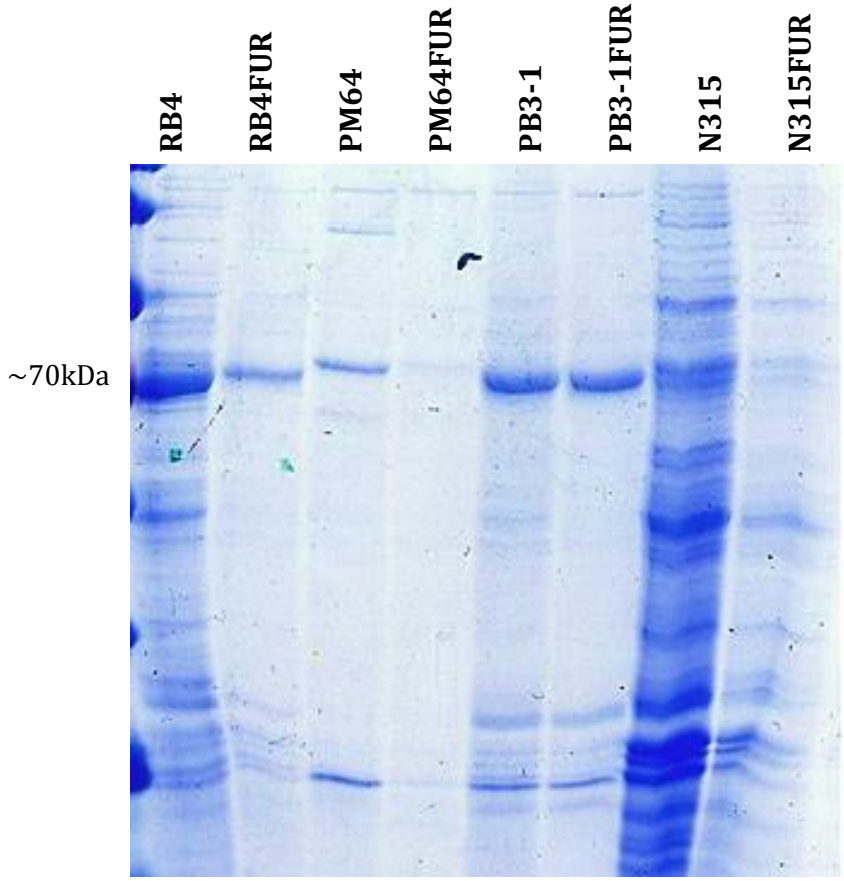


Figure 5-5 Non-covalently bound cell surface protein fraction from MRSA *fur* mutants after 16 hours CRPMI pH 7.0 incubation displayed on 1D SDS-PAGE. The cell surface protein was prepared as previously described from 16 hour CRPMI pH 7.0 cultures. Normalised protein samples were loaded and displayed via 1D SDS-PAGE. Experiments were repeated independently three times to confirm reproducibility. Independent protein bands were identified by MS (PNACL at the UOL).

Table 5-1 Overview of differentially expressed cell surface proteins in RB4*Δfur* compared to its isogenic wild type strain RB4. Relevant proteins which displayed > 0.5 $\log_2$ fold difference between RB4 and RB4*Δfur* are listed below, arranged into functional groups. A full list including fold differences and accession numbers can be viewed in the appendix (Table A).

Down regulated in RB4*Δfur*	**Up regulated in RB4*Δfur***
Gene regulators Staphylococcal accessory regulators (SarA, SarR, SarS) Repressor of toxins (Rot)	Gene regulators Histidine protein kinase (SaeS)
Virulence factors Adhesion proteins (Map/Eap, Emp) Immune evasion (Map/Eap, Sbi) Secreted toxin (LukS)	Virulence factors/Stress response Secreted toxin/antigens (Atl, IsaB) Immune evasion (Scn) Stress response (Csp, Trx) Iron acquisition (SirA, SstD, IsdB, FtnA)
General cellular processes Ribosomal protein (RspA) (~6) tRNA ligases DNA-binding protein HU (~20) Other enzymes (Synthesis, modification, digestion)	General cellular processes Transporter/translocator proteins (MntA, YajC, FhuD, SecDF) (~13) Ribosomal proteins Septation ring formation regulator (EzrA) Transcription termination factor (Rho) DNA repair (RecA) Ribonuclease (NrdF, Rny) ATP-dependent zinc metalloprotease (FtsH) (~15) Other enzymatic reactions (Synthesis, modification, digestion)

Specifically, members of the SaeRS regulon- Eap, Emp and Sbi were down regulated in RB4*Δfur*; however the histidine protein kinase, SaeS was up regulated in RB4*Δfur*. This final point is in direct contrast to previous microarray analysis which showed *saeS* and *saeR* were transcriptionally down-regulated in Newman*Δfur* (Johnson et al., 2011). Other persistence associated proteins, Atl, Csp, Acyl carrier protein and Trx, were up regulated in the *fur* mutant, of which *atl* also displayed up regulation in transcriptional analysis (Johnson et al., 2011). Unusually, numerous cytoplasmic proteins were identified in the cell surface extract, including several Sar proteins and ribosomal sub-units. It is possible that during proteomic preparations a proportion of cells lysed leading to cytoplasmic proteins adhering to the cell surface and identification by iTRAQ. However it is widely known that Staphylococcal cells require enzymatic digestion of the cell wall for lysis to occur.

Overall, numerous cell surface associated proteins which showed persistence specific increases displayed changes in RB4*Δfur*; but results did not accurately reflect persistence data.

5.3.4 Inactivation of *fur* reduces invasion and intracellular persistence capabilities, but does not influence virulence in an insect based model of infection

In this study and previous publications *S. aureus* Fur has been shown to influence the expression of multiple host cell adhesion and extracellular proteins, either directly or indirectly through additional gene regulators e.g. SaeRS (Horsburgh et al., 2001; Johnson et al., 2011). Therefore the ability of the MRSA *Δfur* mutants to adhere, invade and intracellularly persist within a rat myocardium cell line (H9C2) was assessed as previously described (Chapter 2:14) and results presented in Figure 5-6.

No differences were observed between *Δfur* mutants and their isogenic wild type MRSA isolates in their adherence abilities. Interestingly, significant decreases in invaded cell numbers are seen for the SCC*mec* type II *Δfur* mutants PM64*Δfur* and N315*Δfur** but not for RB4*Δfur* or PB3-1*Δfur** (SCC*mec* type IV). All MRSA *Δfur* mutants exhibited significantly lower intracellular persistence numbers compared to their isogenic wild type isolates.

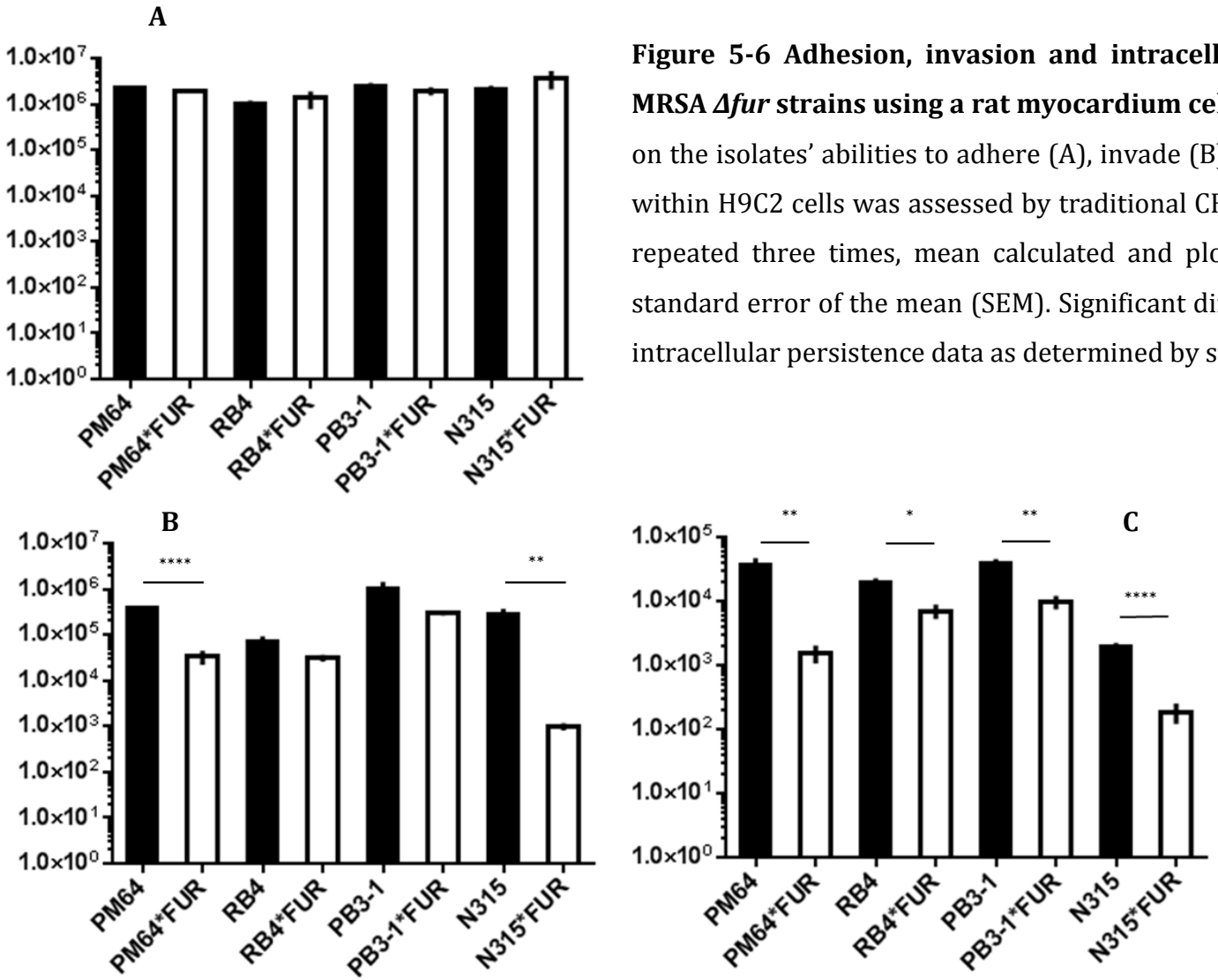


Figure 5-6 Adhesion, invasion and intracellular persistence assay with MRSA *Δfur* strains using a rat myocardium cell line, H9C2. The effect of *Δfur* on the isolates' abilities to adhere (A), invade (B) and persist intracellularly (C) within H9C2 cells was assessed by traditional CFU counting. Experiments were repeated three times, mean calculated and plotted on a $\log_{10}$ scale +/- one standard error of the mean (SEM). Significant differences were observed in the intracellular persistence data as determined by student T-test, indicated by *.

These data provides evidence that Fur is required for intracellular survival for all MRSA strains tested and the effect of Fur on invasion capabilities is dependent on SCC*mec* type.

Another persistence associated trait discovered in this study was virulence attenuation using an insect based model of infection (Chapter 4:4). Fur has been implicated previously in pathogen virulence; specifically *S. aureus Δfur* mutants display attenuated virulence in murine models of pneumonia and skin abscesses (Horsburgh et al., 2001; Torres et al., 2010). Therefore the *Galleria mellonella* model of infection (Chapter 2:15) was used to assess the virulence of the MRSA *Δfur* mutants for a potential association to persistence; results are plotted in Figure 5-7.

No significant differences were observed between any of the MRSA *Δfur* mutants and their isogenic wild type isolates regarding larvae fatalities or bacterial burdens. This suggests Fur is not involved in general MRSA virulence as assessed using an insect model of infection and consequently did not contribute to the previously observed persistence associated attenuated virulence (Chapter 4:4).

In conclusion, Fur does not appear to be involved in MRSA bacteraemia persistence within the cases investigated; however data has indicated novel Fur phenotypes within a MRSA strain background, in particular the ability of *S. aureus* to invade and persists within eukaryotic cells. Interestingly certain phenotypes appear to be SCC*mec* type dependent; a link which has not, to our knowledge, been published previously.

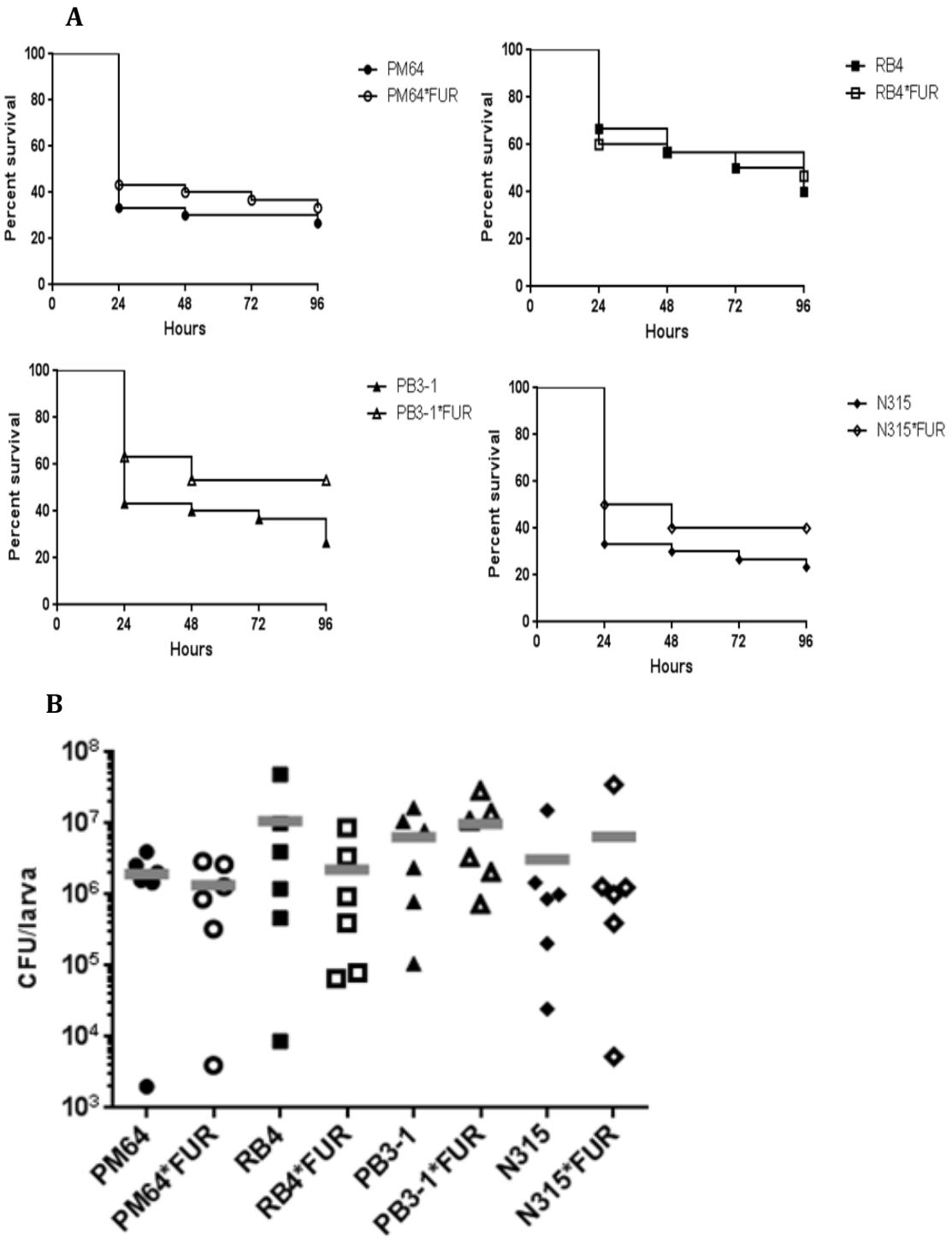


Figure 5-7 Kaplan Meier survival plots (A) and bacterial burden assessment (B) of *Galleria mellonella* larvae infected with MRSA *Δfur*. Experiments were conducted as previously described (Chapter 2:15). Kaplan Meier survival plots were constructed with GraphPad Prism 6 software (A). 48 hour bacterial burdens were analysed traditionally via CFU counts from selective media and plotted on a scatter graph (B) using a $\log_{10}$ scale (dots) including an indication of the mean (line).

5.4 Comparative Genomics using Illumina Next Generation Sequencing

The reproducibility of the phenotypic traits experienced so far in the previous chapters have indicated a stable, genetic basis of persistence which has evolved during two independent cases of MRSA bacteraemia (PB1, PB3). To test this hypothesis, Illumina whole genome sequencing was employed and whole genome comparisons were conducted between persistent and initial isolates within individual infections to identify novel variants associated with persistence development.

5.4.1 SNP and INDEL analysis between initial and persistent isolates within individual persistent bacteraemia cases

All CC22 clinical MRSA isolates were included for Illumina next generation sequencing (NGS), encompassing initial and subsequent persistent isolates for PB1, PB2, PB3, PB5 and all three resolved isolates RB1, RB4 and RB5. Total DNA was extracted for each isolate and assessed for concentration and purity. These samples were then submitted in collaboration (Dr Sam Sheppard and Dr Ben Pascoe) to the Swansea Genome Centre (Swansea, UK) for library preparation and subsequent paired-end high-throughput genome sequencing on the Illumina HiSeq 2500 machine. All subsequent bioinformatic analysis was conducted on SPECTRE (Special Computational Teaching and Research Environment) by Dr Richard Haigh at the UOL. Raw paired-end ends were quality checked and trimmed using the FASTX toolkit, and aligned to the closest available reference genome, HO 5096 0412 (Holden et al., 2013) using Picard, BWA-MEM and SAMtools.

Genetic variation compared to the reference genome and between the sequence data of individual isolates, namely initial and persistent isolate comparisons, was identified using VarScan v2.3.6 with a variant threshold of > 80 % and a > 7 read depth. Moreover, large genomic insertions and deletions unlikely to be identified by traditional variant analysis were examined by eye using the Tablet viewer. Table 5-2 lists the genetic variations found from initial and persistent isolate comparisons contained within or adjacent to genes with putative functions listed in NCBI.

Table 5-2 Overview of significant SNP/INDEL between initial and persistent isolates within individual persistence cases. Gene names, putative function of the encoded protein and the alleles observed in the initial and subsequent persistent isolates are indicated. Genes which exhibit mutations within multiple infections are in **bold.**

PB1

Gene	Putative function	SNP Co-ord	Ref allele	Mutant allele	Amino acid change			
					PB1-1	PB1-15-1	PB1-15-2	Result
NAD	Electron transfer	1860581	G	T	A271D	-	-	Non-polar/acidic
pyrG	CTP synthase	2206316	G	A	-	P412S	-	Non-polar/polar
mprF	Membrane modification enzyme	1343017	C	T	-	P314L	-	Non-polar
		1344552	C	T	-	-	L826F	Non-polar

PB2

Gene	Putative function	SNP Co-ord	Ref allele	Mutant allele	Amino acid change		
					PB2-1	PB2-35	Result
saeQ	Putative membrane protein	730165	C	T	-	W50#	Truncated protein
SAEMRSA15_19920 (*ddl*)	D-alanine D-alanine synthase/ligase	2168537	T	C	-	E71G	Acidic/Non-polar

Continued from previous page...

PB3

Gene	Putative function	SNP Co-ord	Ref allele	Mutant allele	Amino acid change				
					PB3-1	PB3-29 / PB3-32-1	PB3-32-2	PB3-72 / PB3-74	Result
spa	IgG binding protein (Protein A)	98739	G	A	-	D339	-	D339	Synonymous
		98690	T	C	-	-	N356D	-	Polar-acidic
mprF	Membrane modification	1342960	C	T	-	S295L	-	-	Polar/non-polar
		1344552	C	T	-	-	L826F	-	Non-polar

PB5

Gene	Putative function	Insert Co-ord	Base insertion	Frame	PB5-30 / PB5-41
					Insertion
pfk	Pyruvate kinase	328823	A TAT GTT ATG GGA AAG GC	In-frame	K45_E46insYVMGKA
sasA / sraP	Cell wall anchor with Ig domain	2777147	T TTC ACT CAA	Frameshift & insertion	S1218fs#14

The most significant observation was for *mprF* which exhibits mutations between the initial and subsequent persistent isolates originating from two independent cases of persistent bacteraemia, PB1 and PB3. Moreover, each of the infecting strains accumulated at least two distinct *mprF* SNP as demonstrated by each persistent isolate presenting a different mutation. This suggests there was significant selective pressure placed upon MprF function during both cases of persistence leading to the emergence of multiple incidences of *mprF* mutation. In total three distinct C – T transitions are collectively observed all resulting in non-synonymous amino acid changes. MprF (Multiple Peptide Resistance Factor) catalyses the synthesis of lysyl-phosphotidylglycerol (L-PG) by transferring positively charged lysines from lysyl-tRNA to PG and translocating modified L-PG to the cell membrane. (Boyle-Vavra et al., 2011; Oku, 2004; Yang et al., 2013). This has the overall effect of reducing the negative charge displayed by the cell membrane which has been linked to reduced susceptibility to cationic molecules such as CAMP/HDP and daptomycin coupled with calcium (Bayer et al., 2013; Jones et al., 2008).

Additional genes which exhibited mutations within the PB1 infecting strain encode proteins involved in electron transport (*nad*) and nucleotide synthesis (*pyrG*). These SNP however occur in the initial isolate or only within one persistent isolate respectively, suggesting they are not associated with persistence development. An additional PB3 mutated gene (*spa*) codes for immunoglobulin binding protein A (SpA). Interestingly, this gene also experienced two distinct mutations between different PB3 persistent isolates; however only one leads to a non-synonymous amino acid change. The two terminal isolates of the PB3 lineage, PB3-72 and PB3-74, exhibit the same synonymous *spa* alleles as PB3-29 and PB3-32-1, however they exhibit the wild type/initial isolate *mprF* allele. This suggests the *spa* mutation arose prior to *mprF* mutations and therefore occurred before the divergence of the PB3-72 / PB3-74 specific population from the main “persisting” proportion.

Two genes exhibited variations within the PB2 infection; the first being a nonsense mutation in *saeQ* located within the *saeRS* operon. This gene codes for a putative cell membrane protein suggested to work with SaeP to activate SaeS’s phosphatase activity and return SaeR to its dephosphorylated, inactive form (Jeong et al., 2013). This SNP leads to a premature stop codon and truncation in SaeQ, likely negatively

effecting SaeQ's function. The second mutation was within SAEMRSA15_*19920* (*ddl*), a cell wall modification protein. This alteration may have been the result of exposure to cell wall targeting antibiotics, additional research would need to be conducted to ascertain the effects of this mutation.

In contrast, PB5 isolates experienced two insertions within coding regions. The first mutation involved an in-frame insertion of six amino acids within *pfk*, which codes for a pyruvate kinase. The significance of this mutation cannot be hypothesized at present especially as it does not affect the reading frame of this gene, however the extra amino acids may affect protein structure and therefore function. Additional research would need to be conducted to be able to speculate on potential phenotypic consequences. The second insertion resulted in a frameshift within *sasA* leading to a premature stop codon eleven codons downstream. The encoded protein is a cell wall anchor with putative IgG binding capabilities which possibly suggests a role in immune evasion and therefore may have been involved in persistence development during this infection.

To confirm all observed SNP in Table 5-2 were exclusive to persistence development i.e. only found to occur in persistent infecting strains, the resolved isolates' genomic data was scrutinised for the presence of these allele changes. None of the allele changes listed in Table 5-2 are exhibited by either RB1, RB4 or RB5.

Overall, genotypic data retrieved from Illumina genome sequence analysis has indicated *mprF*/MprF played a significant role during persistence of two independent bacteraemia cases (PB1 and PB3). The intensive selective pressure placed upon MprF protein function led to *in vivo* genome evolution within both infecting strains resulting in the accumulation of multiple *mprF* SNP displayed by persistent isolates. Due to the known function of MprF in regards to cell membrane charge, these mutations likely had significant impact on the persistent isolates' sensitivities to a range of cationic molecules, particularly CAMP/HDP and daptomycin (coupled with calcium). The consequence of this will be discussed in detail further in this chapter.

5.4.2 The genetic evolution of the CC22 MRSA lineage

This sequence data has also been used to assess the evolution of the CC22 lineage from the reference EMRSA-15 strain which was used for sequence mapping and assembly (HO 5096 0412). A total of 54 SNP/INDEL were found common to all recent CC22 clinical isolates compared to the reference strain (Appendix Table B). Table 5-3 provides an overview of the mutated genes deemed to have functions associated with virulence and/or cellular processes. The presence of these mutations in all recently infectious clinical CC22 isolates may suggest they have beneficial effects on bacterial fitness and have therefore been maintained throughout evolution of the CC22 EMRSA-15 lineage. Conversely, these mutations may indicate the presence of a Leicester specific clade of CC22 isolates which have experienced regional dependent genetic evolution. More research involving whole genome sequencing of additional CC22 isolates originating from other locations, would need to be conducted to confirm the presence of regional clades.

Several genes encoding DNA modification enzymes, particularly exonucleases and proteins involved in RM systems, contained mutations within this study's CC22 clinical isolates compared to the reference EMRSA-15 strain. This may explain the apparent difficulty experienced in this study when attempting standard genetic manipulation with these isolates compared to the relative ease using lab reference strains and isolates from other CC lineages. Another class of proteins which experienced mutations in their respective genes are involved in bacterial stress response systems, specifically ClpP proteolytic system and oxidative stress. The final functional group related directly to virulence which exhibited genetic alterations included nutrient acquisition i.e. iron (IsdB, IsdH), antibiotic resistance, surface adhesion (SdrD) and immune evasion (SpA) proteins.

Overall, sequencing data has discovered a multitude of "non-reference" EMRSA-15 mutations which have been maintained during CC22 lineage specific genome evolution as demonstrated by the fact that they appear in all recently infectious CC22 clinical isolates. This firstly suggests such genetic alterations provide significant phenotypic fitness advantages hence why they have been selected for and maintained throughout evolution. Moreover, the specific protein functions of affected genes have clear associations with bacterial survival and virulence *in vivo*;

Table 5-3 Overview of "of interest" mutations observed in all recently infectious CC22 clinical isolates (mutant allele) compared to the reference EMRSA-15 strain (HO 5096 0412; ref allele). Gene name, putative function and allele details of each mutation are listed. Genes are grouped according to function i.e. DNA manipulation, stress response and virulence.

Gene name	Putative function	SNP Co-ord	Ref allele	Mutant allele	Amino acid change	Result
DNA replication, modification and repair						
SAEMRSA15_*00460* (*hsdS*)	Type I RM system	60889	G	C	S350T	Polar
deoD1	Purine nucleoside phosphorylation	126477	-	-C	A96fs*?	Translation read through to SAEMRSA15_*01030*
uvrA	Nucleotide excision repair	789882	T	C	I925T	Non-polar/polar
SAEMRSA15_*15530* (*recJ*)	Putative ssDNA exonucleases	1708312	T	C	T514A	Polar/non-polar
dnaB	DNA helicase	1749314	A	G	S440	Synonymous
SAEMRSA15_*17490* (*mre11*)	Putative DNA repair exonuclease	1926268	T	C	I304V	Non-polar
Stress response						
clpB	ClpP APase subunit - protein quality control and degradation	913691	A	G	H417R	Basic
sodA	Superoxide dismutase - Oxidative stress	1627479	A	G	I69T	Non-polar/polar
clpL	ClpP APase subunit - protein quality control and degradation	2646342	A	G	S553G	Polar/non-polar

Continued from previous page…

Virulence factors, adhesion and immune evasion						
spa	Immunoglobulin binding protein	99269	-	-T	Q163fs*?	Frameshift correction enabling complete translation of SpA
sdrD	LPXTG surface protein - fibrinogen adhesion	585810	G	A	G1153D	Non-polar/acidic
isdB	Haemoglobin binding protein	1075448	-	+G	T87fs*?	Frameshift correction enabling complete translation of IsdB
SAEMRSA15_*10270* (*gloA*)	Glyoxalase/bleomycin resistance protein	1143954	A	C	*207S	Correction of premature stop enabling complete translation of GloA
isdH (*harA*)	Haemoglobin / haptoglobin-binding protein	1806877	A	G	L505P	Non-polar

therefore it is postulated their emergence has contributed to observed increase in pathogenicity within this lineage.

5.5 Discussion

This chapter aimed to investigate a potential genetic basis for persistence in the previously characterised MRSA bacteraemia cases using two different strategies. The main assay involved Illumina whole genome sequencing of all clinical isolates, the data of which will be the main focus of this discussion. Additionally, the global gene regulator Fur was investigated for a possible association with persistence. Despite data proving this to be unlikely, novel Fur phenotypes influenced by SCC*mec* type were indicated during this investigation. This section will begin with a brief discussion of the Fur related data including suggestions for further research into this topic. The discussion will then move on to the apparent genome evolution within the CC22 EMRSA-15 lineage as determined by sequencing data. Finally, this chapter will finish by discussing the sequencing data directly relating to persistence development and the possible consequences such observed genetic alterations may have had on infection progression.

5.5.1 Novel Fur associated phenotypes in different MRSA strain backgrounds

Data presented in previous chapters implicated a role for Fur during persistence development particularly as many of the observed persistence associated phenotypes are either directly controlled by Fur e.g. *katA* expression/H_2O_2 sensitivity or indirectly through additional regulators e.g. proteomic data/SaeRS. Overall though, characterisation of two different *Δfur* constructs in one persistent initial isolate (PB3-1) and one resolved bacteraemia isolate (RB4) found limited correlation between Fur function and persistence development. Initial findings did however suggest novel Fur phenotypes in the MRSA isolates characterised potentially influenced by different strain backgrounds/SCC*mec* types. To our knowledge, the effect of Fur in a MRSA strain background has not been published previously, further highlighting the novelty of this data.

Wild type Fur function was found to be required for intracellular persistence within eukaryotic cells in all MRSA strains tested independent of strain background. This

Fur associated phenotype has been previously observed in a MSSA strain background, Newman (Dr Julie Morrissey; unpublished data), indicating a universal Fur function in *S. aureus*. This phenotype could be possibly explained by Fur's role in controlling the oxidative stress response and defence against ROS which is generated as a by-product of metabolism within the eukaryotic cell milieu (Horsburgh et al., 2001; Johnson et al., 2011). Inactivation of *fur* would theoretically render mutants more sensitive to ROS thereby decreasing survival capabilities of these strains.

Interestingly, SCC*mec* type II isolates (PM64, N315) also displayed reduced invasion abilities when *fur* was inactivated using two different *Δfur* constructs. Fur is known to positively regulate invasion factors in *Salmonella enteric* and gene inactivation leads to decreased epithelial cell invasion rates (Leclerc et al., 2013; Teixidó et al., 2011; Troxell et al., 2011). Additionally, Fur has been shown to positively regulate the expression of several virulence factors in *S. aureus* during growth in low-iron conditions, including the well-known adhesion and invasion protein Eap/MAP1 (Haggar et al., 2003; Johnson et al., 2008). One previous study demonstrated that inactivation of *fur* abolished *eap* transcription during low-iron conditions (Johnson et al., 2008). Combined with the findings from this study, it could be hypothesised that *fur* inactivation resulted in reduced Eap expression and potentially other, as yet unidentified, virulence determinants involved in invasion. This subsequently impacted negatively on the invasion capabilities of *fur* mutant strains compared to their isogenic wild type isolates.

In contrast to this hypothesis however, no difference in invasion abilities was observed when *fur* was inactivated in a CC22 SCC*mec* type IV strain background (RB4, PB3-1). This finding demonstrates the substantial effect strain background differences have on the invasion capabilities of different isolates. It is possible recent CC22 isolates such as PB3-1 and RB4 have additional invasion mediators, independent of Fur control, enabling wild type invasion capabilities despite *fur* inactivation. A recent study demonstrated improved invasion abilities for a CC22 SCC*mec* IV clone compared to a previous epidemic clone (ST228 SCC*mec* I) (Baldan et al., 2012). Moreover, the CC22 clone exhibited improved biofilm formation, competitive growth kinetics and increased *in vivo* virulence compared to other

strains (Baldan et al., 2012; Knight et al., 2012). This finding supports the idea that CC22 isolates have additional invasion determinants, potentially independent of Fur function. Further research would need to be conducted to confirm these hypotheses, possibly involving isolates from different CC lineages and observing the effect of *fur* inactivation on eukaryotic cell invasion.

This study's data also indicated a potential Fur influence in the mediation of antibiotic resistance in MRSA strains; specifically oxacillin heteroresistance was exhibited by *fur* mutants compared to full homogenous resistance exhibited by the isogenic wild type isolates. Evidence suggests oxacillin heteroresistance is caused by additional loci independent of the SCC*mec* locus such as *fem* (factors essential for methicillin resistance) and *aux* (auxiliary) coding regions (Berger-Bächi and Rohrer, 2002; Ryffel et al., 1994). This study's data may implicate Fur as another factor influencing the expression of oxacillin resistance either directly through currently unclear mechanisms or indirectly by controlling expression from previously characterised *fem/aux* loci. Additionally, Fur's involvement in oxidative stress and ROS defence may also play a role in antibiotic resistance. Exposure to bactericidal antibiotics has been shown to induce ROS production (Kohanski et al., 2010, 2007; Wang and Zhao, 2009). Therefore, inactivation of *fur* would theoretically lead to increased ROS sensitivity and, by association, increased antibiotic sensitivity. If this was truly the case, *fur* mutants would be expected to display increased sensitivity to the majority of antibiotics tested, but this was only indicated for oxacillin resistance in this study. Moreover, the actual contribution of ROS to antibiotic directed lethality has been disputed by more recent publications (Keren et al., 2013; Liu and Imlay, 2013). Therefore the link between Fur function and antibiotic resistance, particularly for oxacillin in a MRSA strain background, remains unclear and deserves further research.

In conclusion, this study has demonstrated a novel *S. aureus* Fur phenotype in regards to host cell invasion and a strain dependent Fur effect on intracellular persistence capabilities. Moreover, data suggests a possible role for Fur in antimicrobial resistance, although further research is required to confirm this association. Additional investigations would involve the genetic manipulation of *fur* in a diverse MRSA strain cohort and assessing the susceptibility of these mutants to

an array of bactericidal and bacteriostatic antimicrobial agents including oxacillin. Figure 5-8 gives an overview of the novel Fur related phenotypes found in the MRSA strains investigated in this study.

5.5.2 Genome evolution within the epidemic MRSA-15 CC22 lineage

The main aim of this study was to investigate the potential mechanisms of *S. aureus* persistence, inclusive of whole genome sequencing with the aim to identify putative genotypic persistence markers. In addition to this main aim, data generated from whole genome sequencing also enabled comparisons between the CC22 isolates characterised in this study which were recently infectious, to the reference EMRSA-15 isolate used for genome sequence assembly (HO 5096 0412). Through this type of analysis numerous SNP/INDEL were identified indicating substantial genome evolution has occurred within this lineage in recent years, a finding which is supported by several publications (Holden et al., 2013).

Whilst attempting genetic manipulations via phage transduction and *E. coli* transformations it was noted CC22 isolates presented in this study were highly resistant to horizontal gene transfer (HGT). Previous studies have found a combination of type I (SauI) and type IV (SauUSI) RM-systems are responsible for the apparent inability to transfer DNA between divergent *S. aureus* CC lineages and DNA originating from traditional *E. coli* cloning strains (Corvaglia et al., 2010; Murray, 2000; Roberts et al., 2013; Waldron and Lindsay, 2006; Xu et al., 2011). For the purposes of this study, bypassing SauUSI with the use of a *E. coli Δdcm* cloning mutant, developed by Monk *et al* (2012), was found to be sufficient for CC22 genetic manipulations. However, it is the SauI system specifically that is proposed to inhibit genetic transfer between *S. aureus* lineages resulting in intra-lineage specific genome evolution and the observed clonality of *S. aureus* strains (Lindsay, 2014; McCarthy and Lindsay, 2013, 2010). Here we present a non-synonymous mutation between the reference EMRSA-15 strain and the recently infectious CC22 clinical isolates located within *hsdS*, which encodes the SauI specificity sub-unit (HsdS). This observation may suggest a divergence in the recognition of foreign DNA for these CC22 isolates compared to the ancestral EMRSA-15 reference strain which may potentially affect CC22 lineage specific genome evolution via HGT in the future.

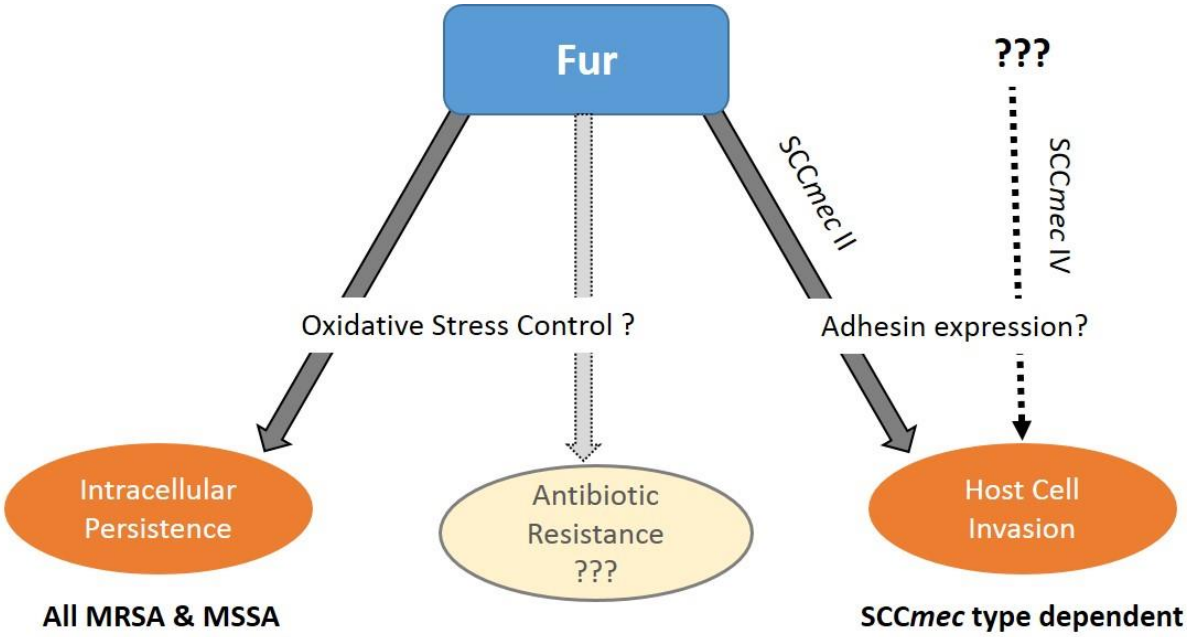


Figure 5-8 A representation of novel Fur associated phenotypes found in MRSA strain backgrounds. Fur was found to positively influence intracellular persistence in all MRSA and MSSA strain backgrounds analysed. Host cell invasion was negatively affected by *fur* inactivation in a SCC*mec* II strain background; however additional invasion mediators exist for CC22 SCC*mec* IV strains independent of Fur. Data also suggested a possible Fur effect during antibiotic exposure potentially via Fur directed oxidative stress response regulation.

Moreover, numerous virulence and stress response associated genes exhibit mutations in the CC22 isolates characterised in this study compared to the reference EMRSA-15 strain. Such functional groups include the ClpP proteolytic system which is responsible for protein quality control (Frees et al., 2014), oxidative stress (*sodA*) (Ballal and Manna, 2009), iron acquisition (*isdB*, *harA*) (Pilpa et al., 2009) and adhesion/immune system evasion (*spa*, *sdrD*) (Atkins et al., 2008; Wang et al., 2013). The precise effect of these mutations on protein structure and function is unclear from just the observed allele alterations. Importantly these mutations are conserved in all CC22 isolates characterised in this study indicating a selective benefit. Previous research has demonstrated high levels of variation typically observed in cell surface proteins (McCarthy and Lindsay, 2010), particularly because they are involved in host-pathogen interactions and are subsequently under a high level of selective pressure (McCarthy and Lindsay, 2013). Moreover, it has been shown the CC22 lineage itself is a continuously evolving population, particularly in regards to healthcare associated specific adaption (Holden et al., 2013); therefore mutations which would theoretically demonstrate colonisation and host survival benefits would be selected for during evolution and maintained within this lineage, as is likely the case for the mutations witnessed in this study.

Overall, genomic sequencing data has demonstrated a significant number of SNP/INDEL which have occurred within the CC22 lineage since the collection of an ancestral EMRSA-15 CC22 strain, HO 5096 0412. Interestingly, but perhaps unsurprisingly, numerous virulence and stress response associated genes were affected. This is likely due to their encoded protein roles during host-pathogen interactions and survival in hostile environments such as those frequently encountered in a healthcare setting. Further research would need to identify the precise effect each of these mutations has on protein function/structure and potentially elude to the reason such mutations were selected for and maintained in the CC22 population. Moreover, additional CC22 isolates originating from different geographical locations would need to be screened for the presence/absence of these mutations. This analysis would investigate if this observed pattern of evolution is common to the majority of clones from the CC22 lineage or is an isolated case of adaptation for UHL isolated CC22 clones. Figure 5-9 provides a summary of the

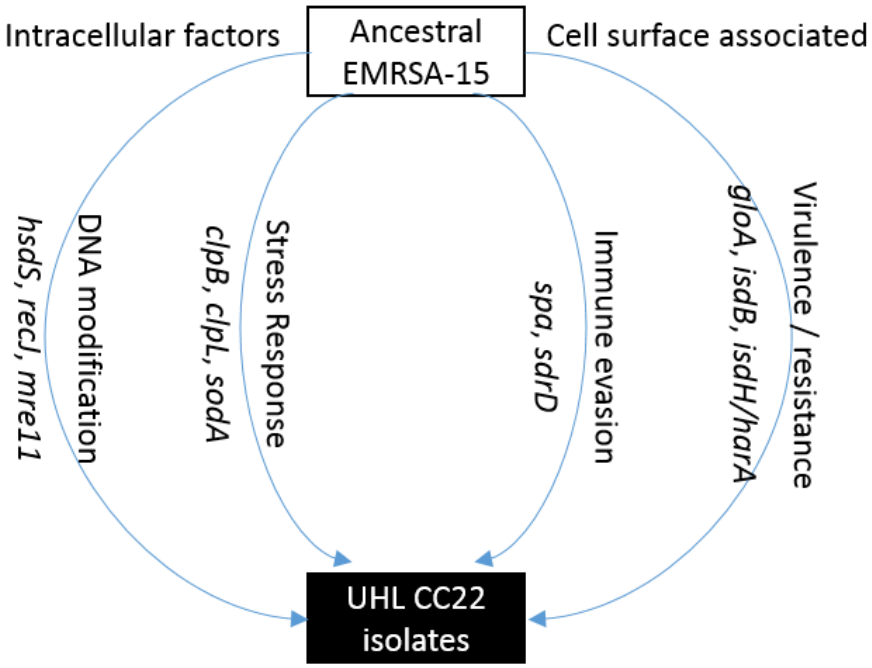


Figure 5-9 Overview of the mutations observed in all UHL associated CC22 MRSA isolates compared to the ancestral EMRSA-15 reference strain. Genome sequence analysis uncovered several genes which experienced mutations during CC22 MRSA lineage evolution. Genes are grouped according to putative functions.

observed evolution of UHL associated CC22 isolates characterised in this study compared to the ancestral EMRSA-15 isolate.

5.5.3 Persistence associated SNP: The significance of MprF and other variant genes

Data generated from whole genome sequencing enabled direct comparisons to be made between initial and subsequent persistent isolates within and between individual infections. This led to the identification of genes affected by *in vivo* genome evolution during infection progression. Additionally, exclusivity of these genetic alterations to persistence development could be confirmed by analysing the genome sequences of resolved bacteraemia isolates. The most significant finding of this analysis was that persistent isolates originating from two independent cases of persistent bacteraemia (PB1 and PB3) displayed multiple incidences of *mprF* mutations. Moreover, distinctly different *mprF* SNP were exhibited by individual persistent isolates within each infecting strain. This truly highlights the intense *in vivo* selective pressure place on MprF function during PB1 and PB3 persistence development. Importantly, PB1 and PB3 persistent isolates also exhibited similar phenotypic signatures presented in previous chapters (Chapter 3 and 4) correlating exactly with the emergence of *mprF* SNP. Consequently this study is the first in its kind to present a relationship between *mprF* mutations, the emergence of distinct virulence phenotypes and the development of persistence during two independent cases of persistent bacteraemia.

MprF catalyses the synthesis of L-PG and translocates the positively charged molecule to the cell membrane which reduces the overall negative charge exhibited by the bacterial cell surface; Figure 5-10 gives a simplistic representation of MprF function (Ernst et al., 2009; Oku, 2004; Peschel et al., 2001; Weidenmaier et al., 2005). Membrane charge alterations are particularly important for HDP/CAMP susceptibility. HDP/CAMP is an indiscriminate antimicrobial strategy against infecting pathogens which initiate cell death via the formation of pores in the microbial membrane (Peschel and Sahl, 2006; Peschel et al., 2001). Interestingly, daptomycin when coupled with calcium, exhibits a similar mode of action to HDP/CAMP where it is able to adhere to the anionic microbial membrane and cause depolarisation leading to cell death (Bayer et al., 2013).

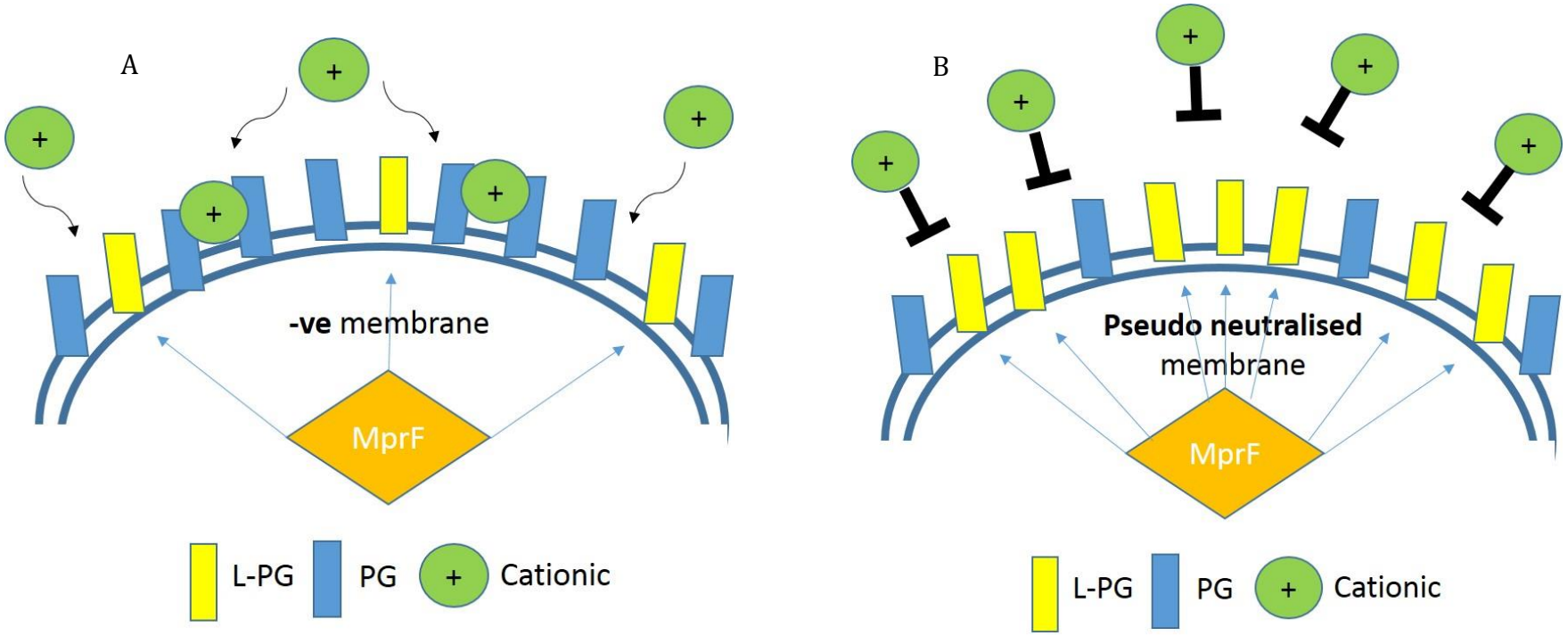


Figure 5-10 Illustration of the PG and L-PG content within the bacterial cell membrane. Under wild type MprF conditions, the cell membrane exhibits a basal negative charge, which is exploited by cationic molecules such as HDP/CAMP and daptomycin (coupled with calcium) (A). In *mprF* variants, there is increased L-PG synthesis and/or translocation to the cell membrane, contributing to the neutralisation of the cell membrane and consequent inhibition of cationic molecule adherence (B).

SNP in *mprF* have been previously associated with the development of daptomycin-nonsusceptibility and increased HDP/CAMP resistance (Bayer et al., 2013; Boyle-Vavra et al., 2011; Weidenmaier et al., 2005; Yang et al., 2013). Such incidences involve gain-of-function (GoF) mutations which exacerbate the neutralisation of the cell membrane either through increased L-PG synthesis and/or translocation to the cell membrane. This reduces the affinity of cationic molecules including HDP/CAMP and daptomycin/calcium complexes to the cell membrane (Bayer et al., 2013; Jones et al., 2008). Reduced susceptibility to HDP/CAMP has been independently associated with persistent *S. aureus* isolates (Fowler et al., 2004; Seidl et al., 2011; Xiong et al., 2009). As has the development of daptomycin resistance in persistent *S. aureus* bacteraemia cases involving prolonged daptomycin treatment (Dortet et al., 2013; Gasch et al., 2014; Sharma et al., 2008). However, none of the above studies have actually presented the combined associations of daptomycin treatment, the subsequent *in vivo* selection of *mprF* mutations, emergence of daptomycin non-susceptibility in combination with HDP/CAMP resistance with incidences of persistent *S. aureus* bacteraemia.

The three distinct *mprF* SNP identified in this study (S295L, P314L and L826F) have been previously presented as causal for daptomycin-nonsusceptibility and increased resistance to HDP/CAMP (Mishra et al., 2013; Peleg et al., 2012; Rubio et al., 2012; Yang et al., 2013, 2009). These particular variants have been shown to display a reduced ratio of PG:L-PG and thicker cell walls when compared to their isogenic wild type isolates, however only one SNP (S295L) was associated with increased *mprF* expression (Mishra et al., 2014, 2013; Peleg et al., 2012; Rubio et al., 2012; Yang et al., 2013, 2009). Two of the amino acid substitutions witnessed in this study occur in the translocation and lysinylation domains (S295L P314L) whereas the third (L826F) occurs exclusively in the lysinylation domain (Peleg et al., 2012). It is therefore easy to hypothesise these amino acid changes lead to increased L-PG synthesis/lysinylation possibly in combination with increased L-PG translocation into the cell membrane. This would have the effect of decreasing PG:L-PG membrane content leading to a reduction in cell membrane negative charge. However, only S295L and P314L have been associated with a total increase in L-PG content (Mishra et al., 2014; Rubio et al., 2012). An alternative hypothesis for *mprF* mutation(s) which do not display a difference in L-PG abundance (e.g. L826F) is they exhibit

increased flippase activity resulting in a membrane bias towards L-PG (Rubio et al., 2012). Rather conversely, only one of these *mprF* mutations (S295L) has ever been associated with reduced cell surface charge (Mishra et al., 2014, 2011; Yang et al., 2013). Therefore a common mechanism for HDP/CAMP resistance and daptomycin non-susceptibility associated with *mprF* mutations is yet to be determined.

Sequence data has also enabled additional conclusions to be made regarding the PB3 infecting strain. The emergence of two *spa* mutations in the persistent population suggests there was also selective pressure placed on SpA during the PB3 infection. SpA or protein A was the first immunoglobulin binding protein to be identified in *S. aureus* associated with multiple immune evasive properties. Such functions include inhibition of phagocytosis and complement fixation in addition to acting as a B-cell super-antigen and reducing the B-cell pool (Atkins et al., 2008; Foster, 2005; Silverman et al., 2005). Mutations in *spa* have been frequently found in *S. aureus* isolated from blood; one particular study found over representation of nonsense transversions resulting in protein A truncations (Khrustalev et al., 2013). However, one of the incidences of *spa* mutation observed in this study was found to be silent/synonymous which subsequently reduces the hypothesised impact SpA had on the progression of the PB3 infection.

So far no defining phenotypic traits have been indicated for either the PB2 or PB5 persistent cases. Subsequently it was impossible to hypothesise on any potential data retrieved from whole genome sequence analysis. There was no one candidate persistence gene common to both cases, however both did display SNP in cell surface related proteins. PB2 displayed a truncation in SaeQ; this could suggest subsequent alterations in the expression of the SaeRS regulon within the PB2 persistent isolate due to SaeQ's putative involvement in SaeR dephosphorylation (Jeong et al., 2013). However there was no indication of a SaeRS effect during previous phenotypic characterisation conducted. The second gene modified in PB2 infection was a peptidoglycan ligase (Ddl), which was likely a result of exposure to cell wall antibiotics during treatment, however there was no associated increased in antibiotic MIC.

Within PB5, a pyruvate kinase (Pfk) experienced an insertion of six amino acids. PfK has been shown to be essential for cell viability in *S. aureus*, demonstrated by the

inability to construct knockout mutations within this gene (Zoraghi et al., 2010). Therefore it is somewhat surprising this mutation has been tolerated in the PB5 infecting strain; this is likely due to the fact that the insertion does not disrupt the reading frame of the gene. The second mutation within *sasA*, sometimes known as *sraP*, led to a protein truncation. SasA is a cell surface associated protein, containing a signal peptide, two serine rich repeats and a cell wall anchor; this particular mutation is hypothesised to terminate translation within the second serine rich region (Roche, 2003; Yang et al., 2014). This protein was been found to be particularly important in endovascular infections where it adheres to human platelets and activate PMP secretion (Fitzgerald et al., 2006; Siboo et al., 2005). Consequently, inactivation of *sasA* could lead to a reduction in platelet associated PMP secretion which would aid an infecting strain overcome one aspect of the host immune system.

Overall, genotypic data has implicated a role for MprF during the development of persistence in two independent cases of MRSA bacteraemia. Moreover, the apparent high level of *in vivo* selective pressure placed upon MprF function was so substantial that multiple *mprF* mutations occurred within the same bacterial population during the course of both infections. There is also evidence *mprF* mutations not only led to daptomycin nonsusceptibility, but also the development of specific virulence signatures which have been observed during the course of this study. This aspect will discussed in detail in the subsequent chapter. Figure 5-11 diagrammatically presents the genes previously mentioned which experienced mutations during each of the persistent infections.

5.5.4 Conclusions

This chapter has encompassed three distinct research areas; the role of Fur in various MRSA strain backgrounds, genetic evolution of the CC22 lineage from an ancestral EMRSA-15 reference strain and identification of putative persistence associated genetic determinants. Despite data not indicating a direct role for Fur in persistence development, results suggest novel Fur related phenotypes within a MRSA strain background such as host cell invasion and intracellular persistence

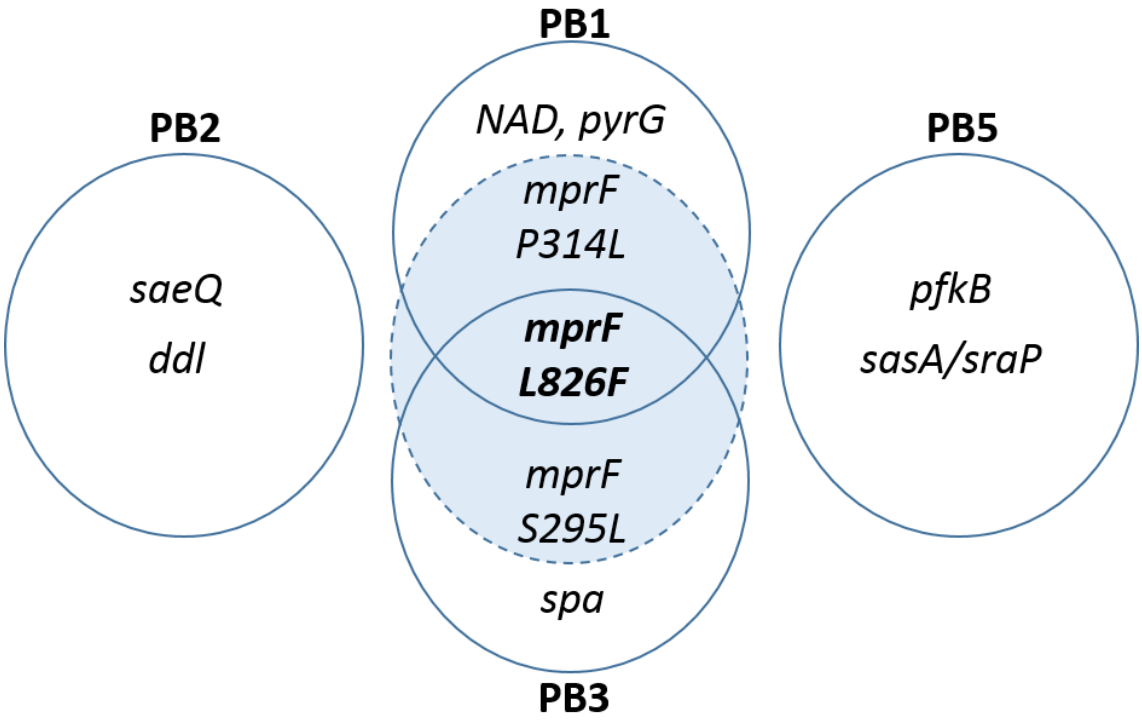


Figure 5-11 A diagrammatical representation of the persistent isolate associated SNP in each of the persistent MRSA bacteraemia cases. Each gene which experienced a mutation between initial and persistent isolate during PB1, PB2, PB3 and PB5 infections are contained in individual circles; if a particular mutation is found in more than one case then the circles overlap i.e. *mprF* L826F

capabilities. It is imperative these novel findings are further investigated as they may lead to a new understanding of the role Fur plays *in vivo*. Moreover, sequence data has provided more evidence for the continuous evolution within the CC22 lineage highlighting the propensity of this epidemic population to acclimatize to new hostile situations. This supports the notion that EMRSA-15 CC22 clones will continue to pose a threat within the healthcare setting and that more work is needed to prevent further evolution and expansion. Finally, *mprF* has been implicated as a major genetic determinant for persistence development in two independent cases of persistent MRSA bacteraemia. The intense selective pressure placed on MprF function specifically cell membrane modifications, is likely a result of antibiotic prescriptions during the infections (i.e. daptomycin). Subsequently, this finding has massive implications for current treatment regimens during complex *S. aureus* bacteraemia and should be of significant clinical concern. Figure 5-12 displays an updated view of persistence that has been presented in previous chapters, which incorporates what has been observed during genomic analyses. The final chapter will collate all the previously presented results relating to *S. aureus* persistence and from this construct an overall model, inclusive of phenotypic and genotypic determinants, for the development of *S. aureus* persistence.

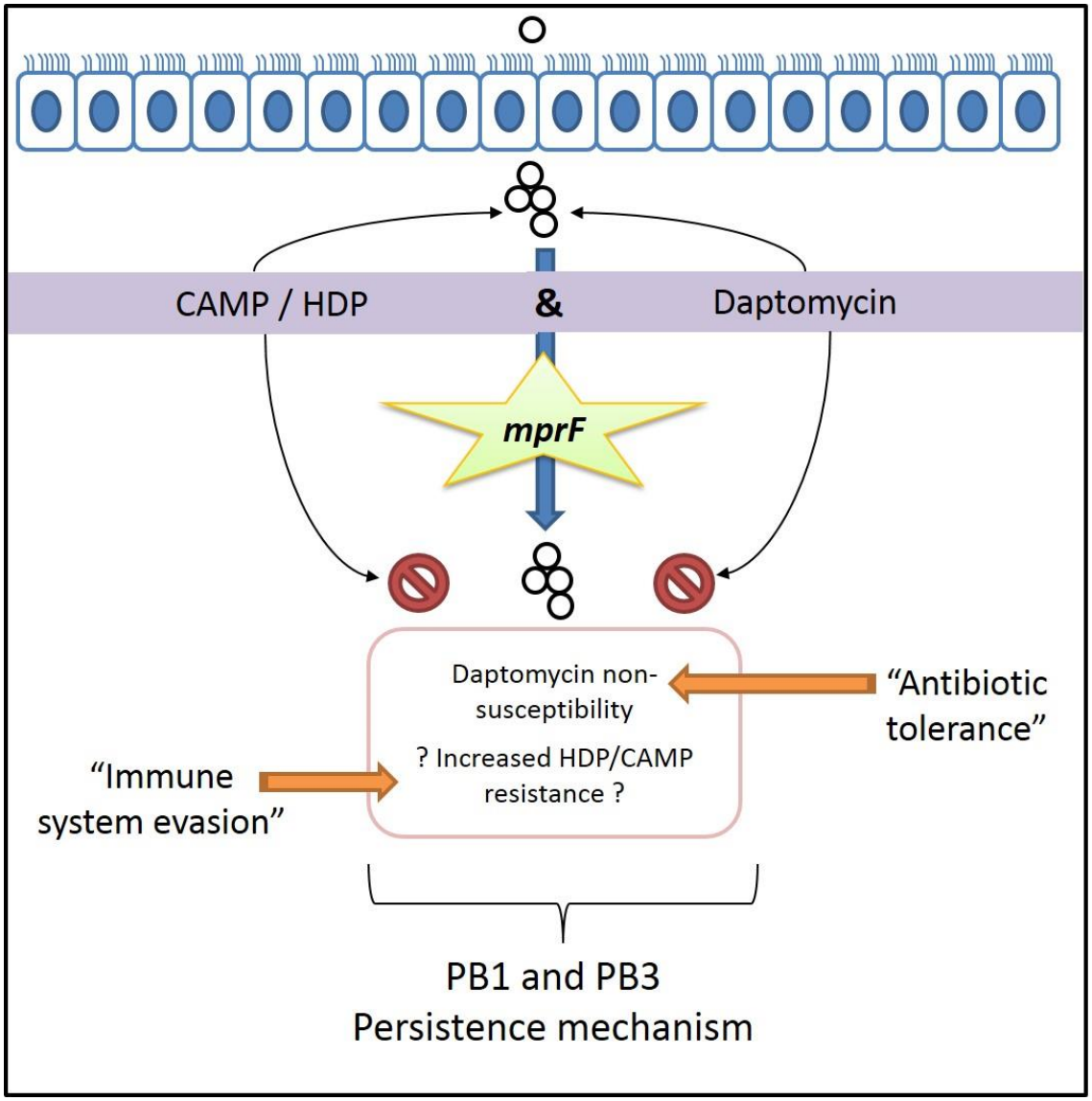


Figure 5-12 Current persistence model presented after Chapter 5's data. This diagram highlights the main mutation witnessed in the PB1 and PB3 persistent bacteraemia cases, *mprF*. It is hypothesised exposure to daptomycin and HDP/CAMP selected for *mprF* GoF mutations which led to daptomycin non-susceptibility and suspected increased HDP/CAMP resistance. These phenotypes fulfil the previously stated prerequisites for bacterial persistence: antibiotic tolerance and immune system evasion.

Chapter 6 Final discussion

6.1 Introduction

The problem of bacterial persistence has been a common trait in numerous chronic infections and is exhibited by a multitude of bacterial species including *S. aureus*. The clinical importance of this phenomenon is attributed to the inherent tolerance displayed by these infecting strains to prescribed antibiotics *in vivo* despite genotypic and observed *in vitro* susceptibility. Biologically, the ability of these infecting strains to overcome the vast number of antimicrobial mechanisms deployed by the host immune system also remains a mystery. It is therefore not surprising that these infections are associated with poorer patient outcomes including prolonged hospital duration, occurrence of metastatic infections and increased attributed mortality rates (Chong et al., 2013; Khatib et al., 2006; Lin et al., 2010; López et al., 2012). All of these factors demonstrate the importance of studies such as this, which aim to uncover the mechanisms behind persistence development with the view to aiding clinical infection diagnosis, management and treatment.

6.2 Aims of this study and the importance of study design

This study firstly aimed to characterise four persistent *S. aureus* bacteraemia which were inclusive of several infecting isolates collected at various time-points during each infection. The analysis of temporally spaced isolates allowed us to ascertain when certain events (phenotypic and genotypic) occurred during the course of individual infections. Additionally, isolates originating from three resolved *S. aureus* bacteraemia were assessed in parallel with persistence derived isolates. This enabled the distinction between traits associated with general infection/bacteraemia adaptation and traits which are exclusively associated with persistence development. Moreover, all isolates from both persistent and resolved infections originated from the same evolutionary lineage (CC22). Therefore the contribution of genetic background differences unrelated to persistence development was minimised. The importance of any traits associated with persistent *S. aureus* isolates was further boosted when they were found in more than

one independent case of persistence; potentially indicating a frequently used mechanism of persistence development.

6.3 MprF and *Staphylococcus aureus* persistence

6.3.1 The significance of MprF during *S. aureus* persistence development

The main finding of this study has been the association of *mprF* mutations with several late infection *S. aureus* isolates originating from two independent cases of persistent bacteraemia (PB1 and PB3). The emergence of these *mprF* SNP occurred as a consequence of *in vivo* genome evolution during both infections. This was concluded because both initial isolates displayed wild type *mprF* gene sequences, whereas isolates collected after the seven day threshold used to define persistence (classified as persistent isolates) displayed various *mprF* SNP. The fact that this phenomenon is witnessed during two separate cases of persistence truly highlights MprF as a major factor during persistence development and potentially a common mechanism for infection progression. Even more significantly, multiple, genotypically distinct *mprF* SNP are observed within both the PB1 and PB3 infecting strains. This finding further demonstrates the strength of selection placed upon *mprF* and its function during persistence development. This subsequently led to multiple expansions of *mprF* variant populations within both infections and persistent isolates displaying genotypically distinct *mprF* alleles. This chapter will now discussion the implications of *mprF* mutations in regards to infection progression and suggest how MprF function could be involved in persistence development.

As discussed in Chapter 5, MprF is responsible for the lysinylation of PG, thereby counteracting the negative charge and converting it to L-PG. MprF is also responsible for the translocation of L-PG to the membrane which subsequently reduces the negative charge and increases the hydrophobic properties exhibited by the bacterial membrane (Ernst et al., 2009). The three *mprF* SNP observed here are said to be GoF as increased synthesis and/or translocation of L-PG are exhibited by *S. aureus mprF* variants (Fischer et al., 2011; Mishra et al., 2013, 2011; Peleg et al., 2012; Rubio et al., 2012; Yang et al., 2013, 2010, 2009). Importantly cationic antimicrobial compounds such as HDP/CAMP and daptomycin, when coupled with

calcium, utilise the wild type cell surface negative charge to adhere to bacterial cells and exert their modes of action. Therefore the increased L-PG synthesis exhibited by *mprF* variants is thought to adversely affect adherence of cationic molecules resulting in reduced susceptibility to HDP/CAMP and daptomycin (Bayer et al., 2013; Jones et al., 2008).

In this study, multiple expansions of *mprF* variant populations correlates well with the emergence of daptomycin non-susceptibility and daptomycin treatment failure in both PB1 and PB3 persistent bacteraemia cases. This finding is supported by previous observations of daptomycin exposure, *mprF* mutations and subsequent daptomycin treatment failure *in vivo* (Bayer et al., 2013; Boyle-Vavra et al., 2011; Weidenmaier et al., 2005; Yang et al., 2013). Interestingly, reduced daptomycin susceptibility has also been linked to mutations within genes other than *mprF*, including *cls2*, *pgsA, rpoB, yycG/walK* (Cui et al., 2010; Patel et al., 2011; Peleg et al., 2012). However, there appears to be bias for *mprF* SNP during *in vivo* exposure. This finding suggests there are additional features exclusively encountered *in vivo* besides daptomycin treatment which leads to *mprF* evolution. One such factor is exposure to the host immune system, specifically HDP/CAMP which is thought to "pre-prime" the infecting isolate to developing daptomycin-nonsusceptibility (Mishra et al., 2012, 2011). Therefore it is hypothesised during the PB1 and PB3 bacteraemia, co-exposure of daptomycin and HDP/CAMP gave rise to highly substantial selective pressure being placed on MprF function; this led to multiple incidences of *mprF* GoF mutations and subsequent *mprF* variant expansion in persistent populations. The combinatory effect of daptomycin and HDP/CAMP on *mprF* evolution is further strengthened by the fact that *mprF* SNP are not observed in persistent infections not treated with daptomycin but still experienced HDP/CAMP exposure whilst *in vivo* (PB2 and PB5). Figure 6-1 provides a simplistic diagram of the different selective pressures and the subsequent genetic variations which have been described in this section.

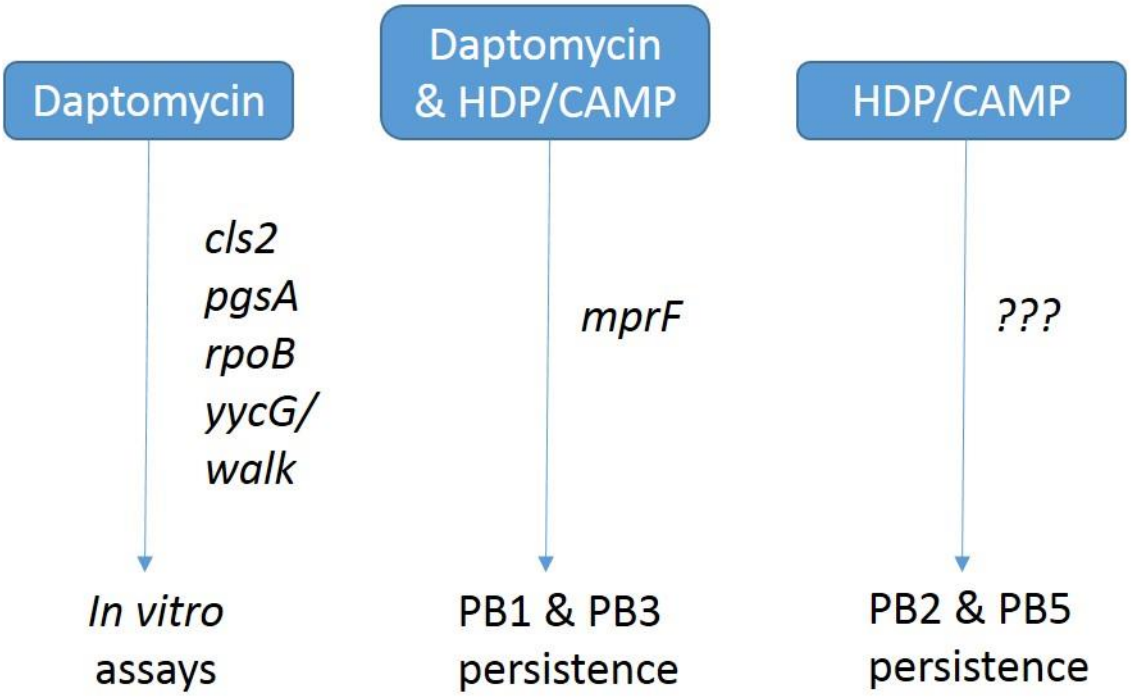


Figure 6-1 Daptomycin and HDP/CAMP associated selective pressures. A simplistic diagram highlighting the selective pressures from daptomycin treatment in vitro or in combination with HDP/CAMP *in vivo*, or singularly from HDP/CAMP without daptomycin treatment in vivo. For each situation a selection of genes which have experienced SNP, if applicable, are listed.

6.3.2 Novel *mprF* variant associated phenotypes related to *S. aureus* persistence

The presence of *mprF* GoF mutations in persistent isolates characterised in this study also correlates with the emergence of novel virulence characteristics not previously published. These MprF associated phenotypes include- growth adaptation in combination with increased abundance of adhesion, immune evasion and stress response cell surface proteins specifically in nutrient and metal ion restricted media; attenuated virulence using a *G. mellonella* larva infection model; increased tolerance to toxic iron levels and solid phase gentamicin induced SCV formation. These traits are in addition to decreased daptomycin susceptibility signified by an elevated daptomycin MIC found in *mprF* variants. Further supporting evidence for the association between MprF and the above virulence characteristics is that they are found in all *mprF* GoF mutants independent of the specific SNP observed. Moreover, these traits are not observed in wild type *mprF* persistent isolates (PB2 and PB5) or resolved isolates originating from daptomycin naïve infections. Therefore this study is the first to demonstrate daptomycin, in combination with HDP/CAMP, induced virulence phenotypes via *in vivo* genome evolution i.e. *mprF*, during two cases of persistent *S. aureus* bacteraemia. These findings highlight the novelty of this study, its data and resulting impact these findings could have on the management of persistent *S. aureus* bacteraemia, particularly in regards to antibiotic treatment choices.

During the proteomic analysis in Chapter 3, it was noted several of the up-regulated cell surface proteins found in persistent *mprF* variant isolates are positively regulated by SaeRS i.e. Eap, Emp and Sbi. SaeRS expression is dependent on the intramembrane histidine kinase, SaeS, which responds to various environment stimuli including salt concentration, pH, antibiotic exposure and oxidative stress (Adhikari and Novick, 2008; Geiger et al., 2008; Novick and Jiang, 2003; Novick, 2003). The specific mechanism of SaeS activation remains unclear but it is likely distortions within the cell membrane led to SaeS kinase activity. Importantly, alterations within the cell membrane, in particular decreased L-PG content exhibited by *ΔmprF* mutants was found to negatively affect the functionality of integral membrane proteins such as SaeS, leading to reduced SaeRS regulon expression including Eap, Emp and Sbi (Sievers et al., 2010). Taken together with

this study's proteomic data it can be hypothesised that the opposite situation is also true. Specifically, an increased abundance of membrane bound L-PG caused by improved MprF function would theoretically promote SaeS activation. This would subsequently result in up-regulation of the SaeRS regulon, i.e. Eap, Emp and Sbi, which is the phenotype observed for the persistent isolates containing *mprF* variants, characterised in this study. Both Eap and Sbi play significant roles in immune system evasion such as interfering with neutrophil function (Chavakis et al., 2002; Haggar et al., 2004; Lee et al., 2002). Eap specifically inhibits leukocyte recruitment, interrupts neutrophil binding to endothelial cells and reduces T-cell proliferation (Chavakis et al., 2002; Haggar et al., 2004; Lee et al., 2002). Sbi binds IgG and β_2-glycoprotein and has also been shown to inhibit neutrophil directed opsonophagocytosis and complement (Haupt et al., 2008; Smith et al., 2011; Zhang et al., 1999, 1998). Moreover, Emp and Eap are both mediators of low iron biofilms and adhesion to a range of host proteins including fibronectin and fibrinogen, known virulence determinants *in vivo* (Hussain et al., 2001; Johnson et al., 2008). Improvement in such functions would enable the infecting strain to colonise the host, develop a niche for proliferation and also evade the host immune system; all factors which are required for bacterial persistence. Moreover, *ΔmprF* mutants also display decreased cell wall biosynthesis protein abundance, namely PBP2a (Sievers et al., 2010), which again correlates with this study's proteomic data. A model depicting the hypothesised interaction between MprF mediated membrane content alterations and SaeS activation can be seen in Figure 6-2.

6.3.3 The effect of *mprF* on global gene regulation

The novel associations between *mprF* GoF mutations and the persistence specific traits observed in this study suggests additional gene regulators are influenced by MprF function either directly or indirectly via changes in the membrane (discussed in more detail later in this chapter). Microarray analysis conducted by Fischer *et al* (2011) using a pair of daptomycin susceptible (dap^S) and non-susceptible (dap^R) isogenic MSSA strains (differing by single nucleotide substitution in *mprF*) revealed interesting transcriptomic differences correlating with one of the *mprF* mutation (S295L) also witnessed in this study (PB3-32-1).

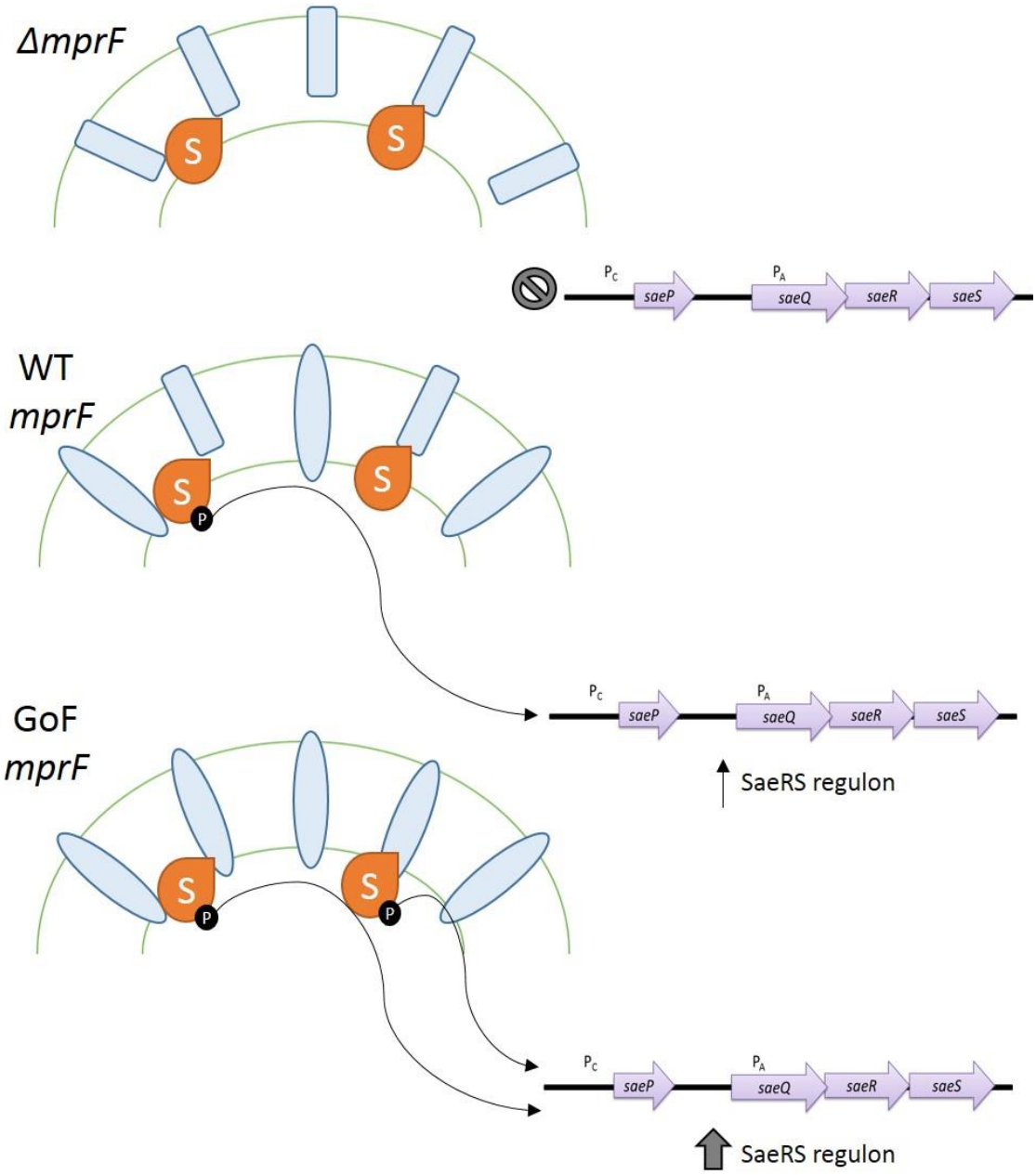


Figure 6-2 The effect of membrane distortion on SaeS activation and control of *saeRS* and SaeRS regulon expression. Inactivation of *mprF* is hypothesised to cause changes in lipid content within the cell membrane negatively affecting SaeS activation which would reduce *saeRS* and SaeRS regulon expression (Sievers et al., 2010). GoF *mprF* mutants are hypothesised to cause contrasting lipid alteration leading to improved SaeS activation, resulting in increased *saeRS* and SaeRS regulon expression.

The similarities between Fischer *et al*'s (2011) findings and this study's data will now be discussed, supported by evidence from additional studies; and potential mechanistic associations between this study's data and findings from previous studies will be speculated upon.

The data from Fischer *et al*'s (2011) study highlighted significant changes between the dap^S and dap^R isolate pairs in regards to the expression of several key gene regulators, further implicating the involvement of MprF in gene and virulence regulation (Fischer et al., 2011). Specifically, up-regulation of *saeR/S*, *rot*, *sarT*, *yycJ/F/I* and numerous ABC transporter encoding genes was observed in the dap^R isolate; together with reduced expression of *agrA/B/D* and *hld* (an indicator of RNAIII expression) (Fischer et al., 2011). Moreover, genes involved in sugar metabolism (*lac*, *tag*, *tre*), cell wall/membrane biosynthesis (*pbp2*, *tag*, *cap*) and adhesion/virulence (*fnb*, *coa*, *hla*, *hlb*) were highly up-regulated in the dap^R isolate (Fischer et al., 2011).

Firstly, the observed *saeRS* and *pbp2* up-regulation in dap^R isolate during Fischer *et al*'s study (2011) is in agreement with our proteomic data and observations made by Sievers *et al* (2010). More interestingly is the differential expression of genes involved global virulence regulation. Increased *rot* (repressor of toxins; Said-Salim et al., 2003) expression, together with decreased AgrA/RNAIII activity could potentially explain the observed attenuated virulence exhibited by persistent *mprF* variant isolates in this study (Fischer et al., 2011). It must be noted that this study found no difference in Agr/RNAIII expression via the qualitative observation of δ-lysin (*hld*) activity. However this particular assay (Chapter 3:6:1) is rather primitive in comparison the quantitative microarray/qRT-PCR data generated by Fischer *et al* (2011); therefore differential expression from the *agr* operon in *mprF* variant isolates leading to reduced virulence cannot be ruled out in this study. At least two independent studies suggested loss of MprF function, via *mprF* knock-out mutants, leads to attenuated virulence in murine infection models (Peschel et al., 2001; Weidenmaier et al., 2005), which is in contrast to this study's observation of attenuated virulence for *mprF* GoF variants. However, attenuated virulence was concluded in these publications by reduced bacterial burdens exhibited by *ΔmprF* strains likely due to improved bacterial clearance by HDP/CAMP. Whereas this

study found decreased larva mortality in combination with equivalent bacterial burdens, when infected with *mprF* variant isolates as compared with their respective initial isolate counterparts.

Increased sugar metabolism and ABC transporter expression presented in the dap^R isolate by Fischer *et al* (2011) could also help explain the observed growth advantage in nutrient and metal ion restricted environments exhibited by persistent *mprF* variant isolates. In support of this association, a separate study found increased expression of the ABC transporter encoding gene, *abcA*, during nutrient limitation (Villet et al., 2014). ABC (ATP binding cassette) transporters are known to be important for the acquisition of essential compounds including amino acids and metals, and also the efflux of toxic substances e.g. antibiotics (Davidson et al., 2008). Their roles in compound import/export also means ABC transporters are critical in maintaining balance of essential but also potentially toxic substances as is the case for intracellular iron concentrations (Klein and Lewinson, 2011). This function suggests an additional involvement of ABC transporters not only during growth in metal ion restricted conditions, but also during exposure to toxic iron levels; this suggestion may have implications for the increased survival in high iron exhibited by persistent *mprF* variants. These hypotheses are purely speculative and based upon the previous knowledge of ABC transporter functions, therefore their involvement in persistence development warrants further research to accept or reject these suggestions.

The differential expression of genes involved in cell wall and membrane metabolism exhibited by the dap^R isolate, particularly the observed up-regulation of *yycJ/F/I* (Fischer et al., 2011) also has potential significance for this study's data. YycGF, aka WalKR, forms a two component regulator system, consisting of a membrane bound sensor kinase (WalK/YycG) and a response regulator (WalR/YycF) which positively regulates a multitude of virulence genes, many of which are also included in the SaeRS regulon (Delauné et al., 2012; Dubrac and Msadek, 2004; Dubrac et al., 2007). Specifically, functions influenced by WalKR include adhesion (*efb*, *emp*, *fnbA/B*), haemolysis (*hla*, *hlb*, *hlg*), immune defence (*scn*, *chp*, *sbi*), autolysis (*atlA*, *lytM*) and cell wall biosynthesis including peptidoglycan turnover (*fmtA/B*, *sle1*, *lytM*, *ssaA*) (Delauné et al., 2012; Dubrac and Msadek, 2004; Dubrac et al., 2007). Many of these

interactions are actually brought about through activation of the SaeRS signalling pathway by WalKR rather than WalR directed transcriptional control (Delauné et al., 2012). Consequently, this study makes a further hypothesis that changes in membrane and/or cell wall content via MprF function not only leads to SaeS activation but similarly WalK. Moreover, WalKR directed SaeSR activation may also occur via structural cell wall changes in a similar way to the MprF and SaeS interaction as suggested by Sievers *et al* (2010).

Constitutive activation of the WalKR system also leads to increased autolysis, attenuated virulence, increased biofilm formation and decreased intracellular persistence in macrophages (Delauné et al., 2012; Dubrac et al., 2007). Three out of four of these phenotypes have been directly assessed in this study, the exception being autolysis, and also shown to correlate with persistent *mprF* variant phenotypes compared to their respective initial isolates. Importantly, data corresponding to biofilm formation and intracellular persistence assays during this study was shown to be significantly affected by background strain differences. Consequently, a definite connection between these phenotypes and persistence development couldn't be confirmed at that time. However, the implication of a role for MprF in the establishment of persistence by Staphylococcal isolates and the subsequent associations between MprF, WalKR, biofilm formation and intracellular survival provides some basis to speculate on the development of phenotypes observed in this study.

Previous literature, in particular Fischer *et al* (2011) and Sievers *et al* (2010)'s studies, which presented transcriptomic, proteomic and phenotypic data based upon the function of MprF and associated proteins provides viable explanations for the persistent *mprF* variant phenotypes observed in this study. Such associations have not be made previously in regards clinical cases of *S. aureus* bacteraemia persistence, in particular the implication of daptomycin treatment on virulence behaviour is extremely novel. It is therefore a priority to investigate such suspected persistence associations further to be able make definite conclusions on persistence development and treatment choices during infection progression.

6.3.4 MprF and Membrane Physical State (MPS)

There is significant evidence to conclude alterations in MprF function have downstream effects on the activation of at least two membrane associated sensor proteins (SaeS, WalK), which subsequently impacts upon global gene virulence via changes in their regulon expression. Therefore it can be speculated changes in membrane lipid content brought about by MprF leads to alterations in the membrane physical state (MPS), which is likely the main stimulus for membrane associated proteins responding to environmental stimuli. It is widely accepted the cell membrane is not only a physical barrier from the extracellular environment, but is also involved in translating environmental conditions contributing to gene regulation. Specifically, environmental factors such as temperature, pH, and osmotic stress alter the structure of membrane bound lipids and overall membrane fluidity; which has been shown to influence the activity of many heat shock proteins (HSP) and many additional stress response systems (Porta et al., 2010a, 2010b; Vigh et al., 2007, 1998). The ClpP proteolytic complex is an example of HSP system involved in general proteome maintenance but is also required for stress survival and virulence expression (Frees et al., 2014; Maurizi et al., 1990; Wickner, 1999).

The ClpP proteolytic system has been shown to influence the activity of several global gene regulators including Agr, Sar, Fur, PerR, SigB and Spx (Frees et al., 2014, 2005; Michel et al., 2006), the precise targets of action are determined by ClpP ATPases e.g. ClpX and ClpC. For example, ClpXP is particularly important for virulence expression where it shows a positive regulatory effect for Agr and subsequently Agr controlled genes e.g. haemolysins (Frees et al., 2005, 2003). Attenuated virulence in murine infection model has also been demonstrated for *clpX*/*clpP* knockout mutants, likely due to the decreased Agr activity exhibit by these isolates (Frees et al., 2005, 2003). Moreover, ClpXP is also required for resistance to HDP/CAMP and cell envelope targeting antibiotics, highlighting its role *in vivo* (McGillivray et al., 2012).

In contrast, ClpCP is known to contribute to the bacterial stress response control, including thermotolerance, oxidative stress, osmotic stress and metal ion homeostasis (Chatterjee et al., 2009). Moreover, ClpCP is essential for anaerobic and nutrient poor growth, intracellular survival, biofilm formation and capsule

biosynthesis (Chatterjee et al., 2009; Frees et al., 2004; Graham et al., 2013; Luong et al., 2011). Importantly, many of these traits are associated with persistent *mprF* variant isolates in this study, potentially implicating ClpCP and possibly ClpXP in persistence development. Moreover, ClpCP is also responsible for antitoxin degradation (Donegan and Cheung, 2009; Donegan et al., 2010), which is interesting as TA systems have been independently associated with persistence development in various bacterial species (Gerdes and Maisonneuve, 2012; Lewis, 2010; Pandey and Gerdes, 2005). Finally, pharmaceutical agents which permanently activate ClpC mediated proteolytic function resulting in nonspecific proteolysis have been suggested as potential drugs for the treatment of persistent/chronic infections (Conlon et al., 2013). Overall, there is significant correlation between the functions of ClpP proteolytic system, in particular ClpC directed specificity, and the phenotypes exhibited by persistent *mprF* variant isolates in this study.

The hypothesis that MPS alterations mediated by MprF could affect membrane associated sensor proteins such as SaeS, WalK, but also stress response systems including the ClpP proteolytic system, has serious implications for this study. Firstly it provides a reputable explanation linking *mprF* GoF mutations with the observed persistent isolate associated phenotypes witnessed in this study. Moreover, it suggests exposure to traditional "antimicrobial agents", daptomycin in particular, has profound effects on the organism's virulence behaviours *in vivo*. Rather than leading to successful infection eradication, antibiotic treatment can result in antimicrobial tolerance not only to pharmaceutical agents (e.g. daptomycin) but also to host immune system defences (e.g. HDP/CAMP). Moreover, such antibiotic induced *in vivo* genome evolution (e.g. *mprF*) can lead to the development of specific virulence determinants likely to cause host deterioration and increase infection duration (e.g. bacterial persistence), as has been observed for the PB1 and PB3 bacteraemia.

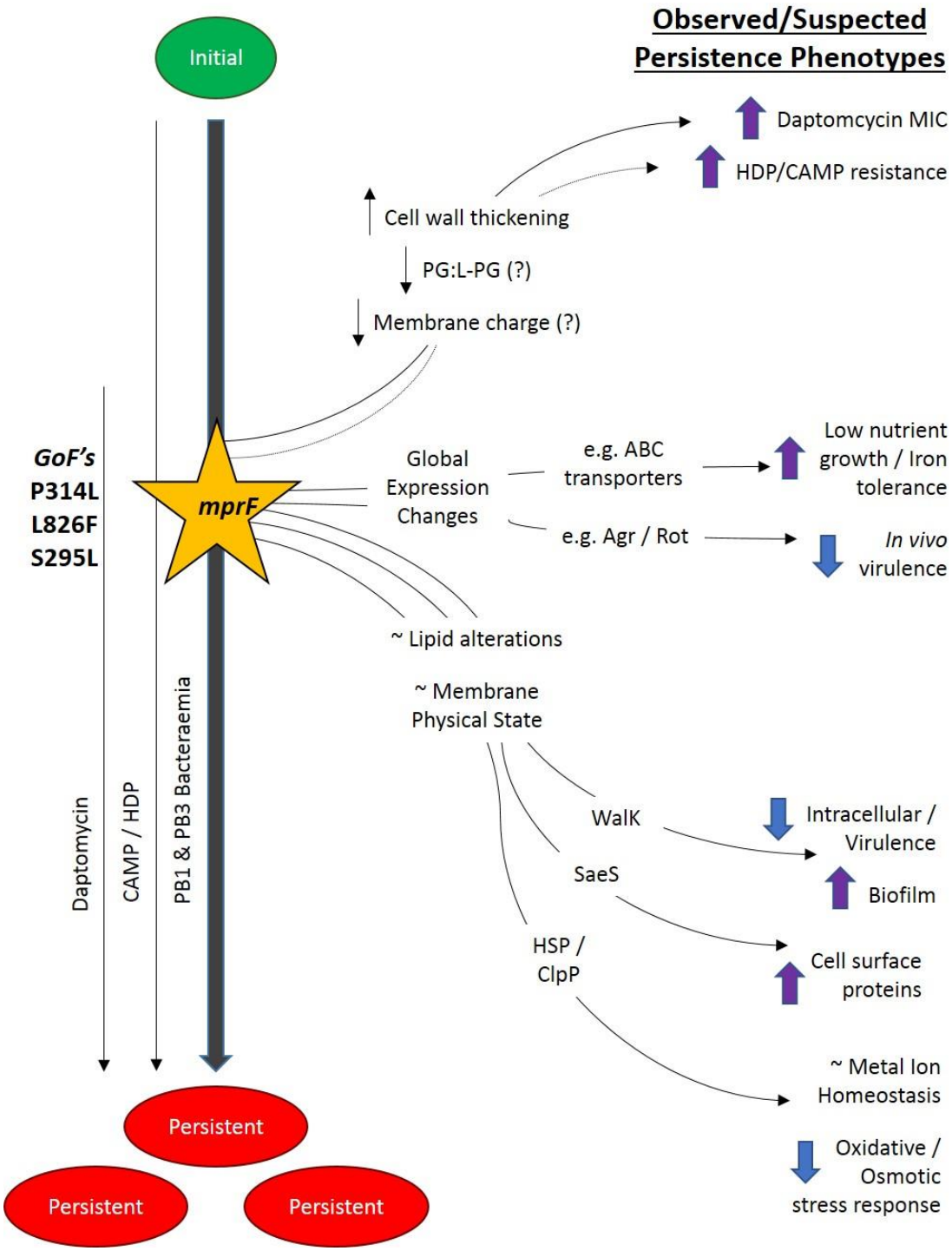


Figure 6-3 Factors leading to mprF GoF mutations and the associated phenotypes observed for the PB1 and PB3 infecting strains. Exposure to daptomycin and HDP/CAMP led to the emergence of multiple mprF GoF mutations within the PB1 and PB3 infecting strains. The resulting persistent mprF variant isolates displayed a range of virulence phenotypes listed on the right. Potential explanations linking mprF variation with the observed persistence associated phenotypic traits are hypothesised.

6.3.5 PB2 and PB5 specific persistence mechanisms

In contrast to the substantial data generated for the PB1 and PB3 persistence cases, there has been no indication of a putative persistence mechanism for either PB2 or PB5 infections. Specifically, there were no defining phenotypic characteristics observed between initial or persistent isolates during the assessments conducted in this study. However, potential findings are solely based upon the assays conducted; it is possible therefore that the PB2/PB5 cases would exhibit differential phenotypic characteristics by implementing different investigative strategies. In each case genotypic analysis identified two genes which experienced SNP during infection progression, which may be individually associated to persistence development.

In regards to the PB2 infection, *saeQ* and *ddl* both exhibited non-synonymous mutations; the former resulting in a protein truncation in the PB2 persistent isolate. SaeQ has been hypothesised to work in conjunction with SaeP and SaeS to dephosphorylate SaeR and terminate the SaeRS phosphorelay (Jeong et al., 2013). Inactivation of SaeQ function would theoretically result in constitutive activation of the SaeRS system. In contrast, a reduction in cell surface associated Eap, which is under SaeRS positive regulation, is observed during 1D SDS-PAGE assessment (Figure 3:14). Moreover, there is no evidence to suggest a major role for SaeQ in *S. aureus* virulence regulation in previous literature. Subsequently the significance of *saeQ* mutation, particularly for persistence development, is still unclear at present. The second protein affected in the PB2 persistent isolate, Ddl, functions as a cell wall modification enzyme. Cell wall modification enzymes and cell wall thickening have been implicated on numerous occasions in regards to mediating antimicrobial resistance, particularly to vancomycin (Cui et al., 2003). However, no difference in vancomycin MIC was observed between initial and persistent isolate. Consequently, there appears to be no association between Ddl function and virulence expression, particularly bacterial persistence.

An in-frame insertion within *pfk* and a frameshift insertion within *sasA*/*sraP* were collectively observed in the PB5 persistent isolate compared to the respective initial isolate. Pfk, a pyruvate kinase, has been found to be essential for *S. aureus* cell viability (Zoraghi et al., 2010) presumably due to roles in ATP synthesis and glycolysis. It could be hypothesised that changes in this protein's function may alter

the persistent isolates metabolism characteristics, however this was not observed during the course of this study. It is possible this insertion does not affect protein function as the reading frame remains intact; therefore this mutation could be circumstantial to persistence development in PB5. The second mutation within *sasA*/*sraP* changes the reading frame, resulting in a truncated protein, therefore this mutation would likely affect protein structure and function. The SasA protein function has been shown to be particularly important during endovascular infection due to its immune evasive properties. Specifically this protein is able to bind human platelets (Siboo et al., 2005) and is hypothesised to mediate host epithelial cell adhesion and invasion (Yang et al., 2014). Its role in host cell adhesion and invasion is difficult to explain as no differences were observed between the PB5 associated isolates during eukaryotic cell invasion assays (Figure 4:4). Whereas, *sasA* inactivation could potentially result in reduced platelet associated PMP secretion thereby aiding host immune system evasion, which is one of the prerequisites for bacterial persistence. As *sasA* variation is only observed in one case of persistence, its global relevance for persistence development is unclear. However, it does provide an alternative mechanism for host immune system avoidance in addition to those described for PB1 and PB3.

6.4 Additional study considerations and further research

The design of this study is based upon retrospectively identifying persistent and resolved bacteraemia cases from hospital records and collecting the relevant frozen bacterial samples stored at the UHL for laboratory characterisation. The standard operating procedures employed during bacteraemia diagnosis meant that prior to being archived, these isolates had already gone through a series of sub-cultures; including dilution and automated incubation of the original blood culture in broth followed by plating onto blood agar plates (see section 1.5.1). Subsequently, the original *in vivo* behaviours of the isolates cannot be ascertained as it is entirely possible, and likely probable that these procedures altered and/or masked potential *in vivo* specific phenotypes. Unfortunately, this factor was unavoidable and therefore this study aimed to employ assays which attempted to replicate the environment in which these isolates persisted *in vivo*. Moreover, this also meant that sampling biases were likely introduced during sub-culturing and only a sub-

population of the infecting bacterial strain could be used to represent the entire infection at any one time point. To compensate for this issue, all available frozen stocks occurring at different time-points during each bacteraemia were used for characterisation. This included occasions where multiple blood cultures were taken a few days apart or on the same day (PB1-15 and PB3-32 for example), resulting in two or more mutually exclusive frozen hospital stocks corresponding to one particular time-point. Phenotypic homogeneity was confirmed between multiple isolates collected within a particular time-frame associated to individual infections; providing evidence for the existence of bacterial population homogeneity, but only after the original blood culture was processed which is the unavoidable caveat.

The only time-point where multiple independent blood cultures were not available and therefore homogeneity within the bacterial population could not be confirmed was during the initial stages of diagnosis i.e. the first blood culture diagnosis plate. However research conducted by Gerlini et al (2014) provided evidence to suggest that Pneumococcal bacteraemia develop as a result of single cell bottlenecks and are largely independent of the size of the inoculum (Gerlini et al. 2014). These single cell bottlenecks are the result of two distinct phases of infection; the first entailing majority clearance via macrophage action leaving a sub-set of cells surviving (Gerlini et al. 2014). This is followed by a stochastic invasion event involving a single surviving cell which, by-chance, reaches the necessary proliferation threshold required to establish a systemic infection (Gerlini et al. 2014). Assuming this had occurred during each of the Staphylococcal bacteraemia, it can be suggested that the initial blood culture contained a clonal population of that one single surviving cell which instigated the bacteraemia. Therefore analysis of a single clone at the initial infection time point could be used as a reasonable representative of the bacterial population in view of there being no optimal alternatives.

Another issue to take into consideration is the time taken from the emergence of infective symptoms within the patient to the prescription of empirical therapy. Moreover, whether the empirical therapy was appropriate for that particular infecting agent is also an important consideration, especially in regards to the progression of each infection. Empirical therapy choices are decided by the primary clinician based upon the clinical presentation of the patient and the current

prevalent pathogens specific to that institution. These drugs typically contain a combination of the following antimicrobials- β-lactams, aminoglycosides, glycopeptides (e.g. vancomycin) and quinolones, and are designed to be "broad-spectrum" as the specific details of the infecting agent are not currently known. Previous studies have shown contradictory data, whereby a delay (i.e. > 48 hours) in "effective" treatment, meaning that inappropriate antibiotics had been used for empirical therapy, is associated with disease complexity such as increased hospital duration and mortality rates (Lodise et al., 2003; Price et al., 2010). However, other studies found no such correlation (Kim et al., 2006; Roghmann, 2000). Taken together, it is still important to bear in mind the empirical therapies administered during each case of persistent bacteraemia included in this study and how these "ineffective" treatments may have influenced infection progression, eventually leading to persistence.

The main advantage to using one location i.e. UHL for the collection of clinical isolates was that it reduced bias from different circulating *S. aureus* strains within different geographical locations. A further research consideration would be to include additional medical trusts each contributing their own set of persistent and resolved infecting isolates all originating from the same lineage. This theoretical cohort collection would also determine if the persistence associated traits discovered in this study are global to all *S. aureus* strains or if different circulating strains i.e. different lineages, have their own distinct mechanisms of persistence development. This would be especially interesting for MRSA and MSSA comparisons, particularly because the latter has demonstrated less clonality compared to the former (Campbell et al., 2008).

Several MSSA bacteraemia cases, inclusive of persistent and resolved types, were also identified and collected from the UHL during the course of this study. However, due to general time and practical constraints this collection has yet to be phenotypically characterised fully as has been completed for the MRSA counterparts. These isolates were subjected to whole genome sequencing in parallel with MRSA strains, which has given an idea of MSSA epidemiology in this geographical location. Preliminary genomic data has suggested there is no one circulating MSSA strain responsible for bacteraemia incidences, as numerous

distinct CC lineages were identified. Moreover, different lineages are found between persistent and resolved infections. This is likely a result of the small cohort collection and not an indication of strain bias for particular infection types. But this may present challenges when attempting to separate persistence specific traits from general strain-to-strain variation caused by genetic background differences. It may be necessary to identify additional MSSA bacteraemia cases associated with a sub-set of lineages enabling persistent/resolved specific comparisons within the same lineage.

Another research aspect currently underway in collaboration with Dr Sam Sheppard, Dr Ben Pascoe (University of Swansea) and Dr Julie Morrissey (University of Leicester) aims to identify persistence specific genetic markers via genome wide association studies (GWAS) using the complete *S aureus* bacteraemia cohort collection. This analysis will utilise all available genome sequencing data from MRSA/MSSA, persistent/resolved, initial/persistent associated isolates; importantly this will be independent of lineage/strain type. From this data the aims are to answer two main biological questions relevant to persistence development-

- Are persistence associated strains predisposed to developing a persistent infection? I.e. markers found in initial and persistent *S. aureus* isolates originating from persistent infections, but not observed in resolved infection isolates
- Are common markers developed as a result of persistence development? I.e. markers found in persistent *S. aureus* isolates but not observed in initial isolates originating from the same persistent infection or resolved infection isolates.

This method has the advantage that it disregards any genetic variation between different strains, allowing persistence specific comparisons. Additionally, it is a robust strategy to find common persistence markers between multiple infections potentially indicating a frequently used mechanism and/or consequence of *S. aureus* persistence. One MSSA (Newman) and one MRSA (PM25) commonly used as lab reference isolates were also included in whole genome sequencing, enabling the

identification of markers specific to recent clinical bacteraemia isolates within MSSA and MRSA strain types.

6.4.1 Future research ideas

Overall, there are many different applications that could be employed to build upon the findings presented in this study. This first and foremost strategy would be to investigate changes in global expression patterns between initial and persistent *mprF* variant isolates, mediated by the hypothesised alterations in gene regulator functions (discussed in detail earlier in this chapter). Such transcriptomic techniques would involve DNA microarrays and/or RNAseq (RNA sequencing). DNA microarray analysis is as high throughput, relatively inexpensive strategy, however it relies upon prior knowledge of the transcriptome; meaning that open reading frames yet to be bioinformatically identified would not be detected, such as small RNA. Moreover, DNA microarrays have difficulty with accurately quantifying both high and low levels of gene expression due to the high level of background signal typically experienced (Malone and Oliver, 2011; Wang et al., 2009). Therefore the sensitivity and resolution of this technique may not be sufficient for this type of study where subtle changes in expression are likely.

An alternative strategy is RNAseq which doesn't require prior knowledge of the transcriptome, therefore an unbiased observation of the whole transcriptome can be made. The sequence of the entire RNA fragment is determined which would aid the discovery of novel small RNA. Additionally, it is more sensitive for the quantification of high and low level transcripts compared to alternative hybridisation techniques (Malone and Oliver, 2011; Wang et al., 2009). The main disadvantage is the relative cost of deep sequencing; but as these techniques become more refined and used more routinely the cost will continue to decrease.

Overall, the assessment of the transcriptome is paramount in taking this research further as it will firstly identify the effects of *mprF* GoF mutations on global expression regulation, specifically within the persistent isolates characterised in this study. Additionally, this type of gene expression assessment would help investigate alternative mechanisms of persistence development which did not involve *mprF* mutations such as in PB2 and PB5 bacteraemia cases.

An additional research aspect would be to investigate the consequences of *mprF* GoF mutations in these persistent isolates on membrane lipid content and subsequently the MPS. To achieve this, a technique which would identify and quantify individual lipids within complex mixtures is required, thereby enabling comparisons to be made between the initial isolate and the persistent *mprF* variant isolates. Traditionally, high performance liquid chromatography (HPLC) has been used to analyse lipid content; however it is rather labour intensive and has relatively low sensitivity and resolution for low abundance lipids. Alternative techniques such as LC-MS have been optimised for lipid characterisation in mixed samples i.e. "lipodomics" (Layre and Moody, 2013). Chromatographic lipid separation prior to MS increases resolution and sensitivity for low abundance lipids and would therefore be appropriate for use during further investigations.

Moreover, the use of animal infection models would help identify the contribution persistence associated, *mprF* mediated phenotypes had on host-pathogen interactions during infection progression. Moreover, the specific environmental conditions experienced during PB1 and PB3 persistence could be replicated, specifically exposure to daptomycin in combination with HDP/CAMP. This type of model could be used to see if PB1 and PB3 specific persistence adaptation could be induced using additional *S. aureus* strains such as lab reference isolates. Moreover, the effect of *mprF* knock-out mutants in PB1 and PB3 persistence isolates could be investigated for a hypothesised inability to persist in a murine bacteraemia model; or conversely the *mprF* GoF alleles could be introduced into PB1 and PB3 initial isolates and their ability to persist measured.

6.5 Conclusions

This study is the first of its kind to investigate multiple persistent MRSA bacteraemia inclusive of numerous temporally spaced infective isolates, in parallel with contemporaneous resolved bacteraemia isolates of the same genetic background. Through this novel work, two distinct mechanisms of *S. aureus* bacteraemia persistence are presented.

The first mechanism involved *in vivo* daptomycin exposure during two independent cases of persistent MRSA bacteraemia which caused the emergence of *mprF* GoF

mutations in both persisting bacterial populations (PB1 and PB3). In addition to the development of daptomycin non-susceptibility, a known consequence of *mprF* mutation, this study also identified several novel virulence phenotypes exclusively associated with both sets of persistent *S. aureus mprF* variant isolates. These phenotypes were not observed in their respective initial MRSA isolates, in isolates originating from daptomycin naïve infections (PB2 and PB5) or resolved bacteraemia isolates. This is the first time, to our knowledge, that MprF has been associated with the emergence of specific virulence determinants as documented in this study and implicated in the development of *S. aureus* persistence.

In contrast, this study was unable to find defining phenotypic or genotypic traits associated with the second persistence mechanism exhibited by PB2 and PB5. It is entirely possible phenotypic differences do exist but more diverse assays would need to be implemented during further investigation. Or indeed, there may be no defining features to be found. In either case, the fact that these two infections were not treated with daptomycin actually provides more supporting evidence for the daptomycin/MprF mediated persistence mechanism previously stated for PB1 and PB3.

Figure 6-4 diagrammatically depicts this study's findings and presents the two previously mentioned *S. aureus* persistence mechanisms. Overall, this study has significant implications for the current clinical treatment regimes implemented during complex *S. aureus* bacteraemia, particularly antibiotic treatment choices (e.g. daptomycin) and potentially for other cases of bacterial infection where persistence is frequently observed.

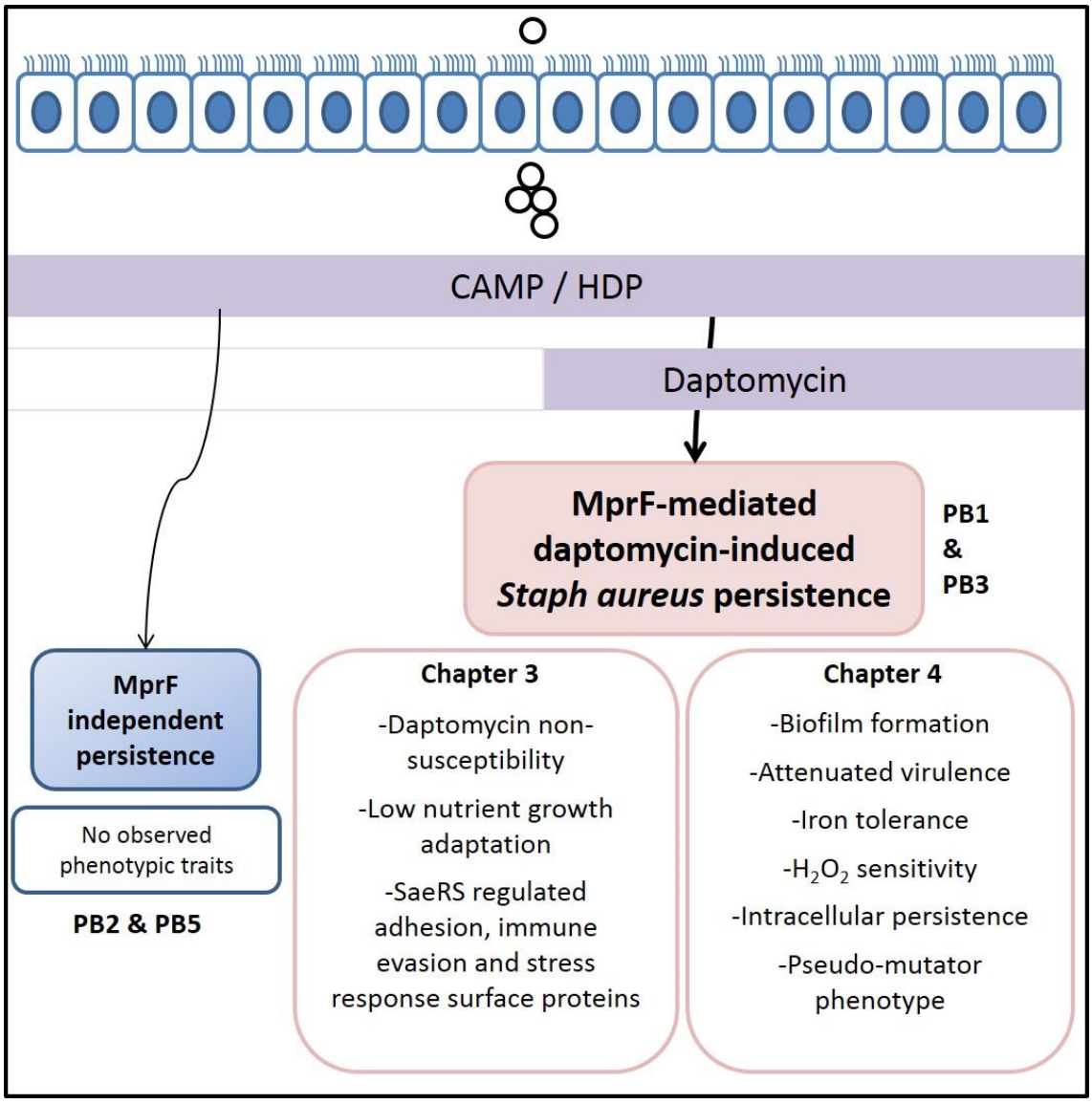


Figure 6-4 Model of daptomycin and HDP/CAMP induced S. aureus persistence mediated by *mprF* GoF. The study presents a model by which daptomycin in combination with HDP/CAMP exposure selects for *mprF* GoF mutants, enabling improved tolerance to antimicrobial agents but also the development of specific virulence signatures which are advantageous to persistent development. This mechanism is observed for PB1 and PB3 cases specifically; whereas no defining characteristics are observed for the PB2 and PB5 infecting strains likely due to the absence of daptomycin exposure during treatment.

Appendix

Table A1 Down regulated cell surface associated proteins in RB4*Δfur* calibrated to the isogenic wild type isolate, RB4, as determined by iTRAQ LC-MS. The proteins which displayed > 0.5 $\log_2$ ratio decrease in RB4*Δfur* compared to the wild type RB4 are listed below, arranged according to protein function. The relative proteins amounts are averages of two biological repeats (each biological repeat inclusive of two technical repeats).

Down regulated in *Δfur* mutant

Accession number	**Protein name/description**	**Fold diff**
Gene regulators		
Q2FJ20.1	Transcriptional regulator SarA	-0.55
Q2FEJ8.3	HTH-type transcriptional regulator SarR	-1.15
D1GND3; Q5HF12.1; Q6GFT9.1; Q99TA4.2; Q9RFJ6.2	HTH-type transcriptional regulator Rot	-1.25
E5QUS2; P0C0R2.1; Q2YUS0; Q6GKJ3.1	Staphylococcal accessory regulator SarS	-0.85
Virulence factors		
Q6GFB8.1	Map protein Eap	-2.85
D2N5J8; Q2FIK4.1; Q99VJ2.1	Extracellular matrix protein-binding protein Emp	-1.3
D2NA02; Q2YVZ4.1	Immunoglobulin-binding protein Sbi	-2.3
Q6G7T7.1	Uncharacterized leukocidin-like protein 2	-0.65

Stress response proteins		
Iron acquisition		
Transporter/Translocator proteins		
Protein synthesis		
Q99U14.1	30S ribosomal protein S1	-0.75
A6U2F0.1; A7X3A6.1; Q2FXP7.1	Threonine--tRNA ligase	-1.35
Q6GGD5.1; Q6G902.1; Q5HFJ5.1; Q2YT14.1; Q2FY08.1; Q2FGF8.1; P99129.1; P67035.1; P67034.1; E5R6C0; D9RK16; D2N7Q6; D1GM94; A8Z4A4.1; A7X2W2.1; A6U237.1; A6QHA8.1; A5IT93.1;	Glycine--tRNA ligase	-0.9
Q6GHP2.1; Q6GA19.1; Q2YXH4.1; E5RB90; D2N6G7; D1GSR8; A7X1D8.1	Isoleucine--tRNA ligase	-0.6
A7WWP0.1; Q2FKP7.1; Q2YUR2.1; Q6GD81.1; Q6GKT6.1; Q8NYY2.1	Serine--tRNA ligase	-0.6
Q6GG85; Q6G8V1.1; Q2YT60.1; Q2FGA8; D2N7V8; A8Z4F6.1; A7X328	Alanine--tRNA ligase	-1.2
A7X3G2; Q5HF45	Tyrosine--tRNA ligase	-0.55
Other cellular processes and general maintanance		
Q5HFV0	DNA-binding protein HU	-1.4

Other enzymatic reactions (Synthesis, modification, digestion)

D1GM42; Q2FK44.1	Formate C-acetyltransferase	-0.55
Q99W07.1; Q2FJ31.1	Alcohol dehydrogenase	-1.25
Q2FDQ4.1	Fructose-bisphosphate aldolase class 1	-0.95
Q2FDQ7.1	L-lactate dehydrogenase 2	-0.95
D9RFF5	Phosphoglycerate kinase	-0.75
Q2FDV8.1; Q2FV74.1; Q6G6C6.1; Q99R88.1	ATP-dependent Clp protease, ATP-binding subunit ClpC	-1.3
D2N6H4; Q5HGN1.1	Dihydroorotase	-0.5
A7X0H6.1; Q2FIB3.1; Q2FZU0.1; Q2YWS3.1	Glucose-6-phosphate isomerase	-0.75
P64269.1; Q2YSE9; Q5HHP2.1; Q6GIL5.1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	-0.55
Q5HIG2.1	Cysteine synthase	-0.65
D2N5Q4; E5R9N1; Q6GIE6	Leucyl aminopeptidase	-0.85
Q6GE17.1; A7X656.1	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	-0.65
Q6GHK4.1; Q6G9Y2.1; Q5HGK2.2; Q2YXI5; Q2FZ53; Q2FHK7; P99093.1; P0A0I0.1; P0A0H9.1; E5RBC8; D9RQ07; D1GT41	3-oxoacyl-[acyl-carrier-protein] reductase	-0.8
Q99V48.1; Q6GAG7.1; Q5HH38.1; Q2YX28	Putative naphthoate synthase	-0.7
Q6GJB4.1	Probable branched-chain-amino-acid aminotransferase	-0.55

Q99V90; Q7A6E5; Q7A1A5; Q6GI85; Q6GAS6; Q5HH84; Q2G1U3; Q2FI76; E5RA09; D1GRQ0	Oligoendopeptidase F	-1
Q8NWQ3.1; Q6GGW9.1; Q6G9C3.1; Q5HFY4.1; Q2YY66.1; Q2FYJ2.1; Q2FH00.1; P99151.1; P63478.1; E5R5R3; E5QVX8; D2N764; D1GUQ2	Alanine dehydrogenase	-0.5
A7X2P6.1	Arginine repressor	-0.85
A7X1E9.1	Aspartate carbamoyltransferase	-1.8
A7X4T7.1	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	-0.75
Hypothetical/Uncharacterised		
Q7A4V3.1	UPF0342 protein SA1663	-0.9
P60359.1	UPF0297 protein SA1445	-0.55
A5IRZ3.1; A6U0S4.1; A7X113.1; D2N680; E5RAS3; Q2YX68.1; Q6GAA8.1; Q6GHX9.1; Q8NX72.1; Q931T8.1; Q99UZ6.1	UPF0637 protein SaurJH9_1166	-1
Q8NYB8; Q2G150	Putative uncharacterized protein	-0.95

Table A2. Up regulated cell surface associated proteins in RB4*Δfur* calibrated to the isogenic wild type isolate, RB4, as determined by iTRAQ LC-MS. The proteins which displayed > 0.5 log_2 ratio increase in RB4*Δfur* compared to the wild type RB4 are listed below, arranged according to protein function. The relative proteins amounts are averages of two biological repeats (each biological repeat inclusive of two technical repeats).

Up regulated in *Δfur* mutant

Accession number	Protein name/description	Fold diff
Gene regulators		
Q840P7.1; Q6GIT7.1; Q2YSM6.1; Q2FIT5.1	Histidine protein kinase SaeS	0.8
Virulence factors		
Q931U5.2; Q2YX22; Q2FI25; D2N627; D1GS40; A7X0T9.2	Bifunctional autolysin Atl	1.05
Q2G2J2.1; Q6G723.1; Q6GED5.1	Secretory antigen	1.2
D1GTX1; D2NAM0; Q6GDG4.1	Immunodominant antigen B IsaB	0.7
Q2FWV6.1; A7X482.1	Staphylococcal complement inhibitor	1.2
Stress response proteins		
Q7A5P3.1; Q7A2R8.1; Q7A0X3.1; Q6GH06.1; Q6G9F9.1; Q5HG18.1; Q2YY16.1; Q2FYN2.1; Q2FH36.1; E5RCB0; D2N730; D1GU99	Cold-shock DNA-binding protein family	2.15
Q99TC0; Q7A527; Q7A0M2; Q6GFW4; Q6G8I3; Q5HF30; Q2YTJ2; Q2FXI6; Q2FFZ4; E5R799; D2N873; D1GNB5; Q2FHT6.1	Thioredoxin	1.05
Q2FJN4.1	Alkyl hydroperoxide reductase subunit C	0.8

Iron acquisition		
Q6GKJ0	Siderophore compound ABC transporter binding protein	0.95
D2N9B9	Ferrichrome ABC transporter lipoprotein	0.8
Q6GHV7.1; D2N6A2	Iron-regulated heme-iron binding protein	2.25
D2N8M6; Q2FFK2.1; Q6GFG4.1	Ferritin	1.85
Transporter/Translocator proteins		
Q6GJ37	ABC transporter ATP-binding protein	0.65
Q99TL5; Q7A585; Q7A0Q4; Q6GG66; Q6G8T1; Q5HFC5; Q2YT92;Q2FXT7; Q2FG89; E5R6S1; D2N7X7; D1GMQ1	Protein translocase subunit yajC	0.7
Q6GEF0	Iron compound ABC transporter, iron compound-binding protein	1.5
Q6GG67; Q2YT93; Q2FXT8; D2N7X6	Preprotein translocase component SecDF	0.65
Protein synthesis		
Q5HDW4.1; A7X5F5.1	30S ribosomal protein S3	0.85
A7X5D8.1; A6QJ75.1	30S ribosomal protein S5	0.7
A7WYW1.1	50S ribosomal protein L1	0.65
Q5HGH6.1; A7X1N3.1; A6U174.1	30S ribosomal protein S2	0.5
Q2FHJ7.1	50S ribosomal protein L19	0.6

A7X5E9.1	50S ribosomal protein L14	0.85
Q99S33.1; Q7A465.1; Q7A083.1; Q6GEJ5.1; Q6G783.1; Q5HDX0.1; Q2YYK9.1; Q2FW18.1; Q2FEQ1.1; E5RA53; D9RPR4; D2N9H3; D1GRK6; A8Z345.1; A7X5E5.1; A6U3W3.1; A6QJ80.1; A5IV22.1	50S ribosomal protein L5	0.75
Q6GFF7.1; Q2YU51.1; Q2FFJ5.1; A6U305.1	Glutamyl-tRNA(Gln) amidotransferase subunit A	0.5
A7X5G6.1; A6U3X6.1	30S ribosomal protein S10	0.8
A7WYX3.1	30S ribosomal protein S7	0.55
A7X5E1.1	50S ribosomal protein L6	0.5
Q5HID0.1; D1GPE6; A7WYX1.1	30S ribosomal protein S12	1
A7X5F2.1	50S ribosomal protein L29	0.75
Q5HID8.2; Q2YSC4.3; A7WYW0.1	50S ribosomal protein L11	0.55
A7X5F6.1	50S ribosomal protein L22	0.7
Other cellular processes and general maintenance		
Q6GFZ0.1; Q2FXK8.1; D2N846; A6U2I4.1	Septation ring formation regulator EzrA	0.5
Q2YX66	Small GTP-binding protein domain:GTP-binding protein TypA	0.95
A8YY86; D2N951; Q99SD7	Transcription termination factor Rho	0.65
Q2FZ09; A7X1S3.1	Protein RecA	0.7
A6QF41	Ribonucleotide-diphosphate reductase beta chain	1.4

A5ISH0.1; A7X1S5.1; D1GT96	Ribonuclease Y	0.95
Other enzymatic reactions (Synthesis, modification, digestion)		
Q99VC2.1; Q7A6H3.1; Q7A1B6.1; Q6GAV8.1; Q5HHB6.1; Q2YWW3.1; Q2FZT4.1; Q2FIA7.1; E5R9X8; D9RNF6; D1GRM0	5-carboxymethyl-2-hydroxymuconate Delta-isomerase	0.75
A7X1J8.1	Acyl carrier protein	2.1
Q7A1R9; Q2YVX4	ATP-dependent zinc metalloprotease FtsH	0.6
Q2YWP4.1; A7X019.1	Glycine cleavage system H protein	1.1
Q5HH02.1	Phosphocarrier protein HPr	0.5
P65895.1	Phosphoribosylamine--glycine ligase	0.6
Q5HEA0.1; Q2FWF4; Q2FF27	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	0.7
Q6G6A5.1; Q5HCY1.1; Q2FV52.1; Q2FDT8.1; P99160.1; P65645.1; P60157.1; E5RBZ4; A7X6T9.1; A6QK59.1	Transglycosylase domain protein	1.05
Q7A484.1; Q2FES9.1; D2N9E7; A8Z315	Possible HAD superfamily hydrolase	0.55
Q6GHI1.1; Q2YXL4.1; D9RQ30; A7X1N1.1; A6U172.1	ATP-dependent protease ATPase subunit HslU	0.6
Q6GG31.1; Q6G8Q1.1; Q2YTB5.1; A7X396.1	ATP-dependent Clp protease ATP-binding subunit ClpX	0.5
Q6GKI8; Q5HJQ2; Q2YUW4	Ornithine cyclodeaminase	1.3
Q99RN0; Q8NV22; Q7A3T8; Q6GE34; Q6G6S2; Q5HDF7; Q2YZ33; Q2FVL9; Q2FE98; E5RB19; D2N9Y4; D1GSH9	Assimilatory nitrite reductase (NAD(P)H) small subunit	1.25

Q2YT12.1; Q2FY06.2; D1GM96; A7X2W5.1	GTPase Era	0.85
Q6GIS5	Allophanate hydrolase subunit 1	0.5
Q2YWV8	Putative ATP-dependent protease protein	0.9
Hypothetical/Uncharacterised		
Q7A531.1	UPF0478 protein SA1560	0.5
Q7A6L9.1; Q2YWN8.1	UPF0337 protein SAB0772	2.25
Q6G9F5.1; Q2YY12.1; Q2FH32.1; P60107.1	Putative uncharacterized protein	0.65
Q99T01.1; Q7A4S2.1; Q7A0I3.1; Q6GFH7.2; Q6G854.2; Q2YU47.2; E5R820; D2N8L3; A7X400.1	Uncharacterized protein	1.4
Q7A5C5.1; Q2YT06.1	UPF0365 protein SAOV_1573	0.65

Table B Full list of mutations observed in all recently infectious CC22 clinical isolates (mutant allele) compared to the reference EMRSA-15 strain (HO 5096 0412; ref allele). Gene reference, putative protein function and allele details of each mutation are listed. Mutations are ordered according to genome coordinates.

Gene Name	Putative Function	SNP Co-ord	Ref allele	Mutant allele
SAEMRSA15_*00460*	Type I restriction-modification system	60889	G	C
spa	Immunoglobulin binding protein	99269	G	-T
deoD1	Purine nucleotide phosphate	126477	G	-C
SAEMRSA15_*01570*	Sucrose-specific PTS transporter	194348	A	G
SAEMRSA15_*01820*	Putative sensory kinase	226980	T	C
SAEMRSA15_*03500*	Hypothetical	413711	G	+T
tmk	Putative thymidylate kinase	481977	G	A
sdrD	LPXTG surface protein	585810 /586681	G / T	A / -A
uvrA	Nucleotide excision repair	789882	T	C
SAEMRSA15_*07090*	Putative lipoprotein	820739	A	+T
SAEMRSA15_*07320*	Nitroreductase	838436	C	T
SAEMRSA15_*07520*	Hypothetical	857003	G	T
SAEMRSA15_*07960*	Hypothetical	898741	T	C
clpB	ATPase, protein quality control	913691	A	G
oppD	Putative oligopeptide transporter	926126	T	C

oppF	Oligopeptide transport	927102	A	+T
-	Integenic	951423	G	T
purH	Purine biosynthesis	1019858	T	C
SAEMRSA15_*09420*	Hypothetical	1058012	T	A
isdB	Iron binding protein	1075448	A	+G
SAEMRSA15_*09700*	Putative ribonuclease	1088103	A	C
tRNA-Arg	Transfer RNA	1125070	T	C
SAEMRSA15_*10270*	Glyoxalase/bleomycin resistance protein	1143954	A	C
fabG	Acyl carrier protein	1183858	A	G
-	Intergenic	1279942	C	T
SAEMRSA15_*11680*	Sensor kinase	1296469	T	C
SAEMRSA15_*11740*	Putative aspartate kinase	1301548	A	G
trpD	Amidotransferase	1353102	G	A
trpB	Tryptophan synthase	1355471	A	G
dfrB	Dihydrofolate reductase	1424504	G	A
SAEMRSA15_*13470*	Putative lipoprotein	1519865	C	T
-	Intergenic	1546831	T	C
SAEMRSA15_*13480*	Phage protein	1567111	A	+T
SAEMRSA15_*14330*	Putative peptidase	1591160	T	C

bfmB	Acyltransferase/ dehydrogenase	1595670	T	C
SAEMRSA15_*14550*	Putative glycine cleavage system	1612410	T	C
sodA	Superoxide dismutase	1627479	A	G
SAEMRSA15_*14550*	Putative DNA exonuclease	1708312	T	C
dnaB	DNA helicase	1749314	A	G
coaE	Dephospho-CoA kinase	1752594	C	T
harA	Haptoglobin-binding surface anchor	1806877	A	G
SAEMRSA15_*17490*	Putative DNA repair exonuclease	1926268	T	C
SAEMRSA15_*17720*	Putative membrane protein	1957456	T	C
-	Intergenic	2030281	C	+A
SAEMRSA15_*18750*	Phage protein	2061810	G	+T
SAEMRSA15_*19200*	Phage repressor protein	2084152	T	G
SAEMRSA15_*22910*	Putative histidine kinase	2470808	T	C
SAEMRSA15_*23700*	Putative short chain dehydrogenase	2561829	T	C
clpL	ATPase Clp protease	2646342	A	G
nrdD	Ribonuclease reductase	2720416	C	T
SAEMRSA15_*25560*	Hypothetical	2770008	T	C
SAEMRSA15_*25560*	Hypothetical	2770556	T	G
-	Intergenic	2823320	T	C

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