Identification of Staphylococcal Genes Involved in Resistance to the Human Antimicrobial Peptide LL-37

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Declarations

I, Ping Zhang, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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I dedicate this study to the treasured memory of my beloved grandmother.

Abstract

Staphylococcus aureus is well-known for its ability to acquire resistance to a broad range of antimicrobial agents and a limited number of commercially available antibiotics exist that are active against multidrug resistant strains. Antimicrobial peptides have been suggested as promising alternatives to current antimicrobials due to their potent antimicrobial activity against a broad range of microorganisms including multidrug resistant bacteria, and a membrane-lytic mode of action that is thought to have low possibility of inducing bacterial resistance. This study describes the identification of *S. aureus* genes involved in resistance to the human cationic antimicrobial peptide LL-37, with a particular interest in the effects of a physiological concentration of bicarbonate on the resistance mechanism. Transposon mutagenesis and recombinase-based *in vivo* expression technology systems were designed to enable genome-wide screening. A *S. aureus* transposon mutant library was screened for increased resistance to LL-37 in the presence of bicarbonate. Mutants with insertions in *yycH* and *yycI*, demonstrated bicarbonate-dependent resistance to LL-37. Both *yycH* and *yycI* form part of a predicted operon *yycFGHI* in *S. aureus*, and have been shown to be suppressors of an essential two component system YycFG in *B. subtilis* that regulates cell wall metabolism. The resistance of *S. aureus* small colony variants (SCVs) to LL-37 was also investigated. SCVs defective in *hemB, menD* or *aroD*, demonstrated bicarbonate-dependent resistance to LL-37. Furthermore, SigB (a global regulator) and TcaR (an activator of protein A) were found to exert opposite effects on resistance to LL-37 in the presence of bicarbonate. Strains defective in TcaR showed bicarbonate-dependent resistance to LL-37, interestingly, this resistance was abolished by either deleting *sigB* or repairing *tcaR* in these strains. These data suggest that YycFG, SigB, TcaR and the SCV phenotype may play important roles in resistance to LL-37 under *in vivo* conditions where bicarbonate is present.

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List of Abbreviations

˚C	Degrees Celsius
Aap	Accumulation-associated protein
ABC	Adenosine triphosphate-binding cassette
Agr	Accessory gene regulator
Amp	Ampicillin
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
ATc	Anhydrotetracycline
ATP	Adenosine triphosphate
Bap	Biofilm-associated protein
bp	Base pair
BP	*attB-attP*
Bra	Bacitracin resistance associated (=Bce)
$CaCl_2$	Calcium chloride
Can	Collagen-binding protein
$CdCl_2$	Cadmium chloride
CFU	Colony-forming unit
CDM	Complete defined medium
$CF_3CO_2^-$	Trifluoroacetate anion
ClfA	Clumping factor A
ClfB	Clumping factor B
CLSI	Clinical and Laboratory Standards Institute
cm	Centimetre
Cm	Chloramphenicol
CO_2	Carbon dioxide
DFI	Differential fluorescence induction
DHB	Dihydroxybenzoate
dH_2O	De-ionised Water
Dlt	D-alanyl-lipoteichoic acid
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Eap	Extracellular adherence protein
EARSS	European Antimicrobial Resistance Surveillance System
ECM	Extracellular matrix
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Efb	Extracellular fibrinogen-binding protein
EGFR	Epidermal growth factor receptor
Emp	Extracellular matrix protein
Erm	Erythromycin
ET	Exfoliative toxin
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
$FMNH_2$	Reduced flavin mononucleotide
FnBPA	Fibronectin-binding protein A
FnBPB	Fibronectin-binding protein B
GM17	M17 broth/agar supplemented with glucose
G-SGM17	GM17 borth/agar supplemented with glycine and sucrose
SGGM17MG	GM17 borth/agar supplemented with sucrose, $MgCl_2$ and $CaCl_2$
Gra	Glycopeptide resistance associated
GRAS	Generally recognised as safe
HBD	Human β-defensin
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate anion
HD	Human α-defensin
HNP	human neutrophil peptide
ICU	Intensive care units
IgA	Immunoglobulin A
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.p.	Intraperitoneal
IPTG	Isopropyl-beta-D-thiogalactoside
i.v.	Intravenous
IVET	*In vivo* expression technology
ITR	Inverted terminal repeat
Kan	Kanamycin
kb	Kilobase pairs
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Potassium monohydrogen phosphate
LAB	Lactic acid bacteria
LB	Luria-Bertani broth
LB agar	Luria-Bertani agar
LL-37	Cathelicidin containing 37 amino acids started with two leucines
Lpp	An outer membrane lipoprotein of Enterobacteriaceae
LPS	Lipopolysaccharide
LPXTG	Leucine-proline-any amino acid-threonine-glycine
LTA	Lipoteichoic acid
Luk	Leukocidins
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
mg	Milligram

mm	Millimetre
mM	Minimolar
ml	Millilitre
Mbp	Mega base pair
MHC II	Class II major histocompatibility complex
MLS_B	Macrolide-lincosamide-streptogramin B
MprF	Multiple peptide resistance factor
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-sensitive *Staphylococcus aureus*
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Sodium dihydrogen phosphate
Na_2HPO_4	Sodium monohydrogen phosphate
Na_3 citrate.$2H_2O$	Sodium citrate dihydrate
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NAG	N-acetylglucosamine
$(NH_4)_2SO4$	Ammonium Sulfate
NAM	N-acetylmuramic acid
ncRNA	Non-coding ribonucleic acid
NET	Neutrophil extracellular traps
NNISS	National Nosocomial Infections Surveillance System
ng	Nanogram
nm	Nanometre
OD	Optical density
PABA	Para-aminobenzoic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG-800	Polyethylene glycol 800

PG	Phosphatidylglycerol
PIA	Polysaccharide intercellular adhesion
PSM	Phenol-soluble modulin
PVL	Panton-Valentine leukocidin
qPCR	Quantitative PCR
R-IVET	Recombinase-based *in vivo* expression technology
RNA	Ribonucleic acid
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
Sae	*Staphylococcus aureus* exoprotein expression
Sar	Staphylococcal accessory regulator
SasG	*Staphylococcus aureus* surface protein G
SCV	Small colony variant
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Staphylococcal enterotoxin
SEM	Scanning electron microscope
SERAM	Secretable expanded repertoire adhesive molecules
SET	Staphylococcal exotoxin-like proteins
SigB (σ^B)	Sigma factor B
SO_4^{2-}	Sulfate anion
SOC	Super Optimal broth with Catabolite repression
SPSS	Statistical program for social sciences
SSL	Staphylococcal superantigen-like proteins
SSS	Scalded skin syndrome
SSSS	Staphylococcal scalded skin syndrome
STM	Signature-tagged mutagenesis
TA	Teichoic acid
TACE	Tumor necrosis factor-α-converting enzyme
TAE	Tris-acetate-EDTA
TCS	Two-component system
TEM	Transmission electron microscopy

Tet	Tetracycline
Tn	Transposon
TNFR-1	Tumour necrosis factor receptor-1
tPMP	Thrombin-induced platelet microbicidal protein
Tris	Tris(hydroxymethyl)aminomethane
Tris-Hcl	Tris-hydrochloride
tRNA	Transfer ribonucleic acid
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSS	Toxic shock syndrome
TSST-1	Toxic shock toxin-1
UPP	Undecaprenyl pyrophosphate
VISA	Vancomycin-intermediate *Staphylococcus aureus*
Vra	Vancomycin resistance associated
VREF	Vancomycin-resistant *Enterococcus faecalis*
VSSA	Vancomycin-susceptible *Staphylococcu aureus*
v/v	Volume to volume
vWbp	Von Willebrand factor binding protein
WHO	World health organization
WTA	Wall teichoic acid
w/v	Weight per volume
X-α-gal	5-Bromo-4-Chloro-3-Indolyl-α-D-galactopyranoside
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
$ZnCl_2$	Zinc chloride

Chapter 1: Introduction

From the time of our birth we are immediately exposed to an enormous number of microbes, and this contact remains throughout our lives. The number of bacteria associated with a healthy human body is approximately 10 times the number of human cells (Wilson *et al.*, 2002). This microbial population is known as the commensal microbiota, which is relatively stable and lives harmoniously with the host. However, these organisms are metabolically active, and therefore have the potential to affect our lives profoundly, for good or bad (Wilson *et al.*, 2002). For example under certain circumstances, such as damage to our skin or suppression of our immune system by drugs, some of the organisms in the commensal microbiota can be harmful to the human body and cause infectious diseases (Wilson *et al.*, 2002). In addition bacteria acquired from the environment which are not part of the commensal microbiota can also cause infectious disease (Wilson *et al.*, 2002). According to the data collected by the world health organization (WHO), infectious diseases caused by microbes are among the leading causes of morbidity and mortality in the world, particularly in developing countries (WHO, 2008). *Staphylococcus aureus* (*S. aureus*) is one of the human commensals that can cause life-threatening infections, such as pneumonia and bacteraemia (Wilson *et al.*, 2002). Prior to the advent of antibiotic therapy the mortality rate of *S. aureus* invasive infection was approximately 90% (Kak and Levine, 1999). However the introduction of antibiotics in 1942 has greatly reduced the mortality rate of *S. aureus* infections. The success of antibiotic treatment was rapidly challenged by the emergence of antibiotic resistance in this organism. To date, novel effective antibiotics or therapeutic strategies are still in urgent need for combating *S. aureus* infections. Despite the fact that this organism has been extensively studied for decades, we still only have a limited view of how it causes infection, and a better understanding of the mechanisms by which *S. aureus* survives and causes disease is desirable for the development of new antimicrobial drugs.

1.1 General characteristics of *S. aureus*

1.1.1 The isolation of *S. aureus*

In 1882, a Scottish surgeon Sir Alexander Ogston discovered that the major cause of surgical abscesses were micrococci and in particular a species exhibiting in clusters (Ogston, 1881), which was therefore designated "staphylococci" from the Greek "staphyle", meaning a cluster of grapes. In 1884, a German surgeon Anton J. Rosenbach isolated two strains of staphylococci; one grew as yellow colonies the other white. He named the yellow-pigmented strain "*Staphylococcus aureus*" from the Latin "aurum", which means gold. The white colonies were designated "*Staphylococcus albus*" from the Latin "albus" meaning white (Rosenbach, 1884). *Staphylococcus albus* is now called *Staphylococcus epidermidis*. To date, 47 species (and 24 subspecies) of staphylococci have been catalogued into the *Staphylococcus* genus (Euzeby, 2012), many of which colonise the human body as commensals (Kloos and Bannerman, 1994). However, *S. aureus* and *S. epidermidis* have the most significant influence on human health, and have been most extensively characterised.

1.1.2 Microbiological identification of *S. aureus*

S. aureus are coccoid with a diameter ranging from 0.7µm to 1.2µm (Somerville and Proctor, 2009) and normally appear as grape-like clusters when viewed under a microscope. They have a Gram-positive cell wall, are non-motile and non-spore forming. In addition, *S. aureus* is able to produce catalase, an enzyme which breaks down hydrogen peroxide to water and oxygen. Catalase-positivity is an important diagnostic phenotype of all staphylococcal species which allows them to be distinguished from the catalase-negative genus *Streptococcus*. Furthermore, *S. aureus* produces coagulase, an enzyme able to clot blood plasma, whereas most of the other staphylococcal species are not able to produce this enzyme, such as *S. epidermidis*. Therefore, the coagulase test is a principal tool to distinguish *S. aureus* from the other staphylococcal species (Kloos and Schleifer, 1975).

In addition to these routine tests, the growth characteristics of *S. aureus* under various conditions can also assist the identification of this organism. They normally

form round and yellow colonies on nutrient agar, and show hemolysis of blood agar due to the production of several hemolysins (Kloos and Schleifer, 1975). Mannitol salt agar (MSA) is often used to differentiate *S. aureus* from other organisms. MSA contains a high salt concentration (7.5% NaCl), which makes it selective for salt-resistant bacteria such as *Staphylococci*. This medium also contains the sugar mannitol and indicator phenol red for the differentiation of organisms fermenting mannitol, such as *S. aureus*. The fermentation of mannitol produces acidic end products which results in pH change in the medium, thereby turning the medium from red to yellow (Chapman, 1945).

Antibiotic susceptibility is also a common test used to identify *S. aureus* isolates that have higher resistance to certain antibiotics, such as methicillin resistant *S. aureus* (MRSA). Media with the combination of mannitol, salt and antibiotics such as oxacillin (Blanc *et al.*, 2003) or cefoxitin (Smyth and Kahlmeter, 2005) were also specifically designed for the identification of MRSA. Apart from these routine clinical tests, PCR tests for species-specific genes are also widely used in research laboratories as well as in a clinical setting, such as PCR detection targeting the *S. aureus*-specific thermostable nuclease gene *nuc* (Hallin *et al.*, 2003), coagulase gene *coa* (Sindhu *et al.*, 2010), and protein A-encoding gene *spa* (Strommenger *et al.*, 2008).

1.1.3 Growth conditions

S. aureus is a facultatively anaerobic organism, but grows optimally under aerobic conditions and in the presence of CO_2 (Somerville and Proctor, 2009). Except for mannitol, *S. aureus* can also utilise glucose, xylose, lactose, sucrose, maltose and glycerol as carbon sources. In addition to sugars, the growth of *S. aureus* also requires inorganic salts such as NaCl, KH_2PO_4 and $MgSO_4$, eleven amino acids (glycine, valine, leucine, threonine, phenylalanine, tyrosine, cysteine, methionine, proline, arginine, and histidine) and three vitamins (thiamine, nicotinic acid, and biotin) (Mah *et al.*, 1967). *S. aureus* normally grows optimally between 35°C and 40°C (Schmitt *et al.*, 1990) at a pH between 6 and 7 (Valero *et al.*, 2009).

S. aureus is a hardy and versatile organism able to tolerate multiple environmental stresses and survive in a wide variety of environmental niches. As mentioned above, *S. aureus* is able to grow at high salt concentration (7.5-10% NaCl) (Somerville and Proctor, 2009). It is also capable of surviving desiccation (Rountree, 1963). *S. aureus* grows at a generally recognised minimum water activity of 0.867 (Valero *et al.*, 2009), but has been found to grow under conditions with water activity as low as 0.83 (Borneman *et al.*, 2009). In addition to water stresses, *S. aureus* can also tolerate a range of temperature and pH stresses, it can grow at temperatures between 7°C and 48°C (Schmitt *et al.*, 1990), and tolerate pH ranging from 4.8 to 9.4 (Somerville and Proctor, 2009).

1.2 *S. aureus* as a human pathogen

S. aureus is the most important human pathogen in the genus of *Staphylococcus*. Asymptomatic *S. aureus* colonisation in humans occurs most commonly in the anterior nasal vestibule, and occasionally on the skin, throat, nails, gastrointestinal tract, axillae, perineum and vagina (Verbrugh, 2009). It is estimated that approximately 20% of healthy people are persistent carriers of *S. aureus* in their noses, and another 60% are colonised by *S. aureus* at least once in their life with no symptoms (Peacock *et al.*, 2001). Healthy people who are *S. aureus* carriers do not usually suffer from, but have an increased risk of developing, staphylococcal infections (Crossley *et al.*, 2009).

Among *S. aureus* carriers, infections can occur once natural physical barriers against *S. aureus* are breached, such as through cuts or wounds on skin and broken mucous membranes (Verbrugh, 2009). Diseases associated with *S. aureus* include infections of skin and soft tissue (e.g. stys, boils, abscess, wound infections, impetigo), blood and the cardiovascular system (e.g. bacteraemia, endocarditis), the musculoskeletal system (such as osteomyelitis, arthritis), the central nervous system (meningitis), and the lung (pneumonia), and diseases mediated by toxins, such as toxic shock syndrome (TSS), food poisoning and scalded skin syndrome (SSS) (Crossley *et al.*, 2009).

In addition to the endogenous infections caused by commensal *S. aureus*, cross-infection with *S. aureus* is also very common, as the bacterium can be transmitted among individuals. Cross-infection of *S. aureus* occurs especially frequently in hospitals due to the vulnerable health status of patients, and the multiple diagnostic and therapeutic procedures provide many opportunities for *S. aureus* to spread and cause infections (Inweregbu *et al.*, 2005). The major nosocomial infections caused by *S. aureus* include surgical wounds infections, lower respiratory tract infections, pneumonia and cardiovascular infections (Richards *et al.*, 1999a & b).

1.3 The *S. aureus* genome

The first published genome sequencing projects were conducted on two methicillin-resistant strains N315 and Mu50 (Kuroda *et al.*, 2001). These sequences revealed that the *S. aureus* genome is a single circular chromosome with an average size of approximately 2.8 mega base pairs (Mbp). There are also other extrachromosomal genetic elements, such as conjugative and non-conjugative plasmids, mobile elements and prophages (Kuroda *et al.*, 2001). Since the sequencing of the above two genome sequences, the complete genome sequences of a variety of *S. aureus* strains have been reported, including MW2 (Baba *et al.*, 2002), MRSA252 and MSSA476 (Holden *et al.*, 2004), COL (Gill *et al.*, 2005), USA300-FPR3737 (Diep *et al.*, 2006), NCTC8325 (Gillaspy *et al.*, 2006), USA300-HOU-MR (Highlander *et al.*, 2007), ET3-1 (Herron-Olson *et al.*, 2007), JH1 and JH9 (Mwangi *et al.*, 2007), Newman (Baba *et al.*, 2008), JKD6008 (Howden *et al.*, 2010), RN4220 (Nair *et al.*, 2011) and T0131 (Li *et al.*, 2011). The availability of the complete genome sequences of *S. aureus* strains facilitates the understanding of this organism and the design of effective research strategies to study the physiology and pathogenesis of this important bacterium.

The extensively used laboratory strain 8325-4 is derived from strain NCTC 8325 by curing three prophages ø11, ø12 and ø13 from NCTC 8325 (Novick, 1967). *S. aureus* NCTC 8325 has a genome of 2,821,361 base pairs (bp) encoding 3,006 genes, including 2,894 protein-coding genes and 112 RNA genes. Of the 2894 protein coding genes, 1177 genes have had their function experimentally determined or predicted, and the remaining 1717 genes encode hypothetical proteins. The 112 RNA genes

include 16 rRNA genes, 61 tRNA genes and 35 other RNA genes, such as non-coding RNAs (ncRNAs) which are crucial regulators enabling the cells to adapt to environmental changes (Repoila and Darfeuille, 2009; Geissmann *et al.*, 2009). The chromosome is A+T rich, with a G+C content of 32.86%. An important feature of *S. aureus* 8325-4 genome is that it is defective in two regulator genes *rsbU* and *tcaR*, which are positive regulators of sigma factor B (SigB) and protein A (Spa) at the transcriptional level, respectively. SigB is a major regulator of the stress response in *S. aureus*, and protein A is an important *S. aureus* virulence factor (Herbert *et al.*, 2010). Therefore, strain NCTC 8325 and its derivative 8325-4 are not ideal models for studying regulation and virulence.

The strain used in the study reported herein is a septic arthritis isolate from a mouse, designated LS-1 (Bremell *et al.*, 1990). The genome sequence of this strain is not currently available. However, when compared to 8325-4, identified differences are that LS-1 possesses functional *rsbU* (Nair *et al.*, 2003) and *tcaR* genes (see chapter 7). In addition it contains a plasmid, which we have designated pLS-1. We have sequenced pLS-1 and found that it is a small plasmid of 3,347bp containing an origin of replication and a cadmium resistance gene (*cad*). Molecular manipulation of *S. aureus* LS-1 in this study was based on the genomic sequence of *S. aureus* NCTC8325, since we have found most other genes that we have examined are identical in these two strains.

1.4 *S. aureus* extracytoplasmic structures

1.4.1 Capsule

The majority of *S. aureus* isolates from humans produce a layer of extracellular capsular polysaccharide, which is a carbohydrate polymer comprised of repeating saccharide units. It is usually called a polysaccharide microcapsule because it can only be detected using electron microscopy. A method for the serological typing of *S. aureus* capsular polysaccharides was designed by Karakawa *et al*. (1985), and is based on bacterial cell agglutination and immunoprecipitation of cell extracts with monospecific antisera (Karakawa *et al.*, 1985). So far, at least 11 types of capsular polysaccharide serotypes have been described for *S. aureus* (Sompolinsky *et al.*, 1985;

Karakawa *et al.*, 1985). Most of the *S. aureus* isolates from humans were found to have capsules serotype 5 or 8 (Sompolinsky *et al.*, 1985; Fournier *et al.*, 1987). These two types of capsular polysaccharides share similar trisaccharide repeating units comprised of N-acetyl mannosaminuronic acid, N-acetyl L-fucosamine, and N-acetyl D-fucosamine, but differ in the linkages between the sugars and in the sites of O-acetylation (Jones, 2005).

1.4.2 Cell wall

The *S. aureus* cell wall exhibits the typical features of a Gram-positive bacterial cell wall. It contains a thick layer (20-40nm) of peptidoglycan, teichoic acids (TA) and surface proteins. Peptidoglycan is the major component which builds the rigid mesh-like framework and accounts for 50% of the cell wall mass (Somerville and Proctor, 2009). Figure 1.1 shows the cross linking between peptidoglycan subunits. Peptidoglycan consists of alternating disaccharide units of β-1-4 linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) which form linear glycan strands. These glycan strands are cross-linked by short peptide chains bound to NAM, which are usually composed of tetrapeptides of L-alanine, D-glutamine, L-lysine and D-alanine. These tetrapeptides are then cross-linked in a second dimension by a pentaglycine bridge consisting five L-glycine residues. The pentaglycine peptides extend from the third amino acid of one of the stem peptides to the last amino acid in the adjacent peptide, therefore cross-linking all the glycan chains and create a three-dimensional macromolecule (Dmitriev *et al.*, 2004; Vollmer *et al.*, 2008; Somerville and Proctor, 2009).

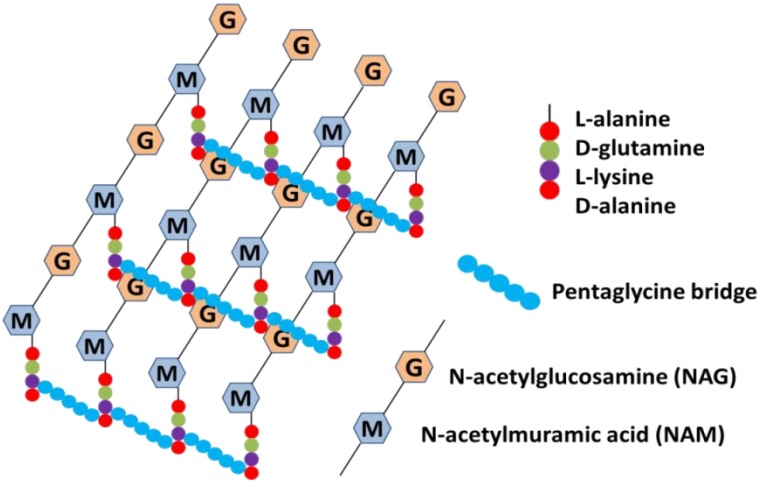

Figure 1.1: Graphical illustration of peptidoglycan structure (based on Fox 2011).

Another component of the *S. aureus* cell wall is the teichoic acids which make up approximately 40% of the cell wall mass. Teichoic acids are long anionic polymers containing ribitol phosphate or glycerol phosphate repeats. Teichoic acids (TAs) can be divided into two groups: wall teichoic acids (WTAs) and lipoteichoic acids (LTAs). WTAs are covalently attached to the peptidoglycan and LTAs are anchored in the outer leaflet of the membrane lipids (Neuhaus and Baddiley, 2003; Somerville and Proctor, 2009). Both TAs extend to the bacterial surface and contribute to the cell surface negative charges that play a role in acquisition of ions and maintaining metal cation homeostasis (Marquis *et al.*, 1976), as well as controlling the interactions between the bacteria and other cells or molecules, such as cationic antimicrobial peptides (AMPs) (Peschel *et al.*, 1999).

In addition to teichoic acids the *S. aureus* cell wall contains a variety of proteins. Some are covalently attached to peptidoglycan and others are attached via non-covalent ionic interactions to peptidoglycan or teichoic acids (Scott and Barnett, 2006). *S. aureus* cell wall associated proteins include an array of surface proteins, exoproteins, peptidoglycan hydrolases, and lipoproteins that anchor to the cell membrane and extend into the cell wall. Some of them play important roles in promoting bacterial interactions with extracellular matrix components and are virulence determinants (Somerville and Proctor, 2009).

1.4.3 Cell membrane

The *S. aureus* cell membrane is a lipid bilayer where lipids are distributed asymmetrically on the inner and outer leaflets. The hydrophobic tails of the lipids face one another inside of the bilayer, and the hydrophilic heads are exposed on the exterior of the bilayer. With the hydrophilic parts contacting the aqueous environment, the cell membrane is a highly impermeable structure that does not allow large or polar molecules to freely pass across the membrane (Zhang and Rock, 2008). The main lipid components of the *S. aureus* cell membrane are phosphatidylglycerol (PG) and lysyl-phosphatidylglycerol (lysyl-PG). The head group of PG is negatively charged, thereby conferring anionic properties to the membrane surface. This property of the membrane is critical for the susceptibility of *S. aureus* to

cationic AMPs and antibiotics (Peschel and Sahl, 2006). However, attaching a L-lysine to the head group of PG adds a positive charge to the lipid. Therefore, the content of lysyl-PG in the outer leaflet of the cytoplasmic membrane is critical for *S. aureus* resistance to cationic AMPs (Peschel *et al.*, 2001).

The *S. aureus* cytoplasmic membrane does not just serve as a barrier between the cell intracellular compartment and its environment, there are proteins assembled on and within the membrane, which are mainly divided into three classes: integral proteins, peripheral proteins and lipid-anchored proteins. Integral proteins, also known as transmembrane proteins, pass through the lipid bilayer and protrude from both the extracellular and cytoplasmic sides of the membrane. Peripheral proteins noncovalently bind to the membrane surface and locate on either the cytoplasmic or extracellular side of the lipid bilayer. Lipid-anchored proteins also locate outside the lipid bilayer on either the extracellular or cytoplasmic surface but are covalently linked to a lipid molecule (Dalbey *et al.*, 2011). These proteins are important for cell functions, such as controlling the exchange of substances across the membrane (Maresso and Schneewind, 2006), participating in the electron transport chain (ETC) and ATP synthesis, and sensing the environment and transmitting signals to intracellular transcriptional regulators to adapt the cells to the environment (Novick and Jiang, 2003).

1.5 *S. aureus* virulence factors

S. aureus possesses a variety of virulence factors that contribute to pathogenicity. These include surface proteins that facilitate attachment and colonisation of the host tissues, factors that inhibit phagocytosis, and toxins that lyse host cells (Foster, 1996).

1.5.1 Adhesins

The attachment of *S. aureus* to host tissue is the first step in colonisation and invasion. This step is facilitated by a group of cell surface components called adhesins. *S. aureus* synthesises a class of adhesins designated Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs), and a recently identified category of adhesins known as Secretable Expanded Repertoire Adhesive Molecules

(SERAMs) (Clarke and Foster, 2006). MSCRAMMs have a common cell wall sorting signal LPXTG motif and are covalently anchored to the cell wall peptidoglycan. This group of proteins can recognise and bind to certain host plasma proteins and extracellular matrix (ECM) components such as fibrinogen, fibronectin and collagens (Foster and Hook, 1998). Analysis of six *S. aureus* genome sequences has identified 21 MSCRAMMs, many of which have not been characterised (Roche *et al.*, 2003). The most well-known adhesins in this group are fibronectin-binding proteins (FnBPA and FnBPB), collagen-binding protein (Cna), clumping factors (ClfA and ClfB) and protein A (Spa) (Foster and Hook, 1998).

1.5.1.1 Fibronectin-binding proteins

Most strains of *S. aureus* produce two fibronectin-binding proteins FnBPA and FnBPB, which are encoded by two closely linked genes *fnbA* and *fnbB* (Greene *et al.*, 1995). Apart from binding to fibronectin, *S. aureus* FnBPs have also been found to interact with fibrinogen (Wann *et al.*, 2000) and elastin (Roche *et al.*, 2004). Binding of fibronectin by FnBPs confers on *S. aureus* the capacity to invade a variety of human cells, such as osteoblast (Ahmed *et al.*, 2001), corneal epithelia cells (Jett and Gilmore, 2002), embryonic kidney cells (Grundmeier *et al.*, 2004), endothelial cells (Schroder *et al.*, 2006b; Edwards *et al.*, 2010), and keratinocyte (Kintarak *et al.*, 2004; Edwards *et al.*, 2011). The invasion process involves *S. aureus*-bound fibronectin interacting with integrin α5β1, which is a membrane protein expressed by the mammalian host cells and serves as a primary receptor for fibronectin. Therefore, fibronectin acts as a bridge facilitating the binding of *S. aureus* to the hosts (Sinha *et al.*, 1999). Both FnBPA and FnBPB are important for the adhesion and invasion of the human embryonic kidney cell line (HEK293 cells). The lack of FnBPA and FnBPB severely impairs the internalization of bacteria, and complementation of either FnBPA or FnBPB in the mutant restores the invasive phenotype (Sinha *et al.*, 1999). Also, strain Newman has a natural stop-codon mutation in *fnbB*, which was subsequently introduced into *fnbA* through sequence replacement of *fnbA* with *fnbB* at the C-domain including the stop-codon mutation, resulting in truncated versions of FnBPA and FnBPB. These mutations lead to weak adherence and host cell invasion in this strain (Grundmeier *et al.*, 2004). Strain 8325-4, which possesses intact *fnbA* and *fnbB*,

also produces low levels of FnBPs due to the deficiency in RsbU, which regulates the expression of FnBPs through SigB (Bisognano *et al.*, 1997; Shinji *et al.*, 2011; Nair *et al.*, 2003). Over-expression of either FnBPA or FnBPB in 8325-4 substantially increases the invasion of HEK293 cells (Sinha *et al.*, 1999). A recent study on FnBPs was conducted using strain SH1000, which was modified from 8325-4 by repairing *rsbU*. Both FnBPs were demonstrated to be important for colonisation and invasion *in vitro* and *in vivo*, with FnBPA playing the major role (Shinji *et al.*, 2011). Heterologous expression of *S. aureus* FnBPs in non-invasive Gram-positive bacteria *Staphylococcus carnosus* and *Lactococcus lactis* confers invasiveness to these strains (Sinha *et al.*, 2000).

1.5.1.2 Collagen-binding protein

The *S. aureus* collagen-binding protein (Cna) mediates the attachment of bacteria to the ECM protein collagen and collagenous tissues and cartilage (Switalski *et al.*, 1993). Cna contributes to virulence in several different animal models. For example, in a rat model of catheter-induced infective endocarditis, a mutant defective in Cna was significantly less virulent than the wild-type (Hienz *et al.*, 1996). Cna has been shown to enhance the adherence of *S. aureus* to the cornea in a rabbit model and promotes inflammation (Rhem *et al.*, 2000). In addition an antibody against Cna has been shown to block the attachment of *S. aureus* to collagen substrates from bovine nasal septum (Visai *et al.*, 2000). Collagen-binding protein has especially been linked to bone and joint infections caused by *S. aureus*, due to the high content of collagen in these tissues. However, Cna is not universally expressed by strains of *S. aureus* isolated from bone and joint infections, with only 38-56% of the isolates being Cna positive (Switalski *et al.*, 1993; Ryding *et al.*, 1997; Smeltzer *et al.*, 1997).

1.5.1.3 Clumping factors

The *S. aureus* clumping factors ClfA and ClfB are fibrinogen-binding MSCRAMMs. ClfA mediates adherence of bacteria to rat fibrinogen and platelet-fibrin clots *in vitro*, and promotes endocarditis in rats (Moreillon *et al.*, 1995). ClfA is an important virulence factor for septic arthritis in mice. A *S. aureus clfA* mutant demonstrated reduced virulence in a murine arthritis model, and passive immunisation of mice with

antibodies against ClfA protects against experimental *S. aureus* arthritis (Josefsson *et al.*, 2001). In addition binding to fibrinogen (Walsh *et al.*, 2008), ClfB is also able to bind to human cytokeratin 10 (Walsh *et al.*, 2004). ClfB is a major virulence determinant for nasal colonisation in mice, and passive immunisation of mice with an anti-ClfB antibody inhibits *S. aureus* nasal colonisation (Schaffer *et al.*, 2006). The crucial role ClfB plays in *S. aureus* nasal colonisation has also been demonstrated in humans, as a *clfB* mutant was less able to colonise human nares compared to the wild-type (Wertheim 2008). In addition, expression of *S. aureus* ClfA or ClfB in *L. lactis* induced the adherence of bacteria to platelet-fibrin clots or human nasal epithelial cells *in vitro* (Que *et al.*, 2001; O'Brien *et al.*, 2002).

1.5.1.4 Protein A

Protein A is a cell surface component produced by most *S. aureus* strains. Its importance in virulence has mainly been attributed to its capacity to inhibit phagocytosis by binding to the immunoglobulin IgG (Forsgren and Sjoquist, 1966). However, it is also recognised as a MSCRAMM mediating the adherence of *S. aureus* to vascular injury sites. It can bind to von Willebrand factor, a large multimeric glycoprotein produced by endothelial cells following endovascular damage, and mediates platelet adhesion to the injury sites. Therefore, protein A facilitates the adherence of *S. aureus* onto endothelium damage sites and promotes endovascular infections (Hartleib *et al.*, 2000; O'Seaghdha *et al.*, 2006). Protein A is able to recognise and bind to gC1qR, which is a 33-kDa C1q receptor produced by platelet or endothelial cells (Peerschke *et al.*, 1994; Nguyen *et al.*, 2000). The surface expression of gC1qR is greatly induced following platelet adhesion to collagen, fibrinogen or fibronectin-coated surfaces (Peerschke *et al.*, 1994), or endothelial cells after being activated by inflammatory mediators (Guo *et al.*, 1999). Protein A is also able to recognise and bind to the tumour necrosis factor receptor-1 (TNFR-1), which is widely distributed on the surface of the respiratory epithelium. This interaction activates proinflammatory signalling and triggers host epithelial inflammatory responses (Gomez *et al.*, 2004). Protein A has also been shown to interfere with host immune response by interacting with the epidermal growth factor receptor (EGFR) to activate the tumor necrosis factor-α-converting enzyme (TACE), which in turn mediates the

shedding of TNFR-1 from the surface of epithelial cells (Gomez *et al.*, 2007). Furthermore, protein A has also been shown to bind to osteoblast TNFR-1 *in vitro* and induce apoptosis of cultured osteoblasts (Claro *et al.*, 2011; Widaa *et al.*, 2012).

1.5.1.5 SERAMs

S. aureus also produces another group of adhesins known as SERAMs, which are secreted proteins bound to the cell surface either covalently or via specific cell surface receptors (Harraghy *et al.*, 2008). The characterised adhesins in this group include the extracellular adhesive protein (Eap) which binds to fibronectin, fibrinogen and prothrombin (Palma *et al.*, 1999), extracellular fibrinogen-binding protein (Efb) (Palma *et al.*, 1998), extracellular matrix protein (Emp) which binds to fibronectin, fibrinogen and vitronectin (Hussain *et al.*, 2001), Von Willebrand factor binding protein (vWbp) (Bjerketorp *et al.*, 2002), and coagulase (Boden and Flock, 1989). Unlike the MSCRAMMs, the SERAMs do not share structural similarity, but possess the function of interacting with the host immune system (Chavakis *et al.*, 2005). For example, Eap serves as an anti-inflammation factor by inhibiting host leukocyte recruitment (Chavakis *et al.*, 2002) and neutrophil adhesion to endothelial cells (Haggar *et al.*, 2004), and Efb facilitates *S. aureus* wound infection by binding to platelets and inhibiting platelet aggregation both *in vitro* and *in vivo* (Shannon and Flock, 2004; Shannon *et al.*, 2005).

1.5.2 Factors inhibiting phagocytosis

S. aureus employs a number of mechanisms that enable it to evade phagocytosis, such as blocking recognition by producing a protective coat capsular polysaccharide, and secreting proteins to block phagocyte receptor.

1.5.2.1 Capsular polysaccharide

The capsular polysaccharide of *S. aureus* is involved in virulence by inhibiting phagocytosis (Peterson *et al.*, 1978). As mentioned in section 1.4.1, most of the *S. aureus* isolates from humans were found to have capsules serotype 5 or 8, which have therefore received the most attention with regard to their role in virulence. The production of capsule polysaccharide serotype 5 has been reported to inhibit

phagocytosis and enhance virulence in a mouse bacteraemia model (Thakker *et al.*, 1998; O'Riordan and Lee, 2004). Capsule polysaccharide serotype 8 has also been found to inhibit phagocytosis *in vitro* by human polymorphonuclear leukocytes and enhance the persistence of the bacteria in the blood stream, the liver and the spleen of mice (Luong and Lee, 2002).

Most *S. aureus* strains also produce polysaccharide intercellular adhesin (PIA) which facilitates biofilm formation (Cramton *et al.*, 1999). PIA is encoded by the intercellular adhesion locus *icaADBC* and has mainly been characterised in *S. epidermidis* (Heilmann *et al.*, 1996). A biofilm is a complex aggregation of bacteria embedded in an extracellular polymeric matrix. The formation of biofilm protects bacteria from phagocytosis or antibacterial substances, and thus is an important mechanism of bacterial virulence (Otto, 2008; Archer *et al.*, 2011). The importance of *S. aureus* biofilms in pathogenesis is described in section 1.5.4.

1.5.2.2 Protein A

Protein A plays another important role in virulence due to its binding activity to IgG on the Fc region (Forsgren and Sjoquist, 1966). Protein A possesses five homologous IgG binding domains at its N-terminal region, and a C-terminal region responsible for anchoring the protein to *S. aureus* cell wall (Kihira and Aiba, 1992). Protein A binds to the Fc end of IgG, which makes the Fc end inaccessible to opsonins, thus impairing opsonisation and phagocytosis of the bacteria. Protein A also interacts with V_H3 region of human IgM, IgA, IgG and IgE (Sasso *et al.*, 1991), and induces the apoptosis of V_H3-bearing B cells (Goodyear and Silverman, 2004; Bekeredjian-Ding *et al.*, 2007). In addition to its adhesive property as described in section 1.5.1.4, Protein A is an important virulence factor facilitating *S. aureus* invasion. For example, protein A mediates *S. aureus* invasion across human airway epithelial cells *in vitro* through activation of RhoA (a key regulator of epithelial cell behaviour) signalling and proteolytic activity of calpain (Soong *et al.*, 2011).

1.5.3 Toxins

1.5.3.1 Cytotoxins

Cytotoxins possess cytolytic activity, which lyses the cytoplasmic membrane of host cells and causes leakage of the cell content. They include alpha-hemolysin, beta-hemolysin, gamma-hemolysin, delta-hemolysin, leukocidins (Luk) and Panton-Valentine leukocidin (PVL) (Kaneko and Kamio, 2004).

Alpha-hemolysin (α-toxin), encoded by *hla*, is the most potent membrane-damaging toxin of *S. aureus*. It is secreted as a monomer, and has high affinity for a variety cells such as rabbit erythrocytes, and human platelets, monocytes and endothelial cells (Bhakdi and Tranumjensen, 1991). Alpha-hemolysin binds to the membrane of a target cell then oligomerises to form heptameric cylinders. Such heptameric cylinders create a central channel, through which cellular contents leak out (Gouaux *et al.*, 1994). Alpha-hemolysin not only lyses host cells, but also interferes with the human immune response. For instance, it induces programmed cell death of human T cells, B cells and monocytes *in vivo* (Nygaard *et al.*, 2012). Alpha-hemolysin is also required for *S. aureus* biofilm formation on plastic catheters under either static or flow conditions (Caiazza and O'Toole, 2003).

Beta-hemolysin (β-toxin) is a sphingomyelinase encoded by the gene *hlb*. Unlike α-hemolysin, β-hemolysin is highly haemolytic for sheep erythrocytes. Hemolytic activity of β-hemolysin is enhanced by incubation at temperatures below 10°C after treatment at 37°C, therefore this toxin is often referred to as the 'hot-cold' hemolysin (Glenny and Stevens, 1935). Beta-hemolysin is an important virulence factor in *S. aureus* infections of the human cornea (OCallaghan *et al.*, 1997) and lung (Hayashida *et al.*, 2009). In addition, β-hemolysin participates in biofilm formation by forming covalent cross-links with extracellular DNA to create an insoluble nucleoprotein matrix as observed *in vitro*, and has been found to strongly trigger biofilm formation in rabbit endocarditis infection models (Huseby *et al.*, 2010).

Delta-hemolysin (δ-toxin), encoded by the gene *hld*, is a small peptide toxin of 26 amino acids and zero net charge (Fitton *et al.*, 1980). It contains 14 hydrophobic

residues and forms an α-helix structure with hydrophobic and hydrophilic domains on opposite sides. It can insert into hydrophobic membranes which leads to cell lysis (Verdon *et al.*, 2009). A *S. aureus* MW2 *hld* mutant has been shown to have significantly attenuated virulence in a mouse bacteraemia model (Wang *et al.*, 2007). Besides its haemolytic activity, purified δ-hemolysin from *S. aureus* has been shown to inhibit colony spreading on agar (Omae *et al.*, 2012), which may indicate its role in promoting efficient colonization.

Unlike the other toxins, the gamma-hemolysin (γ-toxin) and the leukocidins (Luk) are two-component toxins produced by *S. aureus*. Each of these toxins is composed of two separate proteins, which act together to damage membranes of susceptible cells. Leukocidin, consisting of LukF and LukS, is cytolytic toward human and rabbit polymorphonuclear leukocytes and rabbit erythrocytes, but not human erythrocytes (Tomita and Kamio, 1997). The gamma-hemolysin genes are in a cluster containing *hlgA*, *hlgB* and *hlgC*, in which *hlgB* and *hlgC* are co-transcribed, while *hlgA* is separately expressed. The proteins encoded by *hlgB* and *hlgC* are 98.5% and 99.1% identical with LukF and LukS respectively (Cooney *et al.*, 1993). PVL is composed of LukF-PV and LukS-PV, which differ from LukF and LukS, as they are not haemolytic. However, they demonstrate specific cytotoxin activity towards human and rabbit polymorphonuclear leukocytes and monocytes (Tomita and Kamio, 1997).

The γ-hemolysin is produced by almost all *S. aureus* strains, but only about 2% of *S. aureus* express PVL. In spite of this, nearly 90% of *S. aureus* isolates from severe dermonecrotic lesions were found to produce PVL, indicating the importance of PVL in necrotizing skin infections (Foster, 1996). Introduction of the lukF-PV and lukS-PV genes into a PVL-deficient *S. aureus* strain significantly contributed to its pathogenic potential in a mouse pneumonia model (Labandeira-Rey *et al.*, 2007). PVL was also shown to contribute to muscle injury in a mouse model, and intraperitoneal injection with PVL-specific antibodies blocked PVL-associated muscle injury (Tseng *et al.*, 2009).

1.5.3.2 Superantigens

S. aureus superantigens are a class of protein toxins that can bind directly to the class II major histocompatibility complex (MHC II) of antigen-presenting cells, and non-specifically stimulate T cells with appropriate Vβ receptor subsets. Activation of T-cells and antigen-presenting cells by superantigens results in massive cytokine release (Foster, 1996). *S. aureus* superantigens comprise several enterotoxins (SEs) and toxic shock toxin-1 (TSST-1). Enterotoxins are secreted proteins associated with food poisoning and cause diarrhoea and vomiting when ingested (Schelin *et al.*, 2011). Toxic shock toxin-1 is responsible for toxic shock syndrome (TSS), which is marked by the onset of high fever, hypotension, rash, multi-organ failure and desquamation (McCormick *et al.*, 2001).

1.5.3.3 Staphylococcal superantigen-like proteins (SSLs)

Staphylococcal superantigen-like proteins (SSLs) are a family of exoproteins that have previously been known as the staphylococcal exotoxin-like proteins (SETs), because they share sequence similarity and three-dimensional structural similarities to the SE superantigens (Williams *et al.*, 2000; Al Shangiti *et al.*, 2004). However, SSLs do not bind to MHC class II nor induce T cell activation, but interact with components of the immune system, such as immunoglobulin and complement, and human antigen-presenting cells (monocytes and dendritic cells). For example, dendritic cells loaded with SSL7 and SSL9 are able to stimulate T cell proliferation and a SSL-type specific antibody response (Al Shangiti *et al.*, 2005; Langley *et al.*, 2005).

1.5.3.4 Exfoliative toxins (ETs)

The exfoliative toxins are serine proteases that recognise and cleave desmosomal cadherins only in the superficial layers of the skin. They are therefore the causative agent of staphylococcal scalded skin syndrome (SSSS) which is characterised by symptoms of blistering and loss of the epidermis (Plano, 2004). The exfoliative toxins have been found to possess mitogenic activity for murine spleen T lymphocytes, thus induce T cell proliferation (Rago *et al.*, 2000).

1.5.4 Formation of biofilms

An important virulence mechanism that facilitates *S. aureus* infections is the ability to form biofilms. The formation of biofilms is a well-regulated process involving initial attachment, proliferation to form a biofilm and detachment (Otto, 2008). As described in section 1.5.1, *S. aureus* expresses an array of adhesins that allow attachment to human matrix proteins. In fact, *S. aureus* can attach to numerous biotic and abiotic surfaces, and is often found attach to indwelling medical devices (Archer *et al.*, 2011). Attachment is followed by growth and production of an ECM that contains adhesins to promote intercellular adhesion. Intercellular adhesion is predominately mediated by the exopolysaccharide PIA (Gotz, 2002). However, *S. aureus* PIA-independent biofilm formation can also occur via alternative adhesive proteins, such as protein A (Merino *et al.*, 2009), biofilm-associated protein (Bap) (Cucarella *et al.*, 2001), fibronectin binding proteins (FnBPs) (O'Neill *et al.*, 2008) and the *S. aureus* surface protein G (SasG), which is homologous to the accumulation-associated protein (Aap) involved in biofilm formation in *S. epidermidis* (Corrigan *et al.*, 2007). Additionally, extracellular DNA (eDNA) from lysed cells and teichoic acids also facilitate colonisation and biofilm formation probably due to their anionic properties (Gross *et al.*, 2001; Rice *et al.*, 2007). Mature biofilms can detach which enables the spread of bacteria to colonise new infection sites. Quorum-sensing controlled expression of surfactant molecules, such as the phenol-soluble modulin (PSM) peptides, is crucial to biofilm maturation and detachment (Periasamy *et al.*, 2012).

The formation of biofilm contributes significantly to *S. aureus* pathogenesis. For example, a *S. aureus ica* (which encodes PIA) mutant is significantly less able to persist in mouse blood after intravenous (i.v.) or intraperitoneal (i.p.) injection, and shows significantly reduced virulence in mouse renal infection and bacteraemia models (Kropec *et al.*, 2005). Over-expression of *icaADBC* enhances *S. aureus* virulence in *Caenorhabditis elegans* model (Begun *et al.*, 2007). In addition, *S. aureus* biofilms are able to prevent phagocytosis by mouse peritoneal macrophages *in vitro* and biofilms in catheters implanted subcutaneously are capable of attenuating inflammatory responses in a mouse model (Thurlow *et al.*, 2011).

1.5.5 Regulation of virulence determinants

The expression of *S. aureus* virulence factors is regulated by a range of factors, such as bacterial density, pH, and CO_2. The well characterised regulators include the two-component regulatory systems, the accessory gene regulator (*agr*) and *S. aureus* exoprotein expression (*saeRS*), and the DNA-binding proteins, Staphylococcal accessory regulator A (SarA) and the alternative sigma factor (σ^B).

The *agr* quorum sensing system is a two-component regulatory system (TCS). A typical TCS contains a sensor histidine kinase (HK) and a response regulator (RR). The sensor is a membrane protein, which senses environmental signals and transmits the signal through phosphorylation to the response regulator, which mediates the cellular response through regulation of the target genes (Stock *et al.*, 2000). The *agr* quorum sensing system regulates cellular response by secreting and sensing an auto-inducing peptide (AIP). As shown in figure 1.2, the *agr* locus contains two divergent promoters P2 and P3, which control the transcription of transcripts RNAII and RNAIII, respectively. The RNAII transcript contains four genes, *agrBDCA*, which encode functions for AIP biosynthesis and the two-component signal transduction system. AgrD is the AIP precursor which is processed by the integral membrane protein AgrB, resulting in the release of mature AIP of 7-9 amino acids containing a pentapeptide thiolactone macrocycle (Mayville *et al.*, 1999). The other two genes *agrC* and *agrA* encode a histidine kinase and a response regulator, respectively, comprising a two-component signal transduction system. The signal receptor AgrC contains an N-terminal transmembrane sensor domain and a C-terminal histidine protein kinase domain (Lina *et al.*, 1998). When activated by a threshold concentration of AIP, AgrC becomes auto-phosphorylated and activates AgrA through phosphorylation. The activated AgrA directly activates transcription from the P2 and P3 promoters. Activation of the P2 promoter results in the auto-inducing cycle, and activation of the P3 promoter induces the transcription of RNAIII, the effector molecule of the *agr* response that regulates mRNAs at the post-transcriptional level (Reynolds and Wigneshweraraj, 2011).

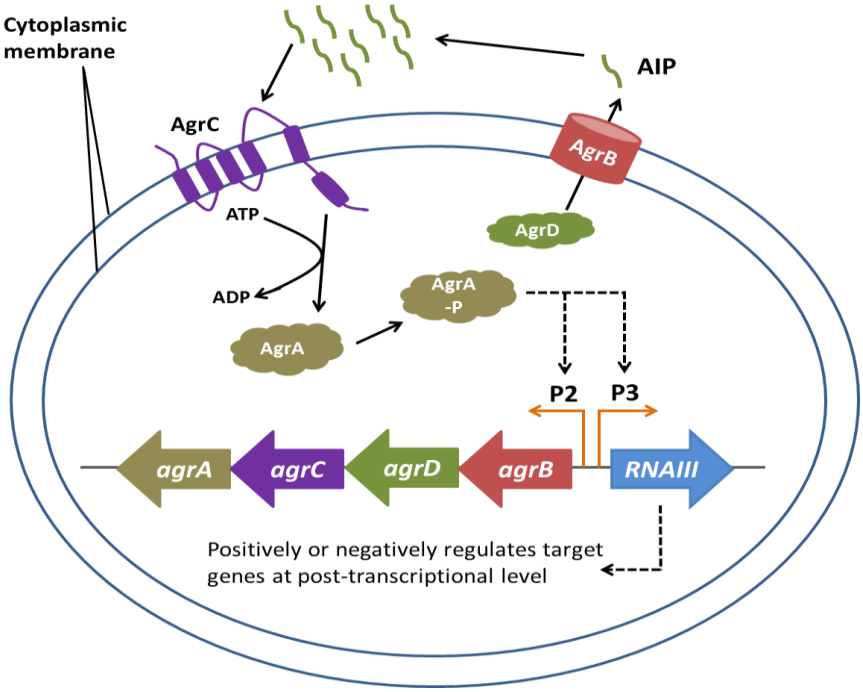

Figure 1.2: The Agr quorum sensing system in *S. aureus* (Camara *et al.*, 2012). Agr: accessory gene regulator. AIP: auto-inducing peptide. P2 & P3: promoters driving the expression of AgrBDCA and RNAIII, respectively. AgrA-P: phosphorylated AgrA. Solid arrows: indication of changes, interaction or reactions. Dashed arrows: indication of regulation of gene expression.

As indicated by microarray and proteomic analysis, the *agr* regulatory system potentially regulates more than 70 genes including 23 known virulence genes (Ziebandt *et al.*, 2004). RNAIII is a large regulatory RNA, which also encodes δ-hemolysin (Janzon and Arvidson, 1990). Thus activating promoter P3 promotes δ-hemolysin expression. In general, RNAIII up-regulates the expression of exoproteins that promote invasion, such as α-hemolysin (Morfeldt *et al.*, 1995), enterotoxin B (Tseng and Stewart, 2005) and serine protease SspA (Oscarsson *et al.*, 2006), and down-regulates the expression of cell wall-associated proteins facilitating adherence to the host and evasion of the immune system, such as protein A (Huntzinger *et al.*, 2005) and FnbAB (SaraviaOtten *et al.*, 1997). The Agr system is activated when the AIP concentration reaches a threshold, which indicates the density of bacteria. A simplistic view is that the Agr system facilitates detachment of bacteria from a site of colonisation when the density reaches a certain level by down regulating adhesins and activates genes like hemolysins that allow the bacteria to evade the immune system and invade other sites. In accordance with this view, Agr was found to be involved in biofilm detachment, and repression of *agr* promotes biofilm formation (Boles and Horswill, 2008).

The SaeRS two-component regulatory system contains four open reading frames *saePQRS*. The genes *saeR* and *saeS* show strong sequence homology to typical bacterial two-component regulators composed of a HK and a RR (Giraudo *et al.*, 1999), and *saeQ* and *saeP* are likely to have regulatory effects on the *sae* operon (Adhikari and Novick, 2008). Proteomic and transcriptomic analyses indicate that the Sae system positively regulates the expression of a range of important virulence factors such as γ-hemolysin, leukocidin, protein A and FnbAB (Rogasch *et al.*, 2006). The role SaeRS plays in regulating virulence factors has been demonstrated in a number of studies. For example, *saeRS* was shown to positively control the expression of α-hemolysin (Goerke *et al.*, 2005), Eap and Emp (Harraghy *et al.*, 2005), and represses the expression of protein A (Giraudo *et al.*, 1997).

The staphylococcal accessory regulator *sarA* operon consists of three overlapping transcripts *sarP1* (0.58kb), *sarP3* (0.84kb) and *sarP2* (1.15kb). These three transcripts share 3' ends, and all of them encode the protein SarA (Bayer *et al.*, 1996). SarA is a global transcriptional regulator that regulates gene transcription by binding as a homodimer structure to the promoter regions of target genes (Chien *et al.*, 1999). It was initially identified as an activator of the *agr* operon, because it binds to *agr* P2 and P3 promoters to promote the transcription of both RNAII and RNAIII (Cheung *et al.*, 1997). Therefore, SarA is able to influence the expression of virulence determinants through *agr*. In addition to this, SarA can also directly regulate a variety of virulence genes, such as positively controlling the expression of FnbAB (Wolz *et al.*, 2000), α-hemolysin, toxin shock syndrome toxin-1 and enterotoxin B (Chan and Foster, 1998), and negatively regulating V8 serine proteases (Chan and Foster, 1998).

S. aureus expresses several SarA homologues, which fall into the family of winged-helix proteins. These Sar homologues include relatively larger proteins that are 29-30kDa (SarS, SarU, and SarY), and small proteins of 13-17kDa (SarA, SarR, SarT, SarX, SarV, sarZ, MgrA and Rot) (Bronner *et al.*, 2004; Cheung *et al.*, 2008). Many of the SarA homologues are involved in regulating virulence determinants and are also under the regulation of the *agr* locus. For instance SarS, which is repressed by the Agr system, has been shown to be an activator of protein A expression (Cheung *et al.*,

2001). Inhibition of Rot has been shown to be an important mechanism by which the Agr system exerts global regulation, as Rot and Agr exert opposite effects on the expression of exoproteins and surface proteins (Said-Salim *et al.*, 2003; Geisinger *et al.*, 2006).

In some bacteria, the RNA polymerase holoenzyme is composed of a core enzyme with four subunits ($\alpha_2\beta\beta'$) and a sigma factor (σ). The holoenzyme recognises specific promoter regions and initiates transcription. There are a number of different σ sigma factors and each σ factor specifically regulates the initiation of transcription of a subsets of genes that participate in related cellular functions (Manganelli *et al.*, 2004). The *S. aureus* alternative sigma factor (σ^B) is responsible for the regulation of gene expression in response to environmental changes or stimulus (Bischoff *et al.*, 2004). The *S. aureus* σ^B operon contains a cluster of four genes *rsbU*, *rsbV*, *rsbW* and *sigB* (Wu *et al.*, 1996). The activity of σ^B is regulated by the three genes upstream of *sigB*. RsbW serves as an anti-sigma factor by binding to σ^B and forming a complex RsbW-σ^B, which is unable to bind to RNA polymerase and initiate the transcription of target genes (Miyazaki *et al.*, 1999). However, RsbV is an antagonist of anti-sigma factor, which can bind to RsbW and lead to the release of σ^B. Both RsbW and RsbU modulate RsbV. RsbW phosphorylates RsbV, in which form RsbV-P is no longer able to bind to RsbW, thus blocking the release of σ^B from the RsbW-σ^B complex. In contrast, the phosphatase RsbU dephosphorylates RsbV-P, restoring its ability to compete with SigB for association with RsbW (Voelker *et al.*, 1995; van Schaik *et al.*, 2005). Therefore, RsbU is an important activator of *sigB* (Giachino *et al.*, 2001).

Microarray analysis indicates that the alternative sigma factor σ^B positively controls 198 genes and negatively regulates 53 genes in *S. aureus* (Bischoff *et al.*, 2004). Virulence factors that are up-regulated in the presence of SigB include cell surface components and proteins such as capsular polysaccharide and clumping factors. Among the genes down-regulated by SigB are those encoding exoproteins and toxins, such as γ-hemolysin, leukocidins and serine protease (Bischoff *et al.*, 2004). Thus SigB may play a role in the colonisation process through regulation of expression of virulence factors in response to changing host environmental conditions. SigB can

also regulate virulence determinants through Agr or SarA, as it up-regulates expression of SarA while it represses expression of RNAIII (Bischoff *et al.*, 2001).

1.6 *S. aureus* resistance to antibiotics

S. aureus is an important human pathogen and a clinical challenge, not only due to its large number of virulence factors that facilitate successful attachment and invasion, but also because of its increasing resistance to antimicrobial agents. Treatment of *S. aureus* infections generally relies on antibiotic therapy, but the increased use of antibiotics has contributed to the rise of antibiotic resistant forms of *S. aureus*. The first antibiotic penicillin was discovered in the early 1940s, it belongs to the category of β-lactam antibiotics that target bacterial cell wall synthesis (Kohanski *et al.*, 2010). The use of penicillin has dramatically reduced the morbidity caused by *S. aureus* infections. However, only one year after its introduction, penicillin-resistant *S. aureus* isolates were identified in hospitals and among the wider community (Rammelkamp and Maxon, 1942). Within 10 years, 75% of *S. aureus* strains isolated in the clinic had become penicillin resistant (Finland, 1955). By the early 1990s, the first-line β-lactam antibiotics, penicillin and ampicillin, failed in the treatment of more than 95% of patients with *S. aureus* infections worldwide (Neu, 1992). *S. aureus* is resistant to penicillin due to the production of β-lactamase, which is able to hydrolase the β-lactam ring, resulting in the inactivation of the antimicrobial activity of penicillin (Gregory *et al.*, 1997).

To combat the β-lactamase-producing strains, methicillin was developed by modifying the side chains of penicillin, which protects the antibiotic from degradation by β-lactamase. However, within 2 years, the first methicillin-resistant *S. aureus* (MRSA) strain emerged (Jevons *et al.*, 1961). The resistance is mediated by an acquired *mecA* gene, which encodes an alternative cell wall synthesis enzyme penicillin-binding protein PBP2a that has a low affinity for the β-lactams (Brown and Reynolds, 1980; Hartman and Tomasz, 1984). This resistance mechanism has resulted in the ineffectiveness of all β-lactam antibiotics. The United States National Nosocomial Infections Surveillance System (NNISS) has monitored the rise of MRSA infections since 1992 and reported that 60% of *S. aureus* clinical isolates from the

USA intensive care units (ICUs) were MRSA between 1992 and 2004 (Cardo *et al.*, 2004). The European Antimicrobial Resistance Surveillance System (EARSS) has compiled similar data since 1999, and reported that MRSA clinical isolates from ICUs account for 25-50% of clinical *S. aureus* isolates in many central and southern European countries from 2007 to 2010 (EARSS, 2011).

MRSA is now a recognised problem in hospitals worldwide. Clinically isolated MRSA are often resistant to multiple antibiotics, it is thus difficult to treat MRSA infections, and lengthy and expensive antibiotic therapy is often required. It has been estimated that MRSA infections cost the UK £3-11 billion every year (Smith *et al.*, 2005). In addition, MRSA also causes significantly higher mortality than methicillin-sensitive *S. aureus* (MSSA) in patients with bacteremina (Cosgrove *et al.*, 2003). Figure 1.3 shows the number of deaths caused by MSSA and MRSA infections in England and Wales between year 1993 and 2010. The mortality caused by MSSA is quite consistent in these 18 years. However, the number of death caused by MRSA increased continuously from 1993 to 2006, and was particularly high between 2005 and 2007. The MRSA-associated mortality has dropped rapidly since 2008 due to appropriate infection control precautions and effective medical care regulations (Liebowitz, 2009).

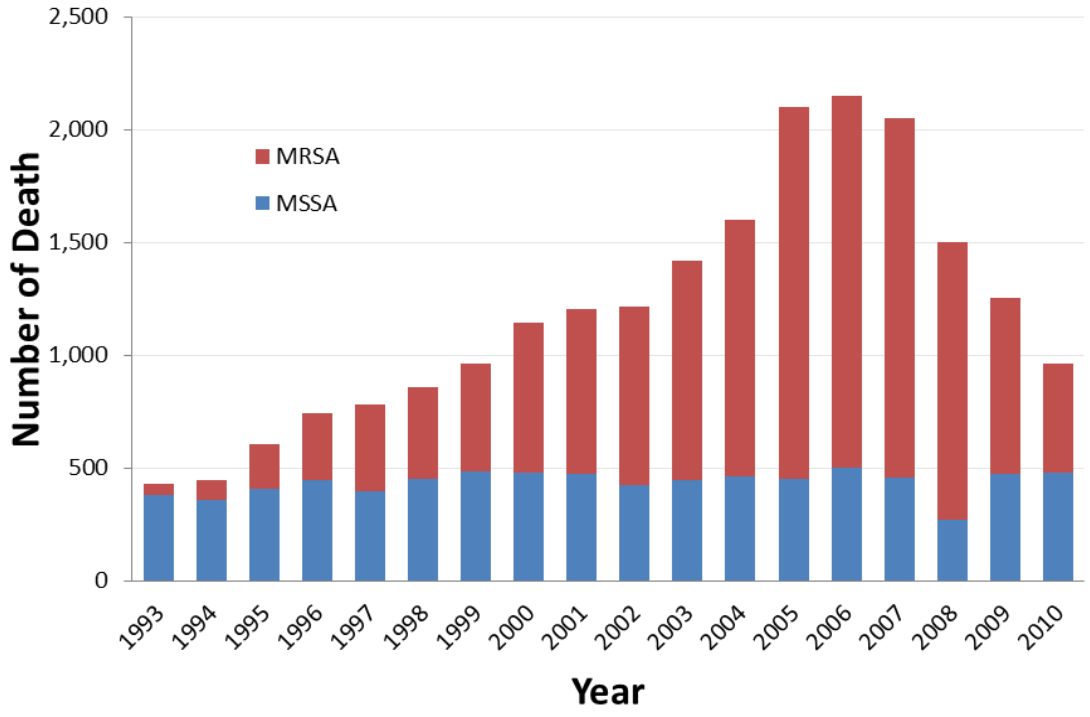

Figure 1.3: Annual number of deaths involving MRSA and MSSA in England and Wales, 1993-2010. (Office for National Statistics, data released on the 23rd August 2011).

Since the 1980s, vancomycin, a glycopeptide antibiotic that also targets bacterial cell wall synthesis, has increasingly been used to treat infections caused by MRSA. The first clinical isolate of *S. aureus* with reduced susceptibility to vancomycin was reported from Japan in 1997 (Hiramatsu *et al.*, 1997), which was soon followed by two additional cases from the USA (Smith *et al.*, 1999). Since then, *S. aureus* with increased resistance to vancomycin has been frequently identified worldwide (Palazzo *et al.*, 2005; Tiwari and Sen, 2006; Aligholi *et al.*, 2008; Sievert *et al.*, 2008; Finks *et al.*, 2009). Resistance to the two most recent new classes of antimicrobial agents against MRSA, the synthetic antibiotic linezolid and the lipopeptide antibiotic daptomycin, was also reported soon after their introduction. The first case of a linezolid-resistant MRSA strain was described a year after the antibiotic was in use in clinic (Tsiodras *et al.*, 2001), and daptomycin-resistant MRSA was reported within two years of its introduction (Mangili *et al.*, 2005).

1.7 Antimicrobial peptides as potential antibiotics

Conventional antibiotics target bacterial cell wall synthesis (β-lactams, glycopeptides and lipopeptides), protein synthesis (aminoglycosides, macrolides, tetracyclines, streptogramins and phenicols) or nucleic acid synthesis (fluoroquinolones, trimethoprim-sulfamethoxazole and rifamycins) (Kohanski *et al.*, 2010). The continued rapid evolution of resistance to nearly all of the antibiotics used against *S. aureus* has highlighted the need for antibiotics that are not only effective at killing *S. aureus*, but also have a low potential for development of resistance. For these reasons, antimicrobial peptides (AMPs) have increasingly been receiving interest as promising potential novel antibiotics (Hancock and Sahl, 2006; Giuliani *et al.*, 2007; Afacan *et al.*, 2012).

AMPs are important components of the innate immune system in most living organisms. In humans, AMPs are often produced by the tissues and cells that are exposed to microbes, such as epithelial cells and neutrophils (Wiesner and Vilcinskas, 2010). AMPs are short peptides that are typically less than 100 amino acids, hydrophobic and many of which are positively charged. They exert direct antimicrobial activity against invading pathogens, and also play regulatory roles in the

immune system (Harder *et al.*, 2007). Many AMPs tested *in vitro* exhibit potent and broad-spectrum antimicrobial activities against bacteria, viruses and fungi (De Smet and Contreras, 2005; Klotman and Chang, 2006; Harder *et al.*, 2007). For example, the human AMPs cathelicidin, α-defensin, β-defensin and histadin effectively inhibit the growth of a range of bacteria and fungi with MICs ranging between 0.4μg/ml and 39.7μg/ml, including MSSA, MRSA, vancomycin-resistant *Enterococcus faecalis* (VREF), *S. epidermidis*, *Bacillus subtilis*, *Listeria monocytogenes*, *Streptococcus mutans*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa* and *Candida albicans* (summarised in a review written by De Smet and Contreras (2005). The potential of AMPs as antibiotics has also been established *in vivo*, for example, oral administration of human lactoferrin B to mice infected with *Escherichia coli* in the urinary tract significantly reduces infection and inflammation (Haversen *et al.*, 2000). Additionally, a derivative of the human cathelicidin LL-37 is effective in eradicating *P. aeruginosa* biofilm in a rabbit sinusitis model (Chennupati *et al.*, 2009).

In addition to their potent antimicrobial activity, some AMPs are thought to be less likely to induce bacterial resistance due to their receptor-independent modes of action. The modes of action of AMPs are not fully established, but a number of AMP targets have been identified, including cell surface components, the cytoplasmic membrane, and intracellular molecules (described in section 1.9) (Park *et al.*, 2011). The main AMP mode of action is believed to be by irreversibly disrupting the bacterial cell membrane. Such activity is due to their structural and chemical properties rather than specific peptide sequences, thus they are less likely to cause the development of resistance among microbes (Hancock, 1997).

1.8 AMPs originated from human and bacteria

1.8.1 Human AMPs

There are a wide variety of human antimicrobial proteins and peptides, but for the most part, human antimicrobial peptides are classified into three types: defensins, cathelicidins and histatins (De Smet and Contreras, 2005). The two main families that have been extensively studied are defensins (Ganz, 2003a), and the cathelicidins (Durr *et al.*, 2006).

1.8.1.1 Defensins

Human defensins are small cationic peptides (29-47 amino acids) rich in arginine, which contributes to their cationic properties. Human defensins fall into two main classes: α-defensins and β-defensins. Both classes of defensins contain six cysteine residues that form three intra-molecular disulphide bonds, resulting in a triple-stranded β-sheet structure. The differences between α and β-defensins are that the disulphide bonds are formed between different cysteine residues, and β-defensins (36-47 amino acids) are generally larger than α-defensins (29-35 amino acids) (De Smet and Contreras, 2005). In humans, six α-defensins have been identified with four being isolated from neutrophils and termed human neutrophil peptides HNP-1 to 4. The remaining two α-defensins, HD-5 and HD-6, are produced by the Paneth cells of human small intestine (Jones and Bevins, 1992, 1993). Six human β-defensins have been identified and designated HBD-1 to 6 (Klotman and Chang, 2006). Both α and β defensins possess a broad spectrum of antimicrobial activity against bacteria, fungi and viruses. For instance, α-defensins are effective against both Gram-positive bacteria such as *S. aureus*, *Bacillus subtilis* and *Listeria monocytogenes*, and Gram-negative organisms *E. coli* and *S.* Typhimurium (Lehrer and Ganz, 1999; De Smet and Contreras, 2005). β-defensins, especially HBD-3, possess potent antimicrobial killing activity against many pathogens, including multidrug-resistant *S. aureus*, *Enterococcus faecium* and *P. aeruginosa* (Harder *et al.*, 2001; Maisetta *et al.*, 2006).

1.8.1.2 Cathelicidins

Cathelicidins are a group of antimicrobial peptides derived from cathelicidin proteins, which are characterised by a highly conserved cathelin domain (acronym for cathepsin-L-inhibitor) (Gennaro and Zanetti, 2000). In humans, only one cathelicidin has been identified and designated LL-37. This peptide is derived from a precursor protein, the human cationic antimicrobial protein (hCAP18) with an approximate mass of 18 KDa. The protein hCAP18 is encoded by the cathelicidin antimicrobial peptide (*CAMP*) gene, and is present mainly in neutrophil specific granules (Sorensen *et al.*, 1997). It is also present in the lymphocytes and monocytes (Agerberth *et al.*, 2000), skin (Reinholz *et al.*, 2012), lung (Bals *et al.*, 1998), epididymis, seminal plasma (Malm *et al.*, 2000), and mammary glands (Murakami *et al.*, 2005). As shown in

Figure 1.4, the protein hCAP18 contains a cathelin domain of 101 amino acid residues, which is flanked by a 30-amino acid N-terminal signal peptide domain and a C-terminal antimicrobial peptide region of 37 amino acids (Larrick *et al.*, 1996; Durr *et al.*, 2006). hCAP18 is an inactive precursor, but becomes active once the C-terminal antimicrobial peptide is released via proteolytic cleavage by protease-3 (Sorensen *et al.*, 2001). The released cathelicidin peptide is called LL-37 because it contains 37 amino acids starting with two leucine residues. The sequence of this peptide is LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES.

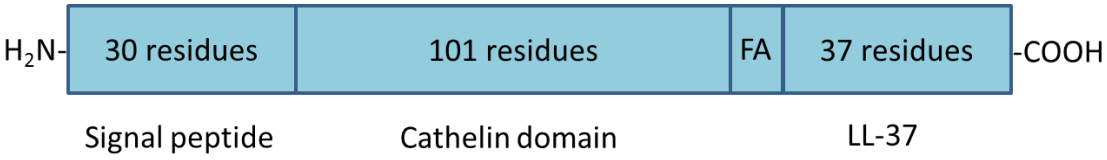

Figure 1.4: A diagram representing the human cathelicidin hCAP18. FA: the amino acids phenylalanine and alanine. Picture is modified from Durr *et al*. (2006).

LL-37 is highly positively charged because it is rich in lysine and arginine. The peptide has 6 lysine and 5 arginine residues that provide 11 positive charges, while 3 glutamate and 2 aspartate residues provide 5 negative charges. Therefore, at physiological pH, LL-37 has a net charge of +6. LL-37 exhibits a disordered conformation in water, but adopts an amphipathic α-helical structure in solutions containing anions, such as SO_4^{2-}, HCO_3^- and $CF_3CO_2^-$, or as the concentration of the peptide is increased (Johansson *et al.*, 1998). The peptide also gains an amphipathic α-helical structure in contact with lipid micelles (Wang, 2008). The antimicrobial activity of LL-37 is correlated with its α-helical structure (Johansson *et al.*, 1998). LL-37 is effective against Gram-positive and Gram-negative bacteria, including MRSA, vancomycin-resistant *E. faecium* and *E. faecalis*, *L. monocytogenes*, *S. epidermidis, B. subtilis* (Turner, 1998), *P. aeruginosa* (Deslouches *et al.*, 2005), and viruses such as herpes simplex virus type 1 (HSV-1) (Gordon *et al.*, 2005). It is less potent at killing *C. albicans* (Turner, 1998; Sigurdardottir *et al.*, 2006) but is still able to reduce *C. albicans* infection by inhibiting adhesion (Tsai *et al.*, 2011). In addition, LL-37 is involved in modulating the immune system by acting as a chemoattractant for neutrophils, monocytes and T-cells by interacting with the receptor formyl peptide receptor-like 1 (Yang *et al.*, 2000), and promoting wound healing (Ramos *et al.*, 2011).

1.8.2 Bacteriocins

Bacteria produce AMPs called bacteriocins to compete against bacteria of the same or closely related species (Cotter *et al.*, 2005). So far 215 different bacteriocins have been reported, most of which are produced by Gram-positive bacteria (BACTIBASE, 2012). Bacteriocins produced by the lactic acid bacteria (LAB) have particularly been extensively studied and LAB bacteriocins have generally been classified into two categories, lanthionine-containing bacteriocins (named lantibiotics) and non-lanthionine-containing bacteriocins (Nishie *et al.*, 2012). Lantibiotics are small peptides (19-38 amino acids), which are characterised by their content of unusual amino acids, such as lantionine and 3-methyllanthionine. These unusual amino acids were introduced by post-translational enzymatic modifications (Lee and Kim, 2011), and they form intramolecular rings, which contribute to the peptide structural stability against heat, protease and a wide range of pH (Abriouel *et al.*, 2011). Non-lanthionine-containing bacteriocins are usually less than 100 amino acids and do not undergo post-translational modification (Cotter *et al.*, 2005).

LAB are generally recognised as safe (GRAS), the bacteriocins produced by this group of bacteria have therefore been the subject of a lot of research interest because of their potential to be safely applied as food preservatives or pharmaceuticals (Nishie *et al.*, 2012). Bacteriocins are normally active against either a narrow spectrum or a broad spectrum of organisms (Abriouel *et al.*, 2011). The lantibiotic nisin, a cationic AMP isolated from *L. lactis*, is active against important Gram-positive foodborne pathogens and spoilage agents; it has therefore been widely used as food preservatives in more than 50 countries (Cotter *et al.*, 2005). Importantly, no significant occurrence of resistance to nisin has been reported over the past 50 years of its usage as a food preservative, which is thought to be because of its special mode of action (described in section 1.9.3) and a relatively narrow spectrum of activity (Jenssen *et al.*, 2006). In addition to food preservatives, bacteriocins are also attractive option as possible new therapeutic agents against multidrug-resistant bacteria. For example, pumilicin 4, a heat-stable bacteriocin derived from *Bacillus pumilus*, is active on both MRSA and VREF (Aunpad and Na-Bangchang, 2007). Some bacteriocins produced by *Staphylococcus*, such as Pep5, epidermin and aureocin A53,

are active against multidrug-resistant *S. aureus* and coagulase-negative *Staphylococci* involved in human infections (Nascimento *et al.*, 2012).

1.9 Mode of action of AMPs

The modes of action of AMPs are not yet fully understood, but the current thought is that AMPs kill microbes through directly disrupting the microbial cell membrane, causing the release of cell contents. However, there are also AMPs that have relatively smaller impact on membrane integrity, but inhibit intracellular processes, such as the synthesis of cell wall, protein, or nucleotides. Regardless of targeting the membrane or intracellular processes, the AMPs need to interact with the microbe membrane, either to lyse it or pass through it. The mechanisms by which AMPs interact with microbes and exert killing are described in the following sections.

1.9.1 Interaction with bacterial cell surface components

The first step by which AMPs target microbes is thought to involve electrostatic attraction between the cationic peptide and negatively charged cell surface components, such as lipopolysaccharides (LPS) in Gram-negative bacteria and teichoic acids in Gram-positive bacteria (Brogden, 2005). In addition, a recently study has identified an outer membrane lipoprotein Lpp of Enterobacteriaceae as a Gram-negative cell surface receptor for cationic AMPs including LL-37 (Chang *et al.*, 2012). Following attraction to the cell surface, the AMPs need to pass through the layers of LPS in the Gram-negative bacteria, and capsular polysaccharides, teichoic acids and lipoteichoic acids in Gram-positive bacteria, to reach the bacterial membrane (Brogden, 2005). So far the mechanisms involved in this process have not been established.

1.9.2 Membrane permeabilisation

Three mechanisms have been suggested for the permeation of bacterial cell membranes by membrane-active cationic AMPs, which are shown in figure 1.5 (Brogden, 2005). A barrel-stave mechanism involves the formation of membrane-spanning transmembrane channels with hydrophobic regions of the peptides facing

the membrane lipids and the hydrophilic domains forming the interior of the pore (Ehrenstein and Lecar, 1977; Yang *et al.*, 2001). The barrel stave mechanism is employed by the peptide alamethicin, which is uncharged and barely soluble in water (Yang *et al.*, 2001). In the carpet-like model, the peptides accumulate on the lipid bilayer of the cell membrane in a carpet-like manner. When a threshold concentration of peptide is reached, the peptides disrupt the membrane into micelles (Shai, 1999). In the toroidal-pore model, the peptides insert into the lipid bilayer and induce the lipid monolayers to bend from the top to the bottom to form a pore lined with both the peptides and the lipid head groups (Yang *et al.*, 2001).

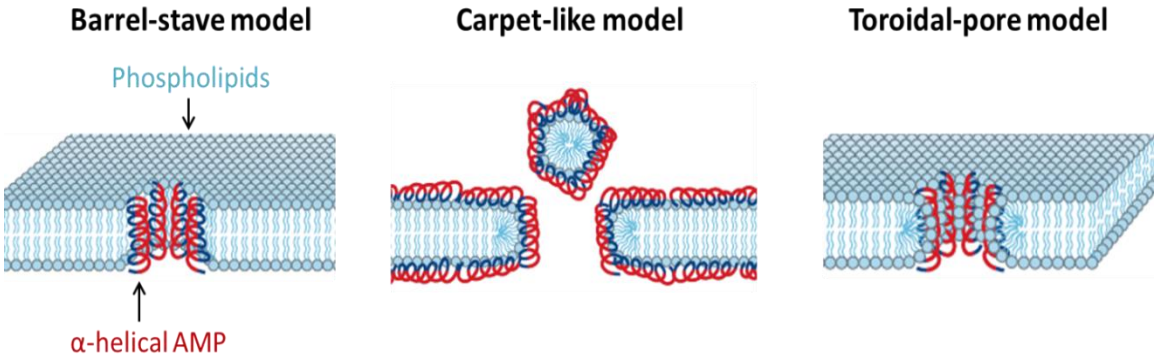

Figure 1.5: Graphic describing the three mechanisms through which α-helical AMPs are proposed to disrupt bacterial cell membranes. Hydrophilic regions of the peptide are shown in red and hydrophobic regions of the peptide are shown in dark blue. Pictures are modified from Brogden (2005).

A recent study which monitored the real-time interaction of LL-37 with *E. coli* using fluorescently labelled LL-37 has revealed the killing process of this peptide. LL-37 binds to and saturates the outer membrane of *E. coli* within one minute. Then the peptide translocates across the outer membrane to the periplasmic space, which is followed by growth inhibition and permeabilization of the cytoplasmic membrane (Sochacki *et al.*, 2011). This real time killing process reveals that LL-37 interacts with bacteria cell surface and exerts a killing effect through disrupting the bacterial membrane. The mode of action by which LL-37 permeabilises cell membrane is controversial, as both the carpet-like mechanism (Oren *et al.*, 1999) and the toroidal pore mechanism (Wildman *et al.*, 2003; Lee *et al.*, 2011) have been suggested.

1.9.3 Inhibition of bacterial intracellular processes

Apart from disruption of the membrane, other mechanisms by which AMPs kill bacteria have been proposed. The lantibiotic nisin is able to form a complex with the membrane-anchored peptidoglycan precursor Lipid II, which promotes the insertion of nisin into the membrane to form pores (Hsu *et al.*, 2004), and also inhibits cell wall biosynthesis (Hasper *et al.*, 2006). Bacitracin, which is a polypeptide antibiotic produced by *B. subtilis* and *Bacillus licheniformis* (Johnson *et al.*, 1945), also inhibits cell wall biosynthesis. Bacitracin binds to undecaprenyl pyrophosphate (UPP) which is a lipid carrier responsible for translocation of cell envelope precursors from the cytosol to the extracellular surface of the cytoplasmic membrane (Stone and Stroming, 1971). In addition, some AMPs that translocate into bacterial cells also disturb cell homeostasis in a variety of ways. For example, the bovine AMP indolicidin and the Asian toad-derived AMP buforin II, penetrate the bacterial membrane without lysing the cells, but exert bactericidal activity by inhibition of DNA, RNA and/or protein synthesis through binding to nucleic acids (Park *et al.*, 1998; Subbalakshmi and Sitaram, 1998). Furthermore, some AMPs can interact with cytoplasmic proteins to interrupt cell function. The insect AMP pyrrhocoricin is capable of binding to the bacterial DnaK heat shock protein, which is crucial for the initiation of bacterial DNA replication, resulting in the impairment of protein activity (Kragol *et al.*, 2001). Microcin B17, a peptide antibiotic produced by *Enterobacteria*, also inhibits DNA replication by targeting DNA gyrase to induce the cleavage of DNA (Pierrat and Maxwell, 2005).

1.10 AMP resistance mechanisms in *S. aureus*

1.10.1 Modification of the cell surface

S. aureus is able to modify some negatively charged cell surface components, such as teichoic acids in the cell wall and phospholipids in the membrane. These modifications result in a reduction in the negative charge of the cell surface, thereby diminishing the electrostatic attraction between the cells and cationic AMPs. For example, the *dltABCD* (D-alanyl-lipoteichoic acid) operon encodes proteins that catalyse the transfer of D-alanine to teichoic acid and lipoteichoic acid. The

incorporation of D-alanine leaves positively charged amino groups exposed, thereby partially neutralising the negative charge on teichoic acids (Peschel *et al.*, 1999). Similarly, MprF (multiple peptide resistance factor) introduces a positively charged L-lysine into phosphatidylglycerol (PG) to counter the negative charges on cell membrane (Peschel *et al.*, 2001). The importance of lysyl-PG for *S. aureus* resistance to AMPs was also established using membrane models containing distinct mixtures of PG and lysyl-PG mimicking the membrane composition of the wild-type and *mprF* mutant (Andra *et al.*, 2011). *S. aureus dltA* and *mprF* mutants have been examined in a rabbit endocarditis model, both mutants exhibited significantly reduced ability to colonise and proliferate within the cardiac vegetations, and the *dltA* mutant was also significantly attenuated in terms of bacterial numbers within renal and splenic abscesses (Weidenmaier *et al.*, 2005).

1.10.2 External trapping of AMPs

In addition to modification of negatively charged surface molecules, *S. aureus* also secretes proteins that neutralise the bactericidal activity of AMPs by forming a complex with AMPs or by enzymatic degradation of the AMPs. For instance, the *S. aureus* exoprotein staphylokinase possess high binding affinity to human α-defensins, and binding to staphylokinase almost completely abrogates the bactericidal effect of α-defensins (Jin *et al.*, 2004). In addition, two major extracellular proteases produced by *S. aureus*, the metalloprotease aureolysin and the V8 protease (serine protease), efficiently inactivate LL-37 through cleavage of the peptide (Sieprawska-Lupa *et al.*, 2004).

1.10.3 Active efflux of AMPs

S. aureus is capable of expelling AMPs using energy-dependent efflux pumps. Some *S. aureus* strains harbour a plasmid pSK1, which encodes resistance to multiple antimicrobial agents including aminoglycosides (Rouch *et al.*, 1987), trimethoprim (Rouch *et al.*, 1989), and organic antimicrobial cations (Mitchell *et al.*, 1998). The resistance to organic antimicrobial cations is mediated by gene *qacA*, which encodes a proton motive force-dependent multidrug efflux pump (Mitchell *et al.*, 1998). This efflux pump is involved in *S. aureus* resistance to thrombin-induced platelet

microbicidal protein-1 (tPMP-1), a small cationic peptide released from mammalian platelets following thrombin stimulation. This was demonstrated by the loss of resistance to tPMP-1 upon the deletion of the *qacA* locus in a pSK1-bearing *S. aureus* strain (Kupferwasser *et al.*, 1999).

1.10.4 AMP-sensing systems

S. aureus resistance to AMPs is also associated with two-component or three component systems, and ABC transporters. These elements cooperatively regulate *S. aureus* resistance to AMPs. Two AMP-sensing and regulation systems have been characterised in *S. aureus*: the GraXRS-VraFG system and the BraRS-BraDE-VraDE system (Gebhard and Mascher, 2011).

Initially, a two-component system GraRS (Gra = glycopeptide resistance associated; R = response regulator; S = sensor kinase) was identified as being involved in resistance to the glycopeptide antibiotic vancomycin (Meehl *et al.*, 2007). This TCS was later recognised as a three-component AMP sensing and regulatory system, because the first gene *graX* in the *graXRS* operon has also been found to be involved in the GraRS conducted AMP sensing and resistance in both *S. epidermidis* and *S. aureus* (Li *et al.*, 2007a & b). This three-component system is also named ApsXRS to indicate its role in antimicrobial peptide sensing (Li *et al.*, 2007a & b). The GraXRS AMP sensing system is composed of a sensor histidine kinase GraS and a DNA-binding response regulator GraR, which constitute a classical two-component system, and a third component GraX, which seems to act through the histidine kinase GraS to transduce the AMP signal to the response regulator GraR (Li *et al.*, 2007b; Falord *et al.*, 2012). The importance of GraRS in resistance to AMPs has been demonstrated in a number of studies. For instance, deletion of *graRS* considerably alters the surface charge of *S. aureus* SA113 and increases susceptibility to LL-37 (Kraus *et al.*, 2008). Moreover, *S. aureus* SG511-Berlin contains a natural insertion in *graS*, which generates a stop codon in this gene. This strain is sensitive to a range of AMPs and susceptibility decreases significantly when the strain is complemented with a functional copy of *graS* (Sass and Bierbaum, 2009).

AMP sensing and regulation by GraRSX involves the cooperation of this three component system with an ATP-binding cassette (ABC) transporter VraFG, encoded by an operon *vraFG* located directly downstream of the *graXSR* genes (Yang *et al.*, 2012). GraRS and VraFG interact and regulate *S. aureus* resistance to the glycopeptide antibiotic vancomycin (Meehl *et al.*, 2007). GraRSX has been demonstrated to strongly up-regulate the *vraFG* operon (Li *et al.*, 2007a), and the expression of *graR* was also found to be dependent on VraFG (Yang *et al.*, 2012), indicating a positive-feedback loop between *graRS* and *vraFG*. A recent study demonstrated that the GraRSX system and the VraFG transporter interact and act as a five-component system regulating cationic AMP resistance in *S. aureus* (Falord *et al.*, 2012). The *vraFG* mutant demonstrates similar level of sensitivity to the peptide antibiotic colistin as the *graRS* mutant, and the VraFG transporter is as essential as the GraRS TCS in regulating cationic AMP-induced genes, such as *mprF*. However, the VraFG transporter does not function alone as a detoxification module, but requires the GraRS TCS to confer resistance to AMPs. In contrast, over-expression of GraR is able to compensate the absence of GraS, GraX or VraFG, and restores wild-type level of resistance to colistin in these mutants. These findings suggest that both GraX and the VraFG ABC transporter may play role in signal transduction through the GraS kinase to activate GraR in response to the presence of cationic AMPs (Falord *et al.*, 2012).

GraRSX-VraFG is able to sense the presence of AMP and to mediate *S. aureus* resistance to AMPs by up-regulating the *dlt* operon for D-alanylation of teichoic acids, and the *mprF* gene for lysylination of bacterial membrane phosphatidylglycerol (Li *et al.*, 2007a; Falord *et al.*, 2012; Yang *et al.*, 2012). It confers resistance to a range of cationic AMPs with diverse structure and origin, such as the mammalian platelet-derived peptide tPMPs, human neutrophil peptide HNP-1, the bacterial peptide polymyxin B (Yang *et al.*, 2012) and LL-37 (Kraus *et al.*, 2008), as well as the cationic antibiotic vancomycin (Meehl *et al.*, 2007) and lantibiotics (nisin, mersacidin and Pep5) (Sass and Bierbaum, 2009). However, this system seems to only sense selected cationic antimicrobial peptide, as the transcription of *mprF* and *dlt* was found to be induced via GraXRS-vraFG only in the presence of RP-1 (synthetic tPMP-1-derived

peptide) and polymyxin B, but not in the presence of HNP-1 or other positively charged antibiotics, such as vancomycin, gentamicin, or calcium-daptomycin (Yang *et al.*, 2012).

In addition to GraXRS-vraFG, a two-component system BraRS (bacitracin resistance associated) was identified as being responsible for resistance to bacitracin and nisin (Hiron *et al.*, 2011). It is also named BceRS according to the locus name of the *B. subtilis* homologs (Kawada-Matsuo *et al.*, 2011) and NsaRS (nisin susceptibility-associated) (Blake *et al.*, 2011; Kolar *et al.*, 2011). As described in section 1.9.3, both bacitracin and nisin possess antimicrobial activity targeting cell wall biosynthesis, suggesting that BraRS may be involved in resistance to cell wall targeted antimicrobials. In fact, BraRS was recognised as a cell-envelope-stress-sensing TCS in *S. aureus*, because it also responds to a variety of cell envelope-targeted antibiotics, including phosphomycin, ampicillin, gramicidin, carbonyl cyanide *m*-chlorophenyl hydrazone and penicillin G (Kolar *et al.*, 2011).

Like the GraXRS three component system, the BraRS TCS also acts through regulating transporters. Two ABC transporters, BraDE and VraDE were identified to be regulated by BraRS, conferring resistance to bacitracin and nisin. The genes *braDE* are located directly downstream of *braRS*, while the *vraDE* genes are located separately from the *bra* locus (Hiron *et al.*, 2011; Kawada-Matsuo *et al.*, 2011). Hiron *et al*. (2011) studied the coordination and regulation in the BraRS-BraDE-VraDE system upon exposure to bacitracin and nisin, and reported a few features of this system. Firstly, the authors confirmed that all three elements, BraRS, BraDE and VraDE, are required for resistance to bacitracin, as *S. aureus* defective in BraRS, or BraDE, or VraDE were all highly sensitive to bacitracin. The BraSR TCS is required for sensing the presence of bacitracin or nisin, and then regulates resistance by inducing the expression of *braDE* and *vraDE*. In the absence of BraRS, the expression of *braDE* and *vraDE* is no longer induced by bacitracin or nisin (Hiron *et al.*, 2011). Secondly, the authors found that BraRS responds much more strongly to bacitracin than to nisin, demonstrated by 9-fold and 500-fold induction of *braDE* and *vraDE* respectively in the presence of 0.5µg/ml bacitracin, whereas no induction of *braDE* and 7-fold induction of *vraDE*

were observed in the presence of 0.5µg/ml nisin (Hiron *et al.*, 2011). Finally, an interesting feature of this system is that the two transporters, BraDE and VraDE, play distinct roles. The BraDE ABC transporter is only required for signalling, and the VraDE ABC transporter directly mediates resistance to bacitracin through the extracellular loop of VraE permease (Hiron *et al.*, 2011).

1.11 Small colony variants

Small colony variants (SCVs) are slow-growing subpopulations of bacteria. As indicated by the name, they form colonies approximately 1/10 the size of normal colonies on agar. SCVs can occur naturally *in vivo* and *in vitro*. *S. aureus* SCVs isolated *in vivo* are frequently associated with persistent and recurrent infections, such as osteomyelitis, implant infections and airway infections in cystic fibrosis patients (von Eiff *et al.*, 2006b), or patients receiving long-term antibiotic treatment (Proctor *et al.*, 1995; von Eiff *et al.*, 1997a, 2001). The occurrence of *S. aureus* SCV *in vitro* is often due to the exposure of the bacteria to antibiotics, especially aminoglycosides (Massey *et al.*, 2001). Clinically isolated *S. aureus* SCVs are often found to be auxotrophic for metabolic substances, such as hemin, menadione, thiamine or thymidine (Kahl *et al.*, 2003).

Among various *S. aureus* SCVs, the most extensively characterised are hemin and menadione auxotrophs (Proctor *et al.*, 1998; McNamara and Proctor, 2000; von Eiff *et al.*, 2004). Both hemin and menadione are required for the biosynthesis of the electron transport chain (ETC) components. As illustrated in figure 7.1, menadione is converted to the electron acceptor menaquinone, which receives electrons from nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$). The electrons from menaquinone are then passed on to the second electron acceptor cytochromes, whose synthesis requires the cofactor hemin (McNamara and Proctor, 2000). Menadione and heme are synthesised by a series of enzymes encoded by *men* genes and *hem* genes, respectively (Nowicka and Kruk, 2010; Heinemann *et al.*, 2008). Therefore, mutations in *men* or *hem* genes may interrupt menadione or hemin biosynthesis, which in turn results in an interrupted electron transport chain.

S. aureus hemin or menadione auxotrophs show phenotypic changes compared to the wild-type, typically they have reduced growth rate, are non-pigmented colonies, unable to ferment mannitol, have low coagulase activity, low haemolytic activity and decreased susceptibility to antibiotics (McNamara and Proctor, 2000). Most of these phenotypic changes are related to a defective ETC. The transfer of the electrons through ETC involves a series of redox reactions which at the same time create a proton gradient across the membrane. This proton gradient provides energy which drives the synthesis of ATP through the ATP synthase F_0F_1ATPase (Stuchebrukhov, 2003). As shown in figure 7.1, an intact ETC is required for maintaining cellular ATP levels, which is essential for many cellular functions, such as cell wall biosynthesis and the uptake of substances essential for bacterial growth (Klein and Lewinson, 2011). These are all prerequisites to ensure normal rapid growth of the cells, and a defective ETC is therefore associated with decreased growth rate. In addition, reduced cell wall biosynthesis as a result of a defective ETC causes increased resistance to cell wall-targeted antibiotics (Garcia *et al.*, 2012). Furthermore, the proton motive force generated from the ETC creates an electrochemical gradient ($\Delta\Psi$) across the cytoplasmic membrane, which is required for the uptake of positively charged antimicrobial substances, such as aminoglycosides (Baumert *et al.*, 2002). Therefore, an interrupted ETC is also associated with the reduced uptake of aminoglycosides, which results in the increased resistance to aminoglycosides in SCVs. Finally, the observation of non-pigmented SCV colonies is also likely to be a consequence of a defective ETC. Electrons are shuttled through the electron transport system to the cytochrome P450 system which is linked to the formation of carotenoid pigmentation (McNamara and Proctor, 2000). Inhibition of electron transport system using piperonyl butoxide or benzo(a)pyrene inhibits cytochrome oxidase o synthesis and represses the synthesis of the carotenoid rubixanthin (Joyce and White, 1971).

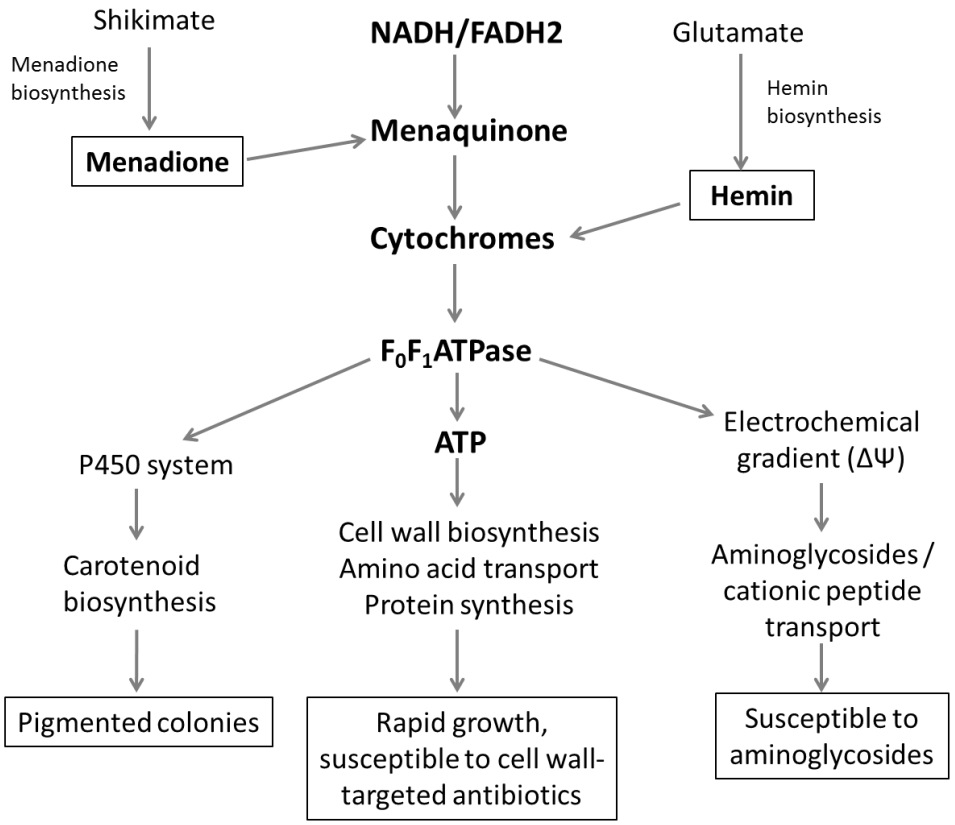

Figure 1.6: Hypothetical relationship between electron transport and *S. aureus* wild-type phenotypes (pigmented colonies, rapid growth and antibiotic susceptibility) that is altered in hemin or menadione SCVs. Figure modified from McNamara and Proctor (2000).

SCVs in a variety of bacteria have been reported to have increased resistance to AMPs than their parental strains. For example, *S. aureus* SCVs are more resistant to cationic AMPs protamine (Sadowska *et al.*, 2002), tPMP (Koo *et al.*, 1996) and bovine lactoferricin B (Samuelsen *et al.*, 2005) than their parental strains. In addition, a range of AMPs, particularly amphiphilic α-helical peptides at sub-lethal concentrations, were shown to trigger the formation of SCVs in *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa* and *S.* Typhimurium indicating that switching into SCV phenotype may be a mechanism of AMP resistance (Berditsch *et al.*, 2012; Pranting and Andersson, 2010). However, SCV phenotype has not been commonly recognised as a mechanism of resistance to AMPs. Given that SCVs are often found to have increased resistance to aminoglycosides due to reduced uptake of these cationic antibiotics conferred by the reduced membrane potential in SCVs (Proctor *et al.*, 2006), it was hypothesised that the reduced membrane potential in SCVs may also be associated with the resistance to cationic AMPs.

1.12 Aim of this study

S. aureus has developed complex metabolic and regulatory pathways to enable survival within the host environment. Resistance to AMPs plays an important role in the ability of *S. aureus* to survive within the host and cause infection (Weidenmaier *et al.*, 2005). Although a number of AMP resistance mechanisms have been characterised, a complete picture of AMP resistance mechanisms and regulation of these resistance remains to be established. The aim of this study was to identify *S. aureus* genes involved in resistance to the only human cathelicidin antimicrobial peptide LL-37. To achieve this goal, three approaches were designed. Firstly, to screen for genes that confer susceptibility or resistance to LL-37 *in vitro*, a tool for transposon mutagenesis (Burrack and Higgins, 2007) of *S. aureus* was constructed, which was then used to construct a genome-wide mutant library of *S. aureus* LS-1. The mutant library was subjected to screening for the mutants with altered resistance to LL-37. Secondly, to identify genes that are induced in the presence of LL-37, a recombinase-based *in vivo* expression technology (R-IVET) was developed. R-IVET is a promoter-trap system capable of monitoring the activity of promoters under different conditions (Angelichio and Camilli, 2002). It could therefore be useful in identifying genes that are up-regulated in the presence of LL-37 and which therefore might be important in resistance. Finally, to examine whether the reduced membrane potential in SCVs contributes to the resistance to cationic AMPs, a range of *S. aureus* SCVs were investigated with regards to their resistance to LL-37. The data generated from these studies was expected to improve our understanding of AMP resistance mechanisms in *S. aureus*, which may be useful for the development effective AMP antibiotics against *S. aureus* infections.

Chapter 2 Materials and Methods

2.1 Bacterial strains and growth conditions

2.1.1 Bacterial strains

The bacterial strains used in this study are listed in table 2.1.

Table 2.1: Bacterial strains used in this study.

Strains	Relevant characteristics / genotype	Source / reference
E. coli DH5α	F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk^-, mk^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Invitrogen Paisley United Kindom
E. coli XL-10 Gold	Tet^rΔ (*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacIqZ*Δ*M15* Tn*10* (Tet^r) Tn*5* (Kan^r) Amy]	Stratagene Leicester United Kindom
L. lactis MG1363	Laboratory wild-type strain	(Gasson, 1983)
S. aureus RN4220	Restriction deficient strain derived from *S. aureus* 8325-4	(Kreiswirth *et al.*, 1983)
S. aureus LS-1	*S. aureus* septic arthritis isolate from mice	(Bremell *et al.*, 1990)
S. aureus 8325-4	Laboratory wild-type strain isolated from a sepsis patient	(Novick, 1967)
S. aureus 8325-4 *sar* :: *lacZ*	*S. aureus* 8325-4 containing *Sar*::*lacZ* reporter fusion	(Pratten *et al.*, 2001)
S. aureus PZ134-2	*S. aureus* LS-1 with *hsdR* replaced by *loxP-erm-Pspac-lacZ-loxP*	This study
S. aureus LS-1 Δ*pyrB*	Assessed in *Galleria mellonella* model (unpublished data)	Dr Nair's laboratory collection
S. aureus LS-1 Δ*purB*	Assessed in *Galleria mellonella* model (unpublished data)	Dr Nair's laboratory collection
S. aureus LS-1 Δ*pyrB* Δ*purB*	Assessed in *Galleria mellonella* model (unpublished data)	Dr Nair's laboratory collection

S. aureus LS-1 Agr$^-$	*S. aureus agr* mutant is attenuated in murine arthritis model (Abdelnour *et al.*, 1993), rabbit endophthalmitis model (Booth *et al.*, 1995), rabbit osteomyelitis model (Gillaspy *et al.*, 1995) and murine abscess model (Wright *et al.*, 2005).	Dr Nair's laboratory collection
S. aureus LS-1 Sar$^-$	*S. aureus sar* mutant is attenuated in murine musculoskeletal model (Blevins *et al.*, 2003).	Dr Nair's laboratory collection
S. aureus LS-1 Agr$^-$ Sar$^-$	*S. aureus agr* and *sar* double mutant is attenuated in rabbit endocarditis model (Cheung *et al.*, 1994), rabbit endophthalmitis model (Booth *et al.*, 1997) and murine brain abscess model (Kielian *et al.*, 2001)	Dr Nair's laboratory collection
S. aureus LS-1 Δ*rsbUVWsigB*	*S. aureus* 8325-4 (SigB$^-$) is attenuated in murine model of arthritis and sepsis infections compared to *S. aureus* SH1000 (SigB$^+$) (Jonsson *et al.*, 2004)	(Nair *et al.*, 2003)
S. aureus LS-1 Δ*bioB*	Assessed in *Galleria mellonella* model (unpublished data)	Dr Nair's laboratory collection
S. aureus LS-1 FnbpAB$^-$	*S. aureus fnbpAB* mutant is attenuated in mouse sepsis model (Shinji *et al.*, 2011)	Dr Nair's laboratory collection
S. aureus LS-1 *atlA*::*Tn*	*S. aureus* AtlA is known to be important for cell wall metabolism and biofilm formation (Biswas *et al.*, 2006)	This study
S. aureus 8325-4 Δ*scdA*	*S. aureus* ScdA affects cell division and morphogenesis (Brunskill *et al.*, 1997)	(Brunskill *et al.*, 1997)
S. aureus 8325-4 Δ*hlg*	*S. aureus hlg* mutant is attenuated in rabbit endophthalmitis model (Supersac *et al.*, 1998)	Dr Nair's laboratory collection
S. aureus SA113	Restriction deficient strain derived from *S. aureus* NCTC 8325	(Iordanescu and Surdeanu, 1976)
S. aureus SA113 Δ*lgt*	*S. aureus lgt* mutant is hyper-virulent in mouse abscess model (Wardenburg *et al.*, 2006)	(Stoll *et al.*, 2005)
S. aureus SH1000	*S. aureus* 8325-4 with *rsbU* repaired	(Horsburgh *et al.*, 2002)
SH1000 Δ*isaA*	SH1000 *isaA*::*tet*	(Stapleton *et al.*, 2007)

SH1000 Δ*sceD*	SH1000 *sceD*::*tet*	(Stapleton *et al.*, 2007)
S. aureus LS-1 Δ*hemB*	*S. aureus* LS-1 *hemB* deletion mutant	(Wright and Nair, 2012)
S. aureus LS-1 Δ*menD*	*S. aureus* LS-1 *menD* :: *erm*	This study
S. aureus LS-1 SCV445	A spontaneous SCV derived from *S. aureus* LS-1 with mutation in *aroD*	This study
S. aureus LS-1 Δ*aroD*	*S. aureus* LS-1 with nucleotides 448bp-717bp deleted in *aroD*	This study
S. aureus 8325-4 Δ*hemB*	*S. aureus* 8325-4 *hemB* :: *erm*	(vonEiff *et al.*, 1997b)
S. aureus 8325-4 Δ*menD*	*S. aureus* 8325-4 *menD* :: *erm*	(Bates *et al.*, 2003)
S. aureus 8325-4 Δ*aroD*	*S. aureus* 8325-4 with nucleotides 448bp-717bp deleted in *aroD*	This study
S. aureus SH1000 Δ*aroD*	*S. aureus* SH1000 with nucleotides 448bp-717bp deleted in *aroD*	This study
S. aureus HG003 Δ*aroD*	*S. aureus* HG003 with nucleotides 448bp-717bp deleted in *aroD*	This study
S. aureus HG001	*S. aureus* NCTC 8325 with *rsbU* repaired	(Herbert *et al.*, 2010)
S. aureus HG002	*S. aureus* NCTC 8325 with *tcaR* repaired	(Herbert *et al.*, 2010)
S. aureus HG003	*S. aureus* NCTC 8325 with *rsbU* and *tcaR* repaired	(Herbert *et al.*, 2010)
S. aureus SH1000 Δ*rsbUVWsigB*	*S. aureus* SH1000 *rsbUVWsigB* :: *erm*	This study
S. aureus SH1000 SasF$^-$	*S. aureus* SH1000 *sasF* :: *erm*	(Kenny *et al.*, 2009)

2.1.2 Bacteriological media

The bacteriological media used in this study are listed in table 2.2.

Table 2.2: The bacteriological media used in this study.

Media	Source / components
Luria-Bertani broth (LB broth)	Supplied by Sigma-Aldrich, L3022 (Dorset, UK) Formula: 1% w/v Tryptone 0.5% w/v Yeast extract 0.5% w/v NaCl
Luria-Bertani agar (LB agar)	LB + 1.5% agar (Sigma-Aldrich, 05039)
Tryptic Soy Broth (TSB)	As supplied by Sigma-Aldrich (T8907) Formula: 1.7% w/v Casein (pancreatic digest) 0.3% w/v Soya peptone (papaic digest) 0.5% w/v Sodium chloride 0.25% w/v Dipotassium phosphate 0.25% w/v Dextrose
Tryptic Soy Agar (TSA)	TSB + 1.5% agar (Sigma-Aldrich, 05039)
Super Optimal broth with Catabolite repression (SOC)	As supplied by Invitrogen (15544-034) 2% w/v Tryptone 0.5% w/v Yeast extract 20mM Glucose 10mM NaCl 2.5mM KCl 10mM $MgCl_2$ 10mM $NgSO_4$ pH = 7
Nutrient broth No.2	Supplied by Oxoid, CM0067 (Hampshire, UK) Formula: 1% w/v `Lab-Lemco' powder 1% w/v Peptone 0.5% w/v Sodium chloride pH = 7.5 ± 0.2

Phage broth	80% Nutrient broth No.2 (Oxoid, CM0067) 1mM $CaCl_2$ (adding after autoclaving)
Top agar	80% Nutrient broth No.2 (Oxoid, CM0067) 0.35% agar bacteriological No.1 (Oxoid LP0011) 1mM $CaCl_2$ (adding after autoclaving)
Base agar	80% Nutrient broth No.2 0.7% agar bacteriological No.1 (Oxoid LP0011) 1mM $CaCl_2$ (adding after autoclaving)
Complete defined medium (CDM)	Adapted from (Pattee and Neveln, 1975) 0.5% w/v Glucose 0.7% w/v K_2HPO_4 0.2% w/v KH_2PO_4 0.04% w/v Na_3 citrate.$2H_2O$ 0.005% w/v $MgSO_4$ 0.1% w/v $(NH_4)_2SO_4$ 0.0001% w/v Thiamine 0.00012% w/v Niacin 0.0000005% w/v Biotin 0.000025% w/v Ca pantothenate 0.05% w/v L-arginine 0.01420325% w/v L-asparagine·H_2O 0.009% w/v L-aspartic acid 0.0125% w/v L-cystine 0.01% w/v L-glutamic acid 0.005% w/v Glycine 0.00375% w/v L-histidine 0.005% w/v Hydroxy-L-proline 0.0125% w/v L-isoleucine 0.0125% w/v L-leucine 0.01% w/v L-lysine·HCl 0.00375% w/v L-methionine 0.00375% w/v L-phenylalanine 0.008% w/v L-proline 0.0075% w/v L-serine 0.005% w/v L-threonine 0.00125% w/v L-tryptophan 0.0057993% w/v L-tyrosine 0.008% w/v L-valine 0.0005% w/v Adenine 0.0005% w/v Guanine 0.0005% w/v Cytosine 0.002% w/v Thymine 0.02% w/v Uracil

GM17 broth	M17 broth is as supplied by Sigma-Aldrich (56156) GM17 broth formula: 1% w/v Glucose 0.05% w/v Ascorbic acid 0.5% w/v Lactose 0.025% w/v Magnesium sulphate 0.5% w/v Meat extract
GM17 agar	GM17 broth + 1.5% agar (Sigma-Aldrich, 05039)
G-SGM17	GM17 + 1.5% glycine + 0.25 M sucrose
SGM17MC	GM17 + 0.5M sucrose + 20mM $MgCl_2$ + 2mM $CaCl_2$

2.1.3 Antibiotics

The antibiotics supplemented in bacteriological media for the selection of bacteria are listed in table 2.3. Antibiotics were dissolved in the indicated solution at 1000 × concentration required for selection of bacteria, followed by filter-sterilisation with 0.22µm filters (Acrodisc® Supor®, VWR International, 514-4131, Leicestershire, UK).

Table 2.3: Antibiotics used for culturing *E. coli*, *S. aureus* and *Lactococcus lactis*.

Antibiotic	**Solution**	**Final concentration (µg/ml)**
Ampicillin (Amp) (Sigma-Aldrich, A9518)	Filtered dH_2O	100
Chloramphenicol (Cm) (Sigma-Aldrich, C0378)	100% ethanol	10 for *S. aureus* 5 for *L. lactis*
Erythromycin (Erm) (Sigma-Aldrich, E7904)	100% ethanol	5
Kanamycin (Kan) (Sigma-Aldrich, K1876)	Filtered dH_2O	50
Tetracycline (Tet) (Sigma-Aldrich, T3383)	70% ethanol	5

2.1.4 Culture of strains

E. coli was grown aerobically in Luria-Bertani (LB) broth or agar at 37°C. *S. aureus* was grown aerobically in Tryptic Soy (TS) broth or agar at 37°C. For growth of *E. coli* or *S. aureus* in liquid culture, shaking at 200rpm was provided. *L. lactis* was grown aerobically in GM17 broth or agar at 30°C without shaking.

2.1.5 Storage of bacterial strains

E. coli, *S. aureus* and *L. lactis* strains were grown overnight in LB, TSB or GM17 broth, respectively. Glycerol (50% v/v) was prepared and sterilised by autoclaving. To store the strains, 0.43ml of 50% glycerol was added to 1ml of culture (final concentration of glycerol = 15%). The mixture was stored at -80°C. Prior to the use of the strains, a loop of the glycerol stock was streaked onto agar plates containing appropriate antibiotics, if applicable, and cultured as described in section 2.1.3 to confirm the colony morphology and absence of contamination.

2.1.6 Growth experiments

Growth experiments in TSB

For the characterisation of *S. aureus* growth dynamics in TSB, a starter culture was prepared by inoculating a single colony of *S. aureus* into 5ml TSB containing appropriate antibiotics and grown for 16 hours to stationary phase. The optical density of the starter culture was measured at 600nm (OD_{600}) using a Pharmacia Biotech Ultrospec 2000 UV / Visible Spectrophotometer (GE Healthcare Life Sciences, Buckinghamshire, UK).), followed by dilution in TSB supplemented with appropriate antibiotics and/or indicated substance to a calculated OD_{600} of 0.05. This was then incubated at 37˚C with shaking at 200rpm. Growth was monitored by measuring the optical density at 600nm at 1-hour intervals, or by determining colony forming units (CFU/ml) every 1 hour or 3 hours by spreading serial dilution of the culture onto TSA and incubating aerobically at 37 ˚C for 24-48 hours before colonies were counted.

Growth experiments in chemical defined media (CDMs)

For the characterisation of *S. aureus* growth dynamics in chemical defined media (CDMs), a starter culture was prepared by inoculating a single colony of *S. aureus* into

5ml TSB containing appropriate antibiotics and grown for 16 hours. The cells were collected by centrifugation at 4000rpm for 5 minutes, washed once with phosphate buffered saline (PBS, pH = 7.4), and re-suspended in 5ml of the CDM used for growth experiments. The optical density of the suspension was measured at 600nm, followed by diluting into CDM to a calculated OD_{600} of 0.05. The remaining steps were as described for the growth experiments in TSB.

2.2 Techniques involving DNA

2.2.1 Plasmids

The plasmids used in this study are listed in table 2.4.

Table 2.4: Plasmids used in this study. G+: Gram positive. G-: Gram negative. Amp^r: ampicillin-resistant. Cm^r: chloramphenicol-resistant. Erm^r: erythromycin-resistant. Kan^r: kanamycin-resistant.

Plasmid	Relevant characteristics	Source / Reference
pUC19	*E. coli* cloning vector, Amp^r	New England Biolabs (NEB), Hitchin, U.K.
pMC38	Contains the *Himar1 mariner* transposon, Cm^r (G- selection), Erm^r and Kan^r (G+ selection)	(Cao *et al*., 2007)
pKOR1	Inducible promoter *Pxyl/tetO* controlling antisense *secY*, lambda recombination sites, *attp*, flanking a *ccdB* gene. Amp^r (G- selection), Cm^r (G+ selection)	(Bae and Schneewind, 2006)
pSK236	Shuttle vector containing pUC19 cloned into the HindIII site of pC194. Amp^r (G- selection), Cm^r (G+ selection)	(Gaskill and Khan, 1988)
pNZ5517	Cm^r (G+ selection), contains *loxP-erm-usp45p-melA-loxP* cassette	(Bachmann *et al.*, 2008)
pNZ5520	Cm^r (G+ selection), SfrI/SmaI restriction site upstream of promoter-less reporter gene *cre*	(Bachmann *et al.*, 2008)
pPZ1-2	pMC38 derivative with *PmrgA* replaced by *Pxyl/tetO,* and *pE194ts ori* removed	This study
pPZ4	pMC38 derivative with *PmrgA* replaced by *Pxyl/tetO*	This study
pPZ111-1	pSK236 derivative with *Pusp45-melA* cloned in MCS	This study

pHCMC05	Shuttle vector containing inducible promoter *lacI-Pspac*	(Nguyen *et al.*, 2005)
pPZ114-5	pPZ4 derivative with -10 sequence of P_{tetR} modified from TAGAGT to TATAAT	This study
pPZ118-1	pKOR1 derivative with *ccdB* replaced by the upstream and downstream sequences of *S. aureus hsdR* gene	This study
pPZ142	pNZ5520 derivative with P_{cat} placed upstream of *cre*	This study
pPZ143-3	pNZ5520 derivative with *Pxyl/terO* placed upstream of *cre*	This study
pPZ144-4	pNZ5520 derivative with *saeP1* placed upstream of *cre*	This study
pPZ138-7	pKOR1 derivative with *ccdB* replaced by the upstream and downstream sequences of *S. aureus aroD* gene	This study
pPZ137-3	pSK236 derivative with *Pspac-aroD* cloned in multiple cloning site	This study
pPZ177-2	pKOR1 derivative with *ccdB* replaced by *menD-erm-menD*	This study

2.2.2 Oligonucleotides

The oligonucleotides used for polymerase chain reactions (PCR) and DNA sequencing in this study are listed in table 2.5. All primers were designed in this study and were assessed using NetPrimer (Premier Biosoft, Palo Alto, CA, USA) with regards to melting temperature (Tm) and possible secondary structure. Designed primers were synthesised by Sigma-Genosys (Haverhill, UK.).

Table 2.5: Oligonucleotides used in this study; (F: forward oligonucleotide; R: forward oligonucleotide; Tm: melting temperature. Underline: restriction endonuclease sites; wave underline: -10 site of P_{tetR}; **bold**: point mutations incorporated into primers; dotted underline: *att*B tails used for lambda recombination with *att*P sites).

Name	Oligonucleotide sequence 5'-3'	Restriction sites	F/R	Tm (˚C)
P1	CCCCCCGAATTCTTAAGACCCA		F	70.5
P2	CCCCCCGGATCCCTCGGTACCATCAAGCTTATTT		R	81.8
P5	GCGGTACCTTAAGACCCACTTTCACATTTAAG	KpnI	F	71.7
P8	ATCCGCATGCTGCAAGGCGATTAAGT	SphI	R	75.6

P9	TTCCATATTCATAAAACTCCTCTGCTTATTTTAAT TATACTCTATCAATG		R	71.3
P10	CATTGATAGAGTATAATTAAAATAAGCAGAGGA GTTTTATGAATATGGAA		F	71.8
P34	CTCTATCAATGATA**T**TA**A**TGTCAACAAAAAGGAG		F	65.9
P35	CTCCTTTTTGTTGACA**T**T**A**TATCATTGATAGAG		R	65.9
P36	TGAAATCGGCTCAGGAAAAGG		F	67.2
P37	TGAGCTATTCACTTTAGGTTTAGGATG		R	63.4
P40	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTT GTGACGAATCAAGGTAGTC		F	82.9
P41	GACGCATGATGATAGAGATTG		R	76.9
P42	CTTTGTATTCTTTCATGTTTCCTC		F	81
P52	GTCATCTAGAAATTCAGGCATGGACACTTTTG	XbaI	F	72.3
P53	GTCAGAATTCCTTAGTCCTTAGCCTTGAAGTAATG	EcoRI	R	70.4
P55	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTC AAATAACACATCATACTCTG		F	83.7
P56	TACTCTAACCACTCGGCTTTTGC		R	65.3
P61	CCAACTTTCGTACAAAAAAGC		F	60.2
P62	ACTTTGTACAAGAAAGCTGGGT		R	60.6
P64	TTCCATGGTTCCGCGGATGATTCAGCCCCCTCGC	NcoI, SacII	F	88.7
P66	TTCCGCGGGATAACTTCGTATAATGTATGCTAT	SacII	R	72.3
P68	CTTCGTATAGCATACATTAT		F	65.9
P71	CTGCTCGAATTCAAGCTTCT		F	61.3
P78	AGTGGATCCTACACAGCCCAGTCCAGAC	BamHI	F	74.2
P79	TCACTCGAGTCACCTCCTTAAGCTTAATTG	XhoI	R	70.1
P80	TTGCTCGAGAATTGATACACTAATGCTTT	XhoI	F	67.9
P81	ATCCTGCAGTTATTTTTGACACCAGACC	PstI	R	70.1
P82	ATCCTGCAGTACACATATTGGGAATCG	PstI	R	70
P83	TAAGCATGCAACTGATGGAACTTTGATT	SphI	R	69.6
P86	ACACCATTCAAGTCCCTC		F	57.3
P94	TTCCATGGTTAGATCTTCATATCCCCTTCCATACACTT	NcoI, BglII	R	75.1
P98	GAGGCTCAACGTCAATA		F	54.2
P99	TTCATAGCACGTCTCCA		R	55.9
P101	ACTGGCAATCACCGCTTAGAT**CT**CTT	BglII	F	69.5
P102	ATATAACTACCGCCTCC**GCG**GATAAC	SacII	R	69.7
P103	TGTTTGGTTGATAATGAACTGTGCTG		R	67.6
P105	AACAGTTGACGATATTC		F	46.7
P106	AATCAGCGTGCCGTCG		R	65.1
P107	TGGTGGTTATGCCGAT		F	58.2
P108	CTGTGAGCCAGAGTTGC		R	58
P109	GTGACCAGCGAATAC		F	48.9
P121	GACATTTGCAGCATATAGAGG		F	58.8
P125	ACTGAAGGATGATTCGTATATTAG		F	57
P126	GCATCAGTTTTCTTTAACCA		R	56.9
P128	CACCCATTGAATATCCAAAC		R	55.8
P130	GACGCAAGTGACTTAATATCAT		R	57.4

P131	TGATGAAGTATTCCATTTAAGTGC		F	60.8
P132	CCTTTATTTAGGTATCCGTCGT		R	59.8
P133	AATGCTGGGCGTACTCTTTTAC		F	63.1
P138	TCGTAAGTACAATAAAGGC		R	51.9
P139	ATTTGCTCGTCCACATC		F	56.8
P140	ATCATCTTCGAGTTGTTGG		R	57.6
P141	TGGTGCGCCTACACTTC		F	61.1
P142	GGCAGTGAAAGACCTGTC		R	58.3
P143	TTTCAGATAACAATGTGGC		F	56
P144	TTATTGAACAACGATGG		R	52.2
P145	CGGTTTTGGAATAATACG		F	55.5
P146	TTGCTGAAATGATTGACGC		R	62.5
P147	TCGCTGGAAGGGATATTAC		F	59.1
P156	CCTCTAATTGATCCTGGTGAAG		F	61.4
P157	TTGCTGCAATGCACCTTG		R	64.9
P159	TGAATAATGTAAATGAAGTAAAGG		R	55.8
P160	GTATCCAACAGATTTAACGGGTAC		F	61.2
P166	GACGCTATGGTTCGTTTTGACTC		F	65.7
P167	CTGATGTGTCCTGAACCTCGG		R	67
P168	GAACCGTTTGGCTTACCTGC		F	65.6
P169	CAATCACTTCAAGCCCTTGATTC		R	65.6
P170	AAGCGTTAGGTTGAATGTATGAG		F	61
P171	TAACAATCGTTAAGTGCAGTATACC		R	59.6
P172	GTCGAAAGCACCGTTGAAGC		F	67
P173	CATTCCGTGATTTGGAGCATC		R	66.5
P174	GAGTAGCTTCGCTTAATGTTTCAG		F	62.1
P175	GTATCAGGTGGTAAACGTCCG		R	63.1
P176	CACTTGACATTGAAATACCCCC		F	64
P177	AAGTCGGTACAAATGTGCAGAC		R	62.9
P178	TTATTTGCTAAAGTTGCGATACG		F	61.8
P179	GCAATAACAATATCCAGCTTCG		R	62.2
P180	CAGTCGTGTTGTTGAAATAGGTG		F	63.2
P181	GTGTTGGCGGTAAAGTGGG		F	65.3
P182	CCAAAATTGTAAAGTGTCTCTCCTAG		R	62
P183	GCAATCATGTTGGCGTCG		F	66.6
P189	CAACATGAGCAGTAATACAGCG		F	62.2
P190	CAGAAGCAGGTACAAAACACG		R	62.3
P192	GGCGTCTAGAATTAGTATAAAGTCACTTGTGC	XbaI	R	67.1
P193	CCAAGGATCCGAAATGAGGGATTCACTATG	BamHI	F	74.5
P194	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*G TCGAAAGCACCGTTGAAGCAAG		F	87.1
P195	CCGCCTCGAGTTAAAAAAATATAAATTGC	XhoI	R	68.6
P196	CCGCCTCGAGTAAAATGAATTAGGTTGCTT	XhoI	F	73
P197	*GGGGACCACTTTGTACAAGAAAGCTGGGT*G CTAAGTCACCTGAACAAGTTG		R	84.8
P199	CCATCCCGGGTCCCCTTTCTATGTATGTTT	SmaI	F	75.7

P200	CGTTCCCGGGTTGATATGCCTCCTAAATT	SmaI	R	75.1
P201	GCTTCCCGGGTTAAGACCCACTTTCACATTT	SmaI	F	76.4
P202	CTACCCCGGGCCTCTGCTTATTTTAATTATAC	SmaI	R	71
P203	CACGCCCGGGTACCTTGATCTTGTGAATTAG	SmaI	F	76.9
P204	CATTCCCGGGTTATTGTGGCAAAAGGTTT	SmaI	R	75.7
P205	TTCTTGCGAACCTCATCACTC		F	64.3
P207	GGTAGGAGGCTTTTGTTATGC		F	61.4
P208	GATGTTATGGAGGACTGGTGG		R	63.2
P209	AAGAAGGTAGAGGCGTAGCG		F	62.4
P210	GTCACAATTATCTGGGGTAGCC		R	63.3
P211	CATGCCGAACACAGAAGTTAAG		F	63.4
P212	CTGAGCCAGGATCAAACTCTC		R	62.9
P213	CGAGACCGCCATTATTATTACC		F	63.1
P214	ACGATTAGGTCATGCAGATGTAC		R	61.8
P215	CATGCCGAACACAGAAGTTAAG		F	63.4
P216	TTACACCTATACCTCATTCCAGG		R	60.7
P217	GATCCCGCTAGTCTCCACC		F	63.7
P218	ATGTGGAGCTGACGAATACTAATC		F	62.1
P219	GCACGTATAATGATGATTTTCAGC		R	63.2
P226	AATTAAAAATACCCCTCGATTTC		F	60
P238	AACTCTCCCCAATTTCTATG		F	57.1
P239	GGCACATATTTCACATTACAG		R	57.1
P240	TTGTAGGAGGATTTTAAGATTATG		F	57.1
P241	GCTTATGCAGTAACCCAATG		R	59.6
P242	GATTTTATGACCGATGATGAAG		F	59.9

2.2.3 Plasmid purification

Plasmid preparation from *E. coli*, *S. aureus* and *L. lactis* was performed by using the Spin Column Plasmid DNA Miniprep Kit (NBS Biologicals, Cambridgeshire, England) according to the manufacturer's instructions. For *S. aureus* and *L. lactis*, an additional cell-lysis step was added after re-suspending the cells. Lysostaphin was added to *S. aureus* cell suspensions at a final concentration of 100µg/ml (Musser *et al.*, 1990), and lysozyme was added to *L. lactis* cell suspensions at a final concentration of 4mg/ml (Bachmann *et al.*, 2008). The cell suspension was then incubated at 37°C for 1 hour before proceeding to the next step. Finally the DNA was eluted using 50µl sterilised dH_2O, and the concentration and purity of the DNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Hemel Hempstead, UK).

2.2.4 Genomic DNA extraction

S. aureus genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Briefly, cells from 3ml of overnight culture were harvested by centrifugation at 7000rpm for 2 minutes, and re-suspended in 180µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH=8). Lysostaphin was added to the cell suspension to 100µg/ml, and the mixture was incubated at 37°C for 1 hour. Subsequently, proteinase K was added as indicated in the manufacturer's protocol of DNA Purification from Tissues, and the remaining purification was performed according to these instructions. Finally the DNA was eluted using 100µl sterilised dH_2O, and the concentration and purity of the DNA was measured using a NanoDrop 1000 Spectrophotometer.

2.2.5 Lyse-and-Go reaction

Bacterial growth was taken from one colony on agar plates and suspended in 20µl lyse and go reagent (Thermo Scientific, Hemel Hempstead, UK), followed by incubation at 65°C for 30 seconds, 8°C for 30 seconds, 65°C for 1 minutes 30 seconds, 97°C for 3 minutes, 8°C for 1 minute, 65°C for 3 minutes, 97°C for 1 minute, 65°C for 1 minute, 80°C for 50 minute and finally paused at 4°C. For PCR amplification reactions, 2µl of the resulting mixture was used as template for a volume of 20µl PCR reaction.

2.2.6 General PCR protocol

All the DNA fragments for use in cloning were amplified using $Vent_R^®$ DNA polymerase [New England Biolabs (NEB), Hitchin, Herts, UK, M0254], while *Taq* DNA polymerase (NEB, M0267) was used in PCRs for analysis and verification. All PCR reactions were performed using a T3000 Thermocycler (Biometra, Goettingen, Germany). PCR reactions and thermal cycling were carried out as described below:

Reaction mix

ThermoPol reaction buffer	1×
dNTPs (NEB, N0447)	0.05mM for each
Forward primer	0.5µM
Reverse primer	0.5µM
Template DNA	~0.2ng / µl
$Vent_R^®$/*Taq* DNA polymerase	0.05 unit / µl

Reaction condition:

1.	Pre-amplification denaturation	95°C	2 minutes
2.	Denaturation	94°C	30 seconds
3.	Annealing	T_m – 5°C	30 seconds
4.	Extension	72°C	1 minute / kilobase (kb)
	Step 2 to 4 were cycled 30 times		
5.	Final extension	72°C	5 minutes
6.	Pause	4°C	

2.2.7 Agarose gel electrophoresis

DNA fragments involved in cloning process were separated by electrophoresis in gels containing 0.8% or 1% agarose (Sigma-Aldrich, A9539), and PCR products from Y-linker PCR reaction (section 2.4.3) were analysed by electrophoresis in gels containing 1.5% agarose. The agarose gels were supplemented with 0.1μg/ml ethidium bromide, and 1 × TAE buffer (40mM Tris-acetate, 1mM EDTA, pH = 8.3). Samples were run in 1 × Loading buffer (0.05% bromophenol blue, 5% glycerol in dH_2O) at 80-100V for 1 hour. The 1kb plus DNA ladder (Invitrogen, 10787-018) or the 2-log DNA ladder (NEB, N3200) was included as a size reference. The DNA bands were visualised under Ultraviolet light at 302nm in Alphalmager (Alpha Innotech, Norfolk, UK).

2.2.8 DNA purification from gel

Following separation on an agarose gel, DNA fragments were visualised on a T2201 UV Transilluminator (Sigma-Aldrich) at 302nm. Desired DNA fragments were excised and purified using the Spin Column Gel Extraction Kit (NBS Biologicals) following the manufacturer's instructions.

2.2.9 Restriction endonuclease digestion of DNA

Restriction endonucleases were purchased from New England BioLabs, and digests were performed according to the manufacturer's instructions for 2-16 hours for different purposes.

2.2.10 Purification of DNA from PCR and restriction digests

DNA from PCR or restriction digest reactions was purified using the Spin Column Reaction Cleanup Kit (NBS Biologicals) according to the manufacturer's instructions.

2.2.11 Dephosphorylation of vectors

To avoid vector self-ligation during cloning, vectors linearised with single restriction endonuclease were treated with Antarctic Phosphatase (NEB, M0289) to remove the 5' phosphates on both strands. The dephosphorylation reaction was performed following the manufacturer's instructions.

2.2.12 Phosphorylation of DNA

For the purpose of ligating fragments which lack 5' phosphates, these fragments were treated with T4 Polynucleotide Kinase (NEB, M0201) to add phosphates to the 5' terminus of the nucleotides. The reaction was performed according to manufacturer's instructions.

2.2.13 Blunting DNA ends

DNA with 3' recessed ends was blunted using Klenow Fragment (NEB, M0212) to fill in the overhangs. Reactions were conducted according to the manufacturer's instructions.

2.2.14 Ligation

Prior to ligation, the concentrations of vector and DNA fragments were determined using a NanoDrop 1000 Spectrophotometer, and the molar concentration ratio of vector to inserts was calculated. Vector and each insert were added in a ratio 1 : 3 in the ligation reaction. Ligation reactions were performed using T_4 DNA ligase (NEB, M0202) following the manufacturer's instructions.

2.2.15 *attB*-*attP* (BP) recombination

The Gateway® Technology (Invitrogen) was used to construct deletion mutants of *S. aureus*. This technology is based on the site-specific recombination properties of

bacteriophage lambda (landy, 1989). The lambda recombination requires two major components: the DNA recombination sites (*att*) and the enzyme that catalyses the recombination (Hsu and Landy, 1984). In this project where this technology was used, the lambda recombination sites *attP* were in plasmid pKOR1 (Bae and Schneewind *et al*., 2006), and the recombination sites *attB* were attached to PCR primers used for the amplification of the insert. The insert with *attB* tails was integrated into pKOR1 through recombination between *attB* sites on the insert and *attP* sites on pKOR1. The BP-recombination reaction was performed using Gateway® BP clonase® II enzyme mix (Invitrogen) according to manufacturer's instructions.

2.2.16 Preparation of electrocompetent cells

2.2.16.1 *S. aureus* electrocompetent cells

S. aureus electrocompetent cells were prepared using a method adapted from that described by Kraemer and Landolo (1990). A starter culture was prepared by growing *S. aureus* overnight in 5ml TSB at 37 °C with shaking at 200rpm. The starter culture was diluted 1 : 100 in 100ml fresh LB broth (menadione was added at 0.3µg/ml if preparing competent cells for menadione auxotrophs). This culture was incubated at 37°C until the optical density at 450nm (OD_{450}) reached 0.2. The growth was stopped by standing the culture on ice for 10 minutes. The cells were harvested by centrifugation at 5000rpm for 10 minutes at 4°C, followed by washing twice with 10ml ice-cold 500mM sucrose. Finally, the cells were re-suspended in 0.85 ml ice-cold 500mM sucrose, and the suspension was split into 0.2ml aliquots and stored at -80 °C.

2.2.16.2 *L. lactis* electrocompetent cells

L. lactis electrocompetent cells were prepared as described by Holo and Nes (1989). A single colony of *L. lactis* was inoculated into 5ml GM17, and 10-fold serial dilutions of this culture from 10^1 to 10^6 were then prepared in 6 × 5ml GM17. All seven cultures were incubated at 30°C overnight. The optical density of each culture was measured at 600nm, and the culture with OD_{600} = 1-2 was selected as a starter culture. The starter culture was diluted into 100ml G-SGM17 to OD_{600} = 0.03, followed by incubation at 30°C to OD_{600} = 0.6. The growth was stopped by standing the culture on ice for 10 minutes. Subsequently, the cells were harvested by

centrifugation at 5000rpm for 10 minutes at 4 °C, followed by washing twice with 15 ml ice-cold washing solution (0.5 M sucrose + 10% glycerol). Finally, the cells were re-suspended in 1ml ice-cold washing solution, and the suspension was distributed into 40µl aliquots and stored at -80 °C.

2.2.17 Transformation

2.2.17.1 Transformation of *E. coli*

Transformation of Subcloning Efficiency™ DH5α™ Chemically Competent Cells (Invitrogen) was performed according to the manufacturer's instruction. Typically, 5µl of ligation reaction was used to transform 50µl of competent cells.

2.2.17.2 Transformation of *S. aureus*

E. coli or *L. lactis* propagated DNA was introduced into *S. aureus* strains via the restriction negative strain RN4220. Electrocompetent *S. aureus* cells (200µl) were thawed on ice and mixed with 1µg of plasmid DNA. The mixture was transferred to a pre-chilled 2mm gap Electroporation Cuvette (VWR International). Electroporation was performed at 2.5KV, 200-400Ω and 25µF using a BioRad Gene Pulser II (Kraemer and Landolo, 1990). The pulsed cells were immediately suspended in 1ml TSB, and cultured at 37°C for 1 hour. The culture was then plated onto TSA with appropriate antibiotic selection and grown overnight.

2.2.17.3 Transformation of *L. lactis*

Transformation of *L. lactis* was performed as described by Holo and Nes (1989). Electrocompetent *L. lactis* cells (40µl) were thawed on ice and mixed with 5µl of ligation reaction. The mixture was transferred to a pre-chilled 2mm gap Electroporation Cuvette (VWR International). Electroporation was performed at 2KV, 200Ω and 25µF using a BioRad Gene Pulser II. Immediately following the discharge, the cells were suspended in 1ml of ice-cold SGM17MC, and cultured at 30°C for 2 hours. The culture was then plated onto GM17 agar with appropriate antibiotic selection and grown at 30°C overnight.

2.2.18 DNA sequencing

DNA sequencing of purified PCR products or plasmids was performed by either the DNA Sequencing Service (University of Cambridge, UK) or the DNA Sequencing Service (Source BioScience, London, UK).

2.3 Cloning, mutant construction and complementation

2.3.1 Construction of plasmid pPZ111-1

A 2.5kb fragment containing *Pusp45-melA* was PCR amplified from pPNZ5517 using primers P52 and P53 with XbaI and EcoRI restriction sites at their 5' ends, respectively. Subsequently, the PCR product was digested with XbaI-EcoRI and purified. Plasmid pSK236 was digested with the same enzymes, and the fragments were separated by gel electrophoresis. The linearised vector pSK236 was purified from the gel and ligated with *Pusp45-melA. E. coli* DH5α was transformed with the ligation mix and grown on LB + 100μg/ml ampicillin. Selected transformants were grown in LB + 100μg/ml ampicillin, and plasmids were purified from the cells. To confirm the success of cloning *Pusp45-melA* into pSK236, the plasmids were digested with XbaI and EcoRI, followed by separating the fragments in the digest by gel electrophoresis. The expected digestion pattern was two fragments at sizes of 5569bp and 2500bp.

2.3.2 Construction of plasmid pPZ122-2 containing *loxP-erm-Pspac-lacZ-loxP*

The construction of *loxP* cassette *loxP*-erm-*Pspac-lacZ-loxP* in pUC19 was achieved through three cloning steps, resulting in plasmid pPZ122-2. In the first step, a 1442bp fragment containing *loxP-erm* was excised from pNZ5517 using EcoRI and BamHI, and the same enzymes were used to linearise vector pUC19. The fragments in the digests were separated by gel electrophoresis, and the fragments corresponding to *loxP-erm* and pUC19 was purified from the gel and ligated. *E. coli* DH5α was transformed with the ligation mix and grown on LB + 100μg/ml ampicillin. Selected transformants were grown in LB + 100μg/ml ampicillin, and plasmids were purified from the cells. To confirm the success of cloning, the plasmids were digested with EcoRI-BamHI, which was then examined by gel electrophoresis. The expected digestion pattern was two

fragments at sizes of 2665bp and 1436bp. One of the successful clones, designated pPZ120-2, was used for the further assembly of *loxP* cassette.

In the next step, the reporter *Pspac-lacZ* was cloned into pPZ120-2 downstream of *loxP-erm*. A 310bp fragment containing the promoter *Pspac* was amplified from plasmid pHCMC05 using primers P78 (BamHI site at 5' end) and P79 (XhoI site at 5' end), and a 3119bp fragment containing *lacZ* was amplified from *S. aureus* 8325-4 *sar* :: *lacZ* (Pratten *et al.*, 2001) genomic DNA using primers P80 (XhoI site at 5' end) and P81 (PstI site at 5' end). The *Pspac* and *lacZ* amplicons were digested with BamHI-XhoI and XhoI-PstI, respectively. DNA was purified from the digests and ligated with BamHI-PstI digested vector pPZ120-2. *E. coli* DH5α was transformed with the ligation mix and grown on LB agar containing 100μg/ml ampicillin and 40μg/ml X-gal. Selected blue colonies were grown in LB + 100μg/ml ampicillin, and plasmids were purified from the cells. To confirm the success of cloning, the plasmids were digested with BamHI-XhoI and XhoI-PstI, followed by separating the fragments in the digests by gel electrophoresis. The expected BamHI-XhoI digestion pattern was two fragments at 7500bp and 310bp, and the expected XhoI-PstI digested pattern was two fragments at 4691bp and 3119bp. The successful clone, designated pPZ121-1, was taken forward for the final cloning step.

In the final step, the second *loxP* recombination site was cloned into pPZ121-1 downstream of *lacZ*. A 478 bp fragment containing this *loxP* recombination site was amplified from pNZ5517 using primers P82 (PstI site at 5' end) and P83 (SphI site at 5' end). The PCR product and pPZ121-1 were then digested with PstI and SphI, followed by purification and ligation. *E. coli* DH5α was transformed with the ligation mix and grown on LB agar containing 100μg/ml ampicillin and 40μg/ml X-gal. Plasmids were purified from blue colonies and the success of inserting the fragment containing the second *loxP* site was confirmed by PCR amplification using primers P82 and P83, which was expected to generate a product of 478bp. To confirm the existence of the entire *loxP* cassette, the *loxP* cassette region in one of the successful clones, designated pPZ122-2, was sequenced using primers P68, P78, P79, P81 and P83 to cover all the ligation joints, *ermB*, *Pspac* and *loxP* sites.

2.3.3 Construction of plasmid pPZ130-4

Firstly, a vector pPZ126-1 containing the upstream and downstream sequences of the *hsdR* gene was generated as shown in figure 2.1. A 1017bp fragment upstream of the *hsdR* gene was amplified using primers P40 and P94, the recombination site *attB* tail was added to the 5' end of primer P40 and restriction sites NcoI and BglII were added to the 5' end of primer P94. Similarly, an 860bp fragment downstream of *hsdR* gene was amplified using primers P64 (NcoI and SacII was added to 5' end) and P55 (*attB* tail was added to 5' end). The two fragments were then digested with NcoI and ligated, followed by BP recombination with pKOR1. *E. coli* DH5α was transformed with the ligation mix and grown on LB agar containing 100μg/ml ampicillin and 40μg/ml X-gal. Selected blue colonies were grown in LB + 100μg/ml ampicillin, and plasmids were purified from the cells. To confirm the success of cloning *hsdR* upstream and downstream fragments into pKOR1, the plasmids were digested with XbaI, followed by separating the fragments in the digests by gel electrophoresis. The expected digestion pattern was two fragments at sizes 4043bp and 5669bp.

Subsequently, the *loxP* cassette, *loxP*-erm-*Pspac*-*lacZ*-*loxP*, was amplified using P101 (with BglII site) and P102 (with SacII site). The PCR product was digested with BglII-SacII and ligated with pPZ126-1 digested with the same enzymes. *E. coli* XL-10 Gold was transformed with the ligation mix and grown on LB agar containing 100μg/ml ampicillin and 40μg/ml X-gal. Selected blue colonies were grown in in LB + 100μg/ml ampicillin, and plasmids were purified from the cells. To confirm the success of cloning the *loxP* cassette into pPZ126-1, the plasmids were digested with XhoI or XbaI, followed by separating the fragments in the digests by gel electrophoresis. The expected XhoI digestion pattern was two fragments at sizes of 7918bp and 6164bp, and the expected XbaI digestion pattern was three fragments at sizes of 6558bp, 5626bp and 1898bp.

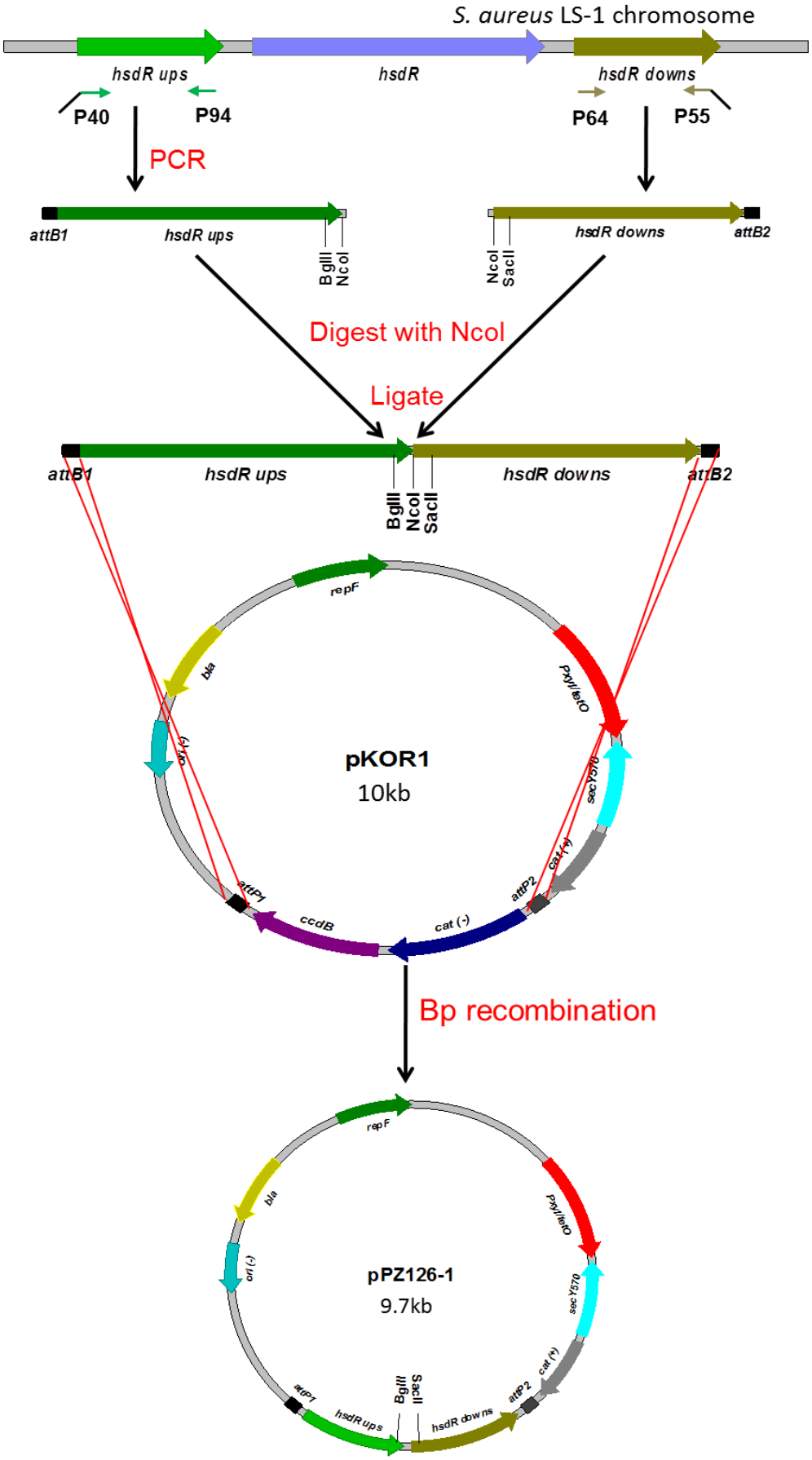

Figure 2.1: Schematic illustration of the introduction of the upstream and downstream sequences of *hsdR* into pKOR1 (not drawn to scale).

One of the successful clones, pPZ130-4, was sequenced across the cloned region *hsdR ups-loxP-erm-Pspac-lacZ-loxP-hsdR downs* using primers P61, P62, P66, P78, P79, P82, P86, P94, P105, P106, P107, P108 and P109 to cover the entire region. The sequencing results of *hsdR* ups and *hsdR* downs were compared to *hsdR* upstream and downstream sequence in *S. aureus* 8325-4, and the *loxP* cassette was compared to pPZ122-2 from which it was amplified.

2.3.4 Construction of *S. aureus* LS-1 *hsdR* :: *loxP* cassette

Vector pPZ130-4 was introduced into *S. aureus* RN4220 by electroporation. The plasmid was then purified from RN4220, and electroporated into *S. aureus* LS-1. The *loxP* cassette was integrated into the chromosome of *S. aureus* LS-1 through allelic replacement using a method adapted from that described previously by Bae and Schneewind (2006). A single colony of *S. aureus* LS-1 pPZ130-4 was inoculated into 10ml TSB + 5μg/ml Erm + 10μg/ml Cm and grown overnight at 30°C to allow plasmid replication. This culture was then diluted 1:1000 into 10ml TSB + 5μg/ml Erm, and incubated at 43°C for 24 hours to prevent the replication of the plasmid. This step was repeated once again to select plasmid-free Erm^r cells. Finally the culture was plated onto TSA + 5μg/ml Erm + 40μg/ml X-gal and incubated at 43°C for 24 hours. The blue colonies were streaked onto TSA + 10μg/ml Cm and TSA + 5μg/ml Erm and cultured at 30°C for two days to select plasmid-free mutant. The colonies confirmed to be Erm^r Cm^s were then grown in TSB + 5μg/ml Erm at 30°C for the preparation of genomic DNA and plasmid DNA. Genomic DNA was used for PCR amplification to confirm the integration of *loxP* cassette into the chromosome of *S. aureus* LS-1, and no plasmid DNA corresponding to pPZ130-4 was expected from the purification.

2.3.5 Insertion of P_{cat}, *Pxyl/tetO* or *saeP1* into pNZ5520

Promoters P_{cat}, *Pxyl/tetO* and *saeP1* were amplified from pSK236, pKOR1 and *S. aureus* LS-1 genomic DNA using primers P199 / P200, P201 / P202 and P203 / P204, respectively. The PCR products and pNZ5520 were digested with SmaI and purified. Linearised pNZ5520 was further treated with Antarctic Phosphatase to remove the 5' phosphates, and purified. The digested PCR fragments were then separately ligated with pNZ5520. The ligation mix was electroporated into *L. lactis* MG1363, and plated

onto GM17 with 5μg/ml Cm. Plasmids were purified from selected transformants, and examined by PCR amplification using P205/P199, P205/P201 and P205/P204 to verify the insertion of P_{cat}, *Pxyl/tetO* and *saeP1*, respectively. The PCR products amplified from the plasmids were examined by gel electrophoresis, and the expected PCR products were at sizes 535bp, 908bp and 528bp for P_{cat}, *Pxyl/tetO* and *saeP1*, respectively. The promoters in the successful clones verified by PCR amplification were then sequenced to confirm the correct sequence and orientation.

2.3.6 Phage transduction

2.3.6.1 Propagation of phage

One of the predominant phage type marker ø85 was used for transducing mutations between *S. aureus* strain. First, ø85 was propagated in *S. aureus* RN4220 as described by Kreiswirth *et al*. (1983). *S. aureus* RN4220 was inoculated into 10ml phage broth, and incubated at 37°C overnight. This culture was diluted 1:200 into 10ml fresh phage broth and incubated at 37°C to mid-exponential phase. Next, 10 × 30μl of phage stock of ø85 was added into 10 × 30μl of phage stock of ø85. The mixture was incubated at room temperature for 30 minutes. Subsequently, 9ml molten top agar was added to each infection sample followed by mixing and pouring over two plates of undried base agar. The plates were incubated base downwards at 37°C overnight. Finally, 1ml of PBS was added to each plate and the top agar was collected into a centrifuge tube using a spreader. The sample was centrifuged at 15,000 rpm for 15 minutes using a SS34 rotor at 4˚C. The supernatant was filter-sterilised using a 0.45μm filter and stored at 4˚C.

2.3.6.2 Precipitation of phage

NaCl and PEG-8000 were added into the phage suspension to final concentrations of 1M and 20% (w/v), respectively. The mixture was incubated at 4˚C for 2 hours or overnight until phage precipitated. The mixture was then centrifuged at 15,000 rpm for 15 minutes at 4˚C to recover phage. Finally, the supernatant was removed and phage was re-suspended in 1ml PBS. Sterilised glycerol was added into the phage to a final concentration of 15% (v/v). This glycerol phage stock was stored at -80˚C.

2.3.6.3 Titration of phage

The prepared ø85 solution was titrated by infecting *S. aureus* RN4220. Mid-exponential phase *S. aureus* RN4220 cells were prepared as described in section 2.3.6.1, and 300µl cell culture was infected with 100µl 10-fold serial dilutions of the phage solution (neat, 10^1 …. 10^6) at room temperature for 30 minutes. Then, 9ml molten top agar was added to each infection sample followed by mixing and pouring over three plates of dried base agar. The plates were incubated base downwards at 37°C overnight. The number of lysed plaques was counted and the size of the phage stock was calculated based on the number of plaques and the dilution factors.

2.3.6.4 Transduction

To transduce a mutation from a donor strain to a recipient strain, the donor strain was first infected with ø85 to propagate the phage. This was done as described in section 2.3.6.1 with slight modifications: I), 300µl of cells was infected with 100µl 10-fold serial dilutions of the ø85 solution; 2), only the phage lysate on plates with complete lysis of bacteria was collected using 0.5ml PBS.

Next, the recipient strain was infected by the resulting phage stock. The recipient strain was grown in 20ml TSB at 37˚C for 18 hours. The cells were then collected by centrifugation at 5000rpm for 10 minutes, followed by re-suspension into 1ml TSB. Subsequently, 0.5ml cell suspension was added to 0.5ml LB broth, and $CaCl_2$ was added into the mixture at a final concentration of 5mM. Next, 0.5ml phage lysate was added to the cell mixture, followed by incubation at 37˚C with shaking for 20 minutes. Finally, 1ml of ice-cold 20mM sodium citrate was added to the mixture, and cells were collected by centrifugation at 5000rpm for 10 minutes at 4˚C. The cells were re-suspended in 1ml of 20mM sodium citrate and plated onto TSA plates containing 20mM sodium citrate and selective antibiotics. The plates were incubated at 37˚C for two days. The arising single colonies were sub-cultured twice onto TSA with 10mM $CaCl_2$ to eliminate contaminating phage. The purified putative transductants were then verified by PCR to confirm the presence of the desired mutations.

2.3.7 Construction of *S. aureus* LS-1 *menD* :: *erm*

A fragment (~3.1kb) containing *menD-erm-menD* was PCR amplified from *S. aureus* 8325-4 Δ*menD* :: *erm* (Bates *et al.*, 2003) using primers P125 and P126. The PCR product was purified and treated with T4 Polynucleotide Kinase to add phosphates to the 5' terminus of fragment. Vector pKOR1 was digested with EcoRI and EcoRV, releasing a fragment containing the *ccdB* gene, which encodes an *E. coli* gyrase inhibitor protein (Bernard and Couturier, 1992). The linearised vector was then treated with Klenow Fragment to fill in the overhangs generated by EcoRI digestion. Finally the vector was ligated with the phosphorylated PCR fragment containing *menD-erm-menD.* The ligation mix was transformed into *E. coli* DH5α, and the propagated plasmid was purified and confirmed by PCR amplification of the fragment containing *menD-erm-menD*.

From one of the successful clones, pPZ177-2 was purified, and integrated into *S. aureus* RN4220 and then LS-1 by electroporation. It was found that the chloramphenicol resistance gene *cat* was somehow inactivated in pPZ177-2 during the cloning process. To integrate *erm* into *menD* in the chromosome of *S. aureus* LS-1 through double cross-over recombination, a single colony of *S. aureus* LS-1 pPZ177-2 was inoculated into 10ml TSB + 5μg/ml Erm and grown overnight at 30°C. This culture was then diluted 1:1000 into 10ml TSB + 5μg/ml Erm, and incubated at 43°C for 24 hours to prevent replication of the plasmid. The culture was then plated on TSA + 5μg/ml Erm + 2μg/ml anhydrotetracycline (ATc) to select Erm^r bacteria but inhibit those containing the plasmid [ATc induces the transcription of antisense *secY* on pPZ177-2, which is lethal to the bacteria (Forsyth *et al.*, 2002)]. Single colonies were selected and grown overnight in TSB + 5μg/ml Erm at 30˚C. Genomic DNA and plasmid DNA were purified from the cells. To identify clones that did not contain the plasmid, plasmid DNA preparations were examined by gel electrophoresis alongside pPZ177-2, and used as template to amplify the *Pxyl/tetO* promoter, which is present in pPZ177-2, using primers P1 and P2. Clones that did not contain a plasmid corresponding to pPZ177-2 in the plasmid preparation and did not result in a PCR product of 780bp representing *Pxyl/tetO* were selected as plasmid-free clones. The insertion of *erm* into *menD* in the chromosome of LS-1 was confirmed by PCR

amplification using primers P125 and P128 (P128 is specific to chromosome but not pPZ177-2). The plasmid-free mutant was verified by the observation of a PCR product of ~4.2kb. These clones were further examined by menadione disk diffusion test as described in section 2.11 to confirm the menadione auxotrophic phenotype.

2.3.8 Construction of *aroD* mutants

Deletion of the *aroD* gene from nucleotide 448bp to 717bp was achieved by using Gateway® Technology followed by alleic replacement similar to that described in figure 2.1 but without intergrating antibiotic resistance marker. This was performed as described by Bae and Schneewind (2006) using vector pKOR1. The upstream and downstream nucleotides of this deletion region were amplified from *S. aureus* LS-1 genomic DNA using primers P194/P195 and P196/ P197. The *attB* sites were designed at the 5' end of primers P194 and P197, and XhoI restriction sites were designed at the 5' end of primers P195 and P196. The two DNA fragments were digested at the XhoI sites, and ligated using T4 DNA ligase. The ligated DNA fragment was integrated into pKOR1 through site-specific recombination in the presence of BP clonase II enzyme (Invitrogen). *E. coli* DH5α was transformed with the recombinant plasmid, and transformants were selected using 100μg/ml ampicillin. Plasmids from the transformants were purified and analysed by sequencing to exclude possible mutations in the cloned region.

The verified plasmid pPZ138-7 was transformed into *S. aureus* RN4220, and then *S. aureus* LS-1, 8325-4, SH1000 or HG003. Truncation of *aroD* gene by homologous recombination was achieved by a series of temperature shift procedures (Bae and Schneewind, 2006) as described below. *S. aureus* strains with pPZ138-7 were grown overnight in TSB + 10μg/ml Cm at 30˚C. These cultures were diluted 1 : 1000 into TSB + 10μg/ml Cm followed by growing overnight at 43˚C. This step was repeated once using TSB + 7.5μg/ml Cm. The resulting cultures were plated on TSA + 10μg/ml Cm followed by incubation overnight at 43˚C. Colonies were selected and grown overnight in TSB at 30˚C. These cultures were then plated onto TSA + 2μg/ml ATc and grown at 30˚C for 4 days. The colonies were selected and streaked onto TSA with or without 10μg/ml Cm, and grown at 30˚C to screen for Cm^S bacteria which lost

plasmid pPZ138-7. The Cm^S colonies were then grown in TSB at 30˚C for the purification of genomic DNA. PCR amplifications from the genomic DNA were performed using primers P172 and P173 flanking *aroD* gene. A PCR product of 991bp was expected for *aroD* mutants with the intended deletion from nucleotide 448bp to 717bp.

2.3.9 Complementation of *aroD* mutants

To complement *aroD* mutants, the *aroD* gene from *S. aureus* LS-1 was amplified using primers P192 and P193. The PCR product was digested using restriction enzyme BamHI and XbaI, and purified. A promoter *lacI-Pspac* was excised from plasmid pHCMC05 (Nguyen *et al.*, 2005) using restriction enzyme SacI and BamHI. The *lacI-Pspac* and *aroD* fragments were then ligated with SacI-XbaI digested pSK236. *E. coli* DH5α was transformed with the ligation mix, and plasmids were purified from the transformants and confirmed by restriction digestion using BamHI-XbaI and SacI-BamHI. One of the successful clones, pPZ137-3, was sequenced at the cloned region to confirm the correct sequence. The verified plasmid pPZ137-3 was transformed into *S. aureus* RN4420 and then the *aroD* mutants.

2.4 Protocols for transposition and transposon mutant verification

2.4.1 Transposition protocol

The transposition protocol was modified from Cao *et al*., (2007). *S. aureus* LS-1 containing pPZ4 or pPZ114-5 was grown overnight in 5ml TSB containing 5µg/ml erythromycin (Erm) and 20µg/ml kanamycin (Kan) at 30°C with shaking at 200rpm. This culture was diluted to OD_{600} = 0.05 into 10ml fresh TSB + 5µg/ml Erm with different concentrations of ATc, followed by incubation at 30°C with shaking at 200rpm for 1 hour. The cells were harvested by centrifugation at 5000rpm for 5 minutes and washed twice with PBS to remove unbound ATc. The cells were subsequently re-suspended into 10ml fresh TSB + 5µg/ml Erm and incubated for 6 hours at 43°C with shaking at 200rpm. Serial dilutions of the culture were plated on TSA and TSA + 5µg/ml Erm, and incubated overnight at 43°C. To assess the plasmid

retention rate, colonies on TSA + 5μg/ml Erm were replicated onto TSA + 50μg/ml Kan and TSA+ 5μg/ml Erm, followed by incubation at 30°C for 48 hours before the number of Erm^r Kan^r and Erm^r Kan^s colonies were counted. In each transposition experiment, the following data was calculated as described below:

- The total number of cells was calculated based on the number of colonies on TSA.
- The number of mutants was considered to be cells that form large colonies on TSA + Erm at 43°C (similar size to LS-1 grown on TSA at 43˚C), and calculated according to the colony counts on TSA + Erm at 43°C.
- The transposition efficiency was calculated by dividing the number of mutants by the total number of cells.
- The plasmid retention rate was based on the number of colonies on the replica plates, TSA + Kan and TSA + Erm, growing at 30°C. Plasmid retention rate = the number of Erm^r Kan^r colonies / the number of Erm^r colonies recovered from 30°C.
- The number of plasmid-free mutants = the number of mutants × (1 - plasmid retention rate).

2.4.2 Southern hybridisation analysis

The number of transposon insertions in *S. aureus* LS-1 transposon mutant derivatives was determined by Southern hybridisation analysis, which was performed using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science, Burgess Hill, UK) according to the manufacturer's instructions. Buffers and solutions used are listed below:

Denaturisation Solution	1.5 M NaCl 0.5 N NaOH
Neutralisation Solution	3M NaCl 1M Tris-HCl pH = 7.5
20X Salt Sodium Citrate (SSC)	3M NaCl 0.3M Sodium citrate pH = 7

Washing Buffer	0.1M Maleic acid 0.15M NaCl 0.3% v/v Tween 20 pH = 7.5
Low Stringency Wash Solution	2 × SSC 0.1% Sodium dodecyl sulphate (SDS)
High Stringency Wash Solution	0.5 × SSC 0.1% SDS
Maleic Acid Buffer	0.1M Maleic acid 0.15M NaCl pH = 7.5
1X Blocking Solution	10 × Blocking Solution diluted in 1× Maleic Acid Buffer
Pre-Hybridisation Buffer	5 × SSC 0.1% Lauryl sarcosine 0.02% SDS 1× Blocking solution
Hybridisation Buffer 1× Blocking Solution	5 × SSC 0.1% Lauryl sarcosine 0.02% SDS 25ng/ml probe DNA
Antibody Solution	Anti-Digoxigenin-AP was diluted to 75 mU/ml in 1 × Blocking Solution
Detection Buffer	0.1M Tris-HCl 0.1M NaCl pH9.5

2.4.2.1 DNA fragmentation and separation

Approximately 3µg of genomic DNA from each mutant was digested with HindIII in a 50µl reaction volume at 37°C for 16 hours. The digest was concentrated in a Speedvac Concentrator (Savant SPD1010) to give a final volume of 20µl, DNA fragments were

electrophoresed on a 0.8% agarose gel in a 13 × 13cm gel tray at 25V for approximately 16 hours. In addition, 200ng of digoxigenin-labeled DNA molecular weight marker VII (Roche Applied Science) was run alongside as a size reference, and a 3.3kb fragment of pPZ4 containing the transposon was included as a positive hybridisation control.

2.4.2.2 Transfer of DNA from gel to membrane

Following electrophoresis, the gel was treated with 0.25M HCl for 40 minutes at room temperature to depurinate DNA fragments. Next, the gel was briefly rinsed twice in dH_2O, followed by incubation for 2 × 20 minutes in Denaturation Buffer to cleave depurination sites. Subsequently, the gel was neutralised in Neutralization Buffer for 30 minutes. DNA fragments were transferred to a positively charged nylon membrane (GE Healthcare, Buckinghamshire, UK) by capillary transfer in 20 × SSC for approximately 24 hours. DNA was covalently linked to the membrane by UV cross-linking at 254nm for 12 seconds.

2.4.2.3 Labelling of the DNA probe with digoxigenin-11-dUTP

A 400bp DNA fragment of the transposon in pPZ4 was PCR amplified using P36 and P37. The PCR product was purified and labelled using the DIG High Prime DNA Labelling Kit (Roche Applied Science) according to the manufacturer's instructions. Prior to hybridization, labelled probes were denatured by heating at 99˚C for 5 minutes, and immediately cooled on ice.

2.4.2.4 Hybridization

The fixed membrane was briefly rinsed in 2 × SSC at room temperature, followed by incubation at 68˚C in pre-Hybridization Buffer for 3 hours with gentle agitation in a hybridization oven. Subsequently, the Pre-Hybridization Buffer was removed and replaced with pre-warmed (68˚C) Hybridization Buffer containing approximately 25ng/ml probe. The membrane was immersed in Hybridization Buffer and incubated at 68˚C with rocking for 16 hours.

2.4.2.5 Immunological detection

Following hybridization, the membrane was washed twice in Low Stringency Buffer for 2 × 15 minutes at room temperature, then twice in High Stringency Buffer for 2 × 15 minutes at 68˚C. The membrane was rinsed briefly in Washing buffer for 5 minutes, and then incubated in 1 × Blocking Solution with gentle agitation for 30 minutes at room temperature. The membrane was then transferred to Antibody Solution, incubated for 30 minutes at room temperature, washed twice in Washing Buffer for 2 × 15 minutes at room temperature, and equilibrated in Detection Buffer for 5 minutes. Finally, the membrane was placed on a plastic folder with the DNA side facing up, and 1 ml of the chemiluminescent substrate CSPD® was applied to the membrane, followed by immediately covering the membrane with the second sheet of the folder, and spreading the substrate evenly without air bubbles over the membrane. Excess liquid was squeezed and the edges of the folder were sealed. The membrane was exposed to X-ray film (GE Healthcare) and images were developed.

2.4.3 Y-linker PCR strategy for detecting transposon insertions

A Y-linker PCR strategy was modified from a protocol described by Kwon and Ricke (2000) for determining the number of transposon insertions in a mutant and also for assessing the possibility of siblings between mutants. The strategy is graphically illustrated in figure 2.2. The Y-linker is composed of two designed oligonucleotides, linker I (TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACAT) and linker II (CGATGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT). The 21bp nucleotides (underlined) on the 3’ end of linker I are complementary to the 21bp nucleotides (underlined) on the 5’ end of linker II, so that they form a Y-shaped fragment when they are annealed together. In addition, a TaqI restriction site was placed at the complementary end so that a TaqI sticky end is available once the two oligonucleotides anneal together, allowing the ligation with TaqI-digested genomic DNA. This ligation mix can then be used as template for PCR amplification using a primer specific to one branch on the Y linker DNA (Y-linker primer) and another primer specific to the transposon (Tn primer). Theoretically, each insertion of a transposon in the genomic DNA of a mutant should result in a single PCR product,

therefore multiple PCR products indicates the presence of multiple transposon insertions.

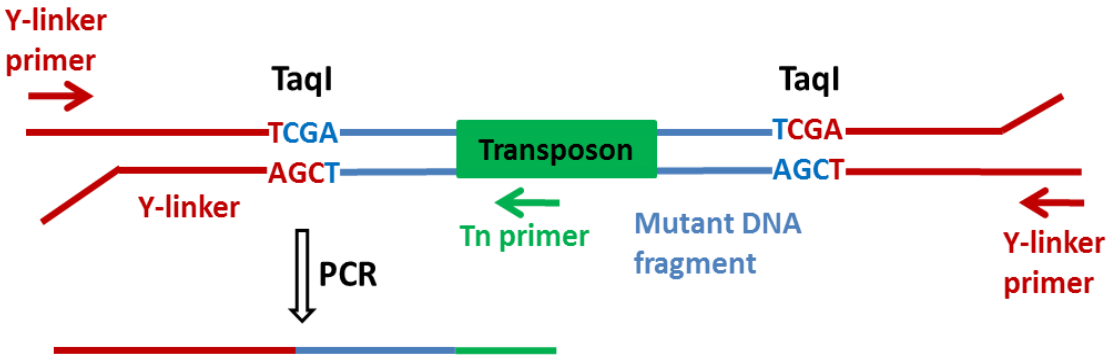

Figure 2.2: Graphical illustration of the Y-linker PCR strategy for the determination of the number of transposon insertions in transposon mutants. Red: Y-linker DNA. Blue: *S. aureus* LS-1 transposon mutant DNA fragment. Green: transposon. Y-linker primer: a primer designed on one of the branch of the Y-linker DNA. Tn primer: a primer specific to transposon sequence.

2.4.3.1 Preparation of Y-linker

To prepare the Y-linker, 3µg of Linker II was first phosphorylated at its 5' end using T4 polynucleotide kinase. The enzyme was then inactivated by heating at 65°C for 20 minutes. Subsequently, 3µg linker I was added, and the mixture was heated at 95°C for 2 minutes, followed by slowly cooling to room temperature to allow annealing of the oligonucleotides, resulting in the formation of the Y-linker.

2.4.3.2 Ligation of the Y-linker with mutant DNA fragments

Genomic DNA from transposon mutants was extracted and approximately 2µg DNA was digested with TaqI at 65°C for 16 hours in 20µl volume. The enzyme was then inactivated at 80°C for 20 minutes prior to the ligation. Approximately 50ng of digested genomic DNA was ligated to 1µg of Y-linker using T4 DNA ligase. The ligation mix was heated at 65 °C for 20 minutes to inactive the ligase.

2.4.3.3 Y-linker PCR

Prior to being used as PCR template, the ligation mix was diluted 10-fold and 1µl of the dilution was used as template for Y-linker PCR. The PCR products were amplified using Y-linker specific promoter P71 and transposon specific primer Tn1 (P103) or Tn2 (P23). PCR reactions are as described below:

Reaction mix

ThermoPol reaction buffer	1×
dNTPs	0.1 mM for each
P71	0.5µM
P103 / P23	0.5µM
1µl of 10-fold dilution of ligation mixture	~5 ng
Taq DNA polymerase	0.05 unit / µl
dH_2O	Top up to 20µl

Reaction condition:

1.	Pre-amplification denaturation	94 °C	2 minutes
2.	Denaturation	94 °C	30 seconds
3.	Annealing	58 °C	30 seconds
4.	Extension	70 °C	2 minutes
	Steps 2-4 were cycled 30 rounds.		
5.	Final extension	70 °C	5 minutes
6.	Pause	4 °C	∞

2.4.4 Construction of a *S. aureus* LS-1 transposon mutant library

2.4.4.1 Transposition

The transposition protocol for generating the *S. aureus* LS-1 mutant library were slightly modified from that described in section 2.4.1. *S. aureus* LS-1 :: pPZ4 was grown overnight in 5 ml TSB + 5µg/ml Erm and 20µg/ml Kan at 30°C with shaking at 200rpm. The overnight culture was then diluted to OD_{600} = 0.005 into 100ml fresh TSB + 5µg/ml Erm + 1µg/ml ATc, and the culture was grown at 30°C with shaking at 200rpm for 1 hour. Subsequently, the cells were collected by centrifugation at 5000rpm for 5 minutes and washed twice with PBS to remove unbound ATc. Following washing, the cells were re-suspended into 100ml pre-warmed (43°C) TSB, and 5µg/ml Erm was added after suspending the cells. This was incubated at 43°C with shaking at 200rpm for 9 hours. To modulate the change of different cell populations during this 9 hours, at time points 0, 3, 6 and 9-hours, 10-fold serial dilutions of the culture was plated onto TSA, TSA + 5µg/ml Erm (two sets) and TSA +

50μg/ml Kan. TSA and one set of TSA + 5μg/ml Erm were incubated overnight at 43°C, and TSA + 50μg/ml Kan and another set of TSA + 5μg/ml Erm were incubated at 30°C for 48 hours. The numbers of colonies on each plate were counted for calculating the following parameters:

- The total number of cells was based on the total number of colonies on TSA.
- The number of Erm^r cells, including mutants with or without pPZ4 and wild-type with pPZ4 was based on the number of colonies on TSA + Erm growing at 30°C.
- The number of mutants, with or without pPZ4, was based on the number of colonies on TSA + Erm growing at 43°C.
- The transposition frequency (the proportion of mutants) was calculated as the number of mutants divided by the total number of cells.
- The number of plasmid-containing cells, including mutants and wild-type which contain pPZ4, was based on the number of colonies on TSA + Kan growing at 30°C.
- The plasmid retention rate was calculated by dividing the number of plasmid-containing cells by the total number of cells.

2.4.4.2 Genome coverage by the transposon mutant library

To estimate the genome coverage by the number of mutants produced by the transposition described in the last section, the minimum number of individual mutants generated in the transposition sample was considered to be the number of mutants at time point 0-hour. The genome coverage by this number of individual mutants was assessed using the formula N = ln (1-P) / ln (1-f) (Orkin, 1990). N is the number of individual mutants required, P is the probability desired for full coverage of the genome, and f is the frequency of insertions within the genome. With a fixed N value (the number of individual mutants obtained from the transposition), the linear relationship between the desired probability and the frequency of transposon insertions within a genome can be calculated. Due to the lack of *S. aureus* LS-1 genome information, *S. aureus* NCTC 8325 genome (GenBank accession CP000253), which has a size of 2,821,361bp containing 3006 genes (average gene size 939bp), was taken as a reference for the analysis.

2.4.4.3 Mutant enrichment and library generation

When generating a mutant library, following the transposition described in section 2.4.4.1, a mutant enrichment step was added by plating 16ml of the 9 hours culture onto TSA + 5μg/ml Erm in 16 square petri dishes (24.5cm x 24.5cm, Thermo Scientific Nunc^{TM}). The plates were incubated at 43°C overnight. The cells on the 16 plates were harvested as a mutant library by washing the agar with 16 × 20ml PBS and collecting the cell suspension. The cells were pelleted by centrifugation at 5000rpm for 15 minutes, and then re-suspended into 282ml TSB with 15% of glycerol. The cell suspension was distributed to 1ml aliquots and stored at -80°C for future use. To determine the numbers of different cell populations in the mutant library, 10-fold serial dilutions of the final cell suspension were plated onto TSA, TSA + 5μg/ml Erm (two sets) and TSA + 50μg/ml Kan. TSA and one set of TSA + 5μg/ml Erm were incubated overnight at 43°C, and TSA + 50μg/ml Kan and another set of TSA + 5μg/ml Erm were incubated at 30°C for 48 hours. The numbers of colonies on each plate were counted to determine the total number of cells, the number of mutants, proportion of mutants and the plasmid retention rate as described in section 2.4.4.1.

2.5 Luminescence assays

Detection of luminescence production was performed according to the method described by Bachmann *et al.* (2007). A single colony of *S. aureus* PZ134-2 with plasmid pNZ5520, or pNZ5520 :: P_{cat}, or pNZ5520 :: *Pxyl/tetO*, or pNZ5520 :: *saeP1* was inoculated into 5ml TSB + 5μg/ml Erm + 10μg/ml Cm, and cultured at 37˚C overnight. The overnight culture was diluted to OD_{600} = 0.05 into 10ml TSB + 5μg/ml Cm (for *S. aureus* PZ134-2 pNZ5520 :: *Pxyl/tetO*, ATc at concentrations 0, 1, or 2μg/ml was supplemented), and the culture was incubated at 37˚C with shaking at 200rpm. The optical density of the culture at 600nm was measured every 3 hours. At the same time, 50μl of the culture was mixed with 150μl of Solution A (1.9% w/v glycerol-2-phosphate disodium salt + 10μg/ml riboflavin dissolved in water) in a 96-well plate (polystyrene). The mixture was incubated at room temperature for 2 minutes, followed by mixing with 10μl of Solution B [0.1% Nonyl aldehyde (Sigma-Aldrich N30803) in 40% ethanol] immediately before the luminescence measurement. Luminescence production was measured at 2-minute intervals over a period of 15

minutes at 37˚C using a Luminoskan Ascent (Thermo Labsystems, Altrincham, UK). The highest value in these measurements was recorded as the luminescence production.

2.6 Antimicrobial assays

2.6.1 Antimicrobial agents, media and buffers

Antibiotics were purchased from Sigma-Aldrich and prepared as described in table 2.3 under section 2.1.3. ATc was dissolved in deionised water and filter-sterilized before using. Antimicrobial peptide LL-37 was purchased from Innovagen (Lund, Sweden) as a powder and dissolved in sterilised deionised water to 1mg/ml before use. The media and buffers used for measuring the activity of antimicrobial agents (antibiotics or antimicrobial peptides) are listed below. All media or buffers containing $NaHCO_3$ were prepared fresh prior to use.

TSB:	Tryptic Soy broth (Sigma-Aldrich, T8907)
Medium I:	20% TSB (v/v) 1mM NaH_2PO_4 10% FBS (Sigma-Aldrich, F0804) 150mM NaCl pH = 7.4
Medium I + C:	Medium I + 50mM $NaHCO_3$ pH = 7.4
Medium II:	20% TSB 1mM NaH_2PO_4 pH = 7.4
Medium II + C:	Medium II + 50mM $NaHCO_3$ pH = 7.4
Solid Medium II + C:	Medium II + C 1% agarose (Sigma-Aldrich, A9539) pH = 7.4

Buffer III:	1mM NaH_2PO_4
	pH = 7.4
Buffer III + C:	Buffer III + 50mM $NaHCO_3$
	pH = 7.4

2.6.2 Minimum inhibitory concentration assays of antibiotics

The minimum inhibitory concentrations (MICs) of antibiotics against *S. aureus* were determined in TSB (Wiegand *et al.*, 2008). Briefly, an overnight culture was prepared by growing a single colony of *S. aureus* in 5ml TSB for 16 hours. The culture was diluted in fresh TSB to approximately 1×10^6 CFU/ml cells. This suspension, typically 50µl, was added to equal volume of TSB containing antibiotics at a range of concentrations 0, 1, 2, 4, 8, 16, 32, 64, 128 and 256µg/ml in polystyrene 96-well plates (Thermo Scientific NUNC). This resulted in a cell density of 5×10^5 CFU/ml and antibiotics being at concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128µg/ml. The microtitre plate was incubated at 37°C with shaking at 200rpm for 24 hours if the strain exhibited a normal growth rate or for 48 hours if the strain was a small colony variant (SCV). Bacterial growth in each well was determined by measuring the optical density of sample in each well at OD_{590} using a DYNEX Technologies MRX-TC Revelation Microtiter plate reader II (Prior Laboratory Supplies, Leicestershire, UK). The MIC was defined as the lowest concentration of antibiotic that resulted in no detectable bacterial growth.

2.6.3 Minimum inhibitory concentration assays of LL-37 in liquid media

The MICs of LL-37 against *S. aureus* in liquid media were technically performed similarly to that was described in section 2.6.2 except for the following modifications according to the methods described by Dorschner *et al*. (2006). 1) *S. aureus* LS-1 was grown overnight in TSB to stationary phase, then diluted 1: 100 into medium I + C and grown to early log phase (OD_{600} ~ 0.5); 2) The cells at early log phase were harvested by centrifugation and re-suspended into assay medium. These cells were inoculated into the MIC assay at a final cell density of 1×10^6 CFU/ml; 3) polypropylene 96-well plates (Costar, Corning 3359, High Wycombe, UK) was used.

2.6.4 Inhibition assays of LL-37 in liquid media

The samples for inhibition assays of LL-37 were prepared as described in section 2.6.3. Bacterial growth in each well was evaluated by measuring the optical density of each well at OD_{590}. The percentage inhibition of bacterial growth was determined as the difference of the OD_{590} in the presence or absence of LL-37 divided by the OD_{590} without LL-37.

2.6.5 Minimum inhibitory concentration assays of LL-37 on agar containing medium II + C

MIC assays of antimicrobial peptides on solid medium were performed as described by Wiegand *et al.* (2008). Medium II + C + 1% agarose was autoclaved and cooled down to 50°C, and 100µl of medium containing LL-37 at concentrations 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128µg/ml was added into each well in a 96-well plate. *S. aureus* grown overnight in TSB was harvested by centrifugation, washed twice in PBS and re-suspended in liquid medium II + C to ~1×10^7 CFU/ml. Following medium solidification, 1µl of cell suspension (~1×10^4 cells) was dropped onto the medium and incubated at 37°C for 24 hours, exceptionally 48 hours for small colony variants. MIC was determined as the lowest concentration of LL-37 that resulted in no visible growth after the incubation.

2.6.6 Twenty four hour minimum bactericidal concentration assays of LL-37

Twenty four hour minimum bactericidal concentration (MBC) assays were performed using a method adapted from the CLSI (Clinical and Laboratory Standards Institute) manual M26-A (CLSI, 1998). The inoculum was prepared as described in section 2.6.3. Approximately 1×10^4 cells were inoculated into 100µl medium II (+/-C) containing LL-37 at 0, 1, 2, 4, 8, 16, 32, 64, or 128µg/ml. Samples were incubated at 37˚C for 24 hours, and the samples without visible growth were plated onto TSA to determine the number of viable cells. The numbers of viable cells in each well were compared to the original inoculum size, and MBC was defined as the lowest concentration resulting in ≥99.9% kill of the original inoculum.

2.6.7 Two hour minimum bactericidal concentration assays of LL-37

Two hour MBC assay samples were prepared as described by Ouhara *et al.* (2008). The inoculum was prepared as described in section 2.6.3 with a modification that an overnight culture was diluted 1:1000 into medium I (+/-C) to allow 4 hours of growth prior to early log phase. Approximately 1×10^4 cells were inoculated into 100µl medium II (+/-C) or buffer III (+/-C) containing LL-37 at 0, 1, 2, 4, 8, 16, 32, 64, or 128µg/ml. Samples were incubated at 37˚C for 2 hours, followed by plating the sample in each well (100µl) onto TSA to determine the number of viable cells. The numbers of the viable cells in each well were compared to the original inoculum size, and MBC was defined as the lowest concentration resulting in ≥99.9% kill of the original inoculum.

2.6.8 Killing dynamics of LL-37

S. aureus LS-1 was grown overnight at 37 °C in TSB with shaking at 200rpm to stationary phase. The cells were then harvested by centrifugation and re-suspended in buffer III + C. This cell suspension was then diluted into buffer III + C to approximately 2×10^5 CFU/ml. Next, 250µl of this cell suspension was added into 250µl buffer III + C containing 0, 4, 8 or 16µg/ml LL-37 in eppendorf tubes (1.5ml), resulting in a cell density of ~ 1×10^4 cells / 100µl and LL-37 at concentrations 0, 2, 4 and 8µg/ml. These eppendorf tubes were incubated horizontally at 37 °C with shaking at 200rpm. The samples were plated on TSA every 1 hour (50µl was plated for the first hour, and 100µl were plated at time 2, 3, 4 and 5 hours) and incubated at 37 °C overnight. The number of viable cells in each sample was determined by the viable counts.

2.7 Screening of mutants with increased resistance to LL-37

2.7.1 Screening of the transposon mutant library

Transposon mutants with potential increased resistance to LL-37 were screened with two rounds of killing by 8µg/ml LL-37, alongside wild-type LS-1 to assess the screening. In the first round, the wild-type cells were harvested from an overnight

culture in TSB, and the mutant cells were collected from the transposon mutant library (described in chapter 3). Both wild-type and mutant cells were collected by centrifugation, washed twice with buffer III, re-suspended in buffer III + C, and diluted to approximately 2×10^5 CFU/ml into 10ml buffer III + C. To screen under the same conditions as those for characterising LL-37 killing dynamics, 250µl of this cell suspension was added into 250µl buffer III + C containing 0 or 16µg/ml LL-37 in eppendorf tubes (20 tubes for each sample), resulting in a cell density of ~ 1×10^4 cells / 100µl and LL-37 concentrations of 0 and 8µg/ml. These eppendorf tubes were incubated horizontally at 37 °C with shaking at 200rpm. All the samples with LL-37 and the dilutions of samples without LL-37 were plated on TSA after 5 hours and incubated at 37 °C overnight. The numbers of viable cells in each sample was determined by viable counts.

In the second round of screening, the colonies, wild-type and mutants, that had survived treatment with LL-37 were grown individually in 100µl TSB for the *S. aureus* LS-1 survivors, and in TSB + 5µg/ml Erm for the mutant survivors in 96-well plates, and incubated overnight at 37 °C with shaking at 200rpm. The optical density of the culture in each well was measured using a DYNEX Technologies MRX-TC Revelation Microtiter plate reader II (Prior Laboratory Supplies) at 590nm. Based on these optical density values, equal numbers of wild-type cells from each well, or mutant cells from each well, were collected into 10ml buffer III. The cells were pelleted, washed twice with buffer III, and re-suspended in buffer III + C to 2×10^5 CFU/ml. As described for the first round screening, 250µl of this cell suspension was added into 250µl buffer III + C containing 0 or 16µg/ml LL-37 in eppendorf tubes, and the viable cells after 5 hours incubation at 37˚C were grown on TSA.

2.7.2 Determination of the susceptibility of mutants to LL-37

To identify mutants with increased resistance to LL-37, the mutants that survived the two-round treatment with LL-37 as described in 2.7.1 were subjected to a LL-37 antimicrobial assay adapted from MBC assay described in section 2.6.7. Each mutant and wild-type LS-1 were grown overnight in TSB, and the cells were harvested and re-suspended into buffer III + C to approximately 2×10^5 CFU/ml. These cell suspensions

were plated onto TSA to determine the initial inoculum size, and 50µl of each cell suspension was added to 50µl buffer III + C with 16µg/ml LL-37 into different wells in 96-well plate (Costar, Corning 3359). The mixture was incubated for 2 hours at 37°C with shaking at 200rpm. Different volumes (10µl and 90µl) of the samples were plated onto TSA and incubated at 37°C overnight to count the number of viable cells. The number of bacteria killed in each sample was determined.

2.8 Sample preparation for TEM and SEM

A single colony of *S. aureus* LS-1 or *S. aureus* LS-1 Δ*yycHI* was grown overnight in 5ml TSB at 37˚C with shaking at 200rpm. This culture was diluted 1 : 200 in 10ml TSB and grown at 37˚C to OD_{600} = 1-1.5. Cells from 1ml of culture were pelleted, washed once with PBS and re-suspended in 0.5ml PBS. This cell suspension was then mixed with 0.5ml of 5% (v/v) glutaraldehyde in sterilised distilled water, and the bacteria were fixed for 24 hours. The sample processing for transmission electron microscopy (TEM) and scanning electron microscope (SEM) analysis and imaging were performed by Dr. Nicky Mordan at the Eastman Dental Institute (UCL).

2.9 Cytochrome c binding assay

Cytochrome c binding assays in buffer III or buffer III + C were performed as described by Peschel *et al.* (1999). A single colony of *S. aureus* was grown overnight in 15ml TSB at 37˚C. The culture was divided equally into two tubes and cells were collected by centrifugation at 5000rpm for 10 minutes. The cells were then washed twice in buffer III, and suspended into 10ml buffer III or buffer III + C. The cell suspensions were incubated at 37˚C for 1 hour, and the cells were harvested by centrifugation and re-suspended into 2 × buffer III or 2 × buffer III + C to OD_{578} = 14 for the wild-types and the mutants. Then, 0.5ml of cell suspension was mixed with 0.5ml of 1mg/ml cytochrome c dissolved in sterile deionised water. This mixture was incubated at 37˚C for 10 minutes followed by centrifugation at 13000rpm for 3 minutes. The supernatant was carefully taken, and the cytochrome c in the supernatant was quantitated photometrically at 530nm using an Amersham Pharmacia Biotech Ultrospec 2000 spectrophotometer. To determine the original cytochrome c content

before binding to cells, 0.5ml of 2 × buffer III or 2 × buffer III + C was mixed with 0.5ml of 1mg/ml cytochrome c and incubated at 37˚C for 10 minutes, followed by centrifugation and measuring OD_{530} for the supernatant. The binding of cytochrome c by a *S. aureus* strain was represented by the difference of OD_{530} between the supernatant of the samples with or without cells.

2.10 *Galleria mellonella* virulence assay

Galleria mellonella (*G. mellonella*) in the larvae stage were purchased from Cornish Crispa (Berkshire, UK), and stored at 12˚C prior to use. Before each assay, *G. mellonella* (wax worms) were weighed to select individuals between 0.2 and 0.3 gram. *S. aureus* strains were cultured in TSB with appropriate antibiotics at 37˚C for 16 hours with shaking at 200rpm. Cells were collected by centrifugation, washed once with PBS, and re-suspended in PBS to approximately ~5×10^8 CFU/ml. Then 10µl of the cell suspension was injected into the haemocoel via the last left pro-leg of each worm using a VanishPoint® syringe (Gao *et al.*, 2010). PBS (10µl) was injected into worms as a negative control and the worms were expected to be alive during the incubation. Colony counts were performed with each cell suspension to determine the inoculum size for each strain. A total of 10 worms were infected with a single strain in one experiment. Once inoculated, worms were incubated at 37˚C for 5 days, and the number of deaths was recorded every day (Gao *et al.*, 2010). Death of the larvae was determined by no response to stimulation (Desbois and Coote, 2011). The experiment was repeated three times on three different days, and the percentages survival of worms inoculated with different strains were presented using Kaplan Meier plots.

2.11 Characterisation of auxotrophy

The disc diffusion assay described previously (Bauer *et al.*, 1966) was modified for characterisation of the auxotrophic phenotype of *S. aureus*. Heme (Fluka), menadione (Sigma) and thymidine (Fluka) were prepared at a range of concentrations 0, 1, 10, 100 and 1000µg/ml according to manufacturers' instructions. Next, 15µL of each substance at different concentrations was inoculated on to a disc

placed in the centre of a TSA plate spread with *S. aureus*. Auxotrophy was identified if a heavy growth zone around the disc was detectable after incubation for 24 h at 37°C.

2.12 DNA sequencing for SCV445

Genomic DNA was prepared from *S. aureus* LS-1 grown in TSB and SCV445 grown in TSB + 50µg/ml kanamycin. The SCV445 culture was plated on TSA to confirm that no reversion had taken place. The primers used for the amplification and sequencing of the menadione biosynthesis genes, chorismate biosynthesis genes, *tyrA* and tRNAs in LS-1 and SCV445 are listed in table 2.6.

Table 2.6: Oligonucleotides used for the amplification and sequencing of the menadione biosynthesis genes, chorismate biosynthesis genes, *tyrA* and tRNAs in LS-1 and SCV445.

	Genes	Locus tag in *S. aureus* 8325-4	Primers used for amplification	Primers used for sequencing
Menadione biosynthesis genes	*menA*	SAOUHSC_00980	P121, P141	P121, P125, P138, P139, P140
	menF	SAOUHSC_00982		
	menD	SAOUHSC_00983		
	menD	SAOUHSC_00983	P142, P130	P142, P143, P144, P145, P146
	menH	SAOUHSC_00984		
	menB	SAOUHSC_00985		
	menC	SAOUHSC_01915	P131, P132	P131, P132, P147, P160
	menE	SAOUHSC_01916		
	gerC	SAOUHSC_01486	P156, P157	P156, P157
	menG	SAOUHSC_01487		
	menG	SAOUHSC_01487	P133, P159	P133, P159
	hepS	SAOUHSC_01488		
		SAOUHSC_01348	P166, P167	P167
		SAOUHSC_02556	P168, P169	P168, P169
Tyrosine biosynthesis genes	*tyrA*	SAOUHSC_01364	P189, P190	P189, P190

	Genes	Locus tag in *S. aureus* 8325-4	Primers used for amplification	Primers used for sequencing
Chorismate biosynthesis genes	*aroA*	SAOUHSC_01481	P178, P179	P178, P179, P180
	aroB	SAOUHSC_01482		
	aroB	SAOUHSC_01482	P181, P182	P181, P182, P183
	aroC	SAOUHSC_01483		
	aroD	SAOUHSC_00832	P172, P173	P172, P173
	aroE	SAOUHSC_01699	P174, P175	P174, P175
	aroG	SAOUHSC_01852	P170, P171	P170, P171
	aroK	SAOUHSC_01635	P176, P177	P176, P177
tRNAs	Glu tRNA	SAOUHSC_T00018	P207, P208	P207
	Ser tRNA	SAOUHSC_T00048	P209, P210	P209
	Glu tRNA	SAOUHSC_T00019	P213, P214	P213
	Lys tRNA	SAOUHSC_T00035	P211, P212	P211
	Leu tRNA	SAOUHSC_T00030		
	Gln tRNA	SAOUHSC_T00016	P215, P216	P215, P216, P217
	Tyr tRNA	SAOUHSC_T00057		
	Ser tRNA	SAOUHSC_T00051		
	Ser tRNA	SAOUHSC_T00052		
	Leu tRNA	SAOUHSC_T00032		
	Lys tRNA	SAOUHSC_T00036		
	Lys tRNA	SAOUHSC_T00037	P218, P219	P218
	Gln tRNA	SAOUHSC_T00017		
	Tyr tRNA	SAOUHSC_T00058		
	Glu tRNA	SAOUHSC_T00020		

2.13 Determination of the reversion rate of SCV445

The rate of SCV445 reversion to rapidly growing cells was measured using fluctuation tests (Luria and Delbruck, 1943). A fresh colony of SCV445 grown on TSA + 50µg/ml Kan was selected and inoculated into 10 ml TSB + 50µg/ml Kan, followed by incubation overnight at 37˚C with shaking at 200rpm. The culture was diluted by 10^6 in PBS, plated on TSA, and incubated at 37˚C. Colonies appearing on agar after 24

hours and 48 hours incubation were visually screened for colonies with evidently larger size than the rest of colonies on the plates. Mutation rate was calculated according to the formula, $\mu = - (1/N) \ln P_0$, where μ is the mutation rate per cell per generation, N is the total number of colonies counted, and P_0 is the proportion of SCV phenotype colonies. Visually identified large colonies were streaked onto TSA alongside LS-1 to compare the colony size and confirm the fast-growth phenotype.

2.14 Statistical analysis

Data are expressed as means ± standard deviations. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, USA). A Shapiro-Wilk test was used for determining the normality of the data distribution. A Levene's test was used for determining the equality of the variance. For data with normal distribution, the significance of the difference between two strains were analysed using t test, and that between a group of strains were analysed using one way ANOVA with a Tukey's honestly significant difference (HSD) test (equal variance) or Games-Howell test (unequal variance). The significance of the difference between the percentage survivals of *G. mellonella* infected with different strains was analysed using the Log Rank test. A p value of less than 0.05 was considered statistically significant.

Chapter 3: Transposon Mutagenesis in *S. aureus* LS-1

3.1 Introduction

As described in chapter 1, *S. aureus*, especially MRSA has created big challenges worldwide. The rapid emergence of *S. aureus* resistance to nearly all of the available antibiotics has highlighted an urgent need for novel drugs against *S. aureus* infections. Screening of natural and synthetic chemical molecules is becoming less efficient in the identification of novel antibiotics, as only two new classes of antibiotics were launched in the past 50 years (Coates *et al.*, 2011). Additional approaches for developing novel antibiotics that are based on a better understanding of bacterial physiology and the mechanisms by which bacteria cause disease are desirable (Wright, 2010). Genes which encode functions contributing to the survival or growth in the host could represent promising potential therapeutic targets, and it is therefore meaningful to identify and study them.

A number of genetic tools are available for the identification of bacterial genes that may be important for virulence, including tools for studying specific genes, such as construction of mutants with deletions or disruptions in genes, inducible promoter systems for studying gene function, antisense oligonucleotides for inhibition of gene expression (Wei and Rubin, 2008), and tools for a large scale screening of genes involved in specific functions, such as genome-wide transposon mutagenesis (Li *et al.*, 2009), *in vivo* expression technology (IVET) (Chiang *et al.*, 1999) DNA microarray (Gresham *et al.*, 2008) and transcriptome (Martin and Wang, 2011). Application of these genetic tools to understand *S. aureus* pathophysiology is now even timely given that multiple *S. aureus* genomes have been sequenced. To identify *S. aureus* genes associated with survival or growth in different environments, we decided to take a genome-wide transposon mutagenesis approach. The aim was to generate a comprehensive library of *S. aureus* transposon mutants defective in all nonessential genes. This mutant library could then be used to identify mutants with altered ability to survive or grow under different environmental conditions.

Transposons are mobile genetic elements that can move from one location to another. They are characterised as DNA fragments flanked by two transposon specific inverted terminal repeat (ITR) sequences. ITRs are short and related or identical sequences located at the ends of a transposon in a reverse orientation. They are critical components contributing to the mobile property of transposons. However, the movement of transposons also requires an enzyme called a transposase, which recognises and binds specifically to the ITR sequences, so as to catalyse the shift of the transposon (Hayes, 2003). Movement of a transposon from one site to another site is achieved by either a cut-and paste mechanism or a replicative mechanism. Transposon Tn*5* exemplifies the cut-and-paste mechanism (Reznikoff, 2003). The transposase binds to the ITR sequences and brings the transposon ends together to form a circular complex, and then excises the complex from the donor DNA. Subsequently, the transposase recognises an insertion site in recipient DNA and catalyses the insertion of the transposon by making a staggered cut and inserting the transposon into the recipient DNA. Host factors repair the excision and insertion sites in the donor and recipient DNA (Reznikoff, 2003). Replicative transposition can be illustrated by transposon Tn*3* (Grindley, 2002). The transposase makes single-stranded breaks at the ends of the transposon in the donor DNA, and staggered double stranded breaks in the target DNA. The 3’ ends of the transposon are then ligated to the 5’ ends of the targeted DNA, followed by the start of replication of the transposon. As replication proceeds, the donor and recipient DNA present as a cointegrate and both of them contain a copy of transposon. Then the cointegrate is separated through recombination between the two copies of the transposon catalysed by the transposon-specific resolvase.

A large number of transposons have been identified and isolated, but only some of these have properties desirable for the use as a genetic tool in bacteria. To choose a transposon for the development of efficient mutagenesis strategy, it is necessary to consider (I), the activity of transposase, which is able to catalyse the transposition in a sufficiently high frequency to allow the generation of thousands of individual insertion mutants; (II), the randomness with which the transposon inserts into the target genome to ensure full coverage of the entire genome; (III), obtaining a high

proportion of mutants with a single insertion of the transposon, to enable identification of single specific genes conferring a certain phenotype. The transposons that have been successfully employed in generating *S. aureus* mutant libraries include Transposon *918* (Tn*918*) (Nakao *et al.*, 2000) the mariner-based transposon *bursa aurealis* (Bae *et al.*, 2004), Transposon *917* (Tn*917*) (Begun *et al.*, 2005), and *Himar 1* mariner transposon (Li *et al.*, 2009). Tn*917* and Tn*918* were both found to have either insertion bias (Garsin *et al.*, 2004) or low transposition frequency (Foster, 1998), while the mariner-based transposons seem to insert randomly and have recently become the favoured mutagenesis tool (Cao *et al*., 2007; Maier *et al.*, 2006; Le Breton *et al.*, 2006).

A variety of different mariner transposons have been isolated, but only two of them have been reported to be active: *Mos1* (Medhora *et al.*, 1988) and *Himar1* (Robertson and Lampe, 1995). Another attractive feature of the mariner transposons is that their requirements for transposition are very simple. Their transposition employs a cut-and-paste mechanism, requiring only their self-encoded transposase rather than other host factors (Munoz-Lopez and Garcia-Perez, 2010). Lampe *et al*. (1999) successfully isolated several hyperactive transposase mutants specific to the *Himar1* mariner transposon (Lampe *et al.*, 1999). These versions of transposase have significantly improved the transposition efficiency of the *Himar1* transposon, rendering this transposon a promising high throughput mutagenesis tool. Moreover, the insertion of the *Himar1* mariner transposon into DNA only requires the dinucleotide TA, making it well-suited for transposition into low G+C content organisms such as *S. aureus*.

Recently *Himar1* mariner transposon was successfully used in another Gram-positive organism *Listeria monocytogenes*. The authors developed two temperature sensitive transposon delivery systems giving plasmids pMC38 and pMC39 (Cao *et al.*, 2007). A map of these two vectors is shown in figure 3.1. The vectors contain the two key elements for transposition: a *Himar1* transposon and a transposase-encoding gene (*tpase*). A Gram-positive erythromycin resistance gene (*ermC*) (Villafane *et al.*, 1987) was integrated into the transposon for the selection of transposon-containing *L.*

monocytogenes upon shifting the bacteria to a temperature which prevented plasmid replication. The transposase-encoding gene *tpase* is under the control of *Bacilllus subtilis* promoter P_{mrgA} in pMC38 and P_{katA} in pMC39. The plasmids contain a Gram-negative replication origin (p15A *ori*) (Chang and Cohen, 1978) and a Gram-positive temperature-sensitive replication origin (*pE194ts ori*), which initiates replication at 30°C but halts at 43°C (Villafane *et al.*, 1987). For selection of these plasmids, they have a Gram-negative chloramphenicol resistance gene (*cat*) (Chang and Cohen, 1978) and a Gram-positive kanamycin resistance gene (*kan*) (Guerout-Fleury *et al.*, 1995) for maintaining the plasmids in Gram-negative or Gram-positive bacteria. Upon introducing pMC38/39 into *L. monocytogenes*, the *tpase* expresses and produces transposase, which then catalyses the jump of transposon from the plasmid into *L. monocytogenes* genome. Subsequently, the plasmids can be eliminated from the cells through temperature shifting from 30°C to 43°C. Removal of the plasmids, which contains *tpase*, from the transposon mutants is important because it stops further movement of the transposon from one site to another in the host genome, thus ensuring the stability of the mutation site. Plasmid-free mutants are those bacteria that are erythromycin-resistant and kanamycin-sensitive.

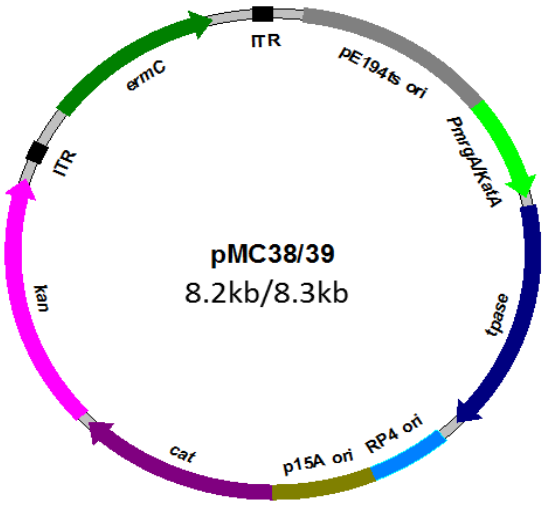

Figure 3.1: Physical map of the mariner-based transposon delivery vectors pMC38 and pMC39 (not drawn to scale). ITR: *Himar 1* mariner transposon inverted terminal repeats. *ermC*: erythromycin resistance gene encoding methyltransferase. *pE194ts ori*: Gram-positive temperature-sensitive replication origin. P_{mrgA} and P_{katA}: *B. subtilis* CU1065 promoters of gene *mrgA* and *katA*, respectively. *tpase*: *Himar 1* mariner transposase-encoding gene. RP4 *ori*: origin of conjugative IncP plasmid RP4 (Pansegrau *et al.*, 1988). P15A *ori*: *Escherichia coli* P15A low copy number replication origin. *cat*: chloramphenicol resistance gene encoding chloramphenicol acetyltransferase. *kan*: kanamycin resistance gene (Cao *et al.*, 2007).

To screen for *S. aureus* genes that are important for survival or growth *in vitro*, this transposon delivery system was chosen to generate a *S. aureus* LS-1 mutant library. Whilst this system was demonstrated to be efficient in generating *L. monocytogenes* mutants, it was reported that 16% of the mutants contained multiple insertions of the transposon in their genome (Cao *et al.*, 2007). Mutants with multiple insertions of the transposon make it difficult to straightforwardly determine which gene plays a role in survival or growth and are therefore not desirable for screening purposes. Taking into account that about 13,000 individual mutants may need to be screened to ensure full coverage of the *S. aureus* genome with every gene being interrupted once with a 99% probability (calculated as described in chapter 2 section 2.4.4.2), a multiple-insertion rate of 16% would also mean that an extra 2,500 mutants would need to be screened to achieve full coverage of the genome. Therefore, it was felt that it was important to reduce the multiple-insertion rate to minimise unnecessary cost and time during screening.

Given that the delivery plasmids are multicopy, the transposon is also present in multicopy within a cell and a high number of mutants with multiple insertions possibly indicates high transposase activity, which catalyses the transposition event at high frequency in a single cell. In an attempt to reduce the multiple-insertion rate, the decision was made to replace the promoter P_{mrgA} that controls *tpase* in vector pMC38 with an inducible promoter, so that the expression of the transposase could be modulated to a desirable level. A widely used Gram-positive inducible promoter, *Pxyl/tetO,* was chosen to achieve this goal. This promoter is a fusion of the *Pxyl* promoter derived from *B. subtilis* and the *tet* operator in conjunction with the *tet* repressor-encoding gene *TetR* from Tn*10* (Geissendorfer and Hillen, 1990). The *tet* repressor can bind to the *tet* operator and inhibit the transcription of genes under the control of *Pxyl* promoter. However, tetracycline, or the derivative anhydrotetracycline (ATc) is also able to bind to the *tet* repressor. Once they form a complex, the *tet* repressor is no longer able to bind to *tet* operator, thus relieving the inhibition of *Pxyl*. Therefore, replacing the native promoter P_{mrgA} in pMC38 with *Pxyl/tetO* was expected to enable inducing the expression of transposase by tetracycline or ATc to a desired level.

3.2 Construction of transposon delivery vector pPZ4

The transposon delivery vector pMC38 (Cao *et al.*, 2007) was modified by replacing the promoter P_{mrgA} with *Pxyl/tetO*. This was first tried by excising P_{mrgA} and ligating *Pxyl/tetO* into the vector backbone. Unfortunately, this strategy failed because it was not possible to identify restriction enzymes that only cut the P_{mrgA} out of the plasmid which was also hampered by the unavailability of pMC38 sequence. After referring to the available information on the sequence of pMC38 (Cao *et al.*, 2007), the plasmid was digested with several restriction enzymes to identify and confirm the number of certain restriction sites in the vector. Based on the information obtained, a cloning strategy, as illustrated in figure 3.2 and 3.4, was designed to replace P_{mrgA} with *Pxyl/tetO*. The sequences of all the primers used in the PCR reactions are listed in Table 2.5 in chapter 2. Cloning techniques such as PCR, restriction digests, ligation and transformation of *E. coli* are also described in chapter 2, section 2.2.

Firstly two PCR products consisting of the DNA sequence for *Pxyl/tetO* and for the *tpase* were individually amplified respectively from vector pKOR1 (Bae and Schneewind, 2006), using primers P5 and P9, and from pMC38 using primers P10 and P8. These primers incorporated, KpnI and SphI restriction sites at the 5' ends of P5 and P8, respectively. Primers P9 and P10 shared 50bp complementary sequences at their 3' ends, allowing a second round of overlap extension PCR to generate a PCR product containing the fusion *Pxyl/tetO-tpase* (Ho *et al.*, 1989).

The PCR products representing *Pxyl/tetO* and *tpase* were confirmed by agarose gel electrophoresis and as shown in figure 3.3 lanes 2 and 3, both PCR products exhibited the expected DNA sizes of 802bp for *Pxyl/tetO* and approximately 1.3kb for *tpase*. The subsequent overlap PCR product containing the fusion of *Pxyl/tetO-tpase* also displayed the expected size at approximately 2.1kb (figure 3.3 lane 5).

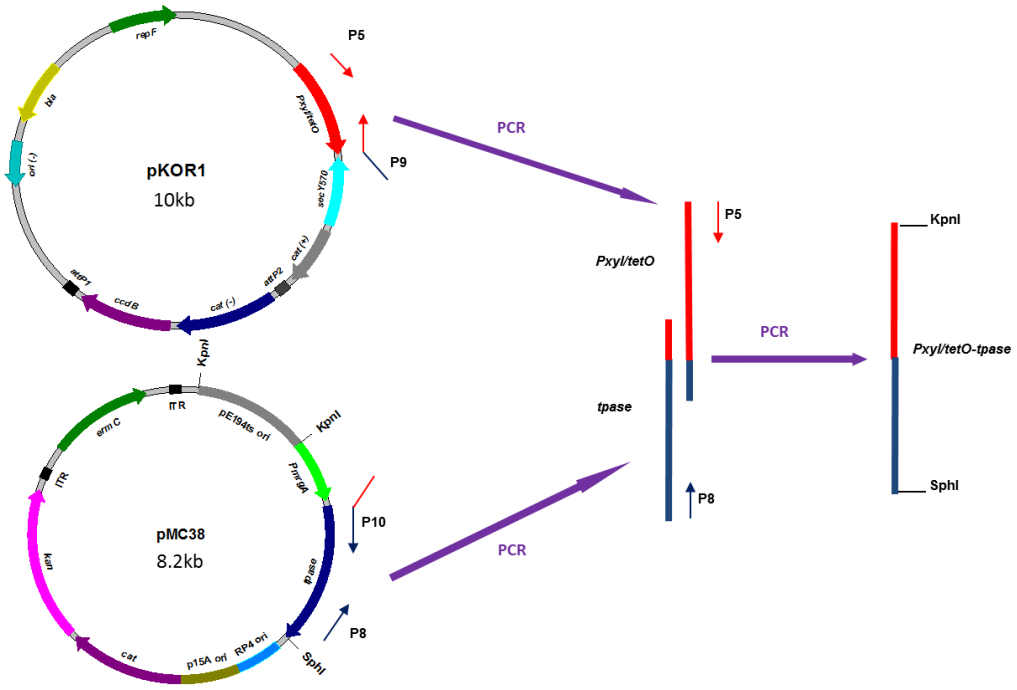

Figure 3.2: Schematic map of PCR amplification of fragment containing *Pxyl/tetO-tpase* with KpnI and SphI restriction sites at each of the end (not drawn to scale).

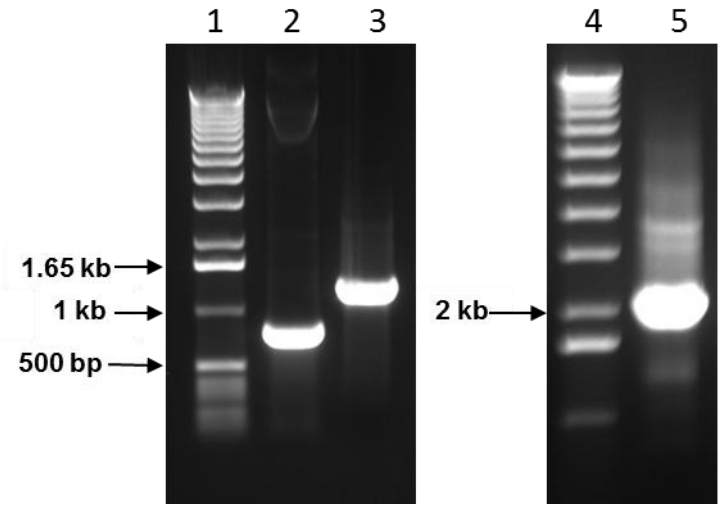

Figure 3.3: Gel electrophoresis images of PCR products. Lanes 1 & 4: 1kb plus DNA ladder (Invitrogen). Lane 2: fragment containing *Pxyl/tetO* (expected size: 802bp). Lane 3: fragment containing *tpase* (expected size: ~1.3kb). Lane 5: fragment containing *Pxyl/tetO-tpase* (expected size: ~2.1kb).

The 2.1 kb band containing *Pxyl/tetO-tpase* was extracted from the gel, purified, digested with KpnI-SphI, and ligated with similarly digested pMC38 as shown in figure 3.4. *E. coli* DH5α was transformed with the ligation mix, and plasmids were purified from three transformants and designated pPZ1-1/2/3. These plasmids were verified by digestion with SphI-KpnI, which was expected to release the fragment containing

Pxyl/tetO-tpase. The digestion pattern of these three plasmids was visualised by gel electrophoresis. As shown in figure 3.5, digesting plasmids pPZ1-2 and pPZ1-3 generated a fragment at around 2.1kb, corresponding to the size of the insert containing *Pxyl/tetO-tpase*.

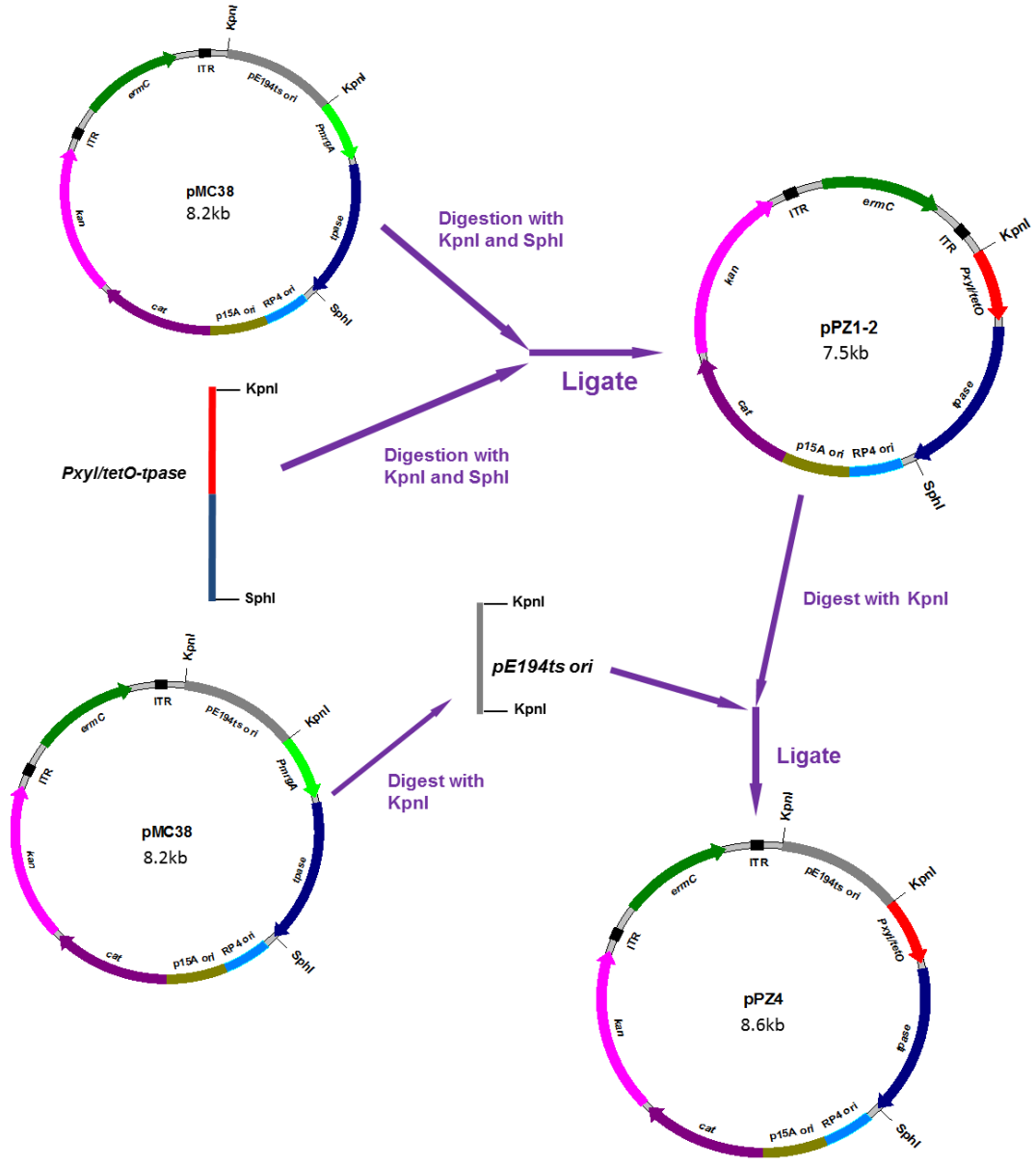

Figure 3.4: Schematic map of the cloning of the fragment containing *Pxyl/tetO-tpase* into pMC38 to replace *pE194ts ori-P_{mrgA}-tpase* (not drawn to scale).

As shown in figure 3.4, *pE194ts ori* in pMC38 is flanked by two KpnI sites. Digesting pMC38 with SphI-KpnI removed both the *pE194ts ori* and *P_{mrgA}-tpase*, while only *Pxyl/tetO-tpase* was cloned into the vector backbone. This resulted in the lack of *pE194ts ori* in pPZ1-2. Since the *pE194ts ori* is essential for replication in Gram-

positives, it was excised from pMC38 by digestion with KpnI, and inserted into KpnI-digested pPZ1-2 (figure 3.4). The resulting vector was verified by digestion with KpnI. As shown in figure 3.5 (lane 5), KpnI digestion of the vector generated a fragment corresponding to the size of *pE194ts ori* (1111bp). This vector was designated pPZ4, and the region consisting of *pE194ts ori*, *Pxyl/tetO* and *tpase* was sequenced to ensure that no mutations were introduced in the cloning process (data not shown).

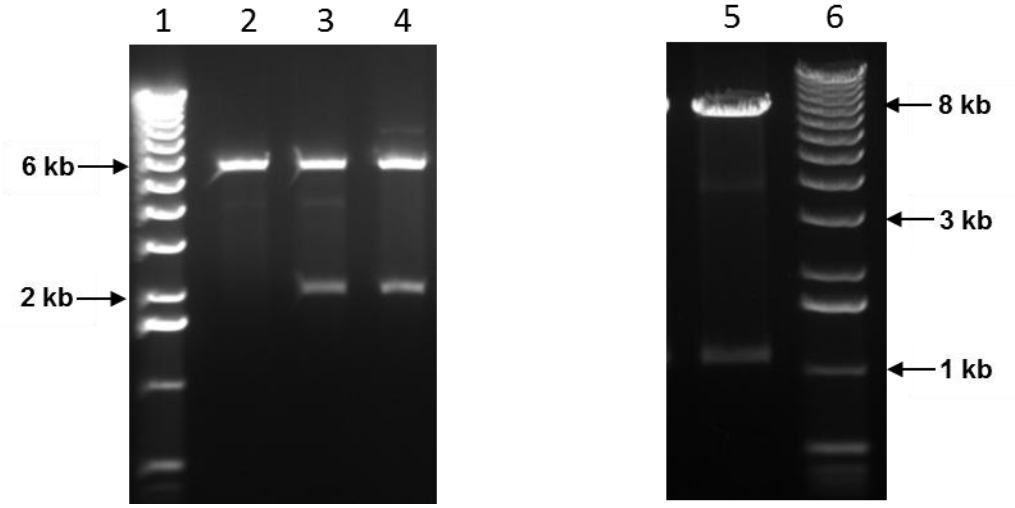

Figure 3.5: Gel electrophoresis picture of plasmid digests. Lane 1 & 6: 1kb plus DNA ladder; lanes 2-4: plasmids pPZ1-1/2/3 digested with SphI-KpnI (expected digestion pattern: two DNA fragments one ~6 kb and one ~2.1 kb). Lane 5: plasmid pPZ4 digested with KpnI (expected digestion pattern: two DNA fragments at ~8 kb and 1111bp).

3.3 Transposon mutagenesis in *S. aureus* LS-1 using pPZ4

Plasmid pPZ4 was introduced into *S. aureus* LS-1 as described in chapter 2 section 2.2.17.2. Transposon mutagenesis in *S. aureus* LS-1 using pPZ4 was conducted according to Cao *et al*. (2007) as described in chapter 2 section 2.4.1. In order to determine the optimal conditions for transposition, a range of concentrations of ATc (0, 0.5, 1, 1.5 and 2µg/ml) were used to achieve different levels of *tpase* expression. To evaluate the transposition frequency and the ability of the system to generate plasmid-free mutants, the total number of cells, the number of mutants, transposition frequency, and the plasmid retention rate was determined as described in chapter 2 section 2.4.1 and the data are presented in table 3.1.

Table 3.1: Data from transposition experiments using different concentrations of ATc. The mean values from three independent experiments are presented with standard deviation included.

ATc (µg/ml)	0	0.5	1	1.5	2
Total number of cells (× 10^8 CFU/ml)	10.2 ± 0.25	7.47 ± 0.08	6.33 ± 0.04	5.31 ± 0.07	1.24 ± 0.02
Number of mutants (× 10^6 CFU/ml)	4.3 ± 0.71	4.52 ± 0.34	4.47 ± 0.84	4.88 ± 1.25	7.24 ± 0.82
Transposition frequency (× 10^{-3})	4.21 ± 0.76	6.07 ± 0.2	7.13± 1.81	9.37 ± 3.16	58.56 ± 2.54
Plasmid retention rate (%)	6.76 ± 1.97	4.96 ± 1.18	5.75 ± 1.19	3.48 ± 1.74	4.60 ± 0.61

The total number of cells decreased with increasing concentrations of ATc. Given that the ATc in solution was removed from the cells and the cells were washed with PBS after induction of the transposition at 30°C, it seems that the ATc retained in the cells was enough to exert inhibition on growth. Despite this, a large number of mutants were generated. Interestingly, the number of mutants generated at each ATc concentration was similar except at 2µg/ml ATc where about 1.5-fold more mutants were generated. An unexpected finding was that even without ATc mutants were generated at high frequency. The plasmid retention rates in mutants from each sample were at similar levels, indicating that the removal of plasmid is not influenced by the concentration of ATc supplemented. The plasmid retention rate at each ATc concentration was similar with around 93-97% of the mutants being plasmid free.

Therefore this system proved efficient at generating transposon mutants. However, an unexpected phenomenon was that the sample without induction demonstrated a high transposition frequency and 4.3 × 10^6 mutants /ml were generated. This suggested that there was probably a fairly high basal expression of *tpase* when no inducer was present, which implied that the promoter *Pxyl/tetO* was not as tightly controlled as expected.

3.4 Southern hybridisation analysis of transposon insertions

As described above, there was likely a high level of basal expression of *tpase* under the regulation of *Pxyl/tetO*. The aim of controlling *tpase* expression was to regulate the transposition events. Ideally, a high transposition frequency is desired, so that a large number of individual mutants can be generated to cover the entire genome of *S. aureus*. On the other hand, it is desirable to have transposition active in each cell for a short period of time, by controlling expression of the *tpase*, so as to minimise the chance of more than one transposon inserting into the genome in each cell. Given that the mutagenesis system using pPZ4 was active without induction by the inducer ATc, it was decided to determine if individual bacteria in the library contained multiple insertions of the transposon.

Genomic DNA was extracted from 39 randomly selected *S. aureus* transposon mutants that had been generated in the absence of ATc and analysed using Southern hybridization, as described in chapter 2 section 2.4.2. The hybridisation probe used for detecting the transposon in mutant DNA was a 400bp fragment containing part of the *ermC* sequence within the transposon. Figure 3.6 shows a representative image of the Southern hybridisation performed on 12 mutants. The bands correspond to the binding of probe to genomic DNA fragments from mutants, indicating the presence of a transposon in those fragments. Hybridisation bands were detected in all 39 mutants demonstrating that the transposon had inserted into the chromosome of *S. aureus* LS-1 or into the plasmid pLS-1 which it harbours. Furthermore, the distribution of the bands indicated that the transposon had inserted at different loci in the genome of each of the 39 mutants (data not shown). Out of these 39 mutants, two hybridisation bands were detected only in one of the mutants (lane 3), which had two insertions of the transposon. Thus the multiple-insertion rate of 1/39, without induction of expression of the transposase, was low and would only increase the library size need to be screened by a few hundred mutants.

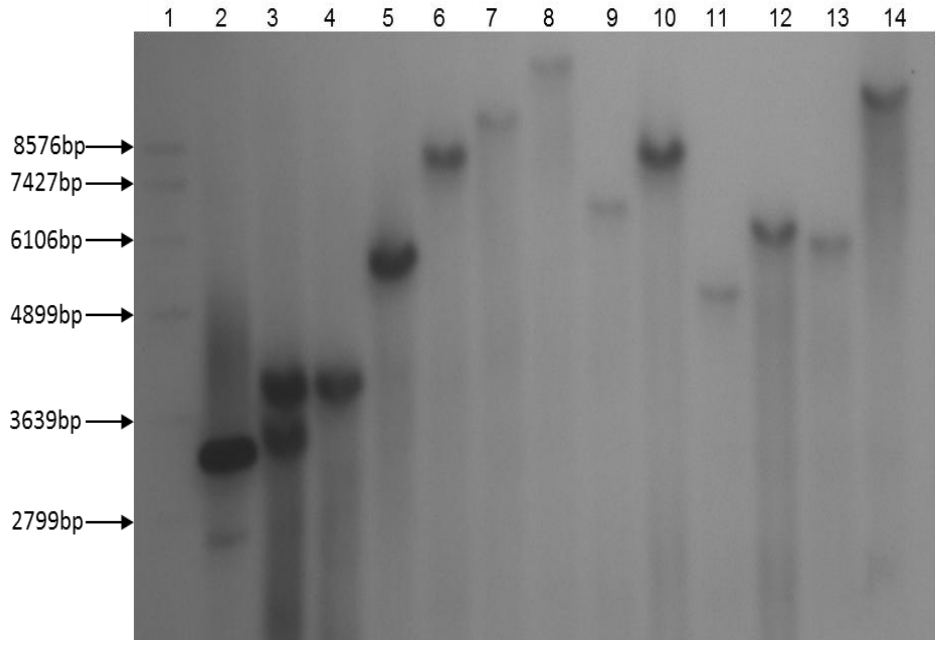

Figure 3.6: Southern blot image of HindIII digested chromosomal DNA from *S. aureus* LS-1 transposon mutants. Lane 1: digoxigenin-labeled DNA molecular weight marker (Roche). Lane 2: a transposon containing fragment excised from pPZ4, expected hybridisation band should be at 3.3kb. Lanes 3-14: *S. aureus* LS-1 mutants genomic DNA digested with HindIII.

3.5 Site-directed mutagenesis of pPZ4

Although pPZ4 could be used to generate a transposon mutant library, we had not achieved our original objective of constructing a tightly regulated *tpase* expression system. During the time when the work described above was being performed, Corrigan and Foster (2009) published a study demonstrating that the control of gene expression by the *Pxyl/tetO*, which had been widely regarded to be very tightly regulated, was in fact to a degree "leaky" (Corrigan and Foster, 2009). The basal expression of genes under the control of *Pxyl/tetO* was determined to be a consequence of low transcription of *tetR*, which in turn causes poor repression of the *Pxyl* promoter. The authors also found that the low transcription of *tetR* was due to an altered -10 sequence (tagagt) of the *tetR* promoter (P_{tetR}), which has two bases differing from the consensus -10 sequence in *B. subtilis* (tataat), the origin of the *Pxyl/tetO* promoter (Geissendorfer and Hillen, 1990).

In an attempt to improve the control of *Pxyl/tetO*, the -10 sequence of the P_{tetR} was modified from TA**G**A**G**T to TA**T**A**A**T in pPZ4. The two base pairs were changed by overlap extension PCR (Ho *et al.*, 1989) as illustrated in figure 3.7. It was known from

previous restriction digestion of pMC38 that there are two SphI restriction sites in pPZ4. As shown in figure 3.7, one is located in *pE194ts ori* and the other one is at the end of the *tpase*. A pair of outer-flanking primer P56 and P8 were designed to amplify a fragment with the two SphI sites at its ends. Primer P56 was designed 106bp upstream from the SphI site in *pE194ts ori*, and primer P8 was designed with the SphI site in the middle of the primer. Another two primers P34 and P35, which are complementary to each other, were designed at the -10 region of P_{tetR}, and had the desired two-base-pair mutation incorporated in.

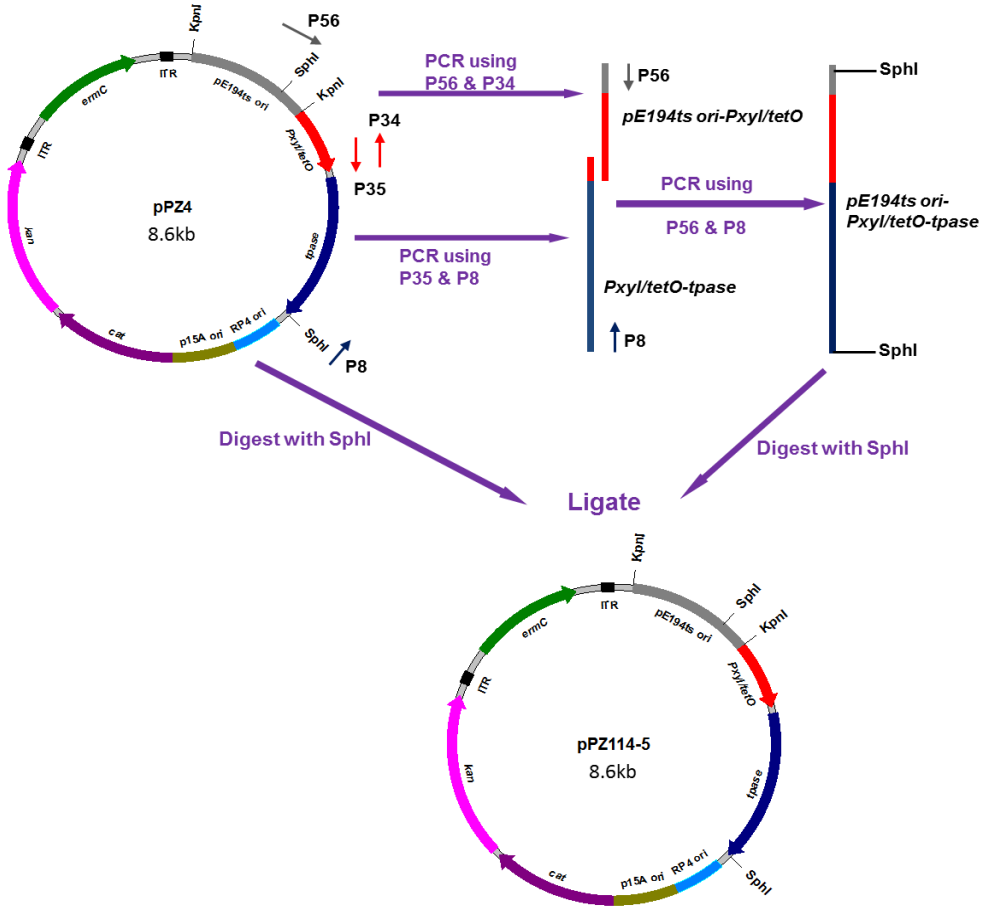

Figure 3.7: Schematic diagram of the strategy for site-directed mutagenesis of the -10 site of P_{tetR} in pPZ4 to give plasmid pPZ114-5 (not drawn to scale).

Two PCR products consisting of *pE194-Pxyl/tetO* and *Pxyl/tetO-tpase* were amplified using primers P56 / P34 and P8 / P35, respectively. The two PCR products were analysed by agarose gel electrophoresis and as shown in figure 3.8 lanes 2 and 3, they had the expected sizes at ~1.2kb (*pE194-Pxyl/tetO*, lane 2) and ~1.3kb (*Pxyl/tetO-tpase*, lane 3). These two PCR products were then fused together by overlap extension PCR using primers P56 and P8. The PCR product was verified to

contain *pE194 ts ori-Pxyl/tetO-tpase* by agarose gel electrophoresis showing a band at the expected size of approximately 2.5kb (figure 3.8, lane 6). Subsequently, the PCR product was digested with SphI and ligated into similarly digested pPZ4. *E. coli* DH5α was transformed with the ligation mix, and plasmids were purified from 6 transformants and designated pPZ114-1 to 6. These plasmids were digested with SphI to verify the presence of the insert, and with KpnI to determine the orientation of insert. Plasmids with insert were expected to release a fragment at ~2.4kb by SphI digestion, and if the cloned insert was in the desired orientation, digestion with KpnI would give a fragment of 1111bp representing *pE194ts ori*. All 6 plasmids were found to contain a 2.4 kb SphI-insert, (figure 3.8 lanes 8-13), whereas only pPZ114-5 and pPZ114-6 harboured the insert in the desired orientation (figure 3.8, lanes 19 & 20). The rest of plasmids either harboured the insert in the opposite orientation (lane 15, 16 & 18), or had two insertions of the SphI-fragment (lane 17). The -10 region of P_{tetR} in pPZ114-5 and pPZ114-6 were sequenced to confirm that they contained the desired point mutations (data not shown).

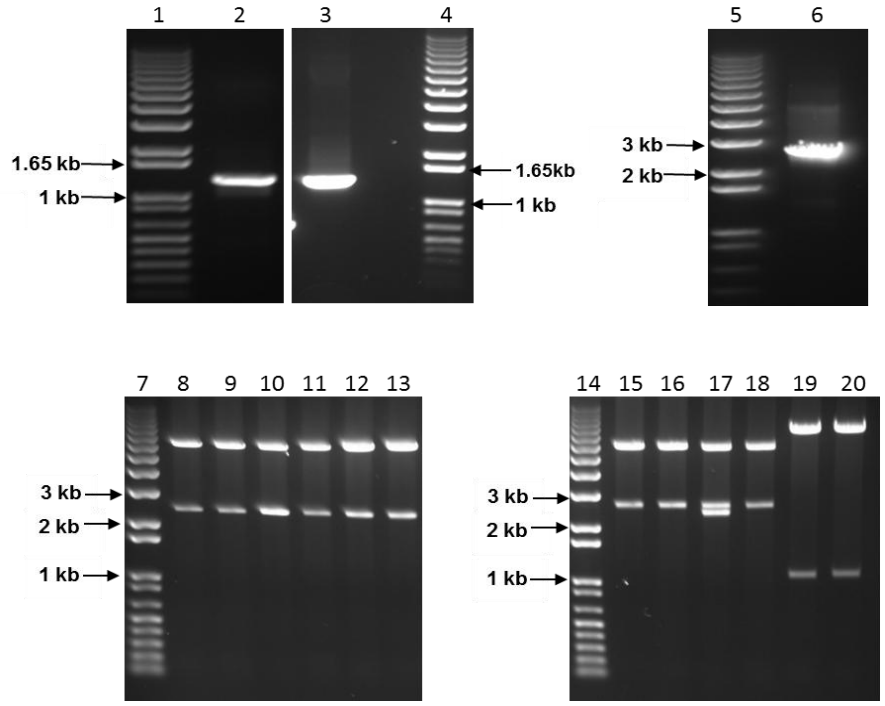

Figure 3.8: Gel electrophoresis photographs of PCR products and restriction digestions of plasmids used in the construction of pPZ114-5. Lanes 1/4/5/7/14: 1kb plus DNA ladder. Lane 2: PCR fragment containing *pE194ts ori-Pxyl/tetO* (expected size: ~1.2kb). Lane 3: PCR fragment containing *Pxyl/tetO-tpase* (expected size: ~1.3kb). Lane 6: PCR fragment containing *pE194ts ori-Pxyl/tetO-tpase* (expected size: ~2.5kb). Lanes 8-13: pPZ114-1 to 6 digested with SphI, desired clones were expected to show a band at ~2.4kb. Lanes 15-20: pPZ114-1 to 6 digested with KpnI, clones with insert in desired orientation were expected to show a band at 1111bp.

3.6 Basal transposition frequency using pPZ114-5

To examine whether the modification of *Pxyl/tetO* turns down the transposition frequency when no inducer was provided, transposition experiments using pPZ114-5 and pPZ4 were conducted in parallel as described in chapter 2 section 2.4.1, except that no ATc was added. The total number of cells, the number of mutants, transposition frequency and the plasmid retention rate in mutants were determined as described in chapter 2 section 2.4.1 and the results are presented in table 3.2.

Table 3.2: Data from transposition experiments without induction using transposon delivery vectors pPZ4 and pPZ114-5. The mean values from three independent experiments are presented with standard deviation included.

Plasmid	**pPZ4**	**pPZ114-5**
Total number of cells (× 10^8 CFU/ml)	8.86 ± 1.24	9.00 ± 1.08
Number of mutants (× 10^6 CFU/ml)	2.89 ± 0.73	0.15 ± 0.02
Transposition frequency (× 10^{-3})	3.24 ± 0.48	0.17 ± 0.01
Plasmid retention rate (%)	6.24 ± 2.64	6.75 ± 2.80

Transposition experiments with pPZ4 or pPZ114-5 had similar numbers of bacterial cells at the end of the experiment, suggesting that the growth of the cells was not influenced by the modification of *Pxyl/tetO*. The number of mutants generated using pPZ114-5 were 19-fold less than that generated using the old vector pPZ4, and there was also a 19-fold decrease in transposition frequency when using pPZ114-5 compared to pPZ4. These findings indicated that modification of *Pxyl/tetO* tightened the control of transposase expression and correspondingly reduced the transposition frequency in the absence of inducer. The plasmid retention rates in the mutants generated using pPZ4 and pPZ114-5 were at a similar level.

3.7 Transposon mutagenesis using pPZ4 or pPZ114-5

In order to compare the transposition frequency using pPZ4 and pPZ114-5 in the presence of inducer, transposition experiments using pPZ4 and pPZ114-5 were performed as described in chapter 2 at section 2.4.1. The total number of cells, the number of mutants, transposition frequency, and the plasmid retention rate was

determined as described in chapter 2 section 2.4.1 and the data are presented in table 3.3.

Table 3.3, Data from the transposition experiments using pPZ4 or pPZ114-5 induced by different concentrations of ATc. The mean values from three independent experiments are presented with standard deviation included.

pPZ4					
Conc. of ATc (μg/ml)	**0**	**0.5**	**1**	**1.5**	**2**
Total number of cells (× 10^8 CFU/ml)	9.78 ± 0.90	8.79 ± 0.57	7.64 ± 1.56	5.56 ± 0.92	1.42 ± 0.18
Number of mutants (× 10^6 CFU/ml)	3.61 ± 0.16	4.12 ± 0.65	4.77 ± 1.27	5.54 ± 1.15	7.28 ± 0.43
Transposition frequency (× 10^{-3})	3.7 ± 0.21	4.73 ± 1.05	6.69 ± 3.35	10.41 ± 3.97	51.43 ± 5.52
Plasmid retention rate (%)	4.51 ± 0.74	4.81 ± 0.78	5.19 ± 0.31	5.67 ± 0.27	4.16 ± 0.97
pPZ114-5					
Total number of cells (× 10^8 CFU/ml)	9.49 ± 0.75	9.16 ± 0.81	8.22 ± 1.07	5.79 ± 0.41	1.48 ± 0.1
Number of mutants (× 10^5 CFU/ml)	1.46 ± 0.22	2.18 ± 0.25	2.74 ± 0.72	4.32 ± 1.54	6.49 ± 3.36
Transposition frequency (× 10^{-4})	1.54 ± 0.27	2.41 ± 0.5	3.34 ± 0.71	7.51 ± 2.79	42.93 ± 19.3
Plasmid retention rate (%)	5.37 ± 1.12	5.53 ± 1.06	5.7 ± 0.53	5.24 ± 0.67	4.43 ± 0.16

Comparing the transposition experiments using pPZ4 and pPZ114-5 using different concentrations of ATc, it is clear that the total number of cells and the plasmid retention rate are at similar levels, suggesting that modification of *Pxyl/tetO* does not affect the growth rate of the cells and the efficiency of removing plasmid from the cells. The number of mutants produced using pPZ114-5 was 11 to 25-fold less than those produced using pPZ4 with the induction by different concentration of ATc. Correspondingly, the transposition efficiencies using pPZ114-5 were also decreased

compared to those using pPZ4. These data suggest that the modification of *Pxyl/tetO* not only reduces leaky expression, but also reduces the degree to which the promoter can be induced.

The data on total bacterial numbers recovered, as presented in tables 3.1 and 3.3 suggested, that ATc at 2μg/ml was inhibitory to growth. Indeed determining the MIC of ATc against *S. aureus* LS-1 pPZ114-5 as described in chapter 2 section 2.6.2 revealed the MIC to be 2μg/ml in three independent experiments. This meant that it would not be possible to try and induce expression of the *tpase* with higher concentrations of ATc and that the vector pPZ4 was superior to pPZ114-5 in the sense that it gave 10-fold higher mutant numbers. Therefore it was decided to concentrate on pPZ4 and determine the optimum conditions that produce sufficient transposition frequency and a low frequency of multiple insertions of the transposon in individual cells.

3.8 The frequency of multiple insertions of the transposon in mutants generated using pPZ4

As presented in table 3.1 and 3.3, the transposition frequencies using pPZ4 with induction by 0, 0.5, 1 and 1.5μg/ml ATc were similar, suggesting that the level of expression of the transposase under these conditions may be similar, which in turn could lead one to assume that the frequency of multiple insertions within the mutants would also be similar. To determine if this was the case, the frequency of multiple insertions of the transposon into the genome of mutants was determined using concentrations of 0, 1 and 2μg/ml of ATc for induction of expression of the *tpase*.

A strategy involving Y-linker PCR (Kwon and Ricke, 2000), described in chapter 2 section 2.4.3, was used to assess the number of transposon insertions present in each mutant. Prior to the use of the Y-linker PCR method, six mutants, which had previously been analysed by Southern hybridisation (described in section 3.4) were selected to test this approach. The Y-linker PCR was conducted as described in

chapter 2 section 2.4.3, and a gel electrophoresis image of the PCR products is shown in figure 3.9. As expected, the mutants which had only one insertion of the transposon in the genome, as determined by Southern hybridisation, gave a single PCR product (lane 3-7), and the mutant with two insertions of the transposon showed two PCR products (lane 2). This data proved that the Y-linker PCR strategy is capable of detecting the presence of transposons in mutant DNA, as well as determining if more than one transposon has inserted into the genome.

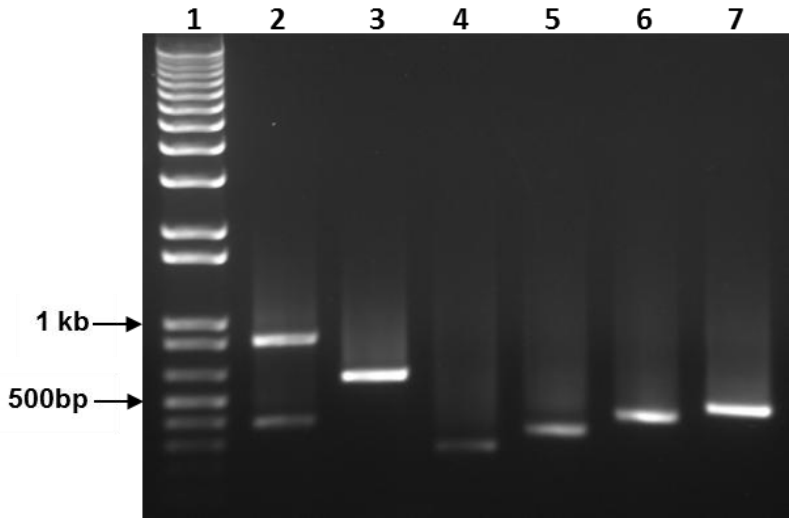

Figure 3.9: the number of transposon insertions determined by Y-linker PCR in 6 mutants. Lane 1: 1kb plus DNA ladder. Lane 2: Y-linker PCR product from a known two-insertion mutant. Lanes 3-7: Y-linker PCR products from mutants with single insertions of the transposon.

Subsequently, 100 transposon mutants generated using pPZ4 with induction using 0, 1 or 2µg/ml ATc were analysed using this strategy. As this data involves a large number of gel images, only a representative image is shown in figure 3.10. Lanes 2-20 exhibit Y-linker PCR products from 19 mutants generated using pPZ4 with induction by 1µg/ml ATc, in which one of the mutants showed two insertions of transposon (lane 7).

The number of mutants with single or multiple transposon insertions and the frequency of multiple insertions for each concentration of ATc are summarised in table 3.4. All three concentrations of ATc gave frequencies of multiple insertions that seemed to be similar, and for practical purposes at least would not make a significant difference to generating a transposon mutant library.

Table 3.4: The number of mutants with one, two or three transposon insertions and the multiple-insertion rate for transposition sample using pPZ4 induced by 0, 1 or 2μg/ml ATc.

Samples	Number of mutants			Multiple-insertion rate
	Single insertion	Two insertions	Three insertions	
No induction	95	5	0	5%
1 μg/ml ATc	95	4	1	5%
2 μg/ml ATc	94	3	3	6%

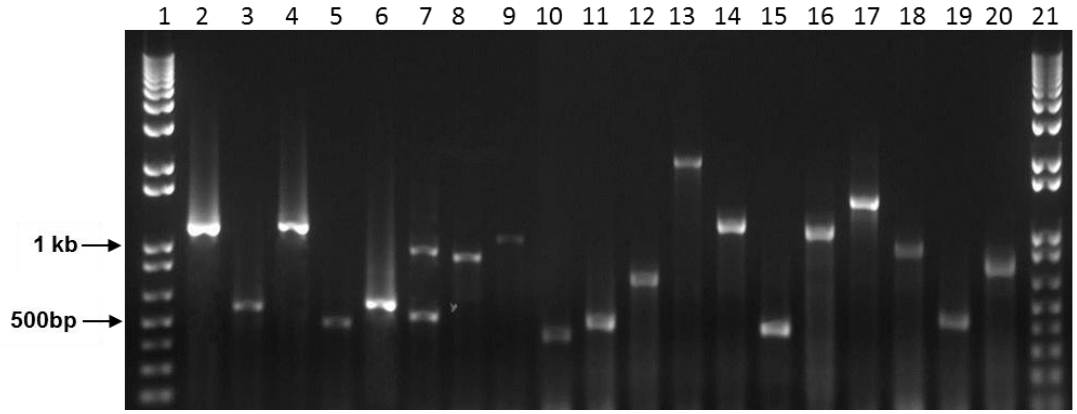

Figure 3.10: Gel electrophoresis image of Y-linker PCR products from 19 mutants generated using pPZ4 with induction by 1μg/ml ATc (lane 2-20). Lane 1: 1kb plus DNA ladder.

3.9 The frequency of siblings in mutants generated using pPZ4

A desired transposon mutant library requires not only mutants with single transposon insertions, but also a sufficient diversity of mutants so that the whole bacterial genome can be covered. Therefore the 100 transposon mutants generated using pPZ4 induced by 0, 1 or 2μg/ml of ATc used for determination of the frequency of multiple transposon insertions, were analysed to determine the occurrence of siblings. To achieve this goal the Y-linker PCR strategy described earlier was adapted as shown in figure 3.11. Simply, another transposon specific primer (Tn 2) was designed facing in the opposite direction to the original transposon specific primer (Tn 1). PCR was then performed using Tn 1 / Y-linker primer, generating PCR product 1, and Tn 2 / Y-linker primer generating PCR product 2. To determine if mutants were siblings, the sizes of PCR product 1 and PCR product 2 were compared for all of the

transposon mutants and when the sizes of both of these products were the same it indicated that such mutants were probably siblings.

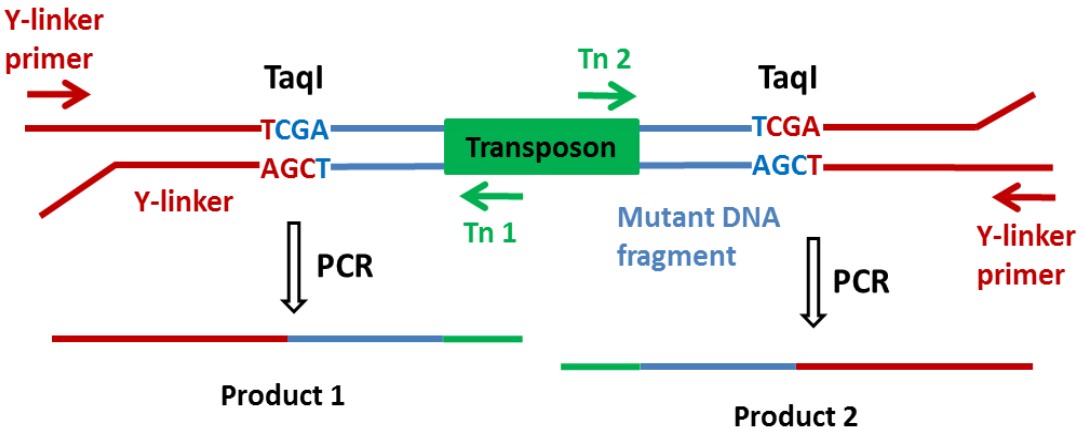

Figure 3.11: Graphical illustration of Y-linker PCR strategy for the determination of the possibility that mutants were siblings. Red: Y-linker DNA. Blue: *S. aureus* LS-1 mutant DNA fragment. Green: transposon. Y-linker primer: a primer designed on one of the branch of the Y-linker DNA. Tn 1 and Tn 2: primers specific to transposon sequence.

Examination of the 100 mutants produced at each concentration of ATc revealed, 13 pairs of probable siblings when ATc was absent, 3 pairs of siblings with 1µg/ml ATc and 13 pairs of siblings with 2µg/ml ATc (gel images are not shown). It was not possible to determine if the difference in the frequency of siblings generated in each library was significant, because of the huge amount of work involved in repeating the experiments in an adequate number of mutants. It was therefore decided to select induction with 1µg/ml ATc for generating a large scale *S. aureus* LS-1 transposon mutant library since it might contain fewer siblings than a library generated without ATc or with 2µg/ml.

3.10 Large scale construction of a *S. aureus* LS-1 transposon mutant library

In order to generate a transposon mutant pool without having to laboriously screen single colonies on agar plates, the transposition protocol was modified slightly for the purpose of producing a large number of mutants and also to minimise the number of bacteria retaining the transposon delivery plasmid. The modified method is described in chapter 2 section 2.4.4.1. The main modifications were as follows:

increasing the volume of the medium 10-fold while maintaining the number of bacteria used and prolonging the time at which the bacteria were incubated at 43°C (non-permissive for plasmid replication) from 6 hours to 9 hours. Keeping the bacterial cell number the same as in previous experiments described in section 3.3, it was expected that similar numbers of transposon mutants would be generated. Increasing the incubation time at 43°C from 6 hours to 9 hours should allow the cells to divide through more generations before reaching stationary phase, so as to increase the portion of plasmid-free cells.

To evaluate the transposition frequency and the proportion of plasmid-free mutants generated using this modified protocol, the total number of cells, the number of mutants, transposition frequency, and the plasmid retention rate was determined as described in chapter 2 section 2.4.4.1 and the data are presented in table 3.5. The transposition frequency using the modified protocol was similar to that using the old protocol, because the same conditions were used for inducing the transposition in both protocols (1µg/ml ATc for 1 hour at 30˚C). As expected, the plasmid retention rate reduced greatly (16-fold) using the modified protocol, giving 99.6% of the mutants being plasmid free. To assess the number of individual mutants generated using this system, the number of mutants after 1 hour ATc induction at 30˚C was considered as the minimum number of individual mutants in the culture, which was determined to be $(3.3 \pm 0.5) \times 10^5$ cells.

Table 3.5: Data from transposition experiments using transposon delivery vectors pPZ4 with induction by 1µg/ml ATc. The mean values from three independent experiments are presented with standard deviation included. (To compare to the data generated using the old protocol, data from table 3.1 with 1µg/ml ATc is included in the table)

Plasmid	**Old protocol**	**Modified protocol**
Total number of cells ($\times 10^8$ CFU/ml)	6.33 ± 0.04	3.2 ± 0.1
Number of mutants ($\times 10^6$ CFU/ml)	4.47 ± 0.84	3.5 ± 0.9
Transposition frequency ($\times 10^{-3}$)	7.13± 1.81	10.9 ± 1.23
Plasmid retention rate (%)	5.75 ± 1.19	0.35 ± 0.1

By using this modified transposition protocol, a large number of individual mutants were generated, and a very low plasmid-retention rate was also achieved. However, as shown in figure 3.5, at the end of the experiments, the major cell population was wild-type cells, with only 1.1% of them being mutants. To generate a mutant library with the majority of the viable bacteria being mutants, an additional mutant enrichment step was performed by spreading the culture on TSA + Erm and incubating at 43°C (described in chapter 2 section 2.4.4.3). These culturing conditions were expected to inhibit the wild-type with or without plasmid pPZ4, only allowing the mutants to grow. The growth on TSA + Erm was harvested as a mutant library and the numbers of different cell population in this library were determined as described in chapter 2 section 2.4.4.3. These numbers were compared to those before plating on TSA + Erm to assess the effect of the mutant enrichment step, and are presented in table 3.6.

As expected, the mutant enrichment step increased the mutant population from 1.8 ± 0.2% to 81.7 ± 0.7%. Surprisingly, the plasmid-retention rate in the populations also increased dramatically by approximately 26-fold. In spite of this, the plasmid free mutants still constituted approximately 75% of the cell population.

Table 3.6: Data from transposition experiments before (0 hour) and after (24 hour) the mutant enrichment step. Number of cells from 1ml of the transposition culture (0 hour), and the number of corresponding cells after growth on agar (24 hours) is shown. Data is from one experiment, and the mean values from three technical replicates are presented with standard deviation included.

Plasmid	**0 hour**	**24 hours**
Total number of cells	$(2.6 \pm 0.3) \times 10^8$	$(9.8 \pm 0.8) \times 10^{11}$
Number of mutants	$(4.7 \pm 0.2) \times 10^6$	$(8.0 \pm 0.5) \times 10^{11}$
Proportion of mutants	(1.8 ± 0.2)%	(81.7 ± 0.7)%
Plasmid retention rate (%)	(0.3 ± 0.1)%	(7.8 ± 0.4)%

It was hypothesised that the huge increase of plasmid-retention rate at non-permissive temperature may have resulted from temperature-permissive mutations in *pE194ts ori* in the plasmid pPZ4. To examine this hypothesis, the mutant library was plated on TSA + Kan and incubated at both 30°C and 43°C. A similar number of colonies were found growing on TSA + Kan at 30°C and 43°C, and the size of these colonies were the same as that of LS-1 cultured on TSA at the same temperature (data not shown). This demonstrated that the cells containing plasmid could also grow rapidly at the non-permissive temperature in the presence of kanamycin, which selects for the presence of plasmid, indicating that the plasmids in these clones have temperature-permissive mutations. This phenomenon has also been found in another two *S. aureus* mutant libraries that we have generated using the same method, suggesting that temperature-permissive mutations are not uncommon and can be selected for under the pressure of antibiotic selection and the non-permissive temperature 43°C.

In order to determine whether the individual mutants in this library is sufficient to cover the whole genome of *S. aureus* LS-1, the minimum number of individual mutants was considered as that after 1 hour ATc induction at 30˚C, which was determined to be $(4.9 \pm 0.9) \times 10^5$ mutants. Using this number of individual mutants in the library, the frequency with which the transposon inserted into the genome was calculated as described in chapter 2 section 2.4.4.2. When constructing a mutant library, the more individual mutants present in the library, the higher the probability that there will be full genome coverage. In this study the *S. aureus* NCTC8325 genome (GenBank accession CP000253), which has a size of 2,821,361bp containing 3006 genes (average gene size 939bp), was taken as a reference for determining genome coverage by 4.9×10^5 individual mutants. Figure 3.12 shows the change in the frequency at which the genome would be inserted into by the transposon when the probability of whole genome coverage increases from 99% to 99.99999%. For example, if a 99.9% probability of full genome coverage is chosen, then theoretically every 40bp of the genome would harbour a transposon insertion, which means each gene in *S. aureus* NCTC 8325 (average size = 939bp) would be inserted into 23 times. Even when a probability of 99.99999% is chosen, theoretically every 93bp of the

genome would be inserted into by the transposon, meaning that each gene would be inserted into 10 times. This data suggested that the number of individual mutants in this library was sufficient to cover the whole genome of *S. aureus* with a very high probability if the transposon inserts randomly into the chromosome.

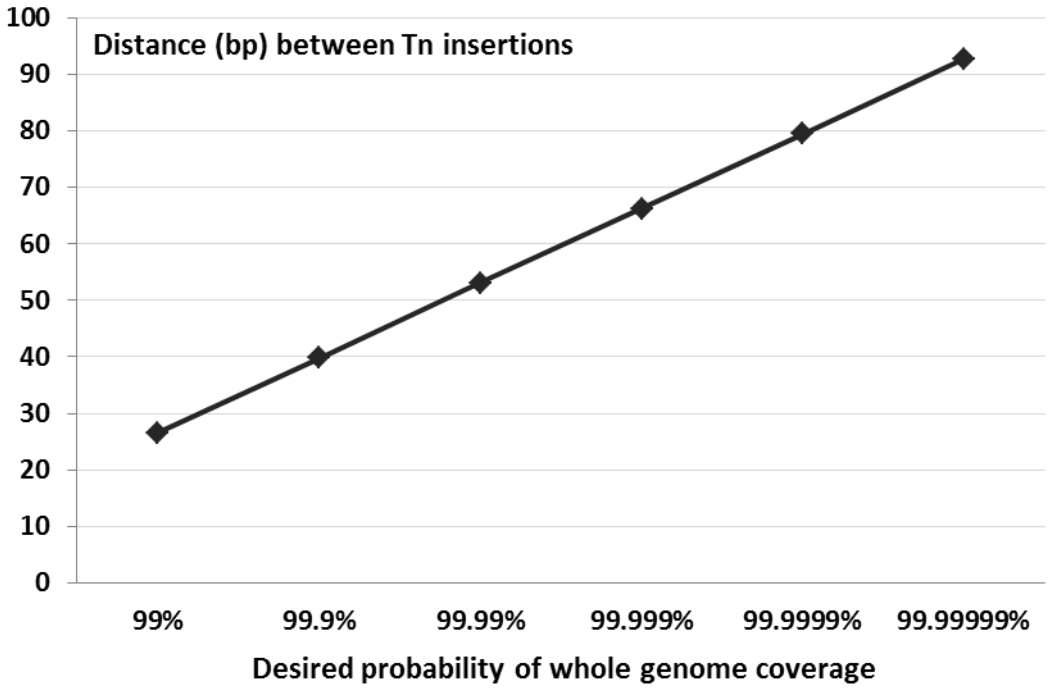

Figure 3.12: The relationship between the desired probability of whole genome coverage and the frequency the *S. aureus* NCTC 8325 genome would be inserted into by the transposon (Tn).

3.11 Discussion

The *Himar1* mariner transposon and its specific transposase have been successfully employed in *S. aureus* for the construction of a mutant library which theoretically gives full coverage of the whole genome. By using an inducible promoter *Pxyl/tetO* for the control of *tpase* expression, the frequency of multiple insertions of the transposon into the genome of *S. aureus* was determined to be 5-6% with induction by 0-2µg/ml ATc, which is lower than that observed with pMC38 system in *L. monocytogenes* (16%) (Cao *et al.*, 2007). However, this could be due to differences in the genomes or physiology of *S. aureus* and *L. monocytogenes.*

In order to try and temporally regulate transposition, a *Pxyl/tetO* promoter derived from vector pKOR1 was used to modulate the expression of the transposase gene in

plasmid pPZ4. However this promoter was found to allow basal expression of the transposase in the absence of inducer. Furthermore, the basal expression of transposase was sufficient to catalyse transposition events and generate a large number of mutants in *S. aureus*. Although the *Pxyl/tetO* promoter has been widely described as being tightly regulated, the promoter was suggested to allow basal expression more than 10 years ago (Zhang *et al.*, 2000), and this problem has been reported more recently (Lei *et al.*, 2011). In 2009, a study on this promoter was published with a description of the reason why this promoter is not tightly controlled. Two point mutations at the -10 site of P_{tetR} result in low expression of *tet* repressor, which in turn leads to insufficient binding of the *tet* operator and thereby incomplete suppression of the promoter *Pxyl*. The authors also demonstrated that modifications at the -10 site of P_{tetR} improved the tightness of the control (Corrigan and Foster, 2009). Modification of the -10 sequence of P_{tetR} in *Pxyl/tetO* in PZ114-5 resulted in a 19-fold decrease in the transposition frequency in the absence of inducer ATc. However inducing the *tpase* using concentrations of ATc as high as 2µg/ml, did not increase the frequency of transposition to levels seen with plasmid pPZ4. According to the study described by Corrigan and Foster (2009), 0.64µg/ml ATc was able to induce the expression of *sasG*, under the control of the modified *Pxyl/tetO*, to 8 fold higher than the basal expression of *sasG* seen with the unmodified *Pxyl/tetO* promoter. In contrast, in our transposition experiments, the transposition frequency using pPZ114-5 with induction by 2µg/ml ATc hardly achieved the basal transposition frequency seen using pPZ4 without induction. However the transcriptional activity of *Pxyl/tetO*, or the expression level of transposase, can not necessarily be linked to the transposition frequency or the number of mutants being generated, as transposition frequency using mariner transposons has been found to be inhibited at high concentrations. For example, the optimum transposition frequency of the *Himar 1* mariner transposon *in vitro* was found when the concentration of transposase was in a narrow range around 10nM (Lampe *et al.*, 1998). In addition, over-expression of the transposase was also found to result in reduced transposition frequency of the mariner transposon *MosI* in *Drosophila* (Lohe and Hartl, 1996).

A transposon mutant library in *S. aureus* LS-1 has been generated using a modified vector based on that described by Cao, *et al*. (2007). This library was characterised to be sufficient to cover the whole genome of *S. aureus* NCTC8325 with a very high probability. By employing an extra mutant enrichment step on agar, the proportion of transposon mutants in the library was increased by 45-fold from 1.8% to 81.7%. One unexpected outcome of the enrichment step was that the proportion of plasmid-containing population was also increased by 26 fold, from 0.3% to 7.8%. However, this still gave a mutant library with 75% of the cells being plasmid free mutants. Therefore this transposon mutagenesis system represents an efficient and high-throughput method for generating transposon mutant libraries for *S. aureus*. This system has been used in the Nair lab (personal communications) to produce transposon mutant libraries of *Enterococcus faecium* and therefore may be applicable to a range of Gram-positive bacteria.

Chapter 4: Construction and Characterisation of a R-IVET System

4.1 Introduction

When studying bacterial virulence determinants and the mechanisms of infectious processes, much of our knowledge comes from experiments conducted under *in vitro* conditions designed to reflect certain aspects of the situation *in vivo*. For example, specific culture media mimicking the physiological properties of host fluids can be used to characterise the survival, growth and pathogenesis of bacteria (Brown *et al.*, 2008). Host antimicrobial components can be supplemented into *in vitro* assays to study the mechanism of bacterial resistance (Li *et al.*, 2007a). In addition, tissue cultures of specific mammalian cells are often used to examine the interaction between bacteria and specific host cell types that bacteria encounter during an infection (Quinn *et al.*, 1996).

Despite the fact that *in vitro* assays have contributed enormously to our understanding of bacterial physiology and pathogenesis, this knowledge cannot be unequivocally applied to *in vivo* conditions, because they do not exactly reproduce the complex host conditions that a bacterium may encounter. During an infection, bacteria may experience a variety of host conditions temporally and spatially. In order to survive and replicate in multiple host environments, bacteria have to produce very different virulence determinants at each time point (Smith, 1998). As a result, the virulence determinants identified under *in vitro* conditions are not necessarily important when the bacteria are exposed to *in vivo* conditions. Conversely, genes that seem dispensable for *in vitro* conditions may play a critical role during a real infection. The levels of bacterial gene expression may also be very different *in vitro* and *in vivo*. One example from *S. aureus* is the *in vitro* and *in vivo* studies on the staphylococcal accessory regulator (Sar). The *sar* locus is composed of three overlapping transcripts, *sarA*, *sarB* and *sarC*, and three distinct promoters P1, P2 and P3 direct the synthesis of these three transcripts. The relative strength of these three promoters was evaluated when *S. aureus* was growing in TSB using transcriptional

fusion with a reporter gene *xylE*, encoding a catechol dioxygenase. The promoter P1 demonstrated the strongest activity, with approximately 50-fold more activity than P2 or P3 (Manna *et al.*, 1998). However, when investigated in a rabbit endocarditis model using a similar transcriptional fusion method, promoter P2 was found to be highly activated compared to P1 in cardiac vegetations (Cheung *et al.*, 1998).

To determine solutions for the prevention or treatment of *S. aureus* infections, it is useful to understand how the bacterium behaves when encountering the host environment. Over the last three decades, there has been an increasing interest in what bacterial genes are expressed *in vivo*. A range of methods for monitoring bacterial gene expression *in vivo* have been developed, such as *in vivo* expression technology (IVET), differential fluorescence induction (DFI), signature-tagged mutagenesis (STM) and DNA microarrays (Hautefort and Hinton, 2000). Among these methods, two strategies have been widely used due to their simplicity and their high throughput nature. These are IVET, designed to identify conditionally-induced bacterial promoters, and STM which enables the identification of genes important for survival or virulence *in vivo* based on comparative hybridization of the input and output libraries (Chiang *et al.*, 1999).

IVET and STM are both potent strategies for large-scale identification of virulence determinants which bacteria express *in vivo*. IVET is a promoter trap strategy based on the transcriptional fusion of bacterial promoters to a reporter gene, thereby identifying promoters that are activated under specific conditions such as in animal models (Angelichio and Camilli, 2002). STM is a transposon mutagenesis-based technology that uses a transposon containing a tag sequence that allows identification of the transposon. The transposon mutants generated for STM are typically subjected to screening in animal models or cell lines to identify genes involved in survival or pathogenesis under those conditions (Mazurkiewicz *et al.*, 2006). The STM method is generally not as simple as IVET strategies, and more importantly, STM is not able to identify virulence genes that are important *in vivo* if they are essential for bacterial survival *in vitro* because mutations in such genes are lethal to the bacteria. To complement the transposon mutagenesis-based strategy

developed as described in chapter 3, it was decided to try an IVET-based method for the screening *S. aureus in vivo*-induced (*ivi*) genes.

Since the first use of IVET in the 1990s, this technology has been adapted in various ways to simplify the process and increase the sensitivity of the screening. The original IVET method used a purine auxotroph of *S.* Typhimurium, which is not able to survive in the murine host unless the strain was complemented with the *purA* gene. In this system random *S.* Typhimurium chromosome fragments were fused with a promoter-less *purA* gene in a plasmid to form a vector library, which was then integrated into the *S.* Typhimurium Δ*purA* chromosome through single crossover recombination. The resulting pool of strains was then screened in BALB/c mice to identify those able to survive within the host. A number of *in vivo*-induced (*ivi*) promoters were identified, and defined mutants of several *ivi* genes were assayed in BALB/c mice and found to have severe virulence defects, validating the capacity of the method to identify genes important for survival *in vivo* (Mahan *et al.*, 1993). However obvious limitations of this method are that it requires an auxotroph which can be complemented, which may not always be readily available, and furthermore that expression of *purA* may be disregulated under the control of a foreign promoter which could produce pleiotropic effects.

To avoid the use of auxotrophs as model strains, the method was modified by using antibiotic resistance markers as reporter genes. Similar to the principle described above, chromosome fragments were fused with an antibiotic marker, and the antibiotic was administered to the host during infection (Mahan *et al.*, 1995). This method requires that the antibiotic administered to the animal model is tolerable and can penetrate to the site of infection. Once this requirement is met, this method can be flexibly adapted to identify *ivi* genes during different stages of infection by administering antibiotic at specific times. This strategy was successfully applied in *Yersinia enterocolitica* infection models, with different genes identified during an early stage of infection in Peyer's patches (Young and Miller, 1997) and a later stage of infection in the liver and spleen (Gort and Miller, 2000).

Although efficient for the identification of *ivi* genes, both of the IVET strategies described above share a disadvantage in that they may not be adequately sensitive to identify genes that are expressed transiently or at a low level, situations in which the selection markers driven by heterologous promoters may not be expressed at an adequate level to maintain the growth of the strain under the selection pressure. To address this problem, a recombination-based *in vivo* expression technology (R-IVET) was developed by using resolvase as the reporter. Resolvase is an enzyme that catalyses irreversible recombination between specific DNA sequences, such as inverted terminal repeats in transposons. When designing an R-IVET screening system, an antibiotic resistance gene, flanked by the resolvase recognition sequences is often introduced into the bacterial chromosome. The expressed resolvase driven by *ivi* promoters is able to permanently resolve the antibiotic marker from the bacterial chromosome, conferring antibiotic-sensitivity back to the strain. The resolution of the antibiotic reporter from the chromosome only requires a small quantity of resolvase, rendering high sensitivity to the R-IVET strategy for the identification of genes expressed at low levels or transiently expressed. Furthermore, R-IVET does not require exposing the bacteria or host to antibiotics during the screening process, thus there is no antibiotic pressure which might interfere with bacterial gene expression (Angelichio and Camilli, 2002).

R-IVET has been used for a number of pathogens to identify virulence genes expressed *in vivo*, including *S. aureus*. For example, Lowe *et al.* (1998) employed a promoter-less *tnpR* (transposon resolvase) as the reporter gene, which encodes a resolvase from the *E. coli* transposon γδ. A kanamycin resistance marker was placed in between the transposon direct repeats serving as resolution site (*res*), and the *res-kan-res* cassette was integrated into the *S. aureus* chromosome. Plasmids containing fusions of *S. aureus* chromosome fragments and the promoter-less *tnpR* were then introduced into this strain. Any *S. aureus* chromosome fragments containing activated promoters leads to the expression of *tnpR*, the product of which in turn acts on the *res* sites and results in the resolution of *res-kan-res* from the chromosome, rendering the strain sensitive to kanamycin. This system was used to identify genes being induced during infection in a murine renal abscess model. A total of 45

staphylococcal genes were induced during infection, including the well-studied virulence gene *agrA*. The novel genes identified were also examined by constructing defined mutants. Seven out of eleven mutants were determined to have significantly reduced virulence in the murine renal abscess model (Lowe *et al.*, 1998). R-IVET is efficient in identifying *ivi* genes, but not all the genes identified are associated with virulence. Therefore, further virulence tests are required to confirm that the genes are involved in virulence.

The R-IVET method described above requires counter-selection using replica plating to identify cells that lose *res-kan-res*. In order to simplify the screening process, the method has been modified further by including another reporter gene, such as a galactosidase-encoding gene, in between the recombination sites to further simplify the process. An established example is the *cre-loxP* system described by Bachmann *et al.* (2008) as shown in figure 4.1. The *cre-loxP* recombination system was derived from the P1 bacteriophage, where *cre* encodes a recombinase which catalyses the recombination between the target sites *loxP* (locus of X-over in P1). The *loxP* recombination site is a 34-bp DNA sequence containing two 13bp inverted repeats flanking an 8bp spacer region. The recombination between *loxP* sites only relies on the recombinase Cre, and leads to irreversible excision of any DNA substrates flanked by *loxP* sites (Sternberg and Hamilton, 1981). The *cre-loxP* system shown in figure 4.1 was designed for the use in *Lactococcus lactis*. A *loxP* cassette with two *loxP* sites flanking two reporter genes *ermB* and *melA* was integrated into *L. lactis* chromosome. Gene *ermB* encodes resistance to erythromycin and *melA* encodes α-galactosidase which catalyses the hydrolysis of 5-Bromo-4-Chloro-3-Indolyl-α-D-galactopyranoside (X-α-gal) to form an insoluble blue pigment 5,5'-dibromo-4,4'-dichloro-indigo, causing a blue colour colony phenotype on X-α-gal containing medium. A promoter-less *cre* gene was cloned into plasmid pPZ5520 as shown in figure 4.1, and *L. lactis* genomic fragments were cloned upstream of *cre*, resulting in a plasmid library of pNZ5520 derivatives containing fusion of *L. lactis* genomic fragments and *cre*. This plasmid library was then introduced into the strain containing the *loxP* cassette to screen for conditionally induced promoters. The activated promoters drive the expression of Cre, which in turn catalyses the resolution of the *loxP* cassette from the chromosome,

rending the strain erythromycin-sensitive and white-colony phenotypes. With *melA* present in the *loxP* cassette, the recovered colonies can be screened by either counter-selection using erythromycin or directly by visualisation of the blue or white colour of the colonies on medium containing X-α-gal.

In addition to allowing direct selection of colonies by colour visualisation, another function of this system is that it is able to quantitatively assess the activity of promoters cloned upstream of *cre*. As shown in figure 4.1, the *luxAB* genes, encoding luciferase, were placed downstream of *cre*. Luciferase is an enzyme that catalyses the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aldehyde in the presence of oxygen, yielding flavin mononucleotide (FMN), a corresponding carboxylic acid, water and light with a peak wavelength at 490nm. The production of bioluminescence can be measured at 490nm in the presence of $FMNH_2$ and the aldehyde to determine the activity of luciferase which is relative to the expression of *luxAB* genes (Nelson *et al.*, 2007). Thus, *luxAB* serves as useful reporter gene to assess the strength of the promoters under different conditions.

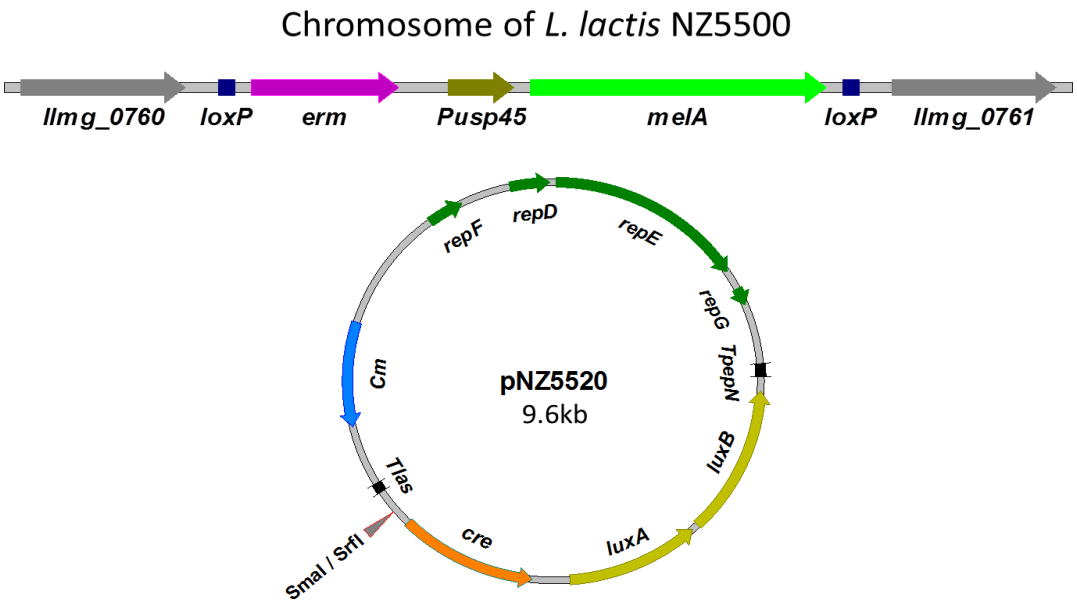

Figure 4.1: Schematic map of the R-IVET system designed by Bachmann et al. (2008) (not drawn to scale). A *loxP* cassette *loxP-ery-Pusp45-melA-loxP* was integrated between loci *llmg_0760* and *llmg_0761* in *L. lactis* MG5267 resulting strain NZ5500. A plasmid pNZ5520 containing a promoter-less reporter genes *cre, luxA* and *luxB* was designed for generating the promoter-reporter gene fusions. The SfrI/SmaI site upstream of *cre* was used for the insertion of *L. lactis* chromosomal fragments. Terminators *Tlas* and *TpepN* were placed flanking the SmaI/SrfI site, *cre* and *luxAB*. Picture is modified from Bachmann *et al.* (2008).

This chapter describes the construction and characterisation of a R-IVET screening system similar to that designed by Bachmann *et al*. (2008) for use in *S. aureus*. Such a system should be useful for screening *S. aureus ivi* genes in animal models. However, it is also applicable for screening genes induced under different *in vitro* environments, such as an environment containing LL-37. Screening the genes induced in the presence of LL-37 could contribute to our understanding of the molecular mechanism of *S. aureus* resistance to LL-37, as well as the mechanism of sensing and regulating this resistance.

4.2 Determination of *Pusp45-melA* activity in *S. aureus*

In order to ascertain whether the *loxP* cassette *loxP*-erm-*Pusp45-melA-loxP* described by Bachmann *et al*. (2008) could be directly applied for use in *S. aureus*, the activity of each component in this cassette was assessed. From published studies, it was known that two components of the system, *cre-loxP*, and the antibiotic resistance marker *ermB* are functional in *S. aureus* (Nicola *et al.*, 1998; Leibig *et al.*, 2008). While the activity of *Pusp45* or *melA* in *S. aureus* had not been reported at the time this work was performed. To determine the activity of *Pusp45* and *melA* in *S. aureus*, the fusion of *Pusp45-melA* was cloned into the multiple cloning sites in pSK236 as described in chapter 2.3.1, generating plasmid pPZ111-1 as shown in figure 4.2.

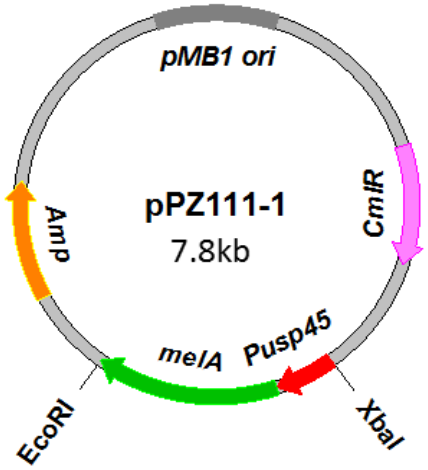

Figure 4.2: Plasmid map of pPZ111-1 (not drawn to scale), a pSK236 derivative with *Pusp45-melA* cloned into the multiple cloning sites. *pMB1 ori*: Gram negative replication origin. *pC194 ori*: Gram positive replication origin. *Amp:* ampicillin-resistance gene for Gram negative selection. *Cm*: chloramphenicol-resistance gene for Gram positive selection.

Figure 4.3 shows an image of the agarose gel electrophoresis of a PCR product containing *Pusp45-melA* (lane 2) with the expected size of 2.5kb, and the EcoRI-XbaI restriction digestions of potential plasmids containing the insert (lanes 4-6) which all exhibit the expected digestion pattern with two fragments at sizes ~6kb and 2.5kb, representing the backbone of pSK236 and the insert containing *Pusp45-melA*, respectively. The resulting vector, designated pPZ111-1, was introduced into *S. aureus* RN4220 via electroporation, and transformants were grown at 37°C on TSA containing 10μg/ml chloramphenicol and 40μg/ml X-α-gal. A large number of colonies appeared after 24 hours incubation, but none were blue, suggesting that *Pusp45-melA* is not functional in *S. aureus*.

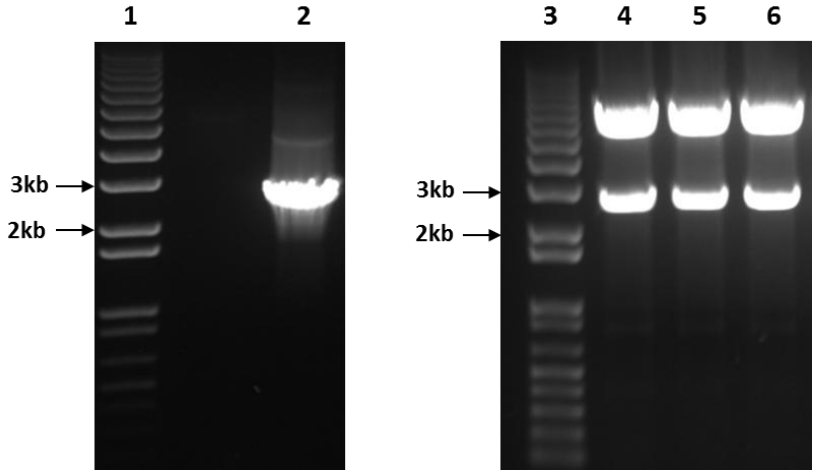

Figure 4.3: Gel electrophoresis images involved in cloning *Pusp45-melA* into pSK236. Lane 2: PCR product containing *Pusp45-melA* (expected size 2.5kb). Lanes 4-6: XbaI-EcoRI digestion of potential plasmids with the insertion of *Pusp45-melA* (expected digestion pattern: two fragments with sizes of 5569bp and 2500bp). Lanes 1 & 3: 1kb plus DNA ladder (Invitrogen).

4.3 Construction of *loxP* cassette *loxP-erm-Pspac-lacZ-loxP*

Since *Pusp45-melA* is not functional as a blue-white screening marker in *S. aureus*, it was decided to replace it with an alternative reporter *Pspac-lacZ*. The inducible promoter and repressor *Pspac-lacI* has previously been used in *S. aureus* to evaluate the essentiality of staphylococcal genes (Jana *et al.*, 2000). This inducible promoter contains a promoter *Pspac* which is inhibited by the repressor encoded by *lacI*. However the inhibition is relieved in the presence of IPTG, which binds to the LacI repressor and inactivates it. Given that an inducible property is not desired for the expression of marker in the *loxP* cassette, only the promoter *Pspac* region was chosen

for cloning as this should drive constitutive expression of the gene under its control. The gene *lacZ*, which encodes β-galactosidase, has previously been used in *S. aureus* by fusing to the *agr* and *sar* promoters as a reporter to determine the expression of these two genes in biofilms (Pratten *et al.*, 2001), demonstrating that it is active in *S. aureus*. Employing the fusion of *Pspac-lacZ* in the *loxP* cassette should allow for blue-white screening to identify *S. aureus* with or without the *loxP* cassette on media with X-gal, the substrate of β-galactosidase.

A straightforward way of replacing *Pusp45-melA* with *Pspac-lacZ* was not available due to the lack of appropriate restriction sites in the plasmid pNZ5517, which contains the *loxP* cassette, *loxP*-erm-*Pusp45-melA-loxP*. Therefore, the decision was made to construct the desired *loxP* cassette *loxP-erm-Pspac-lacZ-loxP* using pUC19, a small *E. coli* plasmid containing multiple cloning sites. The entire *loxP* cassette was then cloned into a plasmid, pKOR1 (Bae and Schneewind, 2006), which replicates in Gram-positive bacteria in a temperature dependent manner. The construction of the *loxP* cassette in pUC19 was achieved through cloning three fragments containing *loxP-erm*, *Pspac-lacZ* and *loxP* in three steps as described in chapter 2.3.2, resulting in plasmid pPZ122-2 shown in figure 4.4.

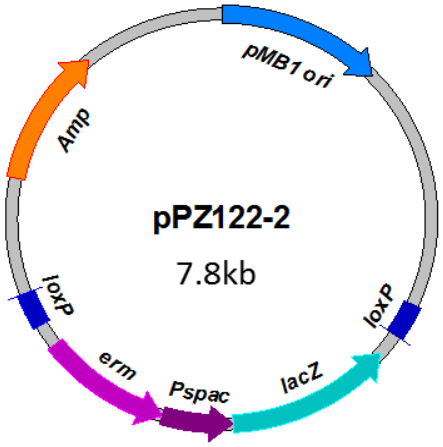

Figure 4.4: Plasmid map of pPZ122-2 (not drawn to scale), a pUC19 derivative with *loxP-erm-Pspac-lacZ-loxP* cloned into the multiple cloning sites. *pMB1 ori*: Gram negative replication origin. *Amp:* ampicillin-resistance gene for Gram negative selection.

The gel electrophoresis images confirming the cloning of each step are shown in figure 4.5. Cloning of the fragment containing *loxP-erm* was confirmed by restriction digestion as shown in lanes 3 and 4, which exhibited expected digestion pattern of

pUC19 backbone at 2665bp and fragment at 1436bp representing *loxP-erm*. The success of introducing *Pspac-lacZ* into the resulting vector is demonstrated by restriction digestion of the plasmids, which are shown in lanes 6 and 9. Lane 6 displays the expected digestion pattern of a fragment at ~8kb (vector backbone) and a fragment at 310bp representing *Pspac*, and lane 9 shows the expected digestion pattern of vector backbone at 4691bp and the *lacZ*-containing fragment at 3119bp. The last step of inserting the second *loxP* site was verified by PCR amplification using primers P82 and P83, which are specific to the insert (all primer sequences involved in this chapter are listed in chapter 2 section 2.2.2). As demonstrated in lanes 13-16, PCR products at the expected size of 478bp were amplified from the successful clones, while it was absent from the vector without the insert (lane 12). One of the resulting plasmids with the correct insert was designated pPZ122-2, and the existence of the entire *loxP* cassette in pPZ122-2 was confirmed by sequencing as described in chapter 2 section 2.3.2. The entire *loxP* cassette, *loxP*-erm-*Pspac-lacZ-loxP*, was successfully assembled and no mutations were found in the *loxP* sites, *ermB* and *Pspac*. Given that *E. coli* colonies harbouring the plasmid containing *Pspac-lacZ* were blue on medium with X-gal, the 3119bp *lacZ* gene was not fully sequenced to confirm its intactness.

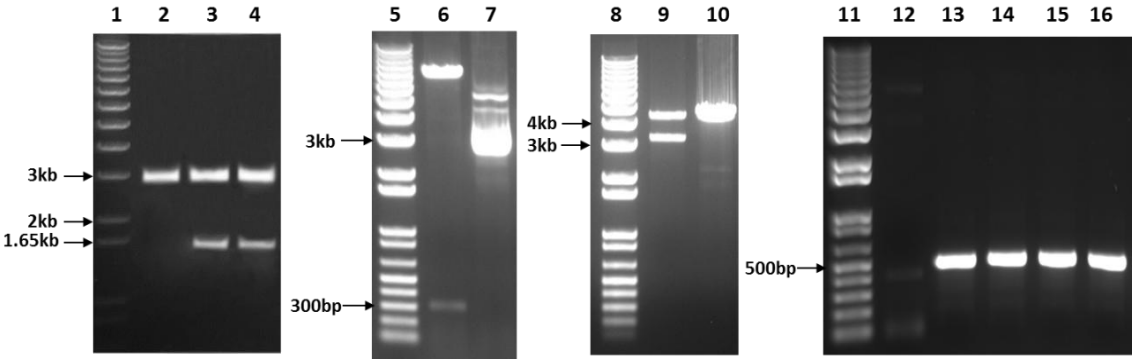

Figure 4.5: Gel electrophoresis pictures confirming the cloning of *loxP-erm-Pspac-lacZ-loxP* into pUC19. Lanes 1/5/8/11: 1kb plus DNA ladder; lanes 2-4: EcoRI-BamHI digested potential pUC19 :: *loxP-erm* (digestion pattern for the expected plasmid: two fragments at sizes 2665bp and 1436bp). Lanes 6 & 7: BamHI-XhoI digested potential pUC19 :: *loxP-erm-Pspac-lacZ* (digestion pattern for the expected plasmid: two fragments at 7500bp and 310bp). Lanes 9 & 10, XhoI-PstI digested potential pUC19 :: *loxP-erm-Pspac-lacZ* (digestion pattern for the expected plasmid: two fragments at 4691bp and 3119bp). Lane 12-16: PCR amplification of fragment containing the second *loxP* site using template pUC19 :: *loxP-erm-Pspac-lacZ* (lane 12, no product was expected) and potential pUC19 :: *loxP-erm-Pspac-lacZ-loxP* (lanes 5-8, expected product size: 478bp).

4.4 Construction of pPZ130-4 for allelic replacement

The entire *loxP* cassette was amplified from pPZ122-2 and cloned into vector pKOR1 for integration into *S. aureus* LS-1 chromosome. Prior to the cloning of *loxP* cassette into pKOR1, an integration site on the chromosome was chosen. It is known that it is very difficult to introduce foreign DNA into *S. aureus* due to a type I restriction-modification systems encoded by the host specificity DNA (*hsd*) genes, *hsdS*, *hsdM* and *hsdR*. The specificity subunit HsdS and the modification subunit HsdM form a complex, in which HsdS recognises a specific DNA sequence from the target host DNA, and HsdM methylates the DNA to protect it from self-digestion. The endonuclease (HsdR) is responsible for the cleavage of unprotected DNA (Murray, 2000). For this reason, introducing foreign DNA, such as *E. coli* DNA, into *S. aureus* requires prior propagation of the DNA in a restriction-negative strain RN4220 which carries a mutation in *hsdR* (Waldron and Lindsay, 2006). Given that pNZ5520 is a *L. lactis*-propagated plasmid that only contains Gram-positive origin of replication and Gram-positive chloramphenicol-resistance gene *cat*, a pNZ5520-derived plasmid library containing random *S. aureus* LS-1 chromosome fragments would be produced in *L. lactis*, and then introduced into the *S. aureus* strain with *loxP* cassette. The efficiency of transformation of *S. aureus* was important to the level of coverage of the *S. aureus* genome. Therefore, since *L. lactis* DNA would not be taken up efficiently by LS-1, we decided to incorporate the *loxP* cassette into *S. aureus* LS-1 at the *hsdR* site. This would theoretically not interfere with virulence and would simultaneously remove this gene and potentially facilitate direct transformation of foreign DNA into the strain.

To insert the *loxP* cassette at the desired site on the *S. aureus* LS-1 chromosome, the *loxP* cassette was placed in between of *hsdR* upstream and downstream sequence in vector pKOR1 (Bae and Schneewind, 2006), on which double cross-over recombination would result in replacement of the *hsdR* with the *loxP* cassette. Figure 4.6 shows the maps of pKOR1 and pKOR1 containing an insert consisting of the *hsdR* upstream region (*hsdR ups*), the *loxP* cassette and the *hsdR* downstream region (*hsdR downs*).

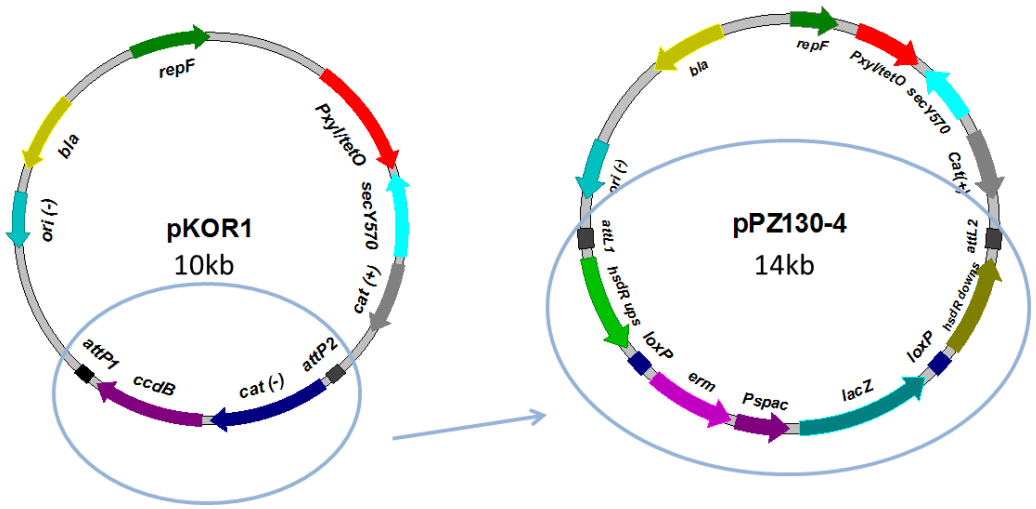

Figure 4.6: Graphic illustration of the integration of *hsdR ups-loxP* cassette-*hsdR downs* into pKOR1, generating vector pPZ130-4 (not drawn to scale). *repF*: replication gene of plasmid pE194ts. *Pxyl/tetO*: ATc-inducible promoter. *secY570*: 570 nucleotides on the 5' end of *secY* including the ribosome binding site. *cat* (+)/(-): Gram-positive/Gram-negative chloramphenicol acetyltransferase. *attP*1/2: phage lambda attachment site. *ccdB* (control of cell death): gene encodes an *E. coli* gyrase inhibitor protein. *ori* (-): Gram-negative replication origin. *bla*: Ampicillin-resistance gene encoding β-lactamase.

The vector pKOR1 possess several features that allow marker-less deletion of genes from Gram-positive bacteria. It contains origins and antibiotic resistance genes for replication and selection in both Gram-positive and Gram-negative bacteria. Its Gram-positive origin *repF* is a temperature sensitive origin from plasmid pE194ts (Villafane *et al.*, 1987) which replicates at 30˚C but not 43˚C, thus enabling plasmid removal from Gram-positive bacteria through temperature shifting. In addition, it has a lambda recombination cassette *attP*1-*ccdB*-*cat*-*attP*2, which allows integration of DNA fragments into this plasmid. The phage lambda attachment sites *attP*1/2 recombine with *attB*1/2 sequences of DNA inserts in the presence of bacteriophage lambda integrase (Hsu and Landy, 1984). The *ccdB* (control of cell death) encodes an *E. coli* gyrase inhibitor protein (Bernard and Couturier, 1992), which renders *E. coli* transformants with non-recombinant plasmid unable to grow. Furthermore, pKOR1 has an antisense *secY* expression cassette *Pxyl/tetO-secY570*, which contains a 570 nucleotide sequence on the 5' end of *S. aureus secY* including the ribosome binding site (Bae and Schneewind, 2006). Since *secY* is an essential secretion gene in *S. aureus* (Forsyth *et al.*, 2002), the expression of antisense *secY* in the presence of inducer ATc inhibits the growth of cells containing the plasmid. These properties of pKOR1 render it an efficient tool for generating marker-less and plasmid-free mutants

of Gram-positive bacteria. However, the antisense component of this plasmid was not used in generating the *loxP* cassette-containing mutant, because the *loxP* cassette contains an *erm* gene, which permits selection of the desired plasmid-free mutants in the presence of erythromycin at non-permissive temperature.

The procedure for integrating *hsdR ups-loxP* cassette-*hsdR downs* into pKOR1 is described in detail in chapter 2 section 2.3.3, which involved firstly introducing sequences for the *hsdR ups-hsdR downs* into pKOR1 through lambda integration, then inserting the *loxP* cassette between the *hsdR* ups and the *hsdR* downs. The success of the last integration step was confirmed by restriction digestion with enzymes that are present in both the insert and pKOR1 backbone. Figure 4.7 lanes 3-6 shows XhoI-digestion patterns of four potential plasmids consisting of pKOR1 :: *hsdR ups-loxP* cassette-*hsdR downs*, three of which exhibited the expected pattern with two fragments at ~8kb and ~6kb, while XhoI-digestion of pKOR1 :: *hsdR ups-hsdR downs* showed an expected single band at approximately 10kb (lane 2). The three plasmid represented by lanes 3, 5 and 6 were further confirmed by restriction digestion with XbaI. As shown in lanes 9-11, all three plasmids demonstrated the expected digestion pattern with three fragments at sizes ~7kb, ~6kb and 1898bp, while digestion of pKOR1 :: *hsdR ups-hsdR downs* with XbaI showed the two expected fragments at 4043bp and ~6kb (lane 8).

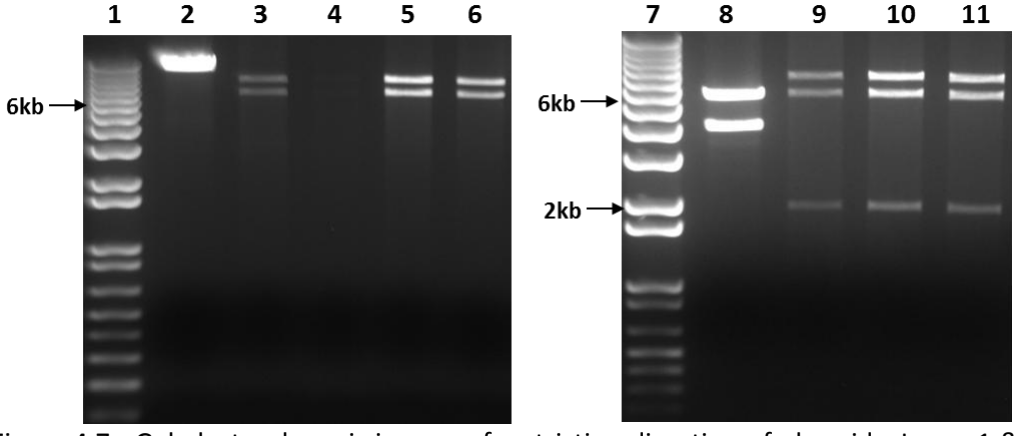

Figure 4.7: Gel electrophoresis images of restriction digestion of plasmids. Lanes 1 & 7: 1kb plus DNA ladder. Lanes 2-6: XhoI-digested pKORI :: *hsdR ups-hsdR downs* (lane 2, expected digestion pattern: one single band at size ~10kb), and potential pKOR1 :: *hsdR ups- loxP* cassette-*hsdR downs* (lanes 3-6, expected digestion pattern: two fragments at sizes 7918bp and 6164bp). Lanes 8-11: XbaI-digested pKORI :: *hsdR ups-hsdR downs* (lane 8, expected digestion pattern: two fragments at sizes 4043bp and 5669bp), and potential pKOR1 :: *hsdR ups-loxP* cassette-*hsdR downs* (lanes 9-11, expected digestion pattern: three fragments at sizes 6558bp, 5626bp and 1898bp).

Prior to integration into *S. aureus* LS-1, pPZ130-4 was sequenced at the cloning region as described in chapter 2.3.3. Sequencing analysis revealed no mutations in the *loxP* cassette but a point mutation in *hsdR* ups and *hsdR* downs, respectively, in comparison to the sequences in strain NCTC 8325 (GenBank accession CP000253). Both mutations are C to T substitutions, with one at the 34th nucleotide in the 1017bp *hsdR ups* and the other one at the 174th nucleotide in the 860bp *hsdR* downs. The mutation site in *hsdR downs* is at the potential promoter region of the gene downstream of *hsdR*. This region was analysed using a promoter prediction program BPROM (Softberry, Inc., Mt. Kisco, NY), which is a bacterial sigma^{70} promoter recognition program with approximate 80% accuracy and specificity (available free at http://linux1.softberry.com/berry.phtml). The analysis revealed that this nucleotide is outside the predicted promoter region. In addition, this nucleotide was found to be a T in most of the other *S. aureus* strains such as Newman, COL and TW20. Given that the *hsdR downs* was amplified from strain LS-1, it is possible that LS-1 also has a T at this site, and the nucleotide T at this site may not be a mutation generated in the cloning process.

Contrarily, the mutation in *hsdR ups* locates in an open reading frame with locus tag SAOUHSC_00160 in *S. aureus* NCTC 8325, and unfortunately, it introduces a stop codon into this gene. Given that the *loxP* cassette in plasmid pPZ130-4 would be integrated into *S. aureus* LS-1 chromosome through recombination of *hsdR* ups and *hsdR* downs between the plasmid and chromosome, mutations in *hsdR ups* on the plasmid may be introduced into the chromosome through recombination. However, the mutation is almost at the edge of this fragment (34th nucleotide of a 1017bp DNA fragment), therefore there was a probability that the crossover would happen downstream of this mutation. Hence it was decided to proceed with the integration of the *loxP* cassette into *S. aureus* LS-1, and screen the resulting strains for those mutants without the mutation in *hsdR* upstream region.

4.5 Integration of the *loxP* cassette into the chromosome of *S. aureus* LS-1

Vector pPZ130-4 was introduced into *S. aureus* LS-1 following modification by passage through strain RN4220. Figure 4.8 shows an overview of the process of integration of the *loxP* cassette into the chromosome of *S. aureus* LS-1. The process of generating the strain *S. aureus* LS-1 *hsdR* :: *loxP* cassette and curing of plasmid pPZ130-4 is described in chapter 2 under section 2.3.4.

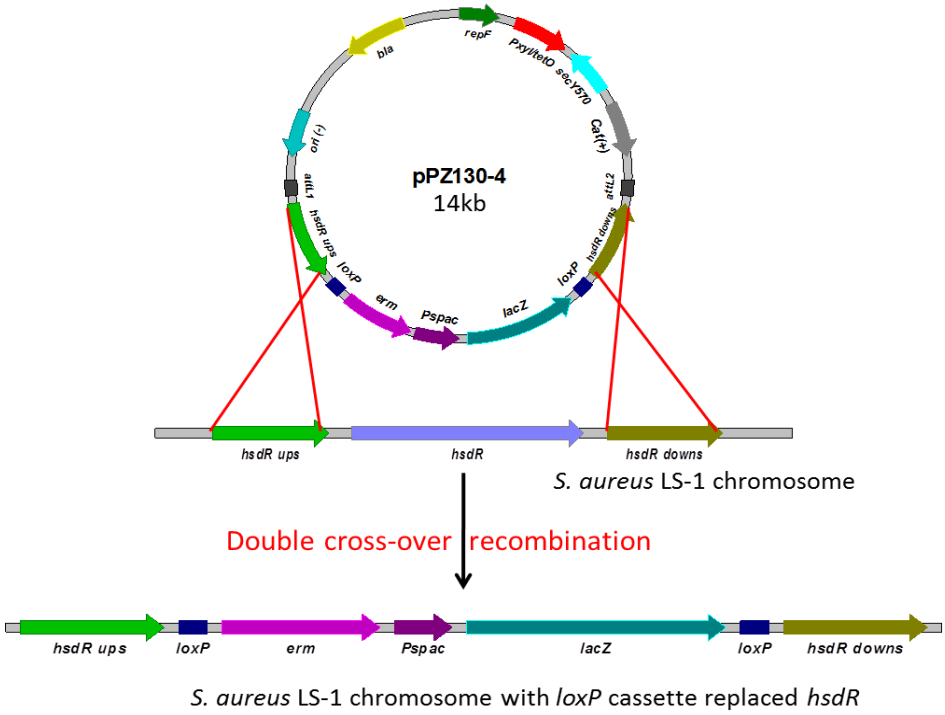

Figure 4.8: Graphic illustration of the introduction of the *loxP* cassette into the chromosome of *S. aureus* LS-1 to replace *hsdR* (not drawn to scale).

To confirm the integration of *loxP* cassette into the chromosome of *S. aureus* LS-1, genomic DNA from four candidates was purified and examined by PCR using primers P41/P79 and P82/P42 to amplify the two junctions of the insertion site. The PCR products were analysed by agarose gel electrophoresis and are shown in figure 4.9. Primer P41 locates 28bp upstream of *hsdR ups* on the chromosome of LS-1 and P79 is specific to *Pspac* in the *loxP* cassette. These two primers were expected to amplify a 2469bp-fragment containing *hsdR up-loxP-erm-Pspac*. As shown in figure 4.9 a PCR product of the correct size was obtained for the candidate clones (lanes 3-6). Whereas the product was absent in an attempted amplification from genomic DNA of

the wild-type strain LS-1 (lane 2). Primer P42 was designed 84bp downstream of *hsdR downs* on the chromosome of LS-1, and P82 locates on the *loxP* cassette downstream of *lacZ*. PCR amplifications using these two primers are shown in lanes 8-12, no product was observed for the wild-type LS-1 (lane 8), and a product was detected for the four potential mutants at the expected size of 1064bp representing a fragment containing *loxP-hsdR downs*. These four mutants were referred to as *S. aureus* PZ134-1 to 4 thereafter, representing *S. aureus* LS-1 *hsdR* :: *loxP-erm-Pspac-lacZ-loxP*. The *hsdR ups* regions on the mutant and the wild-type chromosomes were sequenced and it was confirmed that *hsdR ups* sequences in the four mutants are identical to that in the wild-type, and did not contain the mutation detected in plasmid pPZ130-4.

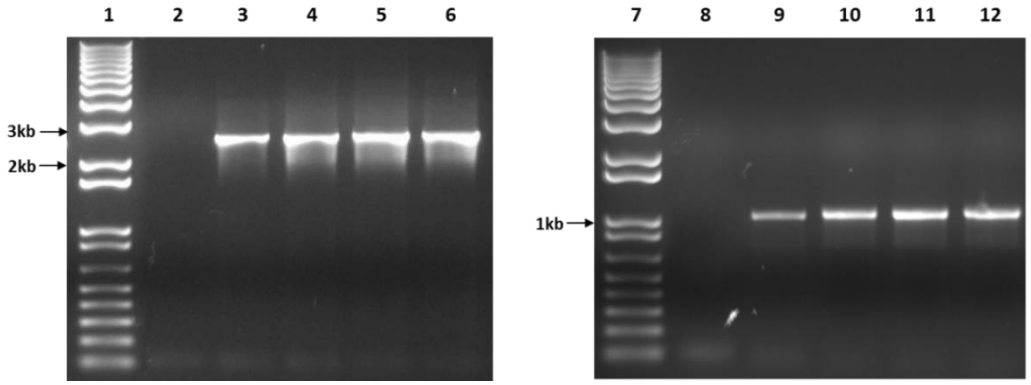

Figure 4.9: Gel electrophoresis images of PCR products. Lane 1 & 7: 1kb plus DNA ladder. lane 2: PCR amplification from LS-1 genomic DNA using primers P41/P79, no product was expected. Lanes 3-6: PCR products amplified from 4 potential mutants using primers P41/P79, expected product size 2469bp. Lane 8: PCR amplification from LS-1 genomic DNA, no product was expected. Lanes 9-12: PCR products amplified from 4 potential mutants using primers P42/P82, expected product size 1064bp.

4.6 Stability of the *loxP* cassette in the chromosome of *S. aureus*

The constructed *cre-loxP* system that theoretically allows the screening of conditionally activated *S. aureus* promoters is graphically shown in figure 4.10, consisting of a strain that harboured the *loxP* cassette and the plasmid pNZ5520 which carries a promoter-less *cre*. Prior to the use of this system, it was important to confirm that no host factors from *S. aureus*, or a promoter-less *cre* on pNZ5520, would result in the resolution of *loxP* cassette from the chromosome of *S. aureus*, so that the event of *loxP* cassette resolution can be only attributed to an activated

promoter upstream of *cre*. Therefore, the stability of the *loxP* cassette in the chromosome of *S. aureus* PZ134-2 (*S. aureus* LS-1 *hsdR* :: *loxP-erm-Pspac-lacZ-loxP*) was assessed in the absence or presence of pNZ5520.

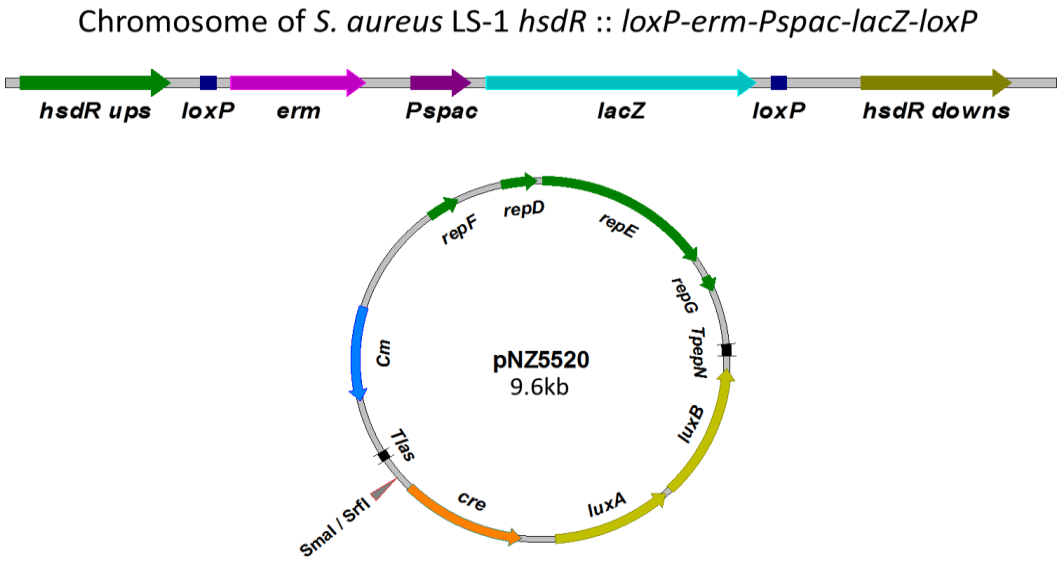

Figure 4.10: Schematic diagram of the *S. aureus* R-IVET system (not drawn to scale). The *loxP-erm-Pspac-loxP* cassette is integrated into the chromosome of *S. aureus* LS-1 between loci SAOUHSC_00161 and SAOUHSC_00163 according to the locus tags in *S. aureus* NCTC 8325. The plasmid pNZ5520 contains the reporter genes *cre, luxA* and *luxB*, and a SfrI/SmaI restriction site upstream of *cre* is for the insertion of chromosomal fragments.

The stability of the *loxP* cassette in the chromosome of *S. aureus* PZ134-2 was assessed according to the methods described by Bachmann *et al.* (2008). Firstly, *S. aureus* PZ134-2 without pNZ5520 was cultured at 37˚C in duplicate in TSB without antibiotic selection. This culture was diluted 1:1000 on a daily basis and cultured for 20 days (approximately 200 generations). On the 21st day the culture was plated on TSA + 40µg/ml X-gal. All the colonies that grew on the plates were visually checked, 9209 and 9216 blue colonies were observed for each culture, respectively, and no white colonies were found for either of the cultures. This indicates that the *loxP* cassette is stably integrated into the chromosome of *S. aureus* PZ134-2, and no *S. aureus* host factors are able to result in the resolution of *loxP* cassette from the chromosome.

The plasmid pNZ5520 was introduced into *S. aureus* PZ134-2 through electroporation, and the stability of *loxP* cassette in the presence of pNZ5520 was also determined as described above. In total, 8845 and 9103 colonies were visually checked and all were

found to be blue in colour. This confirmed the stability of the *loxP* cassette in the chromosome and also demonstrated that no expression of *cre* in pNZ5520 occurred in the absence of a promoter sequence.

4.7 Construction of reference systems

To test the R-IVET system and also produce positive controls for future experiments, previously characterised promoters were cloned upstream of *cre* in pNZ5520. The promoters chosen were P_{cat} which drives the expression of chloramphenicol acetyltransferase (*cat*) in pSK236, the inducible promoter *Pxyl/tetO* from pKOR1, and *saeP1* which drives the expression of the *sae* operon in *S. aureus*. Promoter P_{cat} was planned to serve as a positive control for the system. The inducible promoter *Pxyl/tetO* could be induced to different levels to establish the relationship between promoter activity and the frequency of *cre-loxP* resolution, which may be useful for assessing the sensitivity of this system, and may also serve as a reference to evaluate the strength of the promoters that would be screened based on the frequency of *cre-loxP* resolution. The *sae* operon encodes a global regulatory system for virulence factors and was described in the first chapter. This operon is driven by three promoters P1, P2 and P3, with promoter P1 showing the highest activity (Geiger *et al.*, 2008). The *saeP1* promoter has been demonstrated to respond to different environmental signals such as pH, NaCl concentration, H_2O_2, vancomycin and the human antimicrobial peptide α-defensins (Geiger *et al.*, 2008). Therefore, the *saeP1* promoter may serve as a reference when identifying environmental induced *S. aureus* genes using the R-IVET system.

The insertion of these three promoters into pNZ5520 was performed as described in chapter 2 section 2.3.5. To confirm the success of cloning, as shown in figure 4.11, a primer P205 was designed based on sequence of *cre* in pNZ5520, and was 128bp from the insertion site. PCRs were conducted using P205 and a primer designed to the 5' end of each promoter, so that plasmids with a promoter inserted in the desired orientation should generate a product 128bp larger than the actual size of the promoter. The PCR products were analysed by gel electrophoresis and an image of the gel is shown in figure 4.12. The success of inserting the promoters in the same

orientation as the reporter genes was demonstrated by PCR products in lane 2 (P_{cat}, expected product size 535bp), lane 6 (*Pxyl/tetO*, expected product size 908bp) and lanes 9/12/14/16 (*saeP1*, expected product size 528bp). The cloned promoters in the resulting plasmids were sequenced and confirmed to be the correct sequence and in the desired orientation.

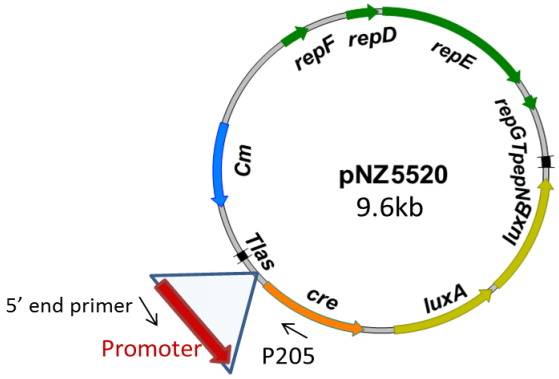

Figure 4.11: Graphical illustration of the insertion of promoter into pNZ5520 upstream of *cre* (not drawn to scale), and PCR amplification examining the correct direction of promoter insertion. P205 is specific to the *cre* gene sequence on pNZ5520 and 5' end primer is specific to the 5' end of the inserted promoter.

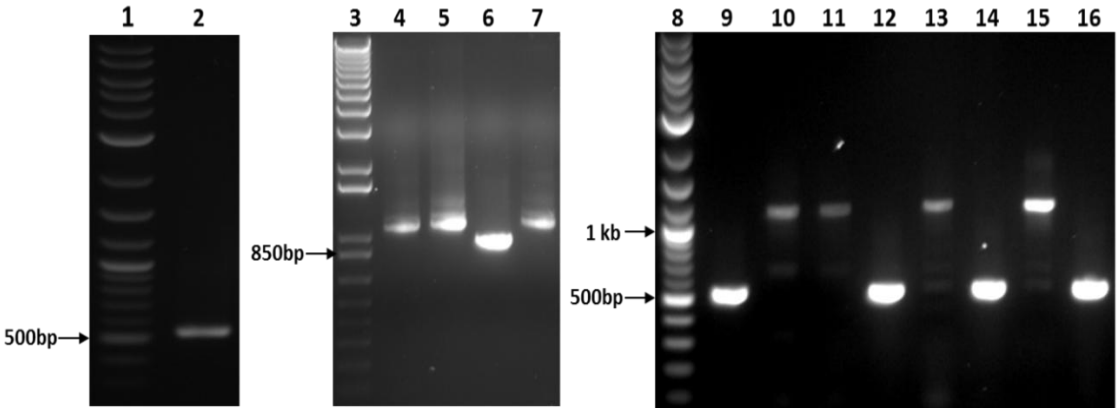

Figure 4.12: Gel electrophoresis images confirming the insertion of P_{cat}, *Pxyl/tetO* and *saeP1* into pNZ5520. Lanes 1 & 8: 1kb DNA ladder (NEB). Lane 3: 1kb plus DNA ladder (Invitrogen) Lane 2: PCR product amplified from potential pNZ5520 :: P_{cat} (expected size 535bp). Lanes 4-7: PCR product amplified from potential pNZ5520 :: *Pxyl/tetO* (expected size 908bp). Lanes 9-16: PCR product amplified from potential pNZ5520 :: *saeP1* (expected size 528bp).

4.8 Activity of reference promoters

Plasmids pNZ5520 :: P_{cat}, pNZ5520 :: *Pxyl/tetO* and pNZ5520 :: *saeP1* were purified from *L. lactis* and introduced into *S. aureus* PZ134-2 by directly electroporating their DNA into this strain. Deletion of the *hsdR* gene in *S. aureus* LS-1 enabled the strain to accept *L. lactis*-propagated DNA, however the transformation efficiency was very low, with only one to four transformants observed for each electroporation. Prior to

assessment of the R-IVET system using these vectors, the activity of the three promoters was determined by measuring luminescence. The growth of strains harbouring each plasmid-promoter construct were examined as described in chapter 2 section 2.1.6 by measuring the optical density at 600nm. The growth curves of the strains in TSB + 10µg/ml Cm with or without ATc are plotted in figure 4.13. The growth curves for each strain exhibit a typical growth cycle consisting of a lag phage, exponential phase and stationary phase. The strains grew at a similar speed in TSB + Cm, demonstrating that the promoters integrated upstream of *cre* did not affect the growth rate of the strains. However, the growth rate of the strain containing pNZ5520 :: *Pxyl/tetO* was inhibited upon the addition of ATc, and the inhibition was dependent on the concentration of ATc.

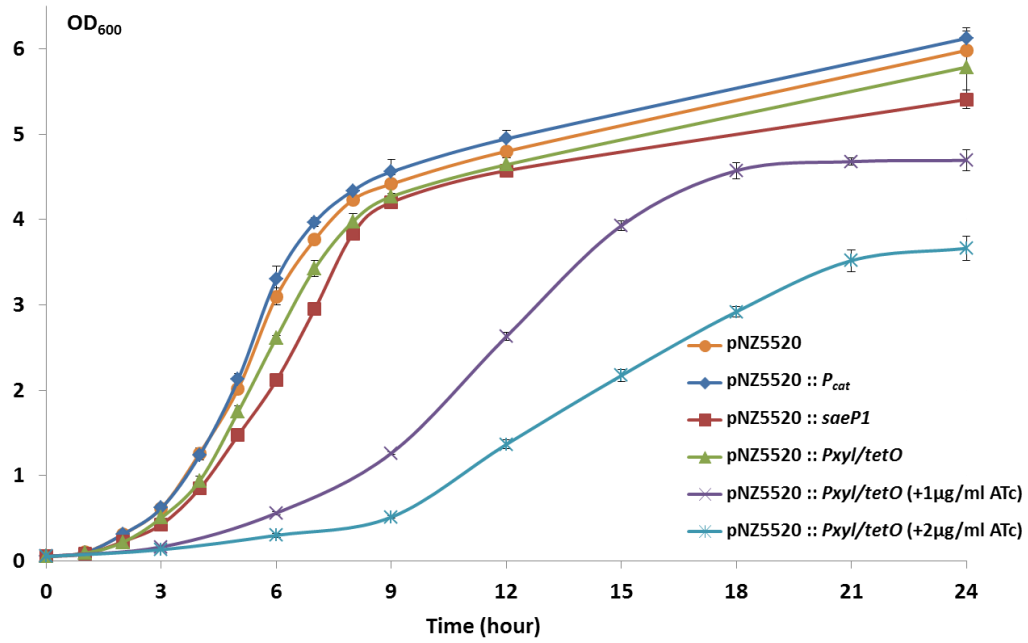

Figure 4.13: Growth curves of *S. aureus* PZ134-2 carrying pNZ5520-derived plasmids in TSB + 10µg/ml Cm. Orange: pNZ5520, dark blue: pNZ5520 :: P_{cat}, green: pNZ5520 :: *saeP1*, red: pNZ5520 :: *Pxyl/tetO*. The strain with pNZ5520 :: *Pxyl/tetO* was also grown in the presence of 1µg/ml (purple) or 2µg/ml (light blue) of ATc. Data presented are the means of three independent experiments with error bars showing the standard deviations.

To examine the promoter strength at different growth phases, the luminescence produced by each strain was measured throughout growth as described in chapter 2 section 2.5. To compare the relative strength of each promoter, and also to determine the growth phase at which they were maximally expressed in individual cells, the ratio of luminescence production over OD_{600nm} was plotted against time in figure 4.14.

The luminescence produced by strain PZ134-2 containing pNZ5520 was almost undetectable throughout the entire growth cycle due to the lack of a promoter driving the expression of *luxAB*. Unexpectedly, luminescence was also not detected from the strain containing pNZ5520 :: P_{cat}, suggesting very low or no activity of the promoter P_{cat}. In the strain containing pNZ5520 :: *Pxyl/tetO*, low level of luminescence production was observed in the absence of induction, indicating that this promoter is not tightly controlled. When this strain was exposed to 1 or 2µg/ml of ATc, the luminescence production was remarkably elevated, especially in the presence of 1µg/ml of ATc. This suggests that *Pxyl/tetO*, although leaky, is highly inducible by ATc. From the growth curves of the strains presented in figure 4.13, it seems that the activity of *Pxyl/tetO* was mainly induced in the early exponential growth phase regardless of the presence of the inducer, which is unexpected for *Pxyl/tetO* with inducer, as the inducer was not removed from the culture at any point during growth. The strain with pNZ5520 :: *saeP1* only produced low levels of luminescence throughout growth with the highest level being produced at the lag growth phase between 0 and 3 hours.

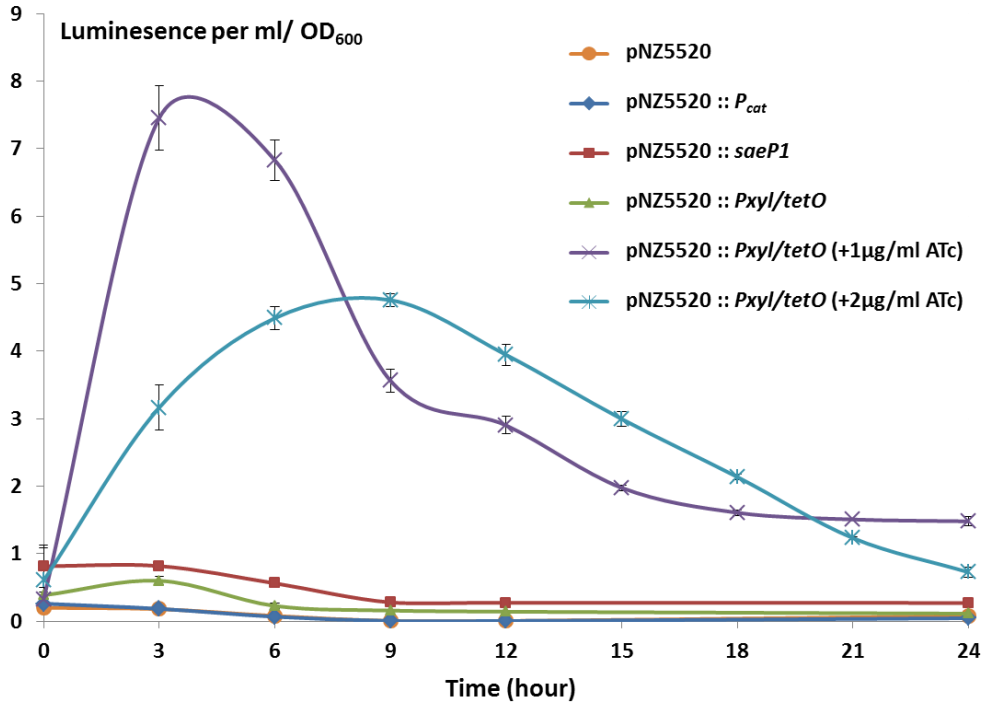

Figure 4.14: The ratio of luminescence production over optical density of *S. aureus* PZ134-2 carrying pNA5520-derived plasmids in TSB + 10µg/ml Cm. Orange: pNZ5520, dark blue: pNZ5520 :: P_{cat}, green: pNZ5520 :: *saeP1*, red: pNZ5520 :: *Pxyl/tetO*. The strain with pNZ5520 :: *Pxyl/tetO* was also grown in the presence of 1µg/ml (purple) or 2µg/ml (light blue) of ATc. Data presented are the means of three independent experiments with error bars showing the standard deviations.

4.9 Sensitivity of the R-IVET system

Following the determination of the promoter activities during different growth phases, the frequency of resolution of the *loxP* cassette from chromosome was estimated when *cre* expression was driven by P_{cat}, *Pxyl/tetO* or *saeP1*. The strains were grown under the same conditions as those in the growth experiments and luminescence assays (TSB + 10µg/ml Cm with or without ATc), and the cultures were plated on TSA + 40µg/ml X-gal every three hours. The proportion of the white colonies among the total number of blue and white colonies grown on the plates was considered as the percentage of the cells in which resolution of the *loxP* cassette has occurred. For strains containing pNZ5520, pNZ5520 :: P_{cat} or pNZ5520 :: *Pxyl/tetO* in the absence of induction, no white colonies were identified in at least 100 colonies over the entire growth cycle, suggesting a very low proportion (< 1%) of cells in which *cre-loxP* resolution occurred. The percentages of the cells in which *cre-loxP* resolution occurred were calculated for strains containing pNZ5520 :: *saeP1* and pNZ5520 :: *Pxyl/tetO* under induction with 1µg/ml or 2µg/ml ATc, and are presented in figure 4.15. At 0 hour, no resolution of *loxP* cassette was detected in at least 100 colonies of strain containing pNZ5520 :: *Pxyl/tetO*. After 3 hours following the addition of 1µg/ml or 2µg/ml of ATc the *loxP* cassette had resolved in 31% and 21 % of the colonies, respectively. The proportion of bacteria in which the cassette had been resolved did not increase overtime. For the strain containing pNZ5520 :: *saeP1*, 31.3% of the cells lost the *loxP* cassette at time 0 hour, and the proportion of the bacteria in which *cre-loxP* resolution occurred increased to 85.4% at 3 hours and did not change significantly over time, indicating that the resolution of *loxP* cassette in this strain mainly happened in the first 3 hours. Given that *saeP1* demonstrated much lower activity than the induced *Pxyl/tetO* in the luminescence assays (figure 4.14), it was unexpected that the *cre-loxP* resolution would occur in a larger proportion of cells with pNZ5520 :: *saeP1* than those with pNZ5520 :: *Pxyl/tetO* in the presence of inducer.

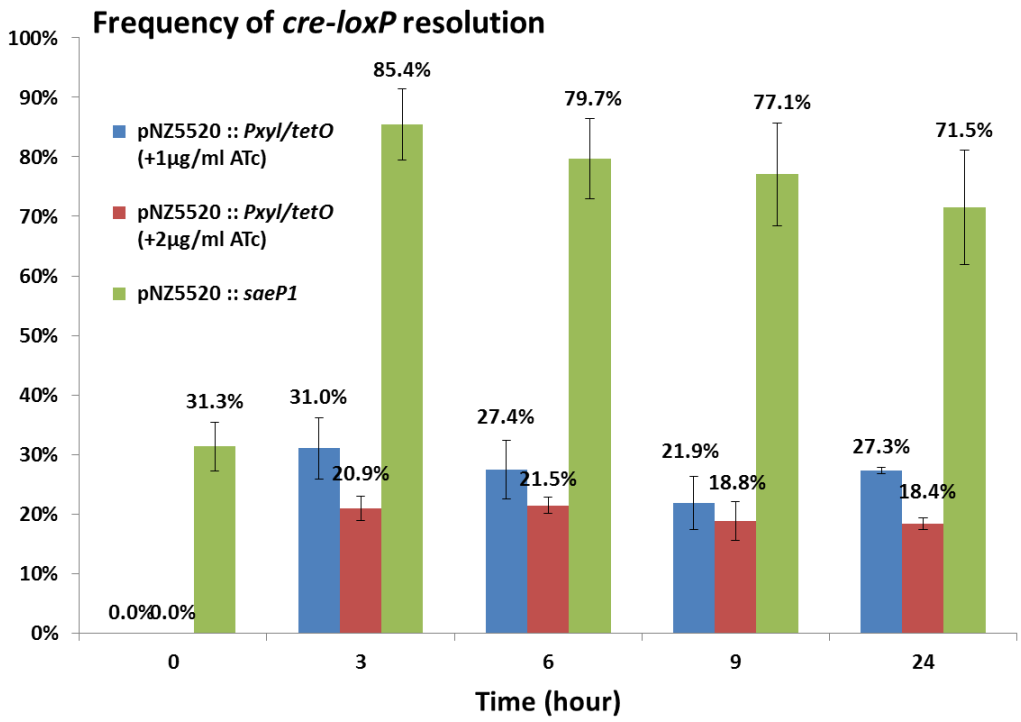

Figure 4.15: The percentage of cells in which the *loxP* cassette resolved from the chromosome of *S. aureus* PZ134-2 in the presence of pNZ5520 :: *Pxyl/tetO* with 1µg/ml (blue bars) or 2µg/ml (red bars) ATc, and pNZ5520 :: *saeP1* (green bars) at different time points throughout growth. The data presented are the means of three independent experiments with error bars showing the standard deviations.

4.10 Discussion

This chapter describes the construction and characterisation of a R-IVET system that could be used to identify *S. aureus* genes that are conditionally induced. This system circumvented the main disadvantage of R-IVET systems previously used with *S. aureus* (Lowe *et al.*, 1998) which employed only an antibiotic resistance gene in the resolution cassette to report resolution, and which required laborious replica plating for the selection of strains of interest. By including a second reporter gene, *lacZ*, in addition to the antibiotic gene *ermB*, our R-IVET system provides a simpler screening method whereby clones in which resolution of the cassette has occurred can be easily identified as white colonies growing on medium supplemented with X-gal. These clones can be further confirmed as having lost the resolution cassette by testing their sensitivity to erythromycin if necessary. In addition, this R-IVET system has been assessed by using characterised promoters and proven to be functional in *S. aureus*. Because of the time limitations and other developments during the project,

screening using this system for *S. aureus* genes activated in the presence of human antimicrobial peptide LL-37 was not performed. However potential strategies for screening will be discussed in chapter 8 as future work.

As mentioned in the introduction, one advantage of R-IVET compared to auxotrophy or antibiotic based IVET is its sensitivity which allows the identification of weakly or transiently expressed genes due to the high activity of Cre resolvase. However the high activity of the resolvase could also be a weakness that results in us missing important groups of genes. Take for example the use of this system to screen for LL-37-induced *S. aureus* genes; pNZ5520 harbouring a variety of *S. aureus* promoters would need to be introduce into *S. aureus* PZ134-2 and the resulting transformants would be propagated in medium containing erythromycin before being exposed to LL-37. In this case, some promoters that have weak activity during propagation of the bacteria may lead to resolution of the *loxP* cassette, conferring erythromycin sensitivity on these cells. This will result in a library lacking in these clones containing these plasmid-promoters constructs, some of which could have potentially been induced by LL-37. The high sensitivity of the R-IVET system is likely to exclude a large number of promoters from the actual screen, as a lot of promoters are activated while bacteria are proliferating. This drawback has been addressed by some researchers by a strategy that involves modification of the ribosome binding site of the recombinase reporter gene to reduce the efficiency of translation from the recombinase mRNA (Lee *et al.*, 1999). Such a post-transcriptional modification is able to turn down the sensitivity of the system by reducing the expression of recombinase regardless of the transcriptional level of the recombinase gene. This could be a way to modify our R-IVET system if it turns out to differentiate too strongly against genes that are expressed during growth *in vitro*, as some of these genes could also be important in the response to LL-37 or other conditions.

In the luminescence assays, it was unexpectedly found that the promoter P_{cat} demonstrated no activity at all and pNZ5520 :: P_{cat} did not lead to the resolution of the *loxP* cassette from PZ134-2. Given that the P_{cat} region in pNZ5520 :: P_{cat} has been sequenced to confirm that no mutations had occurred and the promoter was also

inserted in the vector in the correct orientation, it seems that the lack of activity is due to other reasons. This promoter was PCR amplified from pSK236, in which the transcript P_{cat}-*cat* originated from a small *S. aureus* plasmid pC194. Referring to early studies on pC194 (Horinouchi and Weisblum, 1982; Byeon and Weisblum, 1984), the expression of chloramphenicol acetyl transferase (CAT) from this plasmid seems to be under autogenous control, and the regulator is CAT itself in the presence of chloramphenicol (Horinouchi and Weisblum, 1982). A 37bp inverted complementary repeat sequence was found between the -10 and ribosome binding sequences in P_{cat}-*cat*. It was therefore hypothesised that this 37bp sequence may form a stem in *cat* mRNA, which shares a similar structure with *E. coli* 23S rRNA and is also partially complementary to the sequence of 23S rRNA. As a result, 23S rRNA may bind to this stem in the nascent *cat* mRNA and mask the ribosome binding site, preventing the translation of *cat* mRNA (Byeon and Weisblum, 1984). It is known that the antibiotic chloramphenicol targets bacterial protein synthesis by inhibiting the peptidyl transferase activity of the bacterial ribosome, and it specifically binds to the 23S rRNA of the 50S ribosomal subunit (Mukhtar and Wright, 2005). Therefore, when chloramphenicol is present, it forms a complex with the 50S ribosomal subunit, which alters the conformation of the ribosome and results in the exposure of the *cat* Shine-Dalgarno sequence for interaction with ribosomes (Byeon and Weisblum, 1984). This hypothesis suggests that regulation is at a post-transcriptional level rather than at the transcriptional level, which has been supported by another study on an inducible *cat* from *B. pumilus*. An *E. coli* tryptophan-synthesis gene *tryC* was inserted into the *B. pumilus* CAT structural gene downstream from the CAT ribosomal binding site and was found to be inducible by chloramphenicol, while a gene encoding mouse dihydrofolate reductase inserted upstream from the ribosome binding site was found to express constitutively (Williams *et al.*, 1981). Since the P_{cat} promoter was cloned with the ribosome binding site in pNZ5520 in our study, it should direct the transcription of the *cre* gene, and the addition of chloramphenicol was expected to induce translation of *cre* mRNA. Interestingly, chloramphenicol was actually added in the luminescence assays and *cre-loxP* recombination assays to maintain the pNZ5520-derived plasmids, which carries a chloramphenicol-resistance gene *cat*-86 from *Bacillus pumilus* (Bachmann *et al.*, 2008). However, due to the presence of *cat*-

86, the translation of *cre* mRNA may still be repressed in spite of the supplementation with chloramphenicol, because the enzyme CAT expressed from *cat-86* acetylates chloramphenicol, making it inaccessible to the ribosomes.

Unexpectedly, the induced activity of the promoter *Pxyl/tetO* reflected by luminescence production showed maximum activity only in the early exponential phase (figure 4.14). Given that the strain with pNZ5520 :: *Pxyl/tetO* was incubated in the presence of 1μg/ml or 2μg/ml ATc through the entire growth, it was expected to observe induction of *Pxyl/tetO* throughout the growth cycle. From the transposition experiments with induction by ATc (described in chapter 3), it is known that ATc binds to *S. aureus* cells and cannot be removed by washing with PBS. Therefore, one possibility to explain the decline of luminescence production at the later stage of the growth is that ATc was probably depleted from the medium by binding to the cells, making it unavailable for new generations of cells. This probably also explains why *cre-loxP* recombination in the strain with pNZ5520 :: *Pxyl/tetO* only happened in the first 3 hours after supplementation with ATc.

Discrepancy was found between the activities of the promoters determined by measuring luminescence and those reflected by the proportion of cells in which *cre-loxP* resolution occurred. The induction of *Pxyl/tetO* resulted in considerably higher luminescence production than *saeP1*, while the proportion of cells in which *cre-loxP* resolution occurred for the strain containing pNZ5520 :: *Pxyl/tetO* was much lower than that for the strain containing pNZ5520 :: *saeP1*. There are two possibilities that may explain this result. First, given that the luminescence production was catalysed by LuxAB, and resolution of the *loxP* cassette was mediated by Cre, it is possible that mutations occurred in either the *cre* gene in plasmid pNZ5520 :: *Pxyl/tetO*, or in the *luxAB* in plasmid pNZ5520 :: *saeP1*, during the process of making these constructs. Another possibility is that the efficiency of luminescence production was different between the strains with pNZ5520 :: *Pxyl/tetO* and pNZ5520 :: *saeP1*, therefore luminescence production may not reflect the real activity of *Pxyl/tetO* or *saeP1*. As the luminescence was measured for 15 minutes after adding substrates FMNH2 and aldehyde into the culture, it is possible that uptake of the substrate by bacteria with

pNZ5520 :: *Pxyl/tetO*, cultured in the presence of ATc, was greater compared to that of the bacteria containing pNZ5520 :: *saeP1*. ATc has been found to be able to permeabilise bacterial cell membranes (Rasmussen *et al.*, 1991; Oliva *et al.*, 1992). The increased membrane permeability in the presence of ATc may result in increased uptake of substrates, FMNH2 and aldehyde, for luminescence production. Thereby, the high level of luminescence production by strain with pNZ5520 :: *Pxyl/tetO* may be a consequence of increased substrate inside the cells compared to strain with pNZ5520 :: *saeP1*. In this sense, the frequency of *cre-loxP* resolution, whose catalysis only requires host factors, may be a better parameter for the assessment of promoter activities. However it is more likely that the problem lies with the *luxAB* in pNZ5520 :: *saeP1* since this strain produced hardly any luminescence at any time point.

The *cre-loxP* resolution driven by promoter *saeP1* only occurred in the first 3 hours of growth, suggesting that the promoter was mainly activated during the first 3 hours, which corresponds to the lag growth phase (figure 4.15). This is contradictory to that reported by Steinbuber *et al*., (2003) that maximal activity of this promoter was detected in the post-exponential growth phase of strain Newman and ISP497C by Northern blot analysis (Steinhuber *et al.*, 2003). Strain Newman is characterised by a missense mutation ($^{L}18^{P}$) in the first transmembrane loop of the sensor kinase SaeS, which has been shown to up-regulate the transcript driven by *saeP1* (Mainiero et al., 2010). The mutation was thought to cause increase in the kinase activity of SaeS, which leads to high levels of phosphorylation of SaeR, and SaeR autoregulates *saeP1* (Mainiero *et al.*, 2010). In addition, strain ISP497C is a derivative of strain 8325-4, which is defective in RsbU, an important positive regulator of SigB. It has been shown that complementation of *rsbU* has a substantial inhibitory effect on *sae* expression (Novick and Jiang, 2003). These findings suggest that strains Newman and ISP497C may express *sae* at different growth phases and to different levels compared to most strains.

Chapter 5: Screening of Mutants with Increased Resistance to LL-37

5.1 Introduction

Following the construction of a mutant library as described in chapter 3, the library was subject to screening with the aim of identifying *S. aureus* genes that are important for survival and pathogenesis in the human host. These include genes that are involved in resistance to human immune defences, which comprise of the innate immune defence and the adaptive immune defence systems. The human innate defence system contains physical barriers such as the skin and mucous membranes, and cellular components such as neutrophils, monocytes and macrophages (Goodarzi *et al.*, 2007). Human skin plays a role in preventing bacterial infection due to its low pH, production of antimicrobial peptides (AMPs) and antimicrobial lipids, and its anatomical structures which provide a physical barrier (Goodarzi *et al.*, 2007). Microorganisms that successfully cross through the physical barriers immediately encounter phagocytic cells and are subject to killing via phagocytosis. Phagocytic killing is predominantly accomplished by the antimicrobial cell contents of phagocytic cells, such as antimicrobial proteins and peptides (Nauseef, 2007). Neutrophils are also able to exert phagocytic killing through production of neutrophil extracellular traps (NETs) which contains DNA, histones, granule enzymes and antimicrobial proteins (Brinkmann *et al.*, 2004). The innate immune response represents a fast and non-specific defence against microbes, while the adaptive immune responses are relatively slow and highly specific to particular pathogens that induce them. Upon exposure to a pathogen, it may take between 4-7 days before the adaptive immune responses take effect (Janeway *et al.*, 2001), innate immunity is therefore critically important in protecting the human host from infections during this early period. For *S. aureus* to cause infection it needs to overcome the first line of defence, i.e. the killing mounted by the innate immune system.

In order to identify the molecular mechanisms that *S. aureus* utilises to evade the innate immune response, we have focused on the identification of *S. aureus* genes

that are involved in resistance to one group of key antimicrobial effectors of the innate immune system: AMPs (Ganz, 2003b; Pasupuleti *et al.*, 2012). Human skin is a site *S. aureus* normally colonises and where it can cause infections. The only human cathelicidin, LL-37, is an important AMP produced by the skin, such as in keratinocytes, mast cells, neutrophils, sebocytes and eccrine epithelial cells (Nakatsuji and Gallo, 2012; Reinholz *et al.*, 2012). A range of skin diseases in humans are associated with dysfunctional levels of cathelicidin, such as atopic dermatitis (Ong *et al.*, 2002), chronic ulcers (Heilborn *et al.*, 2003), rosacea (Yamasaki *et al.*, 2007) and psoriasis (Dombrowski and Schauber, 2012). LL-37 is secreted in human sweat (Murakami *et al.*, 2002), providing an inhibitory barrier to pathogens. Additionally, the expression of LL-37 in human skin has been found to be greatly increased following skin injury and declines to pre-injury levels upon wound healing (Dorschner *et al.*, 2001; Heilborn *et al.*, 2003), suggesting an important role of LL-37 in protecting wounds from microbial invasion and / or infection. Furthermore, the importance of AMPs in immune response has also been demonstrated by deletion of the AMP-encoding genes in animal models. For example, the murine cathelicidin antimicrobial peptide CRAMP is encoded by *Cnlp*, a gene which shares 59-76% homology to the human cathelicidin-encoding gene *CAMP* in four exons. Mouse CRAMP also shares a similar α-helical structure and spectrum of antimicrobial activity with human LL-37 (Pestonjamasp *et al.*, 2001), making the murine model a useful tool for the study of human cathelicidin function. Deleting the CRAMP-encoding gene *Cnlp* in mice resulted in the mice exhibiting an increased susceptibility to Group A Streptococcus, as subcutaneous injection of the bacteria caused much larger areas of infection in CRAMP-deficient mice in comparison to the wild-type mice (Nizet *et al.*, 2001).

Nevertheless, *S. aureus* is able to avoid killing by antimicrobial peptides as discussed in chapter 1. *S. aureus* can reduce AMP binding through modification of the cell surface charge by DltABCD or MprF (Peschel *et al.*, 1999, 2001). The bacterium can inhibit the antimicrobial activity of AMPs by secreting enzymes such as staphylokinase (Jin *et al.*, 2004) and a metalloprotease (Sieprawska-Lupa *et al.*, 2004). In addition, AMPs can be expelled from the bacterial cytoplasm by energy-dependent

efflux pumps (Kupferwasser *et al.*, 1999). These are important AMP resistance mechanisms and may contribute significantly to the survival of *S. aureus* in the human body. However as large scale genome screening for *S. aureus* genes involved in resistance or susceptibility to human cationic AMPs had not been conducted at the time of the start of this project, the level of the contribution of the aforementioned mechanisms was not known. In order to more comprehensively explore the molecular mechanisms of *S. aureus* resistance to human cationic AMPs, the decision was made to screen a *S. aureus* LS-1 transposon mutant library for increased resistance to LL-37 as this may give us insights into how the bacterium might regulate resistance or how it might develop resistance in the future.

Prior to screening of a *S. aureus* transposon mutant library, a number of screening conditions and parameters were examined to optimise the screen. These included screening medium or solution, the appropriate concentration of LL-37, and a reasonable time period for the screening process. These parameters were first assessed using the wild-type strain *S. aureus* LS-1 to generate references for designing systems that would be able to identify mutants with altered susceptibility. For instance, the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of LL-37 against *S. aureus* LS-1 are important values for determining the appropriate concentrations of LL-37 required in the screening system. A screening medium containing LL-37 at the MIC for the wild-type would theoretically only allow the proliferation of mutants with increased resistance to LL-37, while the growth of wild-type and mutants with decreased resistance to LL-37 would be inhibited. Given that the peptide LL-37 used in this study was a synthetic peptide, which has a high market price, the design of the screening system needed to fall within a cost-effective range. One way to fulfil this would be to minimise the amount of peptide required by optimising the screening conditions, so that the MICs or MBCs of LL-37 against the wild-type *S. aureus* LS-1 were low.

When considering the optimal MICs or MBCs of LL-37 that could be applied in the screening system, a number of previous studies on the activity of AMPs were considered. Maisetta *et al.* (2008) reported that human cationic AMP β-defensin 3

(HBD-3) exhibits strong antibacterial potential *in vitro*, but its activity is inhibited when examined under conditions that mimic those *in vivo*. For example, supplementation of 20% human serum or physiological concentration of serum salts (NaCl, KCl, $ZnCl_2$, $MgCl_2$ or $CaCl_2$) inhibited the bactericidal activity of HBD-3. Of these salts, physiological concentrations of $CaCl_2$ or NaCl abolished the bactericidal activity of HBD-3 against *S. aureus* to a large extent, with the MBC increasing from 8μg/ml to higher than 256μg/ml (Maisetta *et al.*, 2008). The antimicrobial activity of LL-37 was also found to be inhibited by NaCl (Turner *et al.*, 1998). The inhibition by salt is believed to act through reducing the electrostatic interaction between cationic peptide and negatively charged bacterial surface components, such as teichoic acids and lipoteichoic acids. This is because the cations dissociated from the salts can compete for negatively charged binding site on the cell surface, therefore reducing the chance of cationic peptides binding to the bacterial surface and initiating killing (Kraus and Peschel, 2006). These findings suggest that reducing the concentrations of salts in a medium could be a useful approach to reduce the MICs or MBCs of LL-37 against *S. aureus in vitro*.

The above findings also lead to an interesting question: how do antimicrobial peptides exert their antimicrobial function *in vivo* where there are high concentrations of salts? Several hypotheses have been proposed to explain the killing mechanism *in vivo*: 1) antimicrobial peptides are expressed in high local concentrations in response to inflammation. For instance, LL-37 exists at mucosal surfaces, in the granules of phagocytes, and in most body fluids at 2-5μg/ml (Overhage *et al.*, 2008), but its concentration can be greatly increased at sites of inflammations, such as 15-25μg/ml in bronchoalveolar lavage fluid of infants with pulmonary or systemic infections (Schaller-Bals *et al.*, 2002) and as high as 1.37mg/ml in the psoriatic lesions (Ong *et al.*, 2002). 2) different antimicrobial peptides may act synergistically to exert a desired effect. For example, LL-37 can act with HNP-1 *in vitro* to achieve a much higher killing activity against *S. aureus* and *E. coli* than both individual peptides (Nagaoka *et al.*, 2000). In addition, the expression of multiple antimicrobial peptides such as LL-37 and HBD-3 are induced in human keratinocytes during wound healing (Sorensen *et al.*, 2003).

A study investigating the effect of physiological concentrations of bicarbonate on the antibacterial activity of AMPs suggested that bicarbonate could be a critical ionic factor present in mammalian tissues that enhances the ability of AMPs to kill bacteria in the presence of physiological concentrations of salt (Dorschner *et al.*, 2006). The authors examined the effect of bicarbonate on the antimicrobial activity of LL-37 in a medium constituted of 20% TSB supplemented with 1mM NaH_2PO_4, 10% fetal bovine serum (FBS) and 150mM NaCl (hereafter referred to as medium I). It was found that by adding 50mM $NaHCO_3$ to this medium while maintaining the pH at 7.4, the activity of LL-37 was increased, with MIC reduced from >64μM to 4μM (~18μg/ml) (Dorschner *et al.*, 2006). The authors also tested the effect of bicarbonate on LL-37 in another medium containing 20% TSB + 1mM NaH_2PO_4 (designated medium II), which is medium I devoid of FBS and NaCl. The inhibition of *S. aureus* growth was increased from approximately 5% to 100% by 32μM (144μg/ml) LL-37 upon supplementation with 50mM $NaHCO_3$ (Dorschner *et al.*, 2006). This suggests that for the studies presented in this chapter, addition of bicarbonate may be a promising way to reduce the MIC or MBC of LL-37 against *S. aureus*.

Dorschner *et al*. (2006) have suggested that the increase in antimicrobial activity of LL-37 in the presence of bicarbonate was caused by an action of bicarbonate on the bacteria rather than on the peptide. It was demonstrated that bicarbonate is able to increase the bacterial cell membrane permeability thus promoting the uptake of peptide, which in turn results in the killing of bacteria (Dorschner *et al.*, 2006). The authors found that incubation of bacteria with bicarbonate for at least two hours was necessary in order to achieve increased susceptibility to LL-37, and that incubation with bicarbonate can be prior to, or concurrent with, the addition of LL-37. Bacteria pre-cultured in medium I + 50mM $NaHCO_3$ were susceptible to 57.5μg/ml LL-37 when survival was examined in medium I with or without $NaHCO_3$. In contrast, bacteria that were not pre-cultured with $NaHCO_3$ were resistant to LL-37 regardless of the presence of $NaHCO_3$ in the antimicrobial assay (Dorschner *et al.*, 2006). In light of this, culturing the bacteria in the presence of $NaHCO_3$ prior to the antimicrobial assays may be important to achieve low MICs or MBCs of LL-37.

Since Dorschner *et al*. (2006) did not compare the MICs or MBCs of LL-37 in medium I and II (described above) in the presence of 50mM $NaHCO_3$, this parameter was examined in this study. The activity of LL-37 under the conditions described by Dorschner *et al*. (2006) was also examined in our laboratory. All the media or buffers involved in the LL-37 antimicrobial assays are listed in chapter 2 section 2.6.1.

5.2 Effect of $NaHCO_3$ on the antimicrobial activity of LL-37 in medium I

Dorschner *et al*. (2006) reported that the MIC of LL-37 against *S. aureus* was higher than 64µM (~288µg/ml) in medium I, but it was as low as 4µM (18µg/ml) in medium I with 50mM $NaHCO_3$ (medium I + C) (Dorschner *et al.*, 2006). In order to confirm the effect of $NaHCO_3$ on the activity of LL-37 in medium I, this MIC assay was repeated using *S. aureus* LS-1 as described in chapter 2 section 2.6.3. Unexpectedly, the MICs of LL-37 against *S. aureus* LS-1 in medium I with or without $NaHCO_3$ were both higher than 128µg/ml in three independent experiments. However, the optical density of the cultures showed that the growth of *S. aureus* LS-1 in medium I + C was inhibited to a larger extent than that in medium I. The inhibition of growth was determined as described in section 2.6.4 and is plotted in Figure 5.1. It is clear that LL-37 was more capable of inhibiting the growth of *S. aureus* LS-1 in the presence of $NaHCO_3$ than that in the absence of $NaHCO_3$.

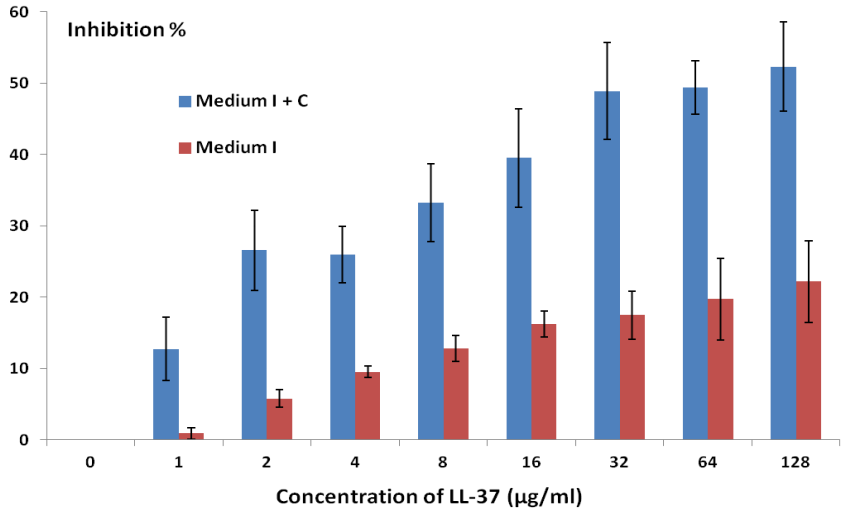

Figure 5.1: Inhibition of *S. aureus* LS-1 growth by a range of concentrations of LL-37 in medium I (red bars) and media I + C (blue bars). The data presented are the means of three independent experiments with error bars showing the standard deviations (SDs).

5.3 Effect of $NaHCO_3$ on the growth inhibitory activity of LL-37 in medium II

The effect of $NaHCO_3$ on the activity of LL-37 in medium II was also examined by Dorschner *et al*. (2006) using an inhibition assay as described in section 2.6.4. The authors found that 32μM (144μg/ml) LL-37 inhibited *S. aureus* growth by approximately 5% in medium II, while complete inhibition was observed in medium II + 50mM $NaHCO_3$ (medium II + C) (Dorschner *et al.*, 2006). This inhibition assay was repeated on strain LS-1 using the same concentration of LL-37. As determined by three independent experiments, the growth of *S. aureus* LS-1 in medium II + C was inhibited by 93.6% ± 3.4% (SD), but by only 5.2% ± 3.2% (SD) in medium II. These results are similar to those reported by Dorschner *et al*. (2006).

5.4 MIC of LL-37 against *S. aureus* in medium II (+/-C)

Repetition of the experiments reported by Dorschner *et al*. (2006) as described above confirmed that supplementation with 50mM $NaHCO_3$ in either medium I or II increases the growth inhibitory activity of LL-37. However, supplementation of medium I with $NaHCO_3$ did not reduce the MIC of LL-37 against *S. aureus* to a level that was practical for screening. It was decided to see if the MIC was reduced in medium II in the presence of $NaHCO_3$. The MIC of LL-37 in medium II (+/-C) was measured as described in section 2.6.3. As shown by three independent experiments, the MIC of LL-37 against *S. aureus* LS-1 in medium II was higher than 128μg/ml, while it was 16μg/ml in medium II + C.

In comparison with medium I + C, medium II + C gave a much lower MIC of LL-37 against *S. aureus* LS-1. These results suggested that medium II + C could be useful for screening the transposon mutant library.

5.5 MIC of LL-37 against *S. aureus* plated on agar plates containing medium II +C

One methodology that could be used to screen for *S. aureus* mutants with decreased resistance to LL-37 is by first plating the library on agar plates without LL-37 to give single colonies and then replica plating them onto agar containing LL-37 at a concentration lower than the MIC. Because agar might alter the MIC of LL-37 against *S. aureus*, the MIC of LL-37 was determined on agar containing medium II + C as described in section 2.6.5. In three independent experiments, the MIC of LL-37 against *S. aureus* LS-1 on agar containing this medium was determined to be in the range of 8-16μg/ml, with a median value of 8μg/ml.

5.6 MBC of LL-37 against *S. aureus* in medium II +C

5.6.1 Twenty four hour MBC assays

To screen for transposon mutants with increased resistance to LL-37 one rapid approach is to grow the mutant library in liquid medium containing LL-37 at a bactericidal concentration against the wild-type bacterium. Most of the bacteria that survive would be expected to have increased resistance to LL-37 unless a secreted resistance factor was produced. Given that the MBC is defined as the concentration which gives ≥ 99.9% kill, this methodology has limitations in that it would only increase the proportion of resistant mutants over wild-type, and not completely eliminate the wild-type if more than 3 $\log_{10}$ bacteria were present in the assay. Nevertheless an increase in the proportion of any mutant present in the surviving population would be an indication that it had increased resistance to LL-37. Therefore the MBC of LL-37 against *S. aureus* LS-1 in medium II + C was determined according to the Clinical and Laboratory Standards Institute (CLSI, guideline M26-A) as described in section 2.6.6. Incubating approximately 1×10^5 CFU/ml of *S. aureus* LS-1 in the presence of a range of concentrations of LL-37 for 24 hours demonstrated that the MBC was 16μg/ml in three independent experiments.

5.6.2 Two hour MBC assays

Given that the medium II contains a low concentration of TSB, by performing the MBC assay over 24 hours as described above there was the added complication that some transposon mutants might grow faster or slower than the wild-type irrespective of any change in resistance change to LL-37. To overcome this possible added complication it was decided to examine the MBC of LL-37 after 2 hours incubation as described by Ouhara *et al*. (2008) who performed LL-37 MBC assays in sodium phosphate buffer (Ouhara *et al.*, 2008). Dorschner *et al*. (2006) reported that at least a 2-hour incubation period with $NaHCO_3$, either prior to or in concurrent with the addition of LL-37, was necessary for the bicarbonate-dependent increased bactericidal activity of LL-37. Therefore in this part of the study bacteria were pre-cultured with or without 50mM $NaHCO_3$ for 4 hours before the 2 hour MBC assay as described in section 2.6.7.

The MBCs of LL-37 in medium II with or without 50mM $NaHCO_3$ were all higher than 128µg/ml regardless of pre-incubation of the cells with $NaHCO_3$. However, the percentage of bacteria killed in each of the samples, as shown in table 5.1, revealed differences in the activity of LL-37 depending on whether $NaHCO_3$ was present in the assay or not. Addition of LL-37 at concentration 0-128µg/ml to medium II did not result in kill of cells pre-cultured with or without $NaHCO_3$. However, LL-37 added at concentration 16-128µg/ml in medium II + C was able to kill more than 90% of the cells regardless of whether they had been pre-cultured in medium containing $NaHCO_3$ (table 5.1). The percentage kill reached 99% at LL-37 concentrations of 64 and 128µg/ml in medium II + C. It is obvious that pre-culturing the bacteria with or without $NaHCO_3$ did not affect the susceptibility of the bacteria to LL-37.

Although the MBCs of LL-37 in medium II or medium II + C were all determined to be higher than 128µg/ml, concentrations of 16-128µg/ml in medium II + C exhibited killing activity against *S. aureus* LS-1, while the same concentrations of LL-37 in medium II failed to result in killing. This confirmed that $NaHCO_3$ enhances the antimicrobial activity of LL-37 against *S. aureus*. However the results presented in

this thesis are different to the findings described by Dorschner *et al*. (2006) that pre-culturing of bacteria in $NaHCO_3$ rendered increased sensitivity to LL-37.

Table 5.1: The percentage of *S. aureus* LS-1 killed after 2 hours incubation with different concentrations of LL-37. The parameters examined were the effect of pre-culturing the bacteria with $NaHCO_3$ on killing, and the effect of $NaHCO_3$ in the killing assay medium on the percentage of bacteria killed. Data presented are the means of three independent experiments with standard deviations included. The significance of the killing was determined using one way ANOVA, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

culturing medium	**Assay medium**	**Concentration of LL-37 (μg/ml)**				
		0 - 8	**16**	**32**	**64**	**128**
Medium I	**Medium II**	0%	0%	0%	0%	0%
	Medium II + C	0%	(92.4 ± 3.4)%	(98.4 ± 1.6)% *	(99.5 ± 0.3)% **	(99.4 ± 0.7)% **
Medium I + C	**Medium II**	0%	0%	0%	0%	0%
	Medium II + C	0%	(96.1 ± 0.8)% **	(98.5 ± 1.4)% ***	(99.4 ± 0.2)% ***	(99.1 ± 0.7)% ***

5.7 Effect of $NaHCO_3$ on the MBC of LL-37 in buffer III

Given that the MBC of LL-37 against *S. aureus* in medium II + C with 2 hours of incubation was higher than 128μg/ml, and the concentration of LL-37 capable of killing ≥99% (2-$\log_{10}$) of the cells was determined to be 64μg/ml, it was decided to try and further optimise the medium system to try and reduce the MBC further. Since NaCl inhibits the antimicrobial activity of LL-37 (Turner *et al.*, 1998), and is present in TSB at 5g/L (~85.5mM), it was hypothesised that removing TSB from medium II + C would reduce the MBC of LL-37. Medium II lacking TSB was designated buffer III, and with the addition of 50mM $NaHCO_3$ was designated buffer III + C.

The activity of LL-37 was examined in buffer III (+/-C) as described in section 2.6.7, using cells pre-cultured with or without $NaHCO_3$ to further examine the necessity of pre-incubating cells with $NaHCO_3$. The MBCs of LL-37 against *S. aureus* in different buffers are summarised in table 5.2. As hypothesised, removing TSB from medium II greatly reduced the MBC of LL-37 against *S. aureus* LS-1, from higher than 128μg/ml to 4μg/ml for all the conditions examined. As observed in MBC assays in medium II (+/-C) described in section 5.6, no difference in susceptibility to LL-37 was found

between cells pre-cultured with or without $NaHCO_3$. Unexpectedly, there was also no difference between the activity of LL-37 in buffer III and buffer III + C. It seems $NaHCO_3$ has no influence on the antimicrobial activity of LL-37 against *S. aureus* LS-1 in the absence of TSB. Nevertheless, both buffer III and buffer III + C would be suitable for screening for mutants with increased resistance to LL-37

Table 5.2: MBCs of LL-37 in buffer III (+/-C) against *S. aureus* LS-1 cells pre-cultured in medium I (+/-C). MBC values outside the brackets are the median values of three independent experiments, and the ranges of the MBCs from the three experiments are presented in brackets.

Pre-culture medium	Assay buffer	MBC µg/ml
Medium I	Buffer III	4 (4-8)
	Buffer III + C	4 (2-4)
Medium I + C	Buffer III	4 (4-8)
	Buffer III + C	4 (4-8)

5.8 Activity of LL-37 against *S. aureus* cultured in TSB

In all the experiments described in the previous sections, *S. aureus* LS-1 was cultured according to Dorschner *et al*. (2006) in medium I (+/-C) to early log phase prior to MIC or MBC assays. Given that we routinely culture *S. aureus* in TSB, the MBCs of LL-37 in buffer III (+/-C) was determined using *S. aureus* LS-1 cultured overnight in TSB to generate a reference for the following experiments. As demonstrated by three independent experiments, the MBC of LL-37 against LS-1 in buffer III was in the range of 4-8µg/ml, with a median value of 4µg/ml, and in buffer III + C was in the range of 8-16 µg/ml, with a median value of 8µg/ml. The MBCs of LL-37 against TSB-cultured LS-1 were still low in buffer III (+/-C) and would allow cost effective screening of mutants in these two buffers.

5.9 Activity of LL-37 against defined mutants of *S. aureus*

Prior to the screening of the transposon mutant library, a collection of defined mutants were tested to characterise their susceptibility to LL-37. These mutants have a deletion or disruption in genes encoding a range of functions including the accessory gene regulator (Agr), the staphylococcal accessory regulator (Sar), the alternative sigma factor (SigB), aspartate transcarbamoylase (PyrB) involved in pyrimidine biosynthesis, adenylosuccinate lyase (PurB) involved in purine biosynthesis, biotin synthase (BioB) involved in biotin biosynthesis, lipoprotein diacylglyceryl transferase (Lgt) involved in lipoprotein biosynthesis, fibronectin binding protein A and B (FnbpAB), gamma-hemolysin (Hlg), the major autolysin (AtlA) and a protein involved in *S. aureus* cell division (ScdA). These mutants were selected from a bank of mutants, possessed by Dr Nair, since they have previously been shown to be attenuated for virulence by other groups or by the Nair group (published and unpublished, summarised in chapter 2 table 2.1). Since neutrophils play an important role in many of the virulence models previously used to examine these mutants it was hypothesised that the deleted genes might confer some resistance to the antimicrobial peptides produced by these immune cells.

To determine if any of these mutants was more or less susceptible to LL-37, MBCs were determined in buffer III in the absence or presence of $NaHCO_3$ (buffer III or buffer III + C). The MBC of LL-37 against each mutant is listed in table 5.3. All the mutants exhibited no more than a 2-fold difference in the level of susceptibility to LL-37 compared to their respective parental strain in buffer III or buffer III + C. This data demonstrates that none of the genes examined were required for resistance to LL-37 *in vitro*.

Table 5.3: MBCs of LL-37 against *S. aureus* parental and mutant strains in buffer III (+/-C). MBC values outside the brackets are the median values of three independent experiments, and the ranges of the MBCs from the three experiments are presented in brackets.

Strains	MBC (µg/ml)	
	Buffer III	Buffer III + C
LS-1	4 (4-8)	8 (8-16)
LS-1 Δ*pyrB*	8 (4-8)	8 (4-8)
LS-1 Δ*purB*	8	16 (8-16)
LS-1 Δ*pyrB*/Δ*purB*	8	8
LS-1 Agr^-	8	8
LS-1 Sar^-	8	8
LS-1 Agr^- Sar^-	8	8
LS-1 Δ*rsbUVWsigB*	8	8 (8-16)
LS-1 $BioB^-$	8 (4-8)	8 (8-16)
LS-1 $FnbpAB^-$	4 (4-8)	8
LS-1 $AtlA^-$	8	8
8325-4	8 (4-8)	16
8325-4 Δ*scdA*	8 (8-16)	8
8325-4 Δ*hlg*	4	8
SA113	8	16
SA113 Δ*lgt*	8 (4-8)	8

5.10 Dynamics of LL-37 killing of *S. aureus* in buffer III + C

Following the characterisation of the susceptibility of the defined mutants to LL-37, the transposon mutant library was screened to identify genes important for resistance to LL-37. Because screening of mutants that had increased sensitivity to LL-37 would be a labour intensive process of replica plating tens of thousands of colonies onto plates with and without LL-37 it was decided to take the less labour intensive approach of screening for mutants with increased resistance to LL-37. Given that bicarbonate is an important ionic factor in the human body, it was decided to screen mutants with increased resistance in buffer III + C.

First, In order to determine an appropriate concentration of LL-37 and a corresponding incubation period for the screening, the killing dynamics of LL-37 at 0, 2, 4 and 8µg/ml against the wild-type *S. aureus* LS-1 over an incubation period of 5 hours was determined as described in section 2.6.8. To avoid electrostatic binding of the positively charge peptide LL-37, eppendorf polypropylene microcentrifuge tubes (1.5ml) were used for these assays, with an initial volume of 0.5ml for each sample. The killing curves of LL-37 at different concentrations are presented in figure 5.2 as the number of viable bacteria in 100µl (the limiting volume of detection) of the samples at each time point. The number of viable cells in the sample without LL-37 decreased slightly over the 5-hour time period examined, but this was not significant as determined using a paired-sample t-test ($p = 0.238$). When *S. aureus* was incubated in buffer containing different concentrations of LL-37, concentration-dependent killing profiles were observed, with 8µg/ml resulting in a greater than 4-$\log_{10}$ kill in 5 hours.

To comprehensively screen for genes involved in resistance to LL-37 on a genome-wide scale, for a genome size similar to *S. aureus* NCTC 8325 (2,821,361bp, 3006 genes), 2.08×10^4 random individual mutant are required for a 99.9% probability of whole genome coverage (calculated as described in chapter 2 section 2.4.4.2). We constructed a *S. aureus* LS-1 mutant library with approximately $(4.9 \pm 0.9) \times 10^5$ individual mutants (described in chapter 3 section 3.10), which is 24 times the number of mutants required for a 99.9% probability of full genome coverage. It was decided to screen 1×10^6 mutants from the mutant library, which should cover all the individual mutants in the library. Given that a 5-hour incubation time with 8µg/ml of LL-37 gave more than 4-$\log_{10}$ kill, these conditions were chosen to screen the library since it would allow us to input 6-$\log_{10}$ mutants and expect that less than 100 of the wild-type and mutants with ≤ wild-type level of resistance to LL-37 would still be present after incubation.

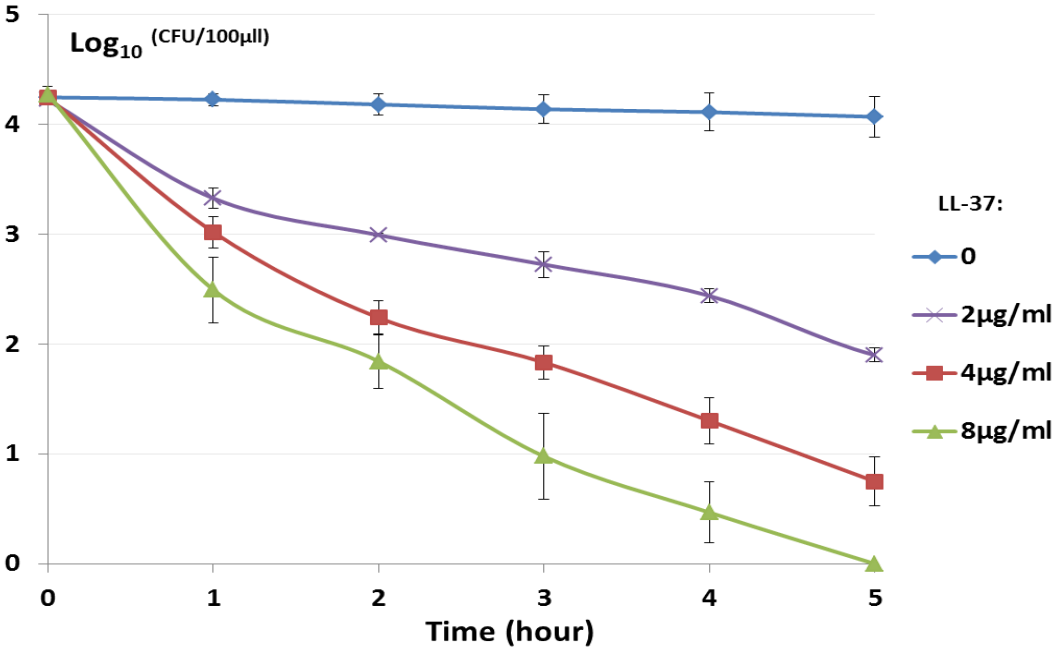

Figure 5.2: Killing curves of LL-37 against *S. aureus* LS-1 in buffer III + C. Curves in the colours blue, purple, red and green represent LL-37 concentrations of 0, 2, 4 and 8µg/ml, respectively. Data presented are the means of three independent experiments with error bars showing the standard deviations.

5.11 Screening for *S. aureus* mutants with increased resistance to LL-37

Mutants with potentially increased resistance to LL-37 were selected for by two rounds of screening in buffer III + C with 8µg/ml LL-37 as described in section 2.7.1. In the first round of selection, 6.11 ± 0.14 (SD) $\log_{10}$ wild-type and 6.15 ± 0.04 (SD) $\log_{10}$ mutants from the transposon mutant library were separately treated with 8µg/ml LL-37 for 5 hours in a total volume of 10ml for each sample. The number of viable cells in the 10ml wild-type or mutant samples with or without LL-37 at time 0 hour and 5 hours are presented in figure 5.3. At 5 hours, both the wild-type and mutants samples incubated with LL-37 contained significantly less viable cells compared to their respective control samples which were not incubated with LL-37. Over the 5-hour period of time, there was no significant change in the number of viable cells in the wild-type sample without LL-37, while there was a significant reduction in the number of viable cells in the mutant sample without LL-37, suggesting that the mutant population is less able to survive in buffer III + C than the

wild-type over this time period. However, significantly more bacteria survived killing by LL-37 in the mutant sample compared to the wild-type sample.

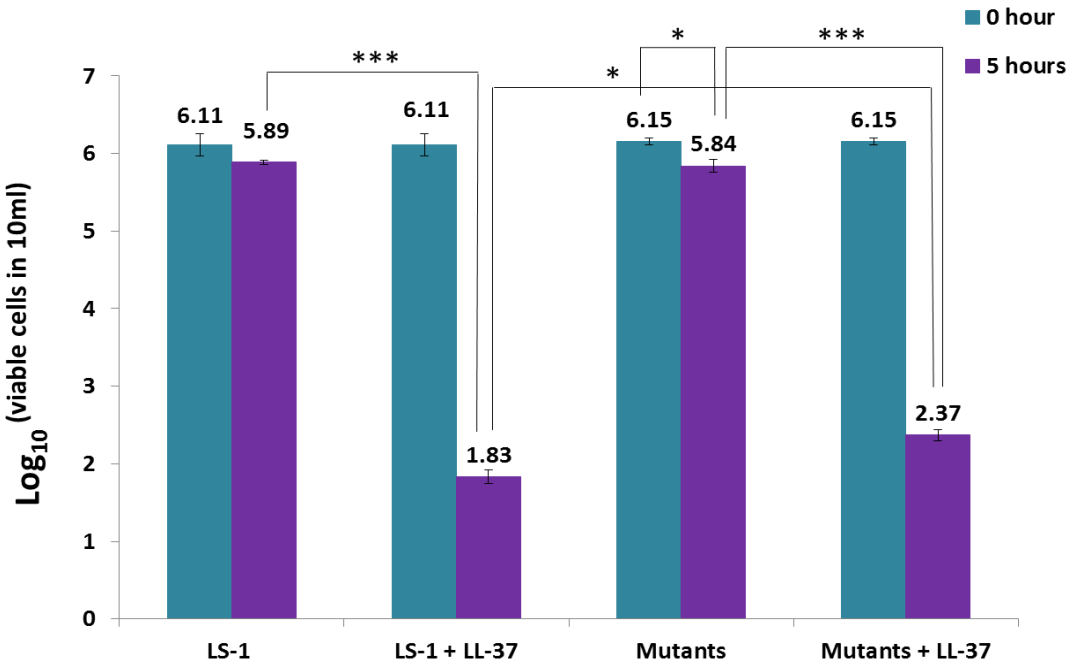

Figure 5.3: The number of viable cells of *S. aureus* LS-1 and mutants before and after the first round of treatment with or without 8µg/ml LL-37 in 10ml buffer III + C. Black bars: number of viable cells at 0 hour. Grey bars: number of viable cells at 5 hours. Data presented are the means of three independent experiments with error bars showing the standard deviations. Significance of the difference between each two samples were analysed using one way ANOVA, *: P<0.05, ***: P<0.001.

Subsequently, the wild-type and mutants that survived the first round of screening were subjected to a second round of screening as described in section 2.7.1. Because only 1.83 ± 0.09 $\log_{10}$ of the wild-type and 2.37 ± 0.07 $\log_{10}$ of the mutants survived the first round of screening, the second round of screening was conducted under the same conditions as those in the first round of screening except using a total volume of 0.5ml for one sample. The bacteria which survived from the first round of screening were propagated in TSB individually, and then introduced into the second round of screening at approximately equal numbers according to their optical density. The wild-type bacteria were introduced at 4.86 ± 0.03 $\log_{10}$ in 0.5ml (4.16 ± 0.03 $\log_{10}$ / 100µl), and the mutants were introduced at 4.82 ± 0.04 $\log_{10}$ in 0.5ml, (4.12 ± 0.04 $\log_{10}$ / 100µl). The number of viable cells in the wild-type or mutant samples with or without LL-37 at time 0 hour and 5 hours are presented in figure 5.4. Over the 5-hour period of time, no significant change in the number of viable cells was found in the

wild-type sample without LL-37, while the number of viable cells in the mutant sample without LL-37 reduced significantly. At 5 hours, the number of viable cells in the wild-type and mutant samples with LL-37 reduced significantly compared to their control without LL-37, and significantly more mutants survived killing by LL-37 than the wild-type.

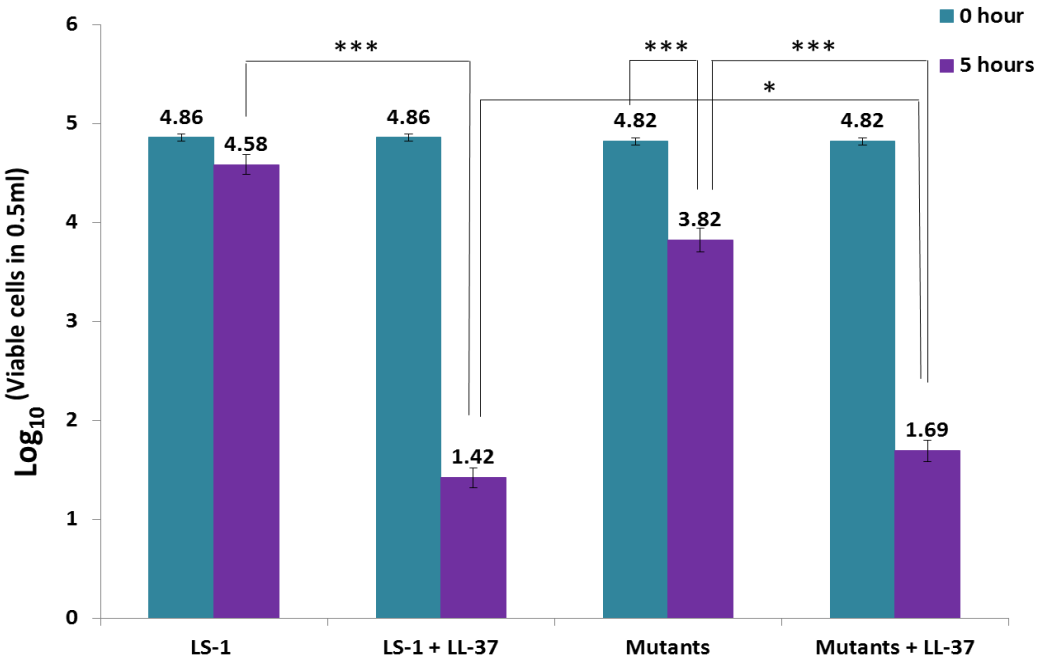

Figure 5.4: The number of viable cells of *S. aureus* LS-1 and mutants before and after the second round of treatment with or without 8µg/ml LL-37 in 0.5ml of buffer III + C. Black bars: number of viable cells at 0 hour. Grey bars: number of viable cells at 5 hours. Data presented are the means of three independent experiments with error bars showing the standard deviations. Significance of the difference between each two samples were analysed using one way ANOVA, *: P<0.05, ***: P<0.001.

5.12. Susceptibility of the surviving *S. aureus* mutants to killing by LL-37

To identify mutants with increased resistance to LL-37, the susceptibility of mutants which survived the second round of screening as described above were characterised. A total of 37 mutants were screened in the third experiment. The susceptibility of these mutants to LL-37 was determined as described in section 2.7.2, using an adapted two-hour MBC assay with only one concentration of LL-37, 8µg/ml (the median MBC of LS-1 in buffer III + C, described in section 5.8). Briefly, approximately 4-$\log_{10}$ cells of each mutant were treated in 100µl buffer III + C with 8µg/ml LL-37 for

2 hours, and the log_{10} kill was calculated. According to the LL-37 killing curves in figure 5.2, it was expected that there would be a 2-3 log_{10} kill of the wild-type over this time period, and the killing of mutants resistant to LL-37 would be less than the wild-type. The numbers of bacteria killed over 2 hours for wild-type and mutants determined in two independent experiments are presented in table 5.4. Unexpectedly, only 1.13-log_{10} kill was detected for LS-1, and of the 37 mutants, only 9 mutants showed less kill than the wild-type, being mutants numbered 5, 8, 10, 13, 21, 22, 25, 32 and 37 (highlighted in bold in table 5.5). The significance of the reduced kills observed for these mutants was analysed using one way ANOVA, and the p values was included in table 5.4. Seven of these mutants, 5, 13, 21, 22, 25, 32 and 37, demonstrated significantly increased resistance to 8μg/ml LL-37.

Table 5.4: The number of bacteria killed in samples containing LS-1 or mutants incubated with 8μg/ml LL-37, and the decreased log_{10} kill for each mutant compared to LS-1. The data presented are the means of two independent experiments with standard deviations included. The significance of the difference between the wild-type and mutants with reduced kill was analysed using one way ANOVA, *: P<0.05, **: P<0.01, ***: P<0.001.

Wild-type and mutant number	Log_{10} kill	Decreased log_{10} kill compared to LS-1
LS-1	1.13 ± 0.05	0.00 ± 0.00
1	1.8 ± 0.09	-0.67 ± 0.15
2	1.6 ± 0.05	-0.47 ± 0.10
3	1.78 ± 0.03	-0.65 ± 0.08
4	1.2 ± 0.02	-0.08 ± 0.04
5	**0.57 ± 0.00**	**0.56 ± 0.05 (p = 0.001) ****
6	1.14 ± 0.04	-0.02 ± 0.02
7	1.24 ± 0.3	-0.12 ± 0.25
8	**0.88 ± 0.15**	**0.25 ± 0.10 (p = 0.14)**
9	1.48 ± 0.35	-0.3 ± 0.30
10	**0.99 ± 0.02**	**0.13 ± 0.04 (P = 0.767)**
11	1.32 ± 0.16	-0.19 ± 0.10
12	1.52 ± 0.54	-0.40 ± 0.49
13	**0.72 ± 0.15**	**0.41 ± 0.09 (p = 0.008) ****
14	1.5 ± 0.07	-0.37 ± 0.02
15	1.33 ± 0.1	-0.20 ± 0.05

Wild-type and mutant number	Log_{10} kill	Decreased log_{10} kill compared to LS-1
16	1.7 ± 0.2	-0.57 ± 0.14
17	1.74 ± 0.08	-0.61 ± 0.02
18	1.82 ± 0.33	-0.69 ± 0.28
19	2.68 ± 0.17	-1.55 ± 0.12
20	1.53 ± 0.59	-0.41 ± 0.64
21	**0.32 ± 0.03**	**0.80 ± 0.03 (p < 0.001) *****
22	**0.7 ± 0.11**	**0.42 ± 0.05 (p = 0.006) ****
23	2.01 ± 1.07	-0.88 ± 1.02
24	2.10 ± 1.01	-0.97 ± 0.96
25	**0.33 ± 0.00**	**0.79 ± 0.05 (p < 0.001) *****
26	1.48 ± 0.08	-0.35 ± 0.13
27	1.36 ± 0.25	-0.24 ± 0.19
28	1.48 ± 0.29	-0.36 ± 0.24
29	1.72 ± 0.34	-0.59 ± 0.28
30	1.96 ± 0.49	-0.83 ± 0.44
31	1.31 ± 0.07	-0.18 ± 0.02
32	**0.48 ± 0.00**	**0.64 ± 0.05 (p < 0.001) *****
33	1.34 ± 0.51	-0.21 ± 0.46
34	1.53 ± 0.37	-0.41 ± 0.32
35	1.31 ± 0.22	-0.19 ± 0.16
36	1.19 ± 0.04	-0.06 ± 0.10
37	**0.31 ± 0.00**	**0.81 ± 0.05 (p < 0.001) *****

5.13 Identification of the transposon insertion sites in mutants with increased resistance to LL-37

To identify the genes disrupted in mutants 5, 13, 21, 22, 25, 32 and 37, the transposon and the flanking chromosomal DNA in each mutant was amplified using Y-linker PCR (Kwon and Ricke, 2000) as described in section 2.4.3. The Y-linker PCR products from these seven mutants were analysed by gel electrophoresis, and the gel image is shown in Figure 5.7. Only one PCR product was detected for mutants 5,

21, 25, 32 and 37, indicating that there was probably a single insertion of the transposon in these mutants. While two PCR products were observed for mutants 13 and 22, which indicates that there were at least two insertions of the transposon in these two mutants. The sizes of the PCR products from mutants 13 and 22 appeared to be similar to each other. Similarly the PCR products from mutants 21, 25 and 37 appeared to be approximately the same size. This suggested that the members of each of these two groups of mutants might be siblings.

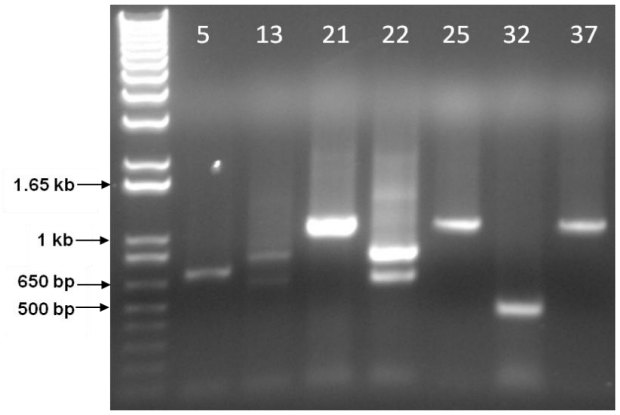

Figure 5.5: Image of the electrophoresis gel on which the Y-linker PCR products from mutants 5, 13, 21, 22, 25, 32 and 37 were separated. The first lane: 1kb plus DNA ladder (Invitrogen).

To identify the insertion sites in these seven mutants, the DNA in each of these bands were excised and purified, followed by sequencing using transposon specific primer P103. The DNA sequences corresponding to the transposon sequence and the Y-linker sequence at both ends of each PCR product was determined and subtracted from the total DNA sequence, thereby theoretically leaving only DNA sequence amplified from the flanking genome sequence. The sequence corresponding to the transposon flanking genome sequence was analysed using BLASTn (available at http://www.ncbi.nlm.nih.gov/BLAST/) and the reference genome sequence of strain *S. aureus* NCTC 8325 (GenBank accession: CP000253). The transposon insertion sites in these mutants are summarised in table 5.5. Mutants 13 and 22 are siblings with transposon insertions in genes with locus tags SAOUHSC_00064 and SAOUHSC_02351. Mutants 21, 25 and 37 are also siblings with a transposon insertion in a gene with locus tag SAOUHSC_00022. The remaining two mutants, 5 and 32, had transposon insertions in genes with locus tags SAOUHSC_02350 and SAOUHSC_00023, respectively. Therefore, only four unique mutants with increased

resistance to LL-37 were identified in this screen. The transposon insertions in these four mutants cluster in two regions, SAOUHSC_02350-SAOUHSC_02351 (*atpB-atpI*) and SAOUHSC_00022-SAOUHSC_00023 (*yycH-yycI*). Genes *atpB* and *atpI* encode the subunit A and subunit I of the F_0F_1 ATP synthase, respectively, and genes *yycH* and *yycI* are part of an operon *yycFGHIJK* and have been suggested to encode two regulators of a two component system encoded by *yycFG* in *B. subtilis* (Szurmant *et al*., 2005, 2007). These data suggest that F0F1 ATP synthase and the two component system YycFG may be involved in resistance to LL-37.

Table 5.5: Transposon insertion sites in mutant 5, 13, 21, 22, 25, 32 and 37. The locus tags are according to strain NCTC 8325 (GenBank accession: CP000253).

Mutant	Locus of Tn insertion	Identity score	Gene	Description
5	SAOUHSC_02350	100%	*atpB*	F_0F_1 ATP synthase subunit A
13/22	SAOUHSC_00064 & SAOUHSC_02351	100%	*norG* *atpI*	Transcriptional regulator NorG F_0F_1 ATP synthase subunit I
21/25/37	SAOUHSC_00022	100%	*yycH*	Regulator of YycFG
32	SAOUHSC_00023	100%	*yycI*	Regulator of YycFG

5.14 Determination of the MBCs of LL-37 against the *S. aureus* mutants identified by screening

The susceptibility of the four types of mutants, recovered from the screening, to killing by LL-37 was characterised using the MBC assay in buffer III and buffer III + C as described in chapter 2 section 2.6.7. The MBCs of LL-37 against each strain in three independent experiments are presented in table 5.6. When measured in buffer III + C, the buffer used in screening for mutants with increased resistance to LL-37, mutants 21 and 32 (LS-1 Δ*yycH* and LS-1 Δ*yycI*) demonstrated greatly increased resistance to LL-37 compared to the wild-type LS-1. However, the other two mutants, LS-1 Δ*atpB* and LS-1 Δ*norG ΔatpI*, had the same susceptibility to LL-37 as the wild-type LS-1. Interestingly, when assayed in buffer III, all the mutants showed the same

level of resistance to LL-37 as the wild-type. Therefore, the *yycH* and *yycI* mutants only demonstrated $NaHCO_3$-dependent resistance to LL-37.

Table 5.6: MBCs of LL-37 against *S. aureus* strains in buffer III with or without $NaHCO_3$. MBC values outside the brackets are the median values of three independent experiments, and the ranges of the MBCs from the three experiments are presented in brackets.

Strains	MBC (μg/ml)	
	Buffer III	Buffer III + C
LS-1	8	16 (8-16)
Mutant 5 (LS-1 Δ*atpB*)	8	16 (8-16)
Mutant 21 (LS-1 Δ*yycH*)	8	>128
Mutant 22 (LS-1 Δ*norG Δatpl*)	8	16 (8-16)
Mutant 32 (LS-1 Δ*yycI*)	8	>128

5.15 Discussion

In order to design a cost effective screening system to identify transposon mutants of *S. aureus* which had altered susceptibility to LL-37, the antimicrobial activity of LL-37 against *S. aureus* LS-1 was tested in a range of media and buffers. It was found that mammalian serum and NaCl inhibited the antimicrobial activity of LL-37, which is consistent with previous studies (Maisetta *et al.*, 2008; Deslouches *et al.*, 2005). Additionally, as reported by Dorschner *et al*. (2006) incorporation of bicarbonate ($NaHCO_3$) into the antimicrobial assay medium, I or II, greatly enhanced the antimicrobial activity of LL-37. The addition of 50mM $NaHCO_3$ into medium II reduced the MIC of LL-37 from higher than 128μg/ml to 16μg/ml, which makes it possible to cost effectively screen for mutants with altered levels of susceptibility to LL-37.

Dorschner *et al*. (2006) have reported that bicarbonate enhances the killing ability of LL-37 through acting on the bacterial cells to increase susceptibility to the peptide, rather than acting on the peptide to alter the activity of the peptide. The authors demonstrated this by showing that *S. aureus* cells pre-cultured in the presence of $NaHCO_3$ exhibited the same level of susceptibility to LL-37 in medium with or without

$NaHCO_3$. In addition, the authors also reported that *S. aureus* grown initially without $NaHCO_3$ were not inhibited by LL-37 even if $NaHCO_3$ was added at the time of LL-37 exposure (Dorschner *et al.*, 2006). However, the data generated from my PhD studies do not support the data published by Dorschner *et al*. (2006). We found that supplementation of $NaHCO_3$ into the antimicrobial assay medium is essential for $NaHCO_3$-enhanced antimicrobial activity of LL-37, rather than culturing the cells with $NaHCO_3$ prior to the antimicrobial assays. Dorschner *et al*. (2006) have demonstrated that bicarbonate enhances the killing ability of LL-37 by increasing bacterial cell membrane permeability, which in turn promotes the uptake of LL-37. Our data may not contradict this mechanism, however we found that the effect of bicarbonate on the bacterial cell membrane is not sustained after the removal of bicarbonate, which is contrary to that reported by Dorschner *et al*. (2006).

An older study correlated the antimicrobial activity of LL-37 with the amphipathic α helical structure of this peptide (Johansson *et al.*, 1998). The authors demonstrated that the α-helical structure of LL-37 is dependent on concentration, pH and anions. The peptide exhibits a disordered conformation in water, whereas it transforms into an α-helical structure when the peptide concentration is above 1μM (4.5μg/ml), the pH is between 5 and 13.2, or in the presence of anions such as SO_4^{2-}, HCO_3^- and $CF_3CO_2^-$ (Johansson *et al.*, 1998). The authors characterised the α-helical content and antimicrobial activity of LL-37 in the presence of a range of anions (SO_4^{2-}, HCO_3^-, Cl^-) at different concentrations, and found that the antimicrobial activity of LL-37 is proportional to the content of amphipathic α helical structure in LL-37.

Although HCO_3^- may increase the α-helical content of LL-37, $NaHCO_3$-enhanced antimicrobial activity of LL-37 has not been demonstrated in all the media and buffers we examined. $NaHCO_3$ was found to enhance the antimicrobial activity of LL-37 in medium I or II, but not in buffer III. Since the only difference between medium II and buffer III is that medium II contains 20% TSB, it suggests that $NaHCO_3$ enhances the antimicrobial activity of LL-37 in the presence of TSB. TSB is a complex medium which may contain components that inhibit the antimicrobial activity of LL-37, and it is possible that $NaHCO_3$ increases the antimicrobial activity of LL-37 in medium I or II

by inhibiting these components. It is known that NaCl, one of the TSB components, inhibits the antimicrobial activity of LL-37 (Turner *et al.*, 1998). However, we have measured the MBCs of LL-37 in buffer III + NaCl (equal to the amount in 20% TSB) in the presence or absence of $NaHCO_3$, and confirmed that the mechanism by which $NaHCO_3$ increases the antimicrobial activity of LL-37 in the presence of TSB is not through attenuating the inhibitory effect of NaCl on LL-37. Given that bacteria incubated in the presence of TSB, medium I or II, are highly metabolically active and dividing, while they are not in buffer III, it is also possible that $NaHCO_3$ only enhances the susceptibility to LL-37 in such cells.

In the mutant screening experiment, 1.41×10^6 bacteria from the transposon mutant library, described in chapter 3, were used to identify mutants with increased resistance to LL-37 in buffer III + C. For a 99.9% probability of full genome coverage 2.08×10^4 transposon mutants would be required and since the library size was actually 4.9×10^5 theoretically this would allow for 24 insertions in every gene in the genome and upon amplification of this number of clones to the number of mutants used for screening each of these would have been represented by three siblings. So in other words, in theory at least, there should have been a very high coverage of the genome by the number of bacteria used in the screen. The mutant library was screened in a pool for two rounds, with a bacterial cell number amplification step between the two rounds. Given that the mutant library was collected from growth on agar (described in chapter 3), this might have resulted in faster growing mutants being present in the mutant library at higher copy numbers than slow growing mutants. Therefore, one drawback of this approach is that it might select against slow growing mutants with increased resistance, while fast growing mutants with decreased resistance may be identified. This may explain why only 7 out of 37 mutants which survived the screening process demonstrated increased resistance to 8μg/ml LL-37.

Nevertheless, the screening method employed successfully identified two mutants with greatly increased resistance to LL-37 in buffer III + C. These two mutants have disruptions in the genes *yycH* and *yycI*. These two genes are in the same operon as

the genes *yycF* and *yycG*, which encode a two component system (TCS) (Szurmant *et al*., 2005, 2007). The functions of YycH and YycI have been characterised in *B. subtilis* where it has been demonstrated that they are suppressors of YycFG (Szurmant *et al.*, 2005, 2007). Transposon insertional disruption of the genes for YycH or YycI would therefore lead to increased activity of YycFG. YycFG is a highly conserved TCS among low GC content Gram-positive bacteria, and has been demonstrated to be essential for these bacteria, including *S. aureus* (Martin *et al.*, 1999), *B. subtilis* (Fabret and Hoch, 1998), *Streptococcus pneumoniae* (Wagner *et al.*, 2002) and *Streptococcus mutans* (Senadheera et al., 2005). In *S. aureus*, YycFG has been reported to play a role in regulating cell wall metabolism (Dubrac and Msadek, 2004; Dubrac *et al.*, 2007; Delaune *et al.*, 2011) and membrane permeability (Martin *et al.*, 1999). In addition, this TCS has also been demonstrated to mediate resistance to a range of antibiotics, such as macrolide-lincosamide-streptogramin B (MLS_B) antibiotics (Martin *et al.*, 1999), daptomycin and vancomycin (Jansen *et al.*, 2007; Howden *et al.*, 2011). Given that LL-37 exerts killing through interacting with negatively charged bacterial cell wall components, such as teichoic acids and lipoteichoic acids, and permeabilisation of bacterial cell membrane (Brogden, 2005), it is possible that the *yycH* and *yycI* mutants are resistant to LL-37 because of alterations in cell wall metabolism or membrane permeability through up-regulation of YycFG. The functions of YycH, YycI and YycFG are further described in the next chapter, including the study on their roles in resistance to LL-37.

Chapter 6 Characterisation of *yycHI* and *yycI* mutants

6.1 Introduction

As presented in the previous chapter, screening of a *S. aureus* LS-1 mutant library for enhanced resistance to LL-37 identified two transposon mutants with insertions in *yycH* and *yycI*, respectively. These two mutants were confirmed to be substantially more resistant to LL-37 (MBC > 128μg/ml) compared to the wild-type strain LS-1 (MBC = 16μg/ml) when 50mM $NaHCO_3$ was present. Interestingly, these two mutants only showed $NaHCO_3$-dependent resistance to LL-37, as they were found to be as sensitive to LL-37 as the wild-type (MBC = 8μg/ml) in the absence of $NaHCO_3$.

The genes *yycH* and *yycI* form part of a probable operon, *yycFGHIJK*, which has been characterised in *B. subtilis*. In this operon, *yycG* and *yycF* encode a histidine kinase and a response regulator, respectively, which constitute a two-component histidine kinase-response regulator signal transduction system (TCS). YycFG is a highly conserved TCS among low GC content Gram-positive bacteria, and has also been designated as WalKR (Dubrac *et al.*, 2007) or VicRK (Wagner *et al.*, 2002). So far, two classes of *yycFG* TCS operon have been identified. Class I is characterised by four genes *yycH*, *yycI*, *yycJ* and *yycK* located downstream of *yycFG*. This seems to be the most wide spread version of the *yycFG* operon, and is present in most of the extensively-studied low GC content Gram-positive bacteria, including *B. subtilis*, *S. aureus*, *E. faecalis* and *L. monocytogenes.* A less common version of the operon, class II, has only *yycJ* in the same operon as *yycFG*, while homologs of *yycH*, *yycI* and *yycK* have not be identified, either downstream of *yycFG* or in the other parts of the genome. A typical representative of class II operon is that described for *Streptococcus pneumoniae* (Ng and Winkler, 2004).

Despite the difference in operon organisation, *yycFG*, as a TCS, has been shown to be essential in nearly all the Gram-positive bacteria in which it has been studied. It is essential for the growth of *B. subtilis* (Fabret and Hoch, 1998), *S. pneumoniae*

(Wagner *et al.*, 2002) and *Streptococcus mutans* (Senadheera et al., 2005). The essentiality of *yycFG* has also been demonstrated in *S. aureus*, as a point mutation in *yycF* in *S. aureus* NT372 is lethal at non-permissive temperatures (Martin *et al.*, 1999). Additionally, attempts to inactivate the *yycFG* genes in *L. monocytogenes* and *E. faecalis* have failed (Kallipolitis and Ingmer, 2001; Hancock and Perego, 2004), suggesting that the system is also essential in these bacteria.

The *yycFG* operon in *S. aureus* is organised in a similar manner to that in *B. subtilis* with *yycH*, *yycI*, *yycJ* and *yycK* downstream of *yycFG*. These four downstream genes have been suggested to encode potential regulators for *yycFG* in *B. subtilis*. However, so far only the regulatory roles played by YycH and YycI have been established in *B. subtilis* (Szurmant *et al.*, 2005, 2007). Deletion of *yycH* or *yycI* in *B. subtilis* seems to result in identical phenotypes. For example, both mutants entered stationary phase earlier than the wild-type and only reached half of the cell density of the wild-type strain at stationary phase. Moreover, the *yycH* and *yycI* mutants also showed cell wall defects, as they exhibited increased susceptibility to SDS-induced lysis, while the wild-type strain was only lysed in the presence of SDS following lysozyme treatment (Szurmant *et al.*, 2005, 2007). Both YycH and YycI have been demonstrated to be suppressors of YycF in *B. subtilis* (Szurmant *et al.*, 2007). This was initially supported by the observations that the cell wall and growth defects observed in *yycH* and *yycI* mutants were also exhibited when YycF was over-expressed. This suggests that the activity of YycF is possibly increased in the *yycH* or *yycI* mutant. Furthermore, a YycF-dependent gene *yocH*, which encodes for a putative autolysin in *B. subtilis*, was found to be elevated 10-fold in both *yycH* and *yycI* mutants (Szurmant *et al.*, 2007).

In *B. subtilis*, both YycH and YycI are exported proteins anchored to the membrane by an N-terminal trans-membrane sequence and face outside the cytoplasm. YycH and YycI do not affect the expression of YycFG, but regulate this TCS by interacting with the sensing domain of the YycG kinase (Szurmant *et al.*, 2007). However, the exact mechanism of action is still unknown. Interestingly, YycH and YycI seem to play the same role in regulating YycFG, as deletion of either resulted in the same phenotype. Nevertheless, they are not able to cross-complement each other, and

deletion of both genes does not cause an additive phenotype. It is therefore likely that they interact and act together to regulate of the YycFG system (Szurmant *et al.*, 2007).

In the transposon mutants *S. aureus* LS-1 *yycH::Tn* and *S. aureus* LS-1 *yycI::Tn*, the mariner transposon carries an erythromycin resistant gene *ermC*, which contains a transcriptional terminator. Therefore, if *yycFGHIJK* are co-transcribed, a transposon inserted in one of the genes would inactivate all the downstream genes. By comparing the *yycFGHIJK* loci in *B. subtilis* with that in *S. aureus*, it was found that the operon organisation in these two bacteria is slightly different, as shown in figure 6.1. It is clear that the six genes are more compactly organised in *B. subtilis* than those in *S. aureus*. To predict whether the six genes are in the same operon in *B. subtilis* and *S. aureus*, the 81bp nucleotides between *yycJ* and *yycK* in *B. subtilis*, the 389bp nucleotides between *yycI* and *yycJ*, and the 227bp nucleotides between *yycJ* and *yycK* in *S. aureus* were analysed for potential promoters using the program BPROM (Softberry, Inc., Mt. Kisco, NY). BPROM is a bacterial promoter recognition program with approximatly 80% accuracy and specificity (available free at http://linux1.softberry.com/berry.phtml). No promoter was predicted between *yycJ* and *yycK* in *B. subtilis*, indicating that the six genes are co-transcribed in the same operon. However, in *S. aureus*, promoters were predicted in the sequence between *yycI* and *yycJ*, as well as that between *yycJ* and *yycK*. This suggests that *yycJ* and *yycK* are possibly transcribed separately from *yycFGHI* in *S. aureus*. Therefore, theoretically at least, the mutant containing a transposon in *yycH* would disrupt both *yycH* and *yycI* and will be referred to as *yycHI* mutant from here on in. On the other hand insertion of the transposon into *yycI* theoretically only disrupts *yycI*, and will be referred to as the *yycI* mutant hereafter. Because *yycH* and *yycI* exert the same regulation role on *yycFG* as demonstrated in *B. subtilis* (Szurmant *et al.*, 2007), most of the work described in this chapter was focused on the *yycHI* mutant.

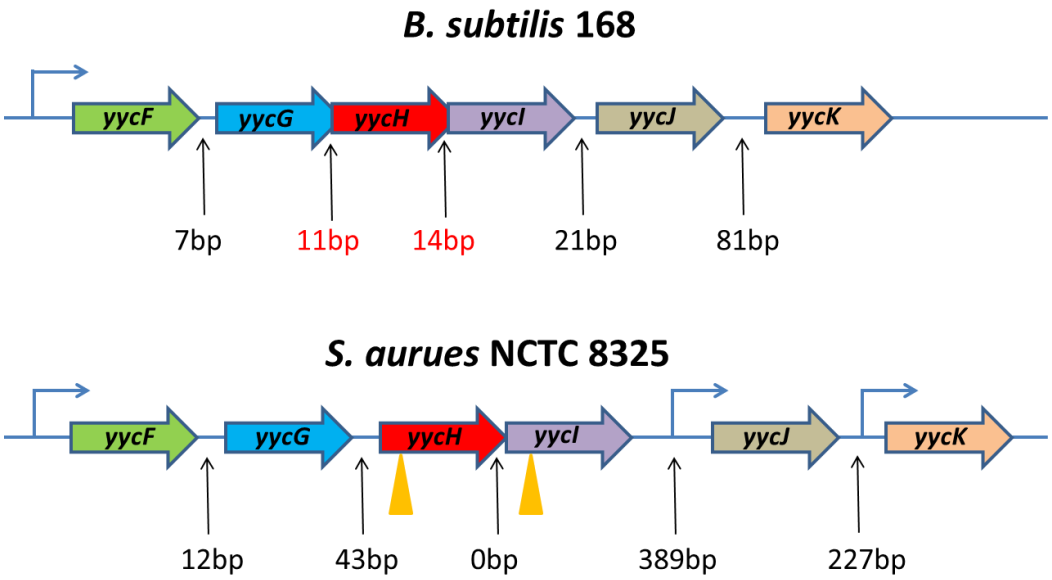

Figure 6.1: The *yycFGHIJK* operon organisation in *B. subtilis* 168 and *S. aureus* NCTC 8325. The distance between each gene is indicated. Black and red letters indicate the distance between genes and the overlap between genes, respectively. The intergenic regions with putative promoters are indicated by blue arrows. The orange triangles point to the approximate position of the transposon insertions in *yycH* and *yycI* genes.

Since the altered resistance to LL-37 observed in the *yycHI* and *yycI* mutants was possibly due to up-regulating the activity of YycFG (Szurmant *et al.*, 2007), it was therefore important to know which genes are controlled by the YycFG TCS. Genes whose expression is dependent on YycF have been reported for a number of Gram-positive bacteria, and differ between bacteria (Fukuchi *et al.*, 2000; Howell *et al.*, 2003; Ng *et al.*, 2003; Dubrac and Msadek, 2004; Bisicchia *et al.*, 2007). The YycF recognition sequence in *B. subtilis* was identified to be a 17bp sequence characterised by two 6bp direct repeats flanking 5 nucleotides (Howell *et al.*, 2003). The YycF regulator in *S. aureus* is 75% identical to that in *B. subtilis*, and the DNA binding domains in these two homologs have 30 out of 31 amino acids identical to each other. Therefore, the YycF recognition sequence in *B. subtilis* has been used by Msadek's group to search the intergenic regions of the *S. aureus* N315 genome to identify putative YycF-regulated genes and these are shown table 6.1 (Dubrac and Msadek, 2004; Dubrac *et al.*, 2007; Delaune *et al.*, 2011). These authors also demonstrated the direct binding of YycF to the promoter regions of three selected genes *lytM*, *isaA* and *ssaA* (Dubrac and Msadek, 2004).

Table 6.1 Nucleotide sequences of putative YycF-regulated promoter regions. The potential YycF-binding sequences are shown in bold. CS: coding strand. NCS: non-coding strand (Dubrac and Msadek, 2004; Dubrac *et al.*, 2007; Delaune *et al.*, 2011). Locus tags are according to strain N315 (GenBank accession BA000018.3).

Gene	Promoter region	Strand	Function
lytM (SA0265)	**TGTAAT**GACAA**TGTAAT**	CS	Peptidoglycan hydrolase
isaA (SA2356)	**TGTAAA**GAAAG**TGTAAT**	CS	Immunodominant antigen A
sceD (SA1898)	**TGTAAT**CACTG**TGTAAA**	CS	Transglycosylase
atlA (SA0905)	**TGTAAA**TTAAG**AGTATA**	CS	N-acetylmuramyl-L-alanine amidase and endo-b-N-acetylglucosaminidas
ssaA (SA2093)	**TGTTAA**CGTTT**TGTAAT** **TGTTAC**AAATT**TGTAAT**	NCS NCS	Staphylococcal secretory antigen
SA0620	**TGTTAT**TATCT**TGTAAT**	NCS	secretory antigen SsaA homologue
SA2097	**TGTTAT**TGATT**TGTAAA**	NCS	secretory antigen SsaA homologue
SA2353	**TGTTAT**CATAA**TGTAAT**	NCS	secretory antigen SsaA homologue
SA0710	**TGTTAT**AACGA**TGTAAT**	NCS	secretory antigen SsaA homologue
Sle1 (SA0423)	**TGTAAT**CATAA**TGTCAT**	CS	N-acetylmuramoyl-L-alanine amidase
prs (SA0458)	**TGTAAT**ATTAA**TGTAAT**	CS	Ribose-phosphate pyrophosphokinase
sdrD (SA0520)	**TGTTAC**AGAAA**TGTAAT**	CS	Sialoprotein-binding protein
hu (SA1305)	**TGTAAT**GCTTG**TGTTAA**	CS	DNA-binding protein II
ebpS (SA1312)	**TGTAAA**ATCAT**TGTAAT**	CS	Elastin-binding protein
phoP (SA1516)	**TGTAAA**AAATG**TGTAAA**	CS	Transcriptional regulatory protein
sak (SA1758)	**TGTTAA**ATATT**TGTTAA**	CS	Staphylokinase precursor
opp-2B (SA1214)	**TGTTAC**TGCAG**TGTAAC**	NCS	Oligopeptide transporter membrane permease domain
ndhF (SA0411)	**TGTAAA**GATTT**TGTAAA**	NCS	NADH dehydrogenase
SA1221	**TGTTAA**GAAAA**TGTAAA**	NCS	Thioredoxin reductase

In this putative *yycFG* regulon, the first 10 genes listed encode proteins with cell wall-metabolising functions, suggesting that *yycFG* plays a major role in cell wall metabolism in *S. aureus* (Dubrac *et al.*, 2007; Delaune *et al.*, 2011). Down-regulation of *yycFG* in *S. aureus* renders the cells more resistant to Triton X-100 induced lysis and lysostaphin-induced lysis, and *yycFG* has also been demonstrated to positively control peptidoglycan biosynthesis and turnover (Dubrac *et al.*, 2007). Additionally, knocking down of *yycFG* in *S. aureus* using an IPTG-dependent inducible promoter

resulted in a thicker cell wall and aberrant division septa, suggesting that the essentiality of *yycFG* could be linked to its role in regulating cell wall metabolism (Delaune *et al.*, 2011). The authors also examined whether over-expression of YycFG-regulated autolysin genes could compensate for the essentiality of *yycFG*, and found that only over-expression of *lytM* or *ssaA* was able to restore cell viability in the *yycFG* knock down mutant (Delaune *et al.*, 2011).

S. aureus YycFG has also been linked to resistance to several antibiotics. Prior to the identification of the putative *yycFG* regulon, an earlier study showed that the YycFG system in *S. aureus* played a role in membrane permeability and a temperature-sensitive mutant of *S. aureus yycF* exhibited increased susceptibility to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics (Martin *et al.*, 1999). Further to this, the authors screened a library of plasmid-based genomic fragments cloned in the *yycF* mutant for the identification of genes that were able to restore the resistance to erythromycin at non-permissive temperature 43˚C, and the YycFG-regulated gene *ssaA* was identified as being responsible for resistance to MLS_B antibiotics. As *ssaA* was down-regulated in *yycF* mutant, over-expression of the SsaA in *yycF* mutant was found to restore the susceptibility of this mutant to the wild-type level, and inactivation of *ssaA* in a strain containing *ermB* resulted in substantially decreased resistance to MLS_B antibiotics (Martin *et al.*, 2002). In addition to MLS_B antibiotics, *S. aureus* YycFG has also been reported to be involved in resistance to the glycopeptide antibiotic vancomycin and the lipopeptide antibiotic daptomycin. A clinically isolated vancomycin-resistant strain SA137/93A carries an insertion element IS256 in the promoter region of *yycFG*, which dramatically up-regulates *yycFG* (>20 fold). The up-regulation of *yycFG* was verified to be responsible for the vancomycin resistance in this strain, as over-expression of YycFG in a vancomycin-sensitive strain SH1000 resulted in increased resistance to vancomycin (Jansen *et al.*, 2007). In agreement with this, a clinically-isolated vancomycin-resistant strain JH6 was found bearing mutations including *yycH* (Mwangi *et al.*, 2007), which encodes an YycFG suppressor (Szurmant et al., 2005). In addition, a clinical isolate with a nucleotide insertion in *yycG* and point mutations in *mprF*, demonstrated increased resistance to daptomycin (Friedman *et al.*, 2006). Furthermore, a recent study has compared the genetic

differences between clinical isolates isolated before and after vancomycin treatment failure, referred to as vancomycin-susceptible *S. aureus* (VSSA) and vancomycin-intermediate susceptibility *S. aureus* (VISA), respectively. Four out of five VISA isolates were found to bear single nucleotide substitutions in either *yycF* or *yycG* compared to their parental VSSA, and introducing these mutations into the parental VSSA led to increased resistance to both vancomycin and daptomycin (Howden *et al.*, 2011).

As discussed in chapter 1, cationic AMPs exert their antimicrobial effect through electrostatically binding to negatively charged cell surface molecules, followed by disrupting the cell membrane and releasing the cell contents (Brogden, 2005). It was hypothesised that the peptidoglycan hydrolase activity regulated by YycFG may be involved in resistance to LL-37 in *yycHI* and *yycI* mutants through altering cell wall metabolism. The ten peptidoglycan hydrolase encoding genes (the first ten genes listed in table 6.1) encode four different types of hydrolytic enzymes, the cleavage sites of which are graphically presented in figure 6.2. The major autolysin gene, *atlA* encodes a bifunctional protein with both amidase and glycosaminidase domains (Oshida *et al.*, 1995). It is therefore able to hydrolyse peptidoglycan at two sites. The N-acetylglucosaminidase activity of AtlA cleaves the glycan chain between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), and the N-acetylmuramyl-L-alanine amidase activity of AtlA hydrolyses the amide bond between NAM and the peptide side chain (Foster, 1995). The amidase activity is also possessed by Sle1 (Kajimura *et al.*, 2005), SsaA, and possibly four structurally related proteins (SA2353, SA2097, SA0620 and SA0710) that contain a common CHAP amidase domain (Dubrac *et al.*, 2007; Delaune *et al.*, 2011). The third type of hydrolase are the transglycosylases such as IsaA and SceD (Stapleton *et al.*, 2007), which cleave the glycan chain between NAM and NAG. Finally, the endopeptidase LytM is a peptidoglycan hydrolase which cleaves the pentaglycine bridges between peptide side chains (Ramadurai *et al.*, 1999).

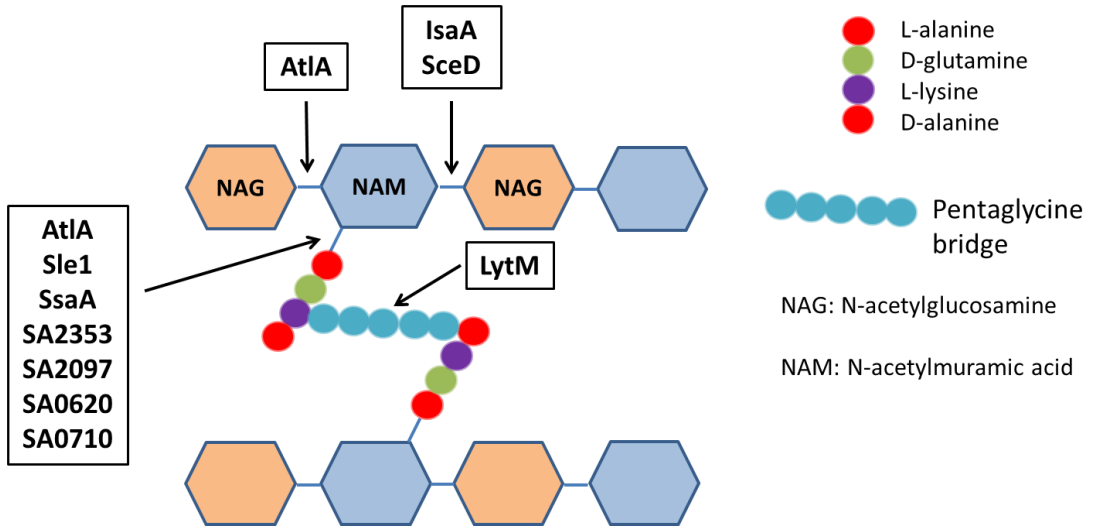

Figure 6.2: Schematic illustration of *S. aureus* peptidoglycan and the cleavage sites of cell wall hydrolases whose gene regulation is putatively under the control of YycFG (Delaune *et al.*, 2011; Dubrac *et al.*, 2007). Locus tags are according to strain N315 (GenBank accession BA000018.3).

YycFG was found to positively regulate all these genes encoding peptidoglycan degradation enzymes (Dubrac *et al.*, 2007), except Sle1, which has only recently been identified to be regulated by YycFG and has not as yet been characterised (Delaune *et al.*, 2011). Given that the increased LL-37 resistance of the *yycHI* and *yycI* mutants was only observed in the presence of $NaHCO_3$, genes that respond to $NaHCO_3$ stress may contribute to $NaHCO_3$-dependent resistance to LL-37. However, there is not much information available on $NaHCO_3$-induced or repressed genes. Relevantly, the expression of *sceD* was found to be greatly induced (approximately 14-fold) in the presence of 1M NaCl (Stapleton *et al.*, 2007). This finding may suggest that *sceD* is involved in responding to sodium chloride, and may be relevant to the $NaHCO_3$-induced resistance to LL-37. In addition, under microaerobic conditions, the gene *isaA* was shown to be up-regulated by a TCS SrrAB (staphylococcal respiratory response regulator), which controls gene expression in response to oxygen availability (Stapleton *et al.*, 2007). Thus IsaA may play a role in adapting to an environment with increased concentration of CO_2, which may potentially suggest its importance in responding to bicarbonate. Therefore, it was decided to determine the roles *sceD* and *isaA* play in $NaHCO_3$-induced resistance to LL-37.

6.2 Growth dynamics of *yycHI* and *yycI* mutants

Growth defects, in particular entry into stationary phase earlier at lower optical density than the wild-type, have been observed in *B. subtilis yycH* and *yycI* mutants (Szurmant *et al.*, 2007). To determine whether the *S. aureus yycHI* or *yycI* mutants exhibit similar growth defects, the growth dynamics of the *yycHI* and *yycI* transposon mutants in the *S. aureus* LS-1 background were examined as described in chapter 2 section 2.1.6. Optical density at 600nm was measured and the numbers of viable cells were also determined by viable counting every hour. The growth dynamics of LS-1 and the two mutants are shown in figure 6.3 and 6.4. Surprisingly, contrary to the growth defects reported for *B. subtilis yycH* and *yycI* mutants, *S. aureus* LS-1 *yycHI* and *yycI* mutants demonstrated a faster increase in optical density than the wild-type (figure 6.3). In comparison to the wild-type LS-1, the two mutants appeared to show longer exponential phases based on optical density and reached higher optical densities at stationary phase. Despite this, the numbers of viable bacteria over time appeared to be slightly lower for the two mutants compared to the wild-type (figure 6.4), which were statistically significant as analysed using one way ANOVA. As shown in figure 6.4, there were significantly fewer viable mutants ($P < 0.001$) at the start of the growth curve (T = 0 hour) than there were of the wild-type, despite having the same starting optical density OD_{600} = 0.05. The growth dynamics of the *yycHI* mutant and the *yycI* mutant were almost identical, without statistically a significant difference, which is consistent with the finding in *B. subtilis* that deletion of both *yycH* and *yycI* did not cause an additive phenotype compared to the single mutants of *yycH* or *yycI* (Szurmant *et al.*, 2007). The difference between the results obtained by measuring cell growth dynamics using either optical density or viable cell numbers demonstrate that the mutants have a higher optical density for a given number of viable cells compared to the wild-type.

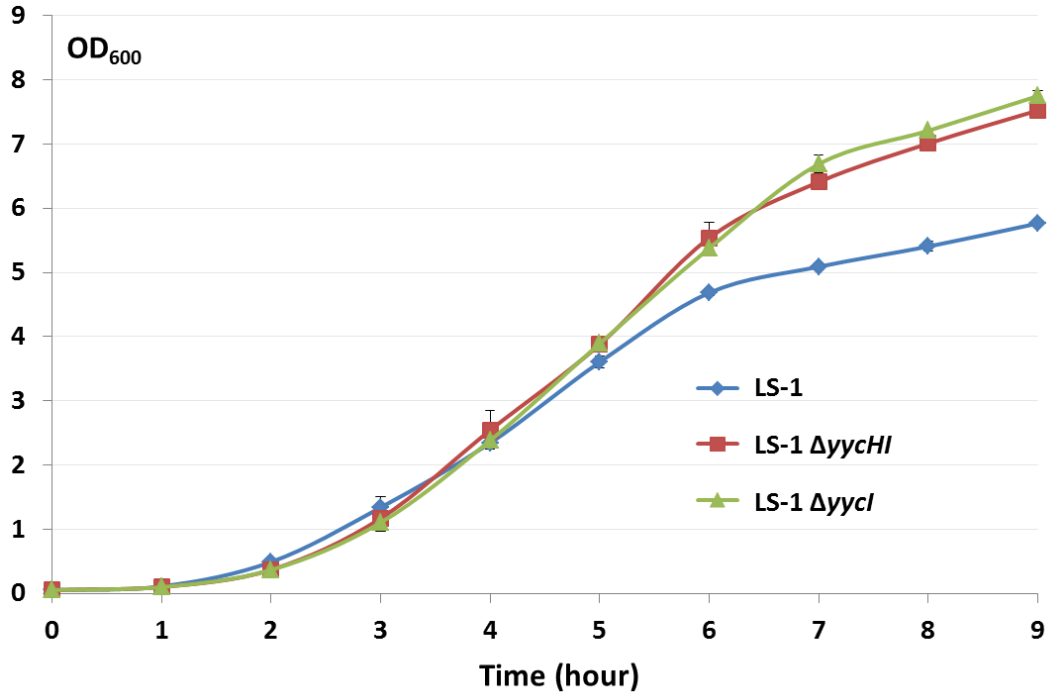

Figure 6.3: Growth curves of *S. aureus* LS-1 (blue) and its *yycHI* (red) and *yycI* (green) mutants showing the change in optical density at 600nm over time. Presented data are the means of three independent experiments with error bars showing the standard deviations.

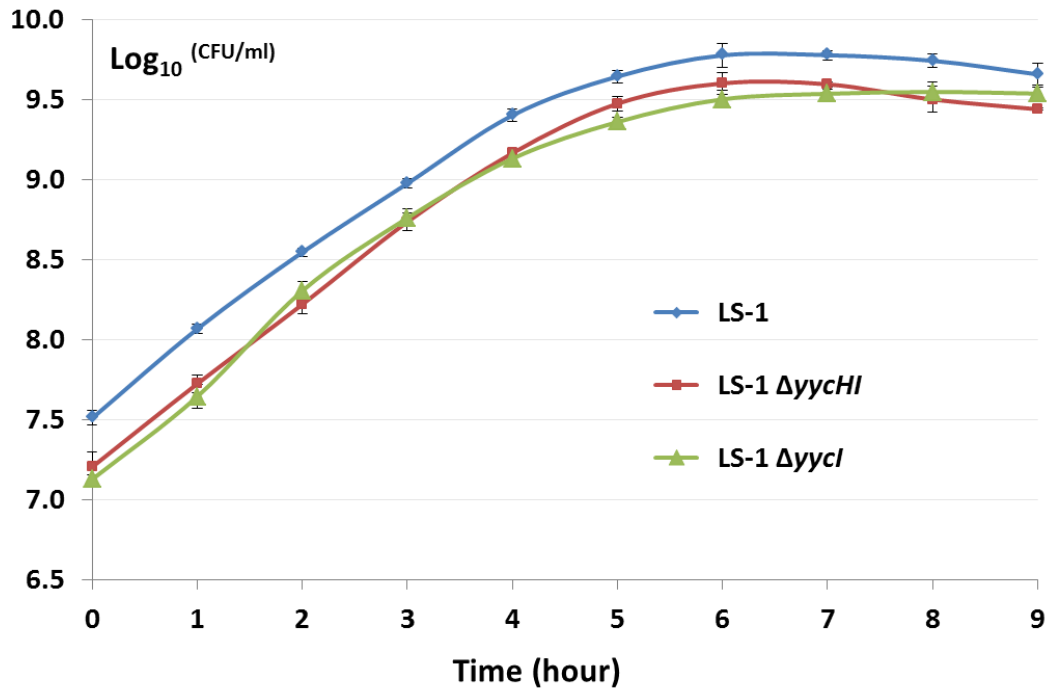

Figure 6.4: Growth curves of *S. aureus* LS-1 (blue) and its *yycHI* (red) and *yycI* (green) mutants showing the change in viable counts over time. Presented data are the means of three independent experiments with error bars showing the standard deviations.

6.3 TEM and SEM analysis of *yycHI* mutant

Since *yycFG* has been shown to regulate cell wall metabolism and cell division (Dubrac *et al.*, 2007; Delaune *et al.*, 2011), we hypothesised that the elevated optical density observed in *yycHI* and *yycI* mutants may be related to altered cell size, thickness of cell surface components, or aggregation of cells. To investigate these possibilities, the wild-type and *yycHI* mutant were grown in TSB to early exponential phase, OD600 = 1-1.5 (Delaune *et al.*, 2011), and the cells were subjected to examination by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) as described in section 2.8. Four representative TEM images of LS-1 and the *yycHI* mutant are shown in figure 6.5. The *yycHI* mutant displayed a similar cell size to the wild-type LS-1, and a normal division septum was observed for all the dividing mutants that were detected. However, as shown in figure 6.5 (bottom right), it appeared that the *yycHI* mutant had a tendency to form very lose chains through cell surface contact. As demonstrated by SEM (figure 6.6), the *yycHI* mutant showed a slightly higher tendency to aggregate than wild-type cells. This data suggests that the higher ratio of optical density to viable counts observed in the *yycHI* mutant compared to LS-1 is possibly a consequence of the increased aggregation of *yycHI* mutant, since the aggregated cells may not be separated while plating on agar and could appear as a single colony. Given that this data was generated from a single experiment, the difference between the *yycHI* mutant and the wild-type strain described above may need to be confirmed by repeating the experiments a few more times.

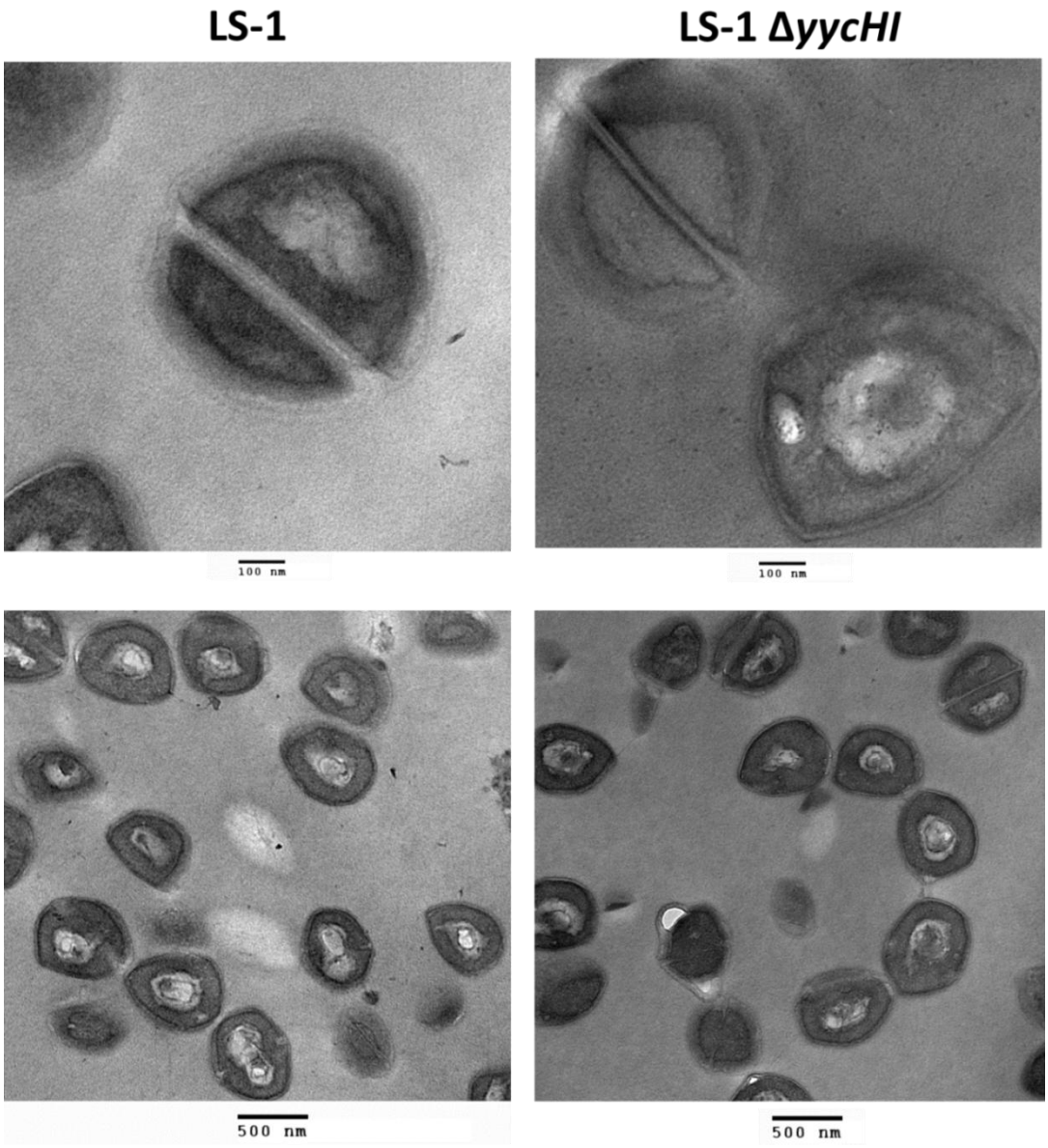

Figure 6.5: Cell morphology of LS-1 and the *yycHI* transposon mutant at exponential growth phase observed by transmission electron microscopy. Data is from one experiment.

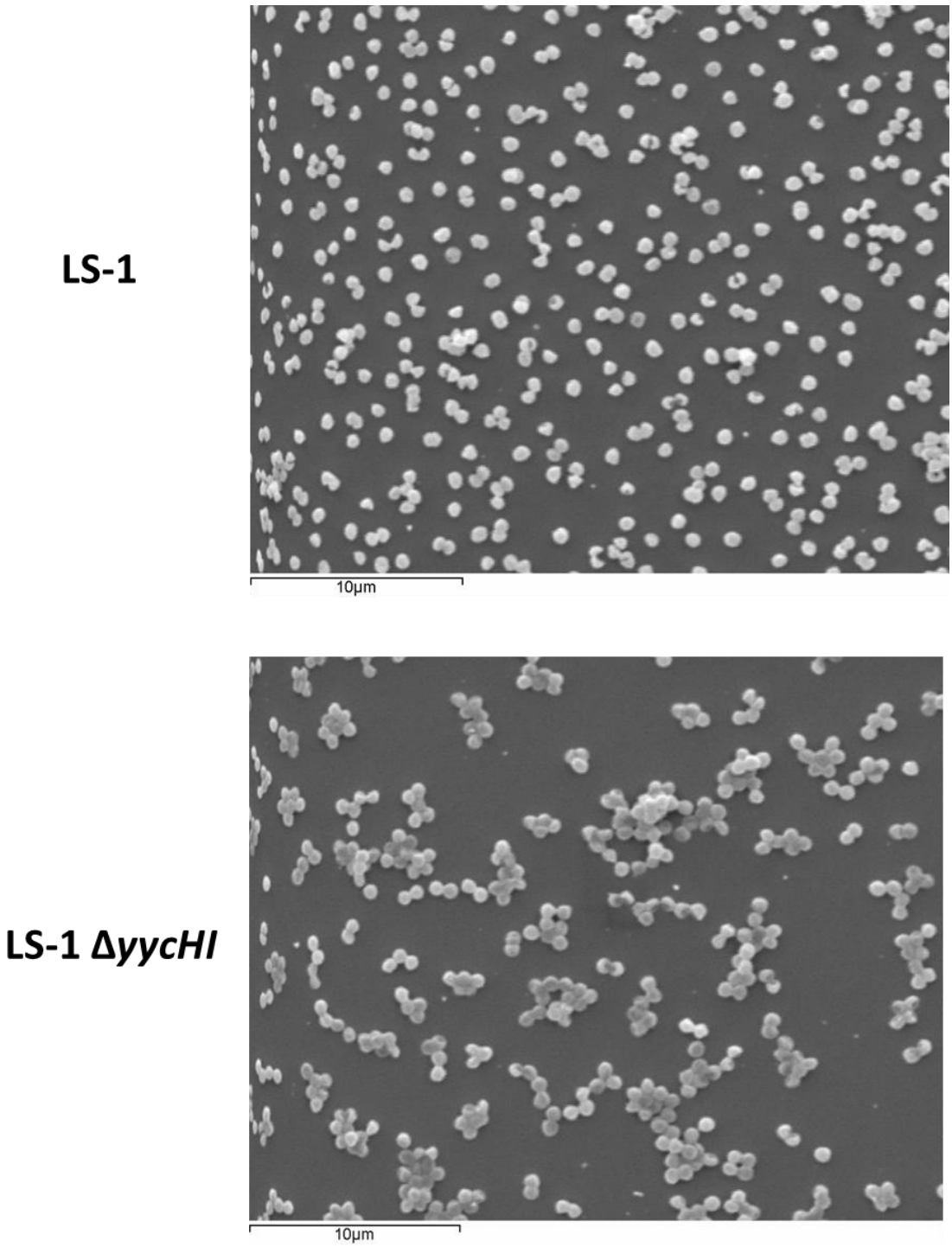

Figure 6.6: The tendency to aggregate of LS-1 and *yycHI* transposon mutant cells at exponential growth phase examined by scanning electron microscopy. Data is from one experiment.

6.4 Construction of *isaA* and *sceD* mutants

To ascertain whether *sceD* and *isaA* are involved in $NaHCO_3$-induced resistance to LL-37 in the *yycHI* mutant, *isaA* and *sceD* mutations were introduced into *S. aureus* LS-1 and the *yycHI* mutant by phage transduction as described in section 2.3.6. The donor strains SH1000 *isaA::tet* and SH1000 *sceD::tet* were obtained from Professor Simon Foster (Stapleton *et al.*, 2007). Potential *isaA* mutants were verified by PCR using primers P238 and P239 (nucleotide sequences of primers are listed in section 2.2.2), which were designed to amplify a region flanking the insertion in the LS-1 chromosome. The wild-type strain should generate a product of 1005bp, while the mutants were expected to generate a product of approximately 2.9kb due to the insertion of *tet*. The gel image of the PCR products from wild-type and the potential mutants is shown in figure 6.7 (image on the left side), all the potential mutants LS-1 Δ*isaA* (lanes 3-5) and LS-1 Δ*yycHI* Δ*isaA* (lanes 6-8) showed a PCR product corresponding to the expected size at ~2.9kb, whereas a PCR product of approximately 1kb was observed from the wild-type LS-1 (lane 2). The potential *sceD* mutants were analysed using primer P240 and another primer P242 locating at the end of the *tet* gene. The PCR products amplified from LS-1 and the potential mutants are displayed in figure 6.7 (image on the right side). PCR amplification from LS-1 failed to generate a product due to the lack of the *tet* gene (lane 10), while amplification from the potential LS-1 Δ*sceD* (lanes 11-13) and LS-1 Δ*yycHI* Δ*sceD* (lane 14) all generated expected products at ~2kb.

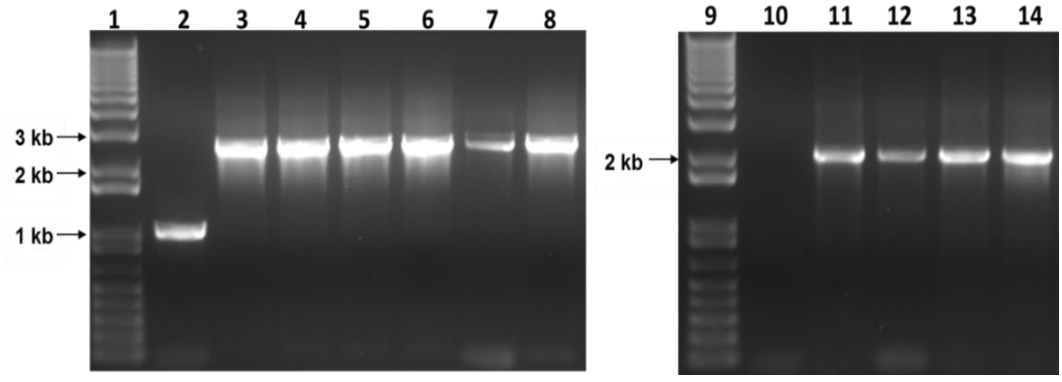

Figure 6.7: Gel electrophoresis images of PCR products amplified from LS-1 and potential mutants. Lanes 1 & 9: 1kb plus DNA ladder (Invitrogen). Lane 2-8: PCR product amplified using primer P238 and P239. Lane 2: from LS-1; lanes 3-5: from potential LS-1 Δ*isaA*; lanes 6-8: from potential LS-1 Δ*yycHI* Δ*isaA*. Lanes 10-14: PCR products amplified using P240 and P242. Lane 10: from LS-1; lanes 11-13: from potential LS-1 Δ*sceD*; lane 14: from potential LS-1 Δ*yycHI* Δ*sceD*.

6.5 Susceptibility of *isaA* and *sceD* mutants to LL-37

The constructed single or double mutants were subjected to LL-37 MBC assays in buffer III (1mM NaH_2PO_4, pH = 7.4) and buffer III + C (buffer III + 50mM $NaHCO_3$, pH = 7.4) as described in section 2.6.7. The MBCs of LL-37 against wild-type and mutants were measured in three independent experiments and are presented in table 6.2. There was no difference in susceptibility to LL-37 in buffer III amongst all of the strains. The resistance to LL-37 was elevated by 4-fold in the wild-type in response to $NaHCO_3$, while the *yycHI* mutant demonstrated substantially increased resistance to LL-37 in the presence of $NaHCO_3$. Unexpectedly, the *isaA* and *sceD* single mutants also showed greatly increased resistance to LL-37 in the presence of $NaHCO_3$, and inactivation of *isaA* or *sceD* in the *yycHI* mutant did not reduce the $NaHCO_3$-dependent resistance to the wild-type level.

Table 6.2: The MBCs of LL-37 against *S. aureus* wild-types and mutants in buffer III and buffer III + C. The median MBC values from three independent experiments were presented, with the MBC ranges from the three experiments included in brackets. MBC values higher than the tested concentration range 0-128 μg/ml were recorded as >128 μg/ml.

Strains	MBC of LL-37 (μg/ml)	
	Buffer III	Buffer III + C
LS-1	4 (4-8)	16 (16-32)
LS-1 Δ*yycH*	4 (4-8)	>128
LS-1 Δ*isaA*	4	128 (128 - >128)
LS-1 Δ*secD*	4	>128
LS-1 Δ*isaA* Δ*yycH*	4	>128
LS-1 Δ*sceD* Δ*yycH*	4	>128

As the MBCs of LL-37 against all of the mutants in buffer III + C were higher than the concentration range tested, to assess the effect of inactivating *isaA* or *sceD* in *yycHI* mutant, the killing of each strain (10^4 cells) by 128μg/ml LL-37 in buffer III + C over 2-hour was quantified by viable counting and is presented in figure 6.8. Compared to

the wild-type LS-1, all of the mutants, except LS-1 Δ*isaA*, demonstrated significantly increased resistance to LL-37 (this significance is not indicated in figure 6.8). The *yycHI* mutant was significantly more resistant to LL-37 compared to the *sceD* mutant, while no significant difference of susceptibility to LL-37 was found between *yycHI* and *isaA* mutants. The *yycHI isaA* and the *yycHI sceD* mutants did not show significantly decreased resistance to LL-37 compared to the *yycHI* mutant, suggesting that the reported up-regulation of *isaA* and *sceD* by YycFG (Dubrac *et al.*, 2007) is not responsible for the increased resistance to LL-37 observed in the *yycHI* mutant. In fact, the *yycHI sceD* mutant demonstrated significantly increased resistance to LL-37 compared to the *yycHI* mutant, indicating that mutation of *sceD* caused additive resistance of the bacterium to LL-37 in buffer III + C.

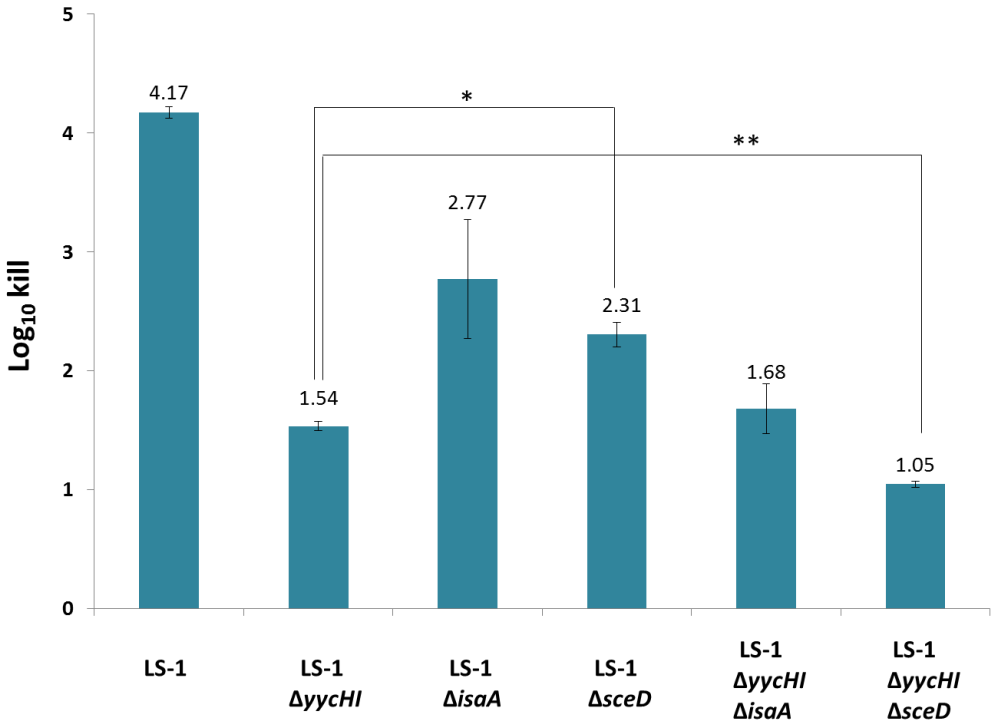

Figure 6.8: Killing of LS-1 and LS-1-derived mutants by 128µg/ml LL-37 in buffer III + C. The data presented are the means of three independent experiments with error bars showing standard deviations. Significance was analysed using one way ANOVA. *: P<0.05, **: P<0.01.

6.6 Comparison of cell surface charge

The proposed mechanism of LL-37 killing involves an initial electrostatic attraction of the peptide to the bacterial cell surface (Brogden, 2005). One of the antimicrobial peptide-resistance mechanisms identified in *S. aureus* is through a reduction of the

cell surface negative charge, thus reducing electrostatic attraction of cationic peptides to the cell surface (Peschel *et al.*, 1999, 2001). Since YycHI and YycI were demonstrated to be regulators of the YycFG system in *B. subtilis* (Szurmant *et al.*, 2007), and YycFG plays a major role in controlling cell wall metabolism in *S. aureus* (Szurmant *et al.*, 2007; Delaune *et al.*, 2011), it is conceivable that they may alter cell surface charge through YycFG. In order to determine whether the increased resistance observed in *yycHI* and *yycI* mutants was due to altered cell surface charge, a cytochrome c binding assay was conducted. Cytochrome c is a highly positively charged protein, which can be quantitatively detected by absorbance at 530nm. The binding of cytochrome c to bacteria is dependent on the availability of cell surface negative charge, hence allowing the comparison of whole cell surface charges between *S. aureus* strains.

The cytochrome c binding assay was performed in buffer III and buffer III + C as described in section 2.9. The amount of cytochrome c bound by the wild-type LS-1 in buffer III was set as 100% binding, and binding of LS-1 in buffer III + C, or other strains under different conditions was calculated relative to this value. The relative binding of cytochrome c by LS-1 and the mutants in buffer III with or without $NaHCO_3$ is plotted in figure 6.9. The amount of cytochrome c bound by the *yycHI* and *yycI* mutants was significantly reduced compared to that bound by the wild-type in either buffer III or buffer III + C. In comparison to the cytochrome c binding in buffer III, both wild-type and mutants bound significantly less cytochrome c in buffer III + C. Given that the MBC of LL-37 towards the *yycHI* and *yycI* mutants was the same as LS-1 in buffer III, despite the reduced binding of cytochrome c in comparison to LS-1 in this buffer, it suggests that a reduction in binding of approximately 50% is not sufficient to increase resistance to LL-37. Furthermore an 80% reduction in binding of cytochrome c was observed for LS-1 in buffer III + C compared to that in buffer III, which only corresponded to a 4-fold increase in MBC of LL-37 in buffer III + C compared to buffer III (table 6.2). On the other hand a reduction in cytochrome c binding of 95-97% was seen with the *yycHI* and *yycI* mutants in buffer III + C, which corresponded to a greater than 64 fold increase in resistance to LL-37. From these data it is clear that there is a difference in the capacity of bacteria to bind to

cytochrome c in the presence or absence of $NaHCO_3$, and the mutants have lower capacity to bind to cytochrome c compared to the wild-type, suggesting that they may have reduced net surface negative charge. Also from this data it is clear that if differences in the net surface charge between the wild-type and the mutants account for the increased resistance then there is a threshold value at which it becomes an important factor for resistance, and that this threshold must be below a reduction in net negative surface charge that would result in a reduction in cytochrome c binding by 50%.

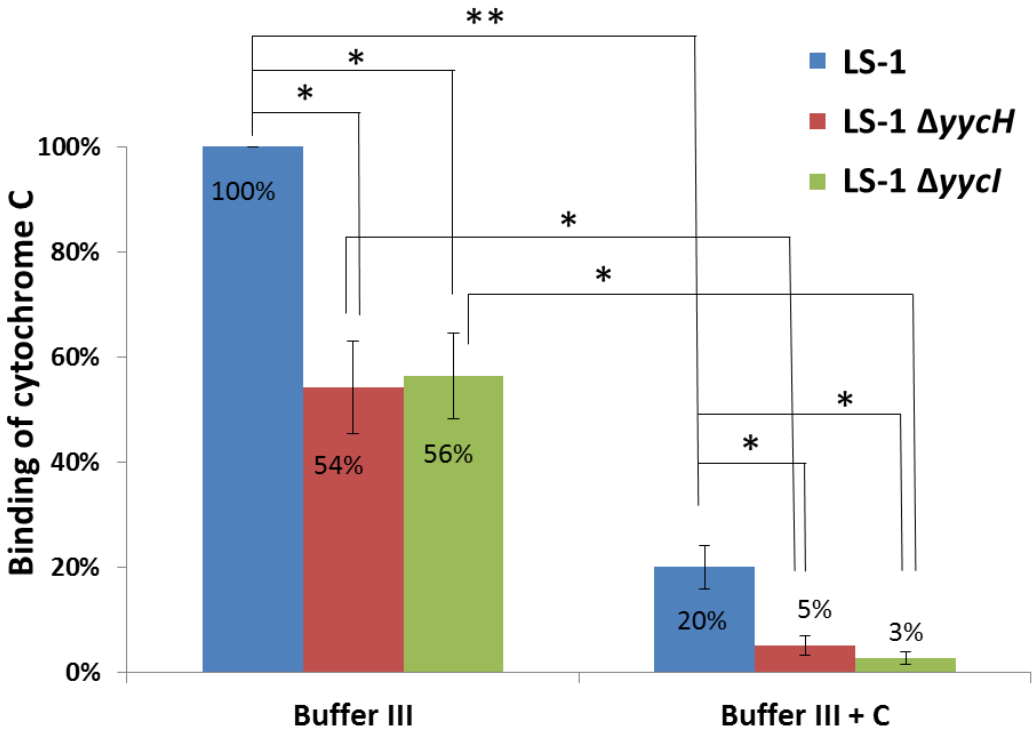

Figure 6.9: The relative binding of cytochrome c between the wild-type LS-1 and *yycHI* and *yycI* mutants in buffer III with or without 50mM $NaHCO_3$. The data presented are the means of three independent experiments with error bars showing standard deviations. Statistical significance was analysed using ANOVA, *: $p < 0.05$, **: $P < 0.01$

6.7 Ionic factors causing $NaHCO_3$-induced resistance to LL-37

The *yycHI* mutant was as susceptible to LL-37 as the wild-type LS-1 in buffer III, while it demonstrated substantially increased resistance compared to LS-1 in buffer III + C. The mechanism of how $NaHCO_3$ induces resistance to LL-37 in the *yycHI* mutant is not known. Both Na^+ and HCO_3^- have previously been suggested to influence

bacterial susceptibility to cationic AMPs. Na^+ was thought to compete for cell surface binding sites with cationic AMP, thus conferring bacterial resistance to cationic AMPs (Ohta *et al.*, 2011). Bicarbonate was demonstrated to increase the permeability of the bacterial membrane, by which means it increases the susceptibility of the cells to LL-37 (Dorschner *et al.*, 2006), which is counter to our findings. In order to determine if the increased resistance to LL-37 of the *yycHI* mutant was due to the sodium cation or the bicarbonate anion, the MBC of LL-37 against LS-1 and the *yycHI* mutant was measured in different assay buffers. In addition to buffer III, buffer III was supplemented with either $NaHCO_3$, or NaCl or NaH_2PO_4-Na_2HPO_4 and adjusted to pH = 7.4. The concentrations of the additional Na^+ resulting from addition of $NaHCO_3$, NaCl or NaH_2PO_4-Na_2HPO_4 was normalised to 50mM.

The MBCs of LL-37 determined in three independent experiments are summarised in table 6.3. As observed previously, LS-1 and the *yycHI* mutant showed the same level of susceptibility to LL-37 in buffer III, and supplementation of $NaHCO_3$ increased the resistance 8-fold for LS-1 and more than 32-fold for the *yycHI* mutant. When supplemented with NaCl, the resistance to LL-37 increased by 4-fold for LS-1 and by 8-fold for the mutant. Taking into account the range of MBCs determined in the three experiments, the susceptibility of LS-1 and the *yycHI* mutant to LL-37 is similar in buffer III supplemented with NaCl, which is also in the same range as the susceptibility of LS-1 to LL-37 in buffer III + $NaHCO_3$. However, the *yycHI* mutant demonstrated increased resistance to LL-37 in buffer III + $NaHCO_3$ compared to that in buffer III + NaCl. These data suggest that Na^+ slightly reduces *S. aureus* susceptibility to LL-37, as demonstrated by the slight increase of MBCs of LS-1 in the presence of NaCl or $NaHCO_3$, while the anion HCO_3^- greatly increases the resistance of the *yycHI* mutant to LL-37. Both the wild-type and the mutant showed highly increased resistance to LL-37 in the presence of NaH_2PO_4-Na_2HPO_4, indicating that phosphate anions, supplemented at approximately 27mM, increases *S. aureus* resistance to LL-37.

Table 6.3: The MBCs of LL-37 against LS-1 and the *yycHI* mutant in different assay buffers. The median values from three independent experiments are presented. The MBC ranges from the three experiments are included in brackets if the results were not consistent in the three experiments. MBC beyond the tested concentration range 0-128 μg/ml are presented as >128 μg/ml.

Assay buffer (pH = 7.4)	**Strain**	**MBC of LL-37 (μg/ml)**
1mM NaH_2PO_4 (Buffer III)	LS-1	4 (4-8)
	LS-1 Δ*yycHI*	4 (4-8)
Buffer III + 50mM $NaHCO_3$	LS-1	32 (16-32)
	LS-1 Δ*yycHI*	>128
Buffer III + 50mM NaCl	LS-1	16 (16-32)
	LS-1 Δ*yycHI*	32 (16-32)
Buffer III + NaH_2PO_4-Na_2HPO_4 (Na^+ supplemented at 50Mm)	LS-1	>128
	LS-1 Δ*yycHI*	>128

Some of the MBC values were out the test range, so in order to evaluate the effect different ions have on the susceptibility of LS-1 and the *yycHI* mutant to LL-37, the killing of cells by 128μg/ml LL-37 over 2 hours in each buffer was determined and is presented in figure 6.10. No significant difference in susceptibility was found between LS-1 and the *yycHI* mutant in buffer III and buffer III + NaCl. However, when assayed in buffer III + $NaHCO_3$ or buffer III + NaH_2PO_4-Na_2HPO_4, the *yycHI* mutant demonstrated significantly increased resistance to LL-37 compared to LS-1. However, supplementation with NaH_2PO_4-Na_2HPO_4 also caused a significant increase in resistance to LL-37 in the wild-type LS-1. This data confirms that the anion HCO_3^- contributes to the resistance of the *yycHI* mutant to LL-37. On the other hand the anions $H_2PO_4^-$-HPO_4^{2-} significantly increased the resistance of wild-type and the *yycHI* mutant to LL-37. This indicates that either phosphate plays a role in enhancing *S. aureus* resistance to LL-37 or that it affects the physiochemical properties of LL-37.

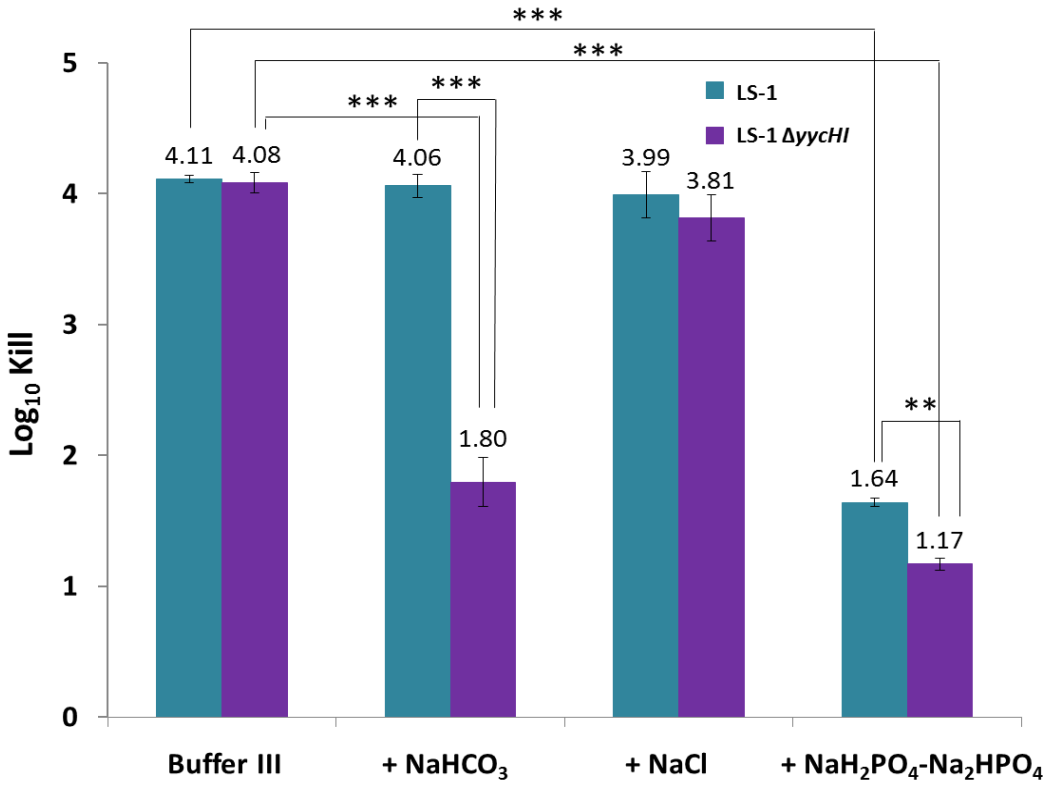

Figure 6.10: Killing of LS-1 and the yycHI mutant by 128µg/ml of LL-37 in buffer III, and buffer III with $NaHCO_3$, or NaCl or NaH_2PO_4-Na_2HPO_4. The data presented are the means of three independent experiments with error bars showing standard deviations. Significance was analysed using one way ANOVA, **: P<0.01, ***: P<0.001.

6.8 *Galleria mellonella* virulence model

To evaluate the virulence of *S. aureus* LS-1 and the *yycHI* mutant, *G. mellonella* in the larval stage were used as an *in vivo* model. This invertebrate model possesses an innate immune system but lacks an adaptive immune system. Like the human innate immune system, the *G. mellonella* immune response contains humoral components producing antimicrobial peptides, and cellular immune responses exerted by hemocytes. *G. mellonella* hemocytes share structural similarities to human neutrophils, and also employ a similar antimicrobial mechanism of engulfing and killing pathogens (Scully and Bidochka, 2006). These properties render *G. mellonella* a useful insect model for studying the virulence of organisms interacting with the innate immune system. The *G. mellonella in vivo* model has previously been used for evaluating the virulence of *S. aureus* (Peleg *et al.*, 2009; Gao *et al.*, 2010) and assessing the efficacy of antimicrobial agents against *S. aureus* (Desbois and Coote, 2011).

The virulence of *S. aureus* LS-1 and the *yycHI* mutant were determined using *G. mellonella* as described in section 2.10, with each strain assayed in 10 worms on three different occasions. The percentage survival of the 30 worms (10 from each of three independent experiments), are presented in figure 6.11 on a daily basis. In comparison to LS-1, the *yycHI* mutant demonstrated decreased virulence in *G. mellonella* model. The decrease is considered to be significant with a p value of 0.007.

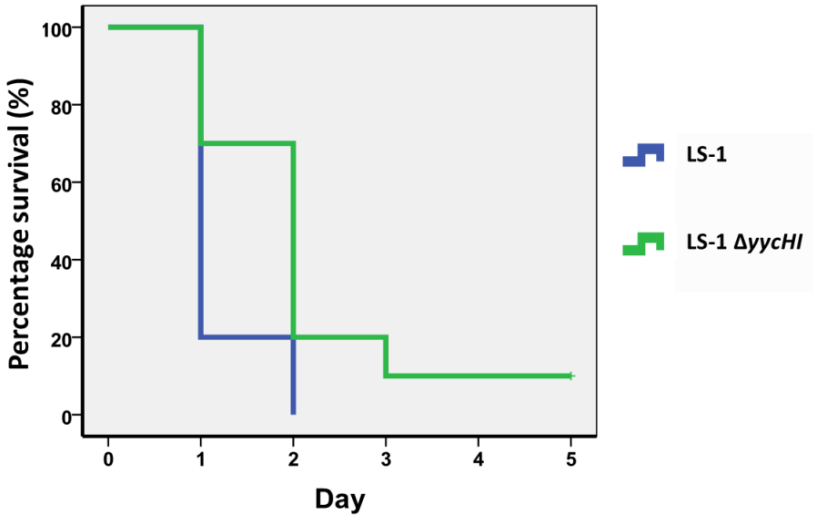

Figure 6.11: The percentage survival of *G. mellonella* infected with *S. aureus* strains during a 5-day incubation. The data presented are the percentages of 30 worms for each strain examined in three different occasions.

6.9 Discussion

Screening of a *S. aureus* LS-1 mutant library for mutants with increased resistance to LL-37 identified two mutants with a $NaHCO_3$-dependent increased resistance to LL-37 as described in chapter 5. These two mutants have transposon insertions in *yycH* and *yycI*, respectively. The genes encoding YycH and YycI form part of a predicted operon *yycFGHI* in *S. aureus*, and have been demonstrated to be negative regulators of the TCS YycFG in *B. subtilis* (Szurmant *et al.*, 2005, 2007). This suggests that the YycFG system is involved in the mechanism of resistance to LL-37 in the presence of $NaHCO_3$.

The YycFG system regulates a range of genes with diverse functions, but its main role seems to be in controlling cell wall metabolism, since half of the genes identified as

being regulated by YycFG encode peptidoglycan hydrolases (Dubrac and Msadek, 2004; Dubrac *et al.*, 2007; Delaune *et al.*, 2011). In an attempt to identify genes that may be responsible for the $NaHCO_3$-dependent resistance to LL-37, attention was focussed on genes encoding peptidoglycan hydrolases, since they have cell wall remodelling and degrading activities that might affect the ability of LL-37 to bind to the cell surface and initiate killing (Brogden, 2005). Amongst these hydrolases the transglycosylase SceD has been demonstrated to respond to NaCl, and the transglycosylase IsaA has been shown to be up-regulated by SrrAB under microaerobic conditions (Stapleton *et al.*, 2007). It was therefore hypothesised that SceD and IsaA might be involved in $NaHCO_3$-dependent resistance to LL-37. In the *yycHI* mutant, YycFG would be expected to have increased activity and lead to increased expression of SceD and IsaA. Therefore, it was hypothesised that the elevated expression of SceD or IsaA in the *yycHI* mutant may contribute to the high level of $NaHCO_3$-induced resistance to LL-37 in this mutant. However, it was found that inactivation of *sceD*, or *isaA* in the *yycHI* mutant did not reduce resistance to LL-37 in the presence of $NaHCO_3$. It suggests that however the *yycHI* mutation increases *S. aureus* resistance to LL-37, it is not through up-regulation of *sceD* or *isaA* directly or indirectly through increasing the activity of YycFG.

It is established that modification of the cell surface to reduce the cell surface negative charge is one of the important mechanisms that render bacteria resistant to cationic antimicrobial peptides. Two well characterised mechanisms of cell surface charge reduction are brought about by DltABCD and MprF which neutralise the negative charge on the major negatively-charged cell surface components techoic acids and phospholipids, respectively (Peschel *et al.*, 1999, 2001). DltABCD catalyse the transfer of D-alanine to teichoic acid and lipoteichoic acid, neutralising the negative charges on techoic acids (Peschel *et al.*, 1999). MprF incorporates a positively-charged L-lysine into phosphatidylglycerol to counter the negative charges on the cell membrane (Peschel *et al.*, 2001). As previously mentioned binding of the cationic protein cytochrome c to the bacterial cell surface is a convenient way to measure surface charge, the greater the net negative charge the greater the binding of cytochrome c to the bacterium. Less cytochrome c bound to the surface of the

yycHI and *yycI* mutants compared to the wild-type LS-1 in buffer III with or without $NaHCO_3$ demonstrating that the mutants have a reduced surface negative charge. It is not yet known how *yycHI* or *yycI* mutations cause this reduction in cell surface negative charge, but it may be because of increased activity of YycFG due to lack of inhibition by YycHI. The YycFG and PhoPR TCSs have been found to act together to repress the teichoic acid biosynthetic genes *tagAB/DEF* in *B. subtilis* in response to phosphate limitation (Howell *et al.*, 2006). This regulation has not been demonstrated in *S. aureus*, but the repression of techoic acid synthesis by YycFG and PhoPR may explain the decreased cell surface charge of the *yycHI* and *yycI* mutants.

However, from my data it is not clear whether the reduced surface net charge is important for resistance to LL-37 in *S. aureus*. As presented in section 6.6, a reduction of as high as 80% in cytochrome c binding is correlated with only a 4-fold increase in resistance to LL-37, while an almost 50% reduction of binding of cytochrome c did not result in increased resistance to LL-37 in the *yycHI* mutant. This suggests that a change in surface charge is either not very important in resistance to LL-37 or that a threshold reduction of surface net charge, more than 50%, is necessary for conferring resistance to LL-37. So far the relationship between changes in cell surface charge of *S. aureus* and susceptibility to cationic AMPs has only been studied in mutants with increased cell surface negative charge, such as the *dlt* mutant (Peschel *et al.*, 1999), *mprF* mutant (Peschel *et al.*, 2001) and *graRS* mutant (Kraus *et al.*, 2008). For example, the capacity to bind to cytochrome c increases 4-fold in a *S. aureus* SA113 *dlt* mutant, which is correlated to a more than 10-fold decrease in resistance to defensin HNP-1 to 3 compared to the wild-type (Peschel *et al.*, 1999). The link between a reduction in cell surface negative charge in the *yycH* and *yycI* mutants and increased resistance to AMPs still requires further investigation to ascertain whether a threshold reduction of surface charge contributes to resistance to LL-37.

Another factor that may be involved in resistance to LL-37 in the *yycHI* mutant could be membrane permeability, as over expression of YycFG in *Streptococcus pneumoniae* has been shown to alter fatty acid biosynthesis and cell membrane

composition (Mohedano *et al.*, 2005). Over-expression of YycF represses the genes involved in generating the fatty acid starter unit β-ketobutyryl-ACP, and elevates the genes responsible for the fatty acid chain elongation, which results in an increased ratio of C_{18} to C_{16} fatty acids in strains over-expressing YycF (Mohedano *et al.*, 2005). Increasing the length of the lipid tails leads to a reduction in membrane fluidity and permeability due to increased hydrophobic interactions between the tails (Zhang and Rock, 2008). Therefore, the interruption of *yycHI* may increase the activity of YycF, which in turn may result in reduced membrane permeability. However, it is unknown whether the increased resistance to LL-37 observed for *yycHI* mutant in the presence of bicarbonate is associated with decreased membrane permeability. It is interesting that bicarbonate was actually suggested to increase cell membrane permeability in both Gram-positive and Gram-negative bacteria (*S. aureus* and *E. coli*), thus increasing their susceptibility to LL-37 (Dorschner *et al.*, 2006), which is counter to our data. The effect bicarbonate has on membrane permeability and AMP resistance under our experimental conditions needs further investigation.

It was found that both LS-1 and the *yycHI* mutant demonstrated significantly increased resistance to LL-37 upon supplementation of buffer III with phosphate (table 6.3 and figure 6.9). In *B. subtilis*, it has been shown that under phosphate-limited conditions, the synthesis of cell wall components, peptidoglycan and teichoic acids, were both repressed, and the expression of genes responsible for cell wall metabolism were also altered through cooperative regulation by YycFG and PhoPR (Howell *et al.*, 2006; Bisicchia *et al.*, 2010; Botella *et al.*, 2011). This suggests that phosphate content in the environment may be a signal that alters expression of genes associated with the metabolism of cell wall components, which may be linked with resistance to AMPs (Brogden, 2005). Phosphate is an important anion present in the human serum at concentrations between 0.81 and 1.45mM (Tonelli *et al.*, 2005), which corresponds to the concentration of phosphate in buffer III (1mM NaH_2PO_4). Given that high levels of resistance to LL-37 were only observed when buffer III was supplemented with extra phosphate (27mM), it suggests that a physiological concentration of phosphate does not contribute to the resistance to LL-37.

The YycFG system has been reported to be involved in antimicrobial resistance, to MLS_B antibiotics (Martin *et al.*, 1999), daptomycin (Friedman *et al.*, 2006) and vancomycin (Jansen *et al.*, 2007). In the present study, we have shown that mutation of *yycHI* in *S. aureus* is associated with increased resistance to the human cationic AMP LL-37 in the presence of bicarbonate. Since YycHI inhibits the essential TCS YycFG, this indicates that YycFG may be an important factor regulating *S. aureus* resistance to antimicrobial peptides in the presence of bicarbonate. Given that bicarbonate is a major substance maintaining the acid-base balance in the human body (Cordat and Casey, 2009), the link between increased YycFG activity and bicarbonate enhanced resistance to LL-37 may indicate an important role for YycFG *in vivo* in avoiding killing by AMPs. However, the *yycHI* mutant also demonstrated reduced virulence in the *G. mellonella* virulence model. This is in accordance with a recent finding published while I was writing this thesis, showing that when YycF was expressed in a constitutively active form which did not require phosphorylation by YycG, *S. aureus* was strongly attenuated in a murine infection model (Delaune *et al.*, 2012). Reduced virulence in a murine model was also observed when the *yycFG* operon was over-expressed in *Streptococcus pneumoniae* (Wagner *et al.*, 2002). These findings suggest that further investigation into the role played by YycFG in resistance to AMPs in an environment corresponding to the host environment are necessary to assess its importance in resistance to AMPs *in vivo*. Interestingly, reduced or unaltered virulence has also been reported for *yycG* or *yycF* mutants of bacteria that only have an essential requirement for the response regulator or the sensor kinase. A *yycG* mutant of *Streptococcus pneumoniae* displayed a similar level of virulence as the wild-type in a mouse respiratory tract infection model (Throup *et al.*, 2000), and an *yycF* insertion mutant in *Streptococcus pyogenes* was unable to grow in human blood and serum, and exhibited attenuated virulence in mice (Liu *et al.*, 2006). These diverse findings indicate the importance of YycFG in regulating virulence and that disregulation of their expression or activity, either negatively or positively, probably brings about pleiotropic effects that negatively impact on the virulence of bacteria.

Chapter 7: Characterisation of a Spontaneous *S. aureus* LS-1 Small Colony Variant

7.1 Introduction

The isolation of a *S. aureus* SCV from the clinic was reported nearly 60 years ago (Jensen, 1957), but it is only more recently that the importance of SCVs in persistent and recurrent infections has been recognized (Proctor *et al.*, 1995). There are several important features of SCVs that are thought to enable them to cause persistent infections. SCVs are better able to resist antibiotic treatment due to their slow growth rate and reduced membrane potential (Proctor *et al.*, 2006; Sendi and Proctor, 2009). In addition, SCVs are capable of enhanced persistence in non-phagocytic host cells such as epithelial cells and endothelial cells (Schroder *et al.*, 2006a; Atalla *et al.*, 2010; vonEiff *et al.*, 1997b). Furthermore, SCVs are capable of reversion to rapidly growing and virulent wild-type phenotypes, potentially resulting in the recurrence of the infections (Garzoni and Kelley, 2009).

The pathogenic potential of *S. aureus* SCVs auxotrophic for hemin or menadione has been investigated in a variety of animal models and insect models, and various levels of virulence have been reported in these models. For instance, a *hemB* mutant of *S. aureus* Newbould, a strain associated with bovine mastitis, exhibited a substantially reduced capacity to colonize mouse mammary glands (Brouillette *et al.*, 2004), while a *hemB* mutant of *S. aureus* Newman demonstrated increased virulence in a murine model of septic arthritis compared to its parental strain, as higher frequency and more severe arthritis were caused by the *hemB* mutant than the wild-type (Jonsson *et al.*, 2003). In addition, the virulence of *hemB* and *menD* mutants derived from *S. aureus* 8325-4 has been assessed in a rabbit model of endocarditis, and equal virulence was found for the mutants and the wild-type, as demonstrated by similar 95% infectious dose (2×10^6 CFU) for the mutants and wild-type to seed heart valves or other tissues (kidney and spleen) (Bates *et al.*, 2003). In a *Caenorhabditis elegans* infection model, significantly reduced virulence was observed in a range of *S. aureus* hemin or menadione auxotrophs compared to their respective parental strain,

including clinically isolated SCVs auxotrophic for hemin or menadione, defined *hemB* and *menD* mutants of *S. aureus* 8325-4, a *hemB* mutant of *S. aureus* Newman and a *hemB* mutant of *S. aureus* COL (Sifri *et al.*, 2006).

S. aureus heme or menadione auxotrophs have also been found to have increased resistance to host cationic AMPs *in vitro* compared to their respective wild-type strains, these includes protamine (Sadowska *et al.*, 2002), thrombin-induced platelet microbicidal protein (tPMP) (Koo *et al.*, 1996) and bovine lactoferricin B (Samuelsen *et al.*, 2005). The enhanced resistance to host antimicrobial peptides of SCVs may be another potential mechanism by which they circumvent the host immune response. The reduced membrane potential in SCVs due to a defective ETC is thought to confer resistance to cationic antibiotics aminoglycosides (Proctor *et al.*, 2006). It was hypothesised that the reduced membrane potential in SCVs may also contribute to the resistance to cationic AMPs. However, there are only limited studies on the susceptibility of *S. aureus* SCVs to AMPs, especially human-derived AMPs, thus more investigation is needed to ascertain whether an interrupted ETC can also cause decreased susceptibility to cationic AMPs. To examine this, the susceptibility of *S. aureus* hemin and menadione auxotrophs to the human cationic AMP LL-37 was determined as described in this chapter.

While numerous hemin or menadione autotrophs of *S. aureus* have been isolated from patients (Kahl *et al.*, 2003; Sadowska *et al.*, 2002), the genetic mutations responsible for the SCV phenotypes, the rate of the reversion, and the genetic basis for the reversion have rarely been characterised. This information may be important in understanding SCV-associated persistent and relapsing infections. In the study reported in this chapter, a spontaneous *S. aureus* SCV was isolated *in vitro* and characterised with regards to its auxotrophy, the genetic basis of the SCV phenotype, the reversion rate and the genetic and phenotypic characteristics of the revertants.

7.2 Isolation of a *S. aureus* LS-1 SCV using kanamycin

The aminoglycoside antibiotic kanamycin was used to select for *S. aureus* LS-1 SCVs. The MIC of kanamycin against *S. aureus* LS-1 was determined and found to be

16µg/ml in three independent experiments (methods are described in section 2.6.2). Kanamycin resistant *S. aureus* LS-1 mutants were isolated *in vitro* by growing *S. aureus* LS-1 in TSB + 50µg/ml Kan, a concentration usually used for the selection of kanamycin-resistance *S. aureus* strains that harboured kanamycin-resistance genes. The culture was then plated onto TSA with 50µg/ml Kan and incubated at 37˚C for 48 hours. One small and non-pigmented colony was observed on the agar. This colony was designated SCV445 and streaked onto TSA, alongside the wild-type *S. aureus* LS-1, to compare their colony sizes. As shown in figure 7.1, SCV445 formed pinpoint colonies with reduced pigmentation, about 1/10th of the size of *S. aureus* LS-1, after 48 hours of incubation at 37˚C.

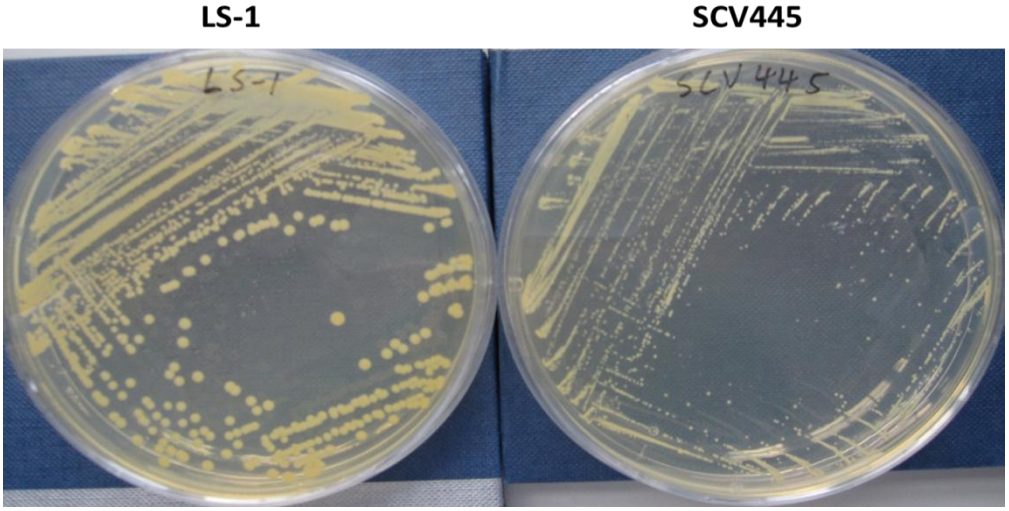

Figure 7.1: *S. aureus* LS-1 and SCV445 grown on TSA at 37˚C for 48 hours.

7.3 Confirmation of the origin of SCV445

To confirm that SCV445 was indeed an LS-1 derivative, rather than a contaminant, a range of tests, including Gram stain, catalase test and coagulase test, were conducted. As expected, SCV445 was found to be Gram-positive and formed typical grape-like clusters when examined under the microscope (data not shown). In addition, the bacterium was catalase-positive and coagulase-positive, indicating that it was likely to be *S. aureus*. To confirm that it was a *S. aureus* LS-1 derivative, advantage was taken of the endogenous plasmid pLS-1 which is present in *S. aureus* LS-1. Previous sequencing of this plasmid found that it contains an origin of

replication and a cadmium resistance gene *cad*. Plasmid DNA was extracted from LS-1 and SCV445, and analysed by gel electrophoresis, confirming the presence of a plasmid of similar size in both strains, as shown in figure 7.2 (lanes 2 and 3). Additionally, two primers P98 and P99 were designed to PCR amplify a 1557bp fragment from pLS-1 (primer sequences are listed in section 2.2.2). The plasmid DNA from LS-1 or SCV445 was used as template for the PCR amplification, and the products examined by gel electrophoresis. PCR products corresponding to the expected size of 1557bp were generated from both templates as show in figure 7.2 (lanes 5 and 6).

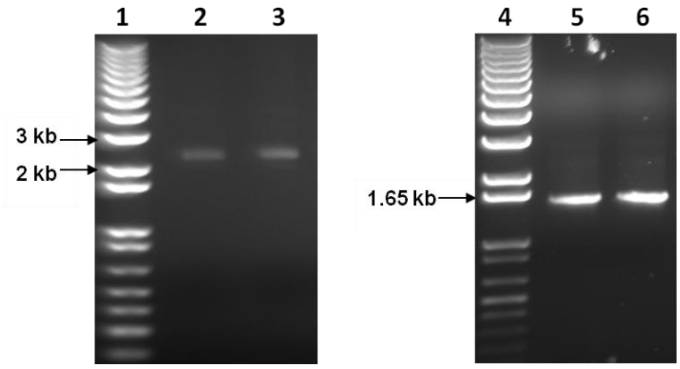

Figure 7.2: Gel electrophoresis images of plasmid DNA extracted from LS-1 (lane 2) and SCV445 (lane 3), and PCR products amplified using primers P98 and P99 from LS-1 plasmid DNA (lane 5) and SCV445 plasmid DNA (lane 6). Lanes 1 & 4: 1kb plus DNA ladder (Invitrogen).

The cadmium resistance profiles of LS-1 and SCV445 were also determined, together with that of *S. aureus* 8325-4, which served as a cadmium sensitive control strain. The MICs of cadmium chloride ($CdCl_2$) against LS-1, SCV445 and 8325-4 were determined in TSB as described in section 2.6.2. Three independent experiments demonstrated that the MICs of $CdCl_2$ against LS-1 or SCV445 were 64µg/ml, which is much higher than that against 8325-4 (8µg/ml). This suggests that SCV445, like LS-1, also contains a cadmium resistance gene. These data all suggest that LS-1 and SCV445 contain the same plasmid and provide strong evidence that SCV445 is a SCV derivative of *S. aureus* LS-1.

7.4 Kanamycin resistance of *S. aureus* SCVs

In order to quantify the increased kanamycin resistance of SCV445, susceptibility to this antibiotic was measured by determining the MIC of kanamycin in TSB as described in section 2.6.2. In addition to SCV445, defined SCVs auxotrophic for hemin or menadione were also tested for comparison with SCV445. These SCVs included two hemin auxotrophs in different strain backgrounds, *S. aureus* LS-1 Δ*hemB* (Wright and Nair, 2012) and *S. aureus* 8325-4 Δ*hemB* (vonEiff *et al.*, 1997b), and one menadione auxotroph, *S. aureus* 8325-4 Δ*menD* (Bates *et al.*, 2003). The MICs of kanamycin against the wild-type strains and their corresponding SCVs are listed in table 7.1. Compared to their respective wild-types, all the SCVs showed greatly increased resistance to kanamycin. SCV445 demonstrated higher resistance to kanamycin compared to the LS-1 *hemB* mutant. This implies that SCV445, similar to *hemB* or *menD* mutants, may bear an interrupted electron transport chain, which causes increased resistance to the aminoglycoside kanamycin, although at this point in the study the mutation(s) responsible for the SCV phenotype were not known.

Table 7.1: The MICs of kanamycin against *S. aureus* wild-types and SCVs in TSB. The median values from three independent experiments are presented. The MIC ranges from the three experiments are included in brackets if the results were not consistent in the three experiments.

Strains	**MICs of kanamycin (μg/ml)**
LS-1	16
LS-1 Δ*hemB*	128
SCV445	256 (128-256)
8325-4	16
8325-4 Δ*hemB*	128
8325-4 Δ*menD*	128

7.5 Determining the auxotrophic phenotype of SCV445

The auxotrophic phenotype of SCV445 was determined using a disk diffusion assay as described in section 2.11. SCV445 was tested on TSA with disks containing the metabolites for which SCVs are usually auxotrophic, hemin, menadione and thymidine. SCV445 was auxotrophic for menadione as shown by growth around disks with menadione after 24 hours of incubation at 37˚C (figure 7.3). Strain 8325-4 Δ*menD* was included as a reference of a defined menadione auxotroph. When manadione is absent from the disks, the two wild-type strains LS-1 and 8325-4 exhibited heavy growth, while very little growth of SCVs, 8325-4 Δ*menD* and SCV445 was observed. However, when 1mg/ml menadione was added onto the disks, growth zones of 8325-4 Δ*menD* and SCV445 were observed around the disks. No growth stimulation of SCV445 was apparent when hemin or thymidine-impregnated disks were used (data not shown). Thus SCV445 is auxotrophic for menadione, but not hemin or thymidine.

As shown in figure 7.3, there were inhibition zones around the disks on all the plates, particularly on plates with disks containing 1mg/ml menadione. The small inhibition zone around disks without menadione was due to the inhibition by ethanol, which was applied as a control because menadione was prepared in ethanol. It is known that menadione is required for normal growth of bacteria, but it is also toxic to the cells as it can reduce oxygen to superoxide radicals, which creates oxidative stress damaging proteins such as the elongation factor G and the β-subunit of F_0F_1-ATPase (Tamarit *et al.*, 1998). The larger inhibition zones around disks with 1mg/ml menadione are likely to be a consequence of the toxicity of menadione at such a high concentration.

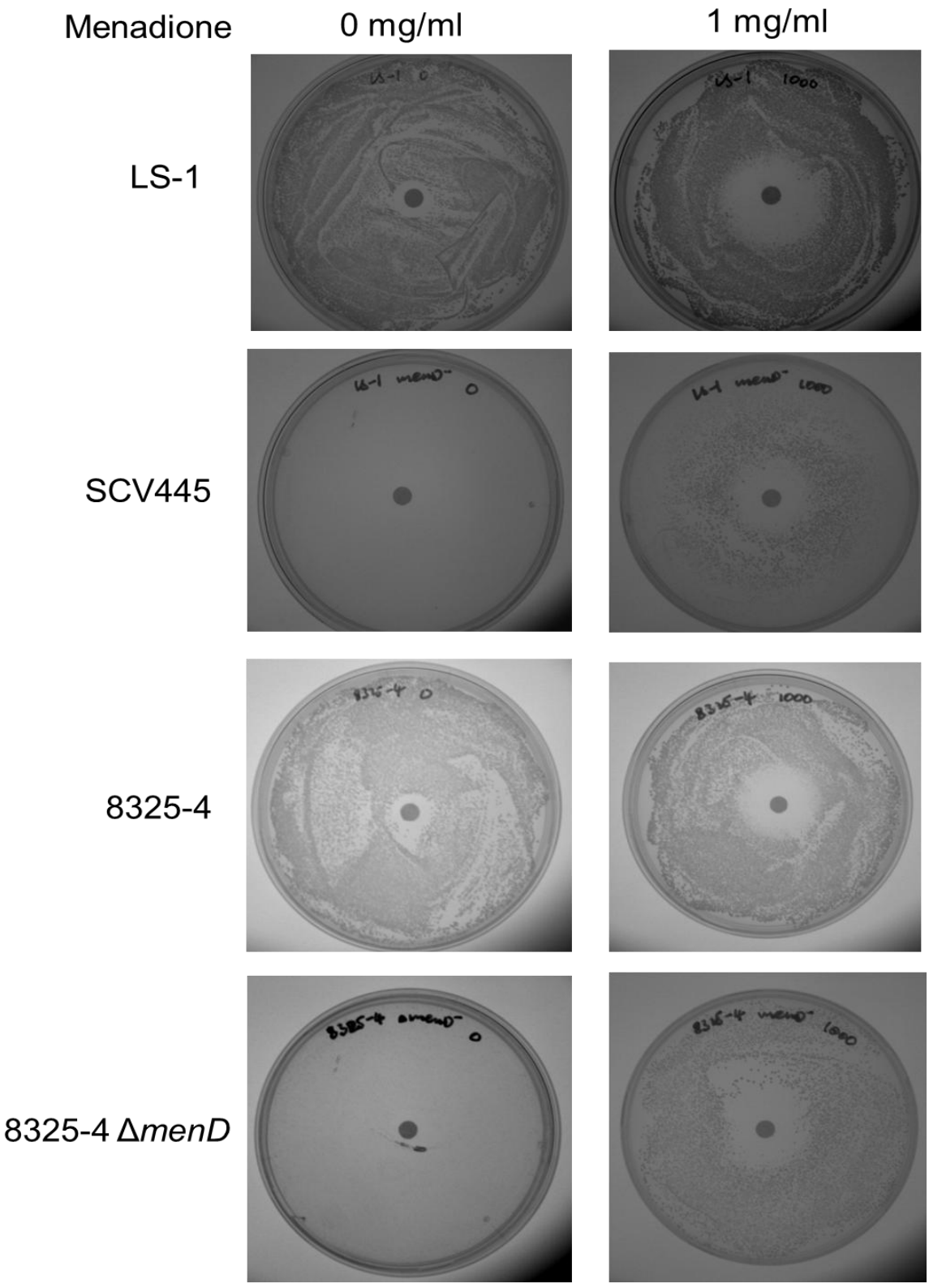

Figure 7.3: Disk diffusion images indicating that SCV445 is auxotrophic for menadione. Plates on the left panel: TSA + disk without menadione but with ethanol. Plates on the right panel: TSA+ disk with 1mg/ml menadione.

7.6 Determination of the optimum concentration of menadione for SCV445 growth

Since SCV445 is auxotrophic for menadione, which is also toxic to the cells, an optimal concentration of menadione is required to restore growth while minimising toxicity. In a previous study, a menadione concentration of 0.375µM (0.06µg/ml) was found to confer the maximal growth restoration on *S. aureus* 8325-4 Δ*menD* (von Eiff *et al.*, 2006a). To determine the optimum concentration of menadione for restoring the growth of SCV445, growth experiments were performed as described in section 2.1.6, using TSB supplemented with a range of concentrations of menadione from 0 to 1µg/ml. Concentrations of menadione between 0.3µg/ml to 0.5µg/ml were found to impart the maximal growth rate for SCV445. Growth curves of *S. aureus* LS-1 and SCV445 in TSB in the presence and absence of 0.3µg/ml menadione are presented in figure 7.4.

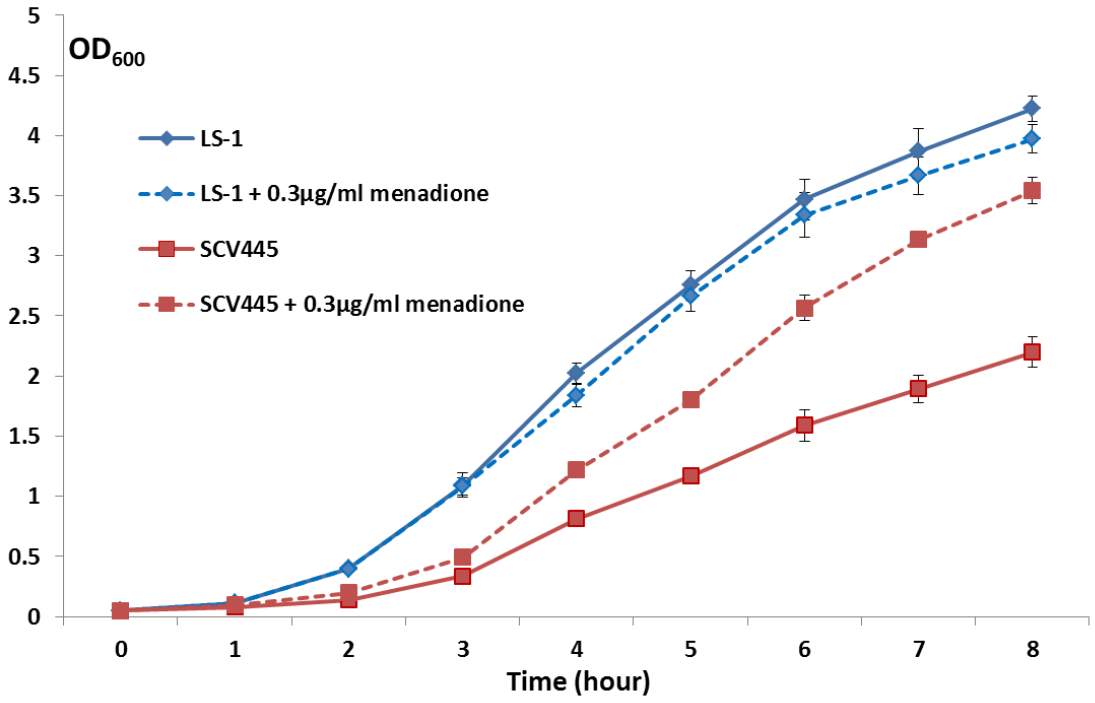

Figure 7.4: Growth curves of *S. aureus* LS-1 and SCV445 at 37˚C in TSB with or without 0.3µg/ml menadione. Data presented is the mean of three independent experiments with error bars showing standard deviations.

7.7 Identification of the genetic mutation in SCV445

To identify the genetic basis of the menadione auxotrophy of SCV445, menadione biosynthesis genes and putative promoter regions were amplified from both LS-1 and SCV445, and sequenced as described in section 2.12. These genes included previously identified menadione biosynthetic genes *menA, menB, menC, menD, menE, menF* (Nowicka and Kruk, 2010), a *gerC* locus (also named *hepT-menG-hepS*), which has been suggested to be involved in menadione biosynthesis in *B. subtilis* (Leatherbarrow *et al.*, 1998), and SAOUHSC_01348 and SAOUHSC_02556, which are the homologues of a recently identified thioesterase-encoding gene that participates in menadione biosynthesis in cyanobacterium *Synechocystis* sp. PCC6803 (Widhalm *et al.*, 2009). No differences were identified between these genes or their promoters in SCV445 compared to LS-1.

Analysis of the biosynthetic pathways upstream of the menadione biosynthetic pathway (figure 7.5) led to a hypothesis that the mutation may be located in the shikimate pathway (figure 7.5, black bold arrows). As shown in figure 7.5, the shikimate pathway leads to the formation of chorismate, which is a branching point for several biosynthetic pathways, including ubiquinone, menaquinone, folate, siderophore group nonribosomal peptides and aromatic amino acids. A mutation causing a disruption of shikimate pathway would be expected to disrupt all the biosynthesis pathways downstream of chorismate. To test the hypothesis that SCV445 is auxotrophic for menadione due to mutation in the shikimate pathway, the decision was made to examine whether the other biosynthetic pathways downstream of chorismate are disrupted. Given that amino acids are essential for protein synthesis, interruption of the aromatic amino acids biosynthetic pathways should be lethal to bacteria under conditions were aromatic amino acids are not available from the environment. Therefore, chemical defined media (CDM) with or without aromatic amino acids was used to measure the growth of SCV445 and LS-1 as described in section 2.1.6.

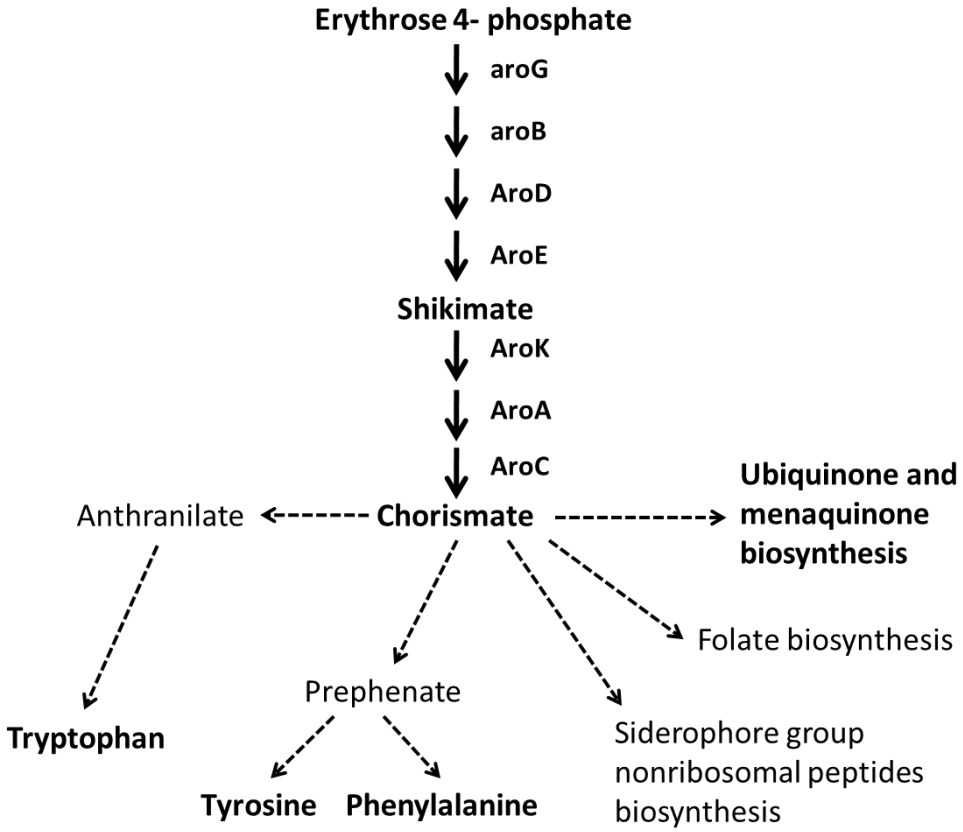

Figure 7.5: Schematic outline of the shikimate pathway and downstream biosynthetic pathways in *S. aureus*. The pathway is modified from KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway: phenylalanine, tyrosine and tryptophan biosynthesis, which is available at http://www.genome.jp/kegg/pathway/map/map00400.html. Black filled arrows: biosynthetic pathways towards chorismate biosynthesis. Dashed arrows: biosynthetic branches downstream of chorismate.

SCV445 was grown in CDM, CDM without aromatic amino acids (CDM-AAAs), and CDM or CDM-AAAs supplemented with 0.3µg/ml menadione. The growth curves are displayed in figure 7.6. In CDM, the wild-type LS-1 demonstrated normal fast growth rate, while SCV445 showed much slower growth rate. However, enhanced growth of SCV445 was observed in CDM in the presence of menadione, confirming its menadione auxotrophic phenotype. As expected, no growth of the SCV445 was detected in CDM-AAAs within the 9 hour experimental period, regardless of supplementation with menadione. However, it was surprising to find that *S. aureus* LS-1 was also not able to grow in CDM-AAAs. Therefore *S. aureus* LS-1 is auxotrophic for one or more aromatic amino acids. Since *S. aureus* LS-1 was not auxotrophic for menadione, it was likely that the aromatic amino acid biosynthesis pathway was disrupted due to a mutation(s) at a point downstream of chorismate.

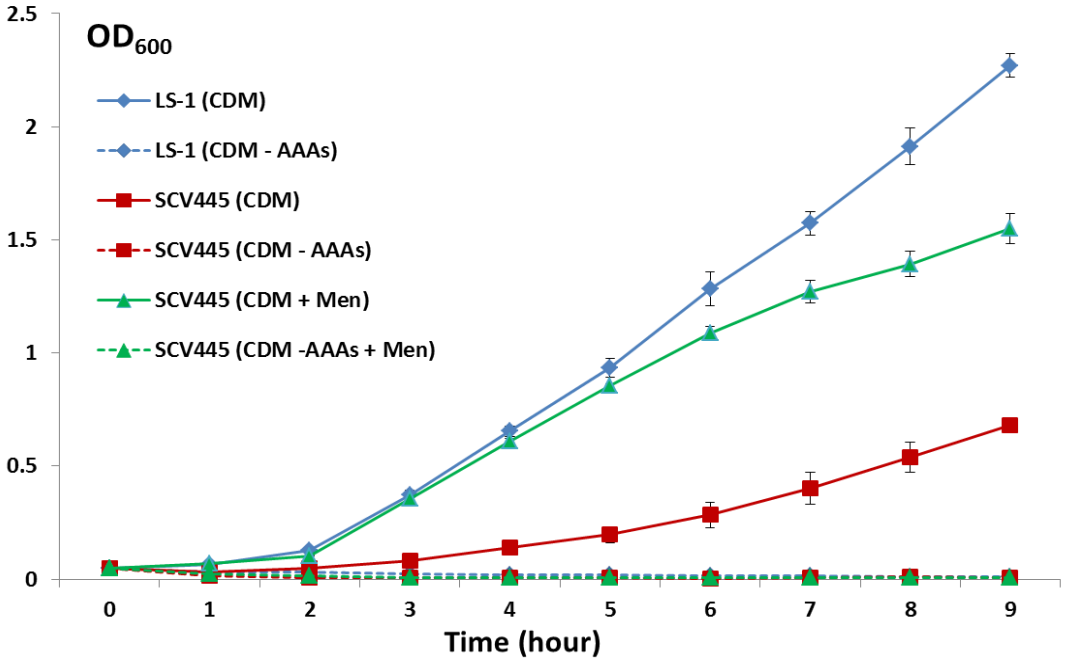

Figure 7.6: Growth curves of *S. aureus* LS-1 and SCV445 in CDM, CDM without aromatic amino acids (CDM - AAAs), CDM with 0.3µg/ml menadione (CDM + Men) or CDM - AAAs with 0.3µg/ml menadione (CDM - AAAs + Men). Presented data is the mean of three independent experiments with error bars showing the standard deviations.

To locate the mutations that caused the aromatic amino acid auxotrophies in SCV445 and *S. aureus* LS-1, their growth was subsequently measured in CDM deprived of individual aromatic amino acids. Figure 7.7 shows the growth dynamics of LS-1 and SCV445 in CDM with and without combinations of tyrosine, tryptophan, or phenylalanine. *S. aureus* LS-1 was only auxotrophic for tyrosine, as it was able to grow albeit at a reduced rate in CDM without phenylalanine (blue dashed line with triangle marker) or tryptophan (blue dashed line with cross marker), but not in CDM without tyrosine (blue dashed line with square marker). Unlike *S. aureus* LS-1, SCV4445 was not able to grow in the absence of any one of the three aromatic amino acids (red dashed lines). These data suggest that SCV445 possesses a mutation(s) in the pathway upstream of chorismate, which results in the inactivation of both menadione biosynthesis and aromatic amino acids biosynthesis.

To confirm this, the seven *aro* genes involved in chorismate synthesis (black bold arrows in figure 7.5) were PCR amplified and sequenced as described in section 2.12. Sequence alignment between *S. aureus* LS-1 and SCV445 revealed an A^{448} to T^{448} substitution in the *aroD* gene of SCV445, resulting in the 150^{th} codon changing from

AAA (lysine), to a stop codon TAA. The *aroD* gene encodes 3-dehydroquinate dehydratase (Duncan *et al.*, 1986), which is indispensable for the synthesis of chorismate. This mutation is likely to be responsible for the SCV phenotype of SCV445. The disruption of *aroD* leads to an interrupted chorismate biosynthetic pathway, which in turn results in the lack of the precursor for all of the biosynthetic pathways downstream of chorismate, thereby accounting for the menadione and aromatic amino acids auxotrophy phenotype.

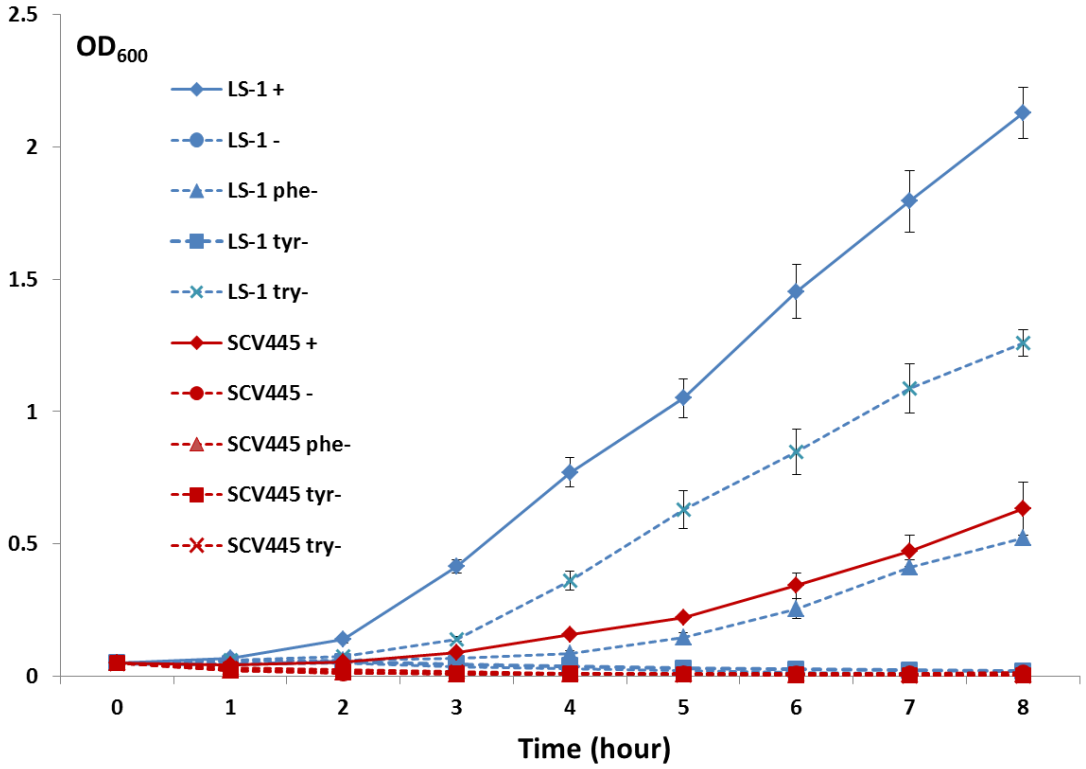

Figure 7.7: Growth curves of *S. aureus* LS-1 and SCV445 in CDM with or without aromatic amino acid(s). Blue lines: *S. aureus* LS-1. Red lines: SCV445. Filled lines: CDM. Dashed lines: CDM without one or three aromatic amino acids. Diamond: CDM (+); circle: CDM without all three aromatic amino acids (-). Triangle: CDM without phenylalanine (phe-). Square: CDM without tyrosine (tyr-). Cross: CDM without tryptophan (try-). Data presented is the mean of three independent experiments with error bars showing standard deviations.

7.8 The genetic basis of tyrosine auxotrophy in *S. aureus* LS-1

Since *S. aureus* LS-1 is auxotrophic for tyrosine, but not tryptophan or phenylalanine, a mutation(s) in the gene(s) responsible for tyrosine biosynthesis may count for this phenotype. The extensively used laboratory strain of *S. aureus*, 8325-4, was

examined for auxotrophy to aromatic amino acids, and found to be able to grow in the absence of any aromatic amino acids (data not shown). Therefore, the decision was made to compare the sequences of tyrosine biosynthetic genes of *S. aureus* LS-1 to those of 8325-4. The aromatic amino acid biosynthesis pathways are schematically presented in figure 7.8. Chorismate is converted to prephenate, which is a branching point for tyrosine synthesis and phenylalanine synthesis. At this point, two enzymes, prephenate dehydrogenase (Bonvin *et al.*, 2006) and prephenate dehydratase (Zhang *et al.*, 1998) convert prephenate to 4-hydroxy-phenylpyruvate and phenylpyruvate, respectively, which are precursors for tyrosine and phenylalanine. The following steps, which convert 4-hydroxy-phenylpyruvate to tyrosine and phenylpyruvate to phenylalanine are catalysed by the same enzyme, histidinol-phosphate aminotransferase (Hsu *et al.*, 1989).

Given that *S. aureus* LS-1 is auxotrophic for tyrosine rather than phenylalanine, the location of the mutation responsible for tyrosine auxotrophy was expected to be in the prephenate dehydrogenase-encoding gene (*tyrA*), which specifically participates in tyrosine biosynthesis, but not phenylalanine. Therefore, the *tyrA* gene was PCR amplified from *S. aureus* LS-1, SCV445 and 8325-4, and sequenced as described in section 2.12. The sequences of *tyrA* in LS-1 and SCV445 were identical. However, they differ from 8325-4 *tyrA* at four positions (table 7.2). The 107th nucleotide was found to be a cytosine (C) in all the other *S. aureus* strains, whose genome sequences are available at NCBI (http://www.ncbi.nlm.nih.gov/genome/?term=S.%20aureus), indicating that this substitution may not cause the inactivation of TyrA. In addition, the difference at 525th nucleotide was silent as both GCT and GCC encode alanine. Therefore, the mutation imparting the inability to synthesise tyrosine could possibly be the 68th and/or the 673th nucleotide substitution.

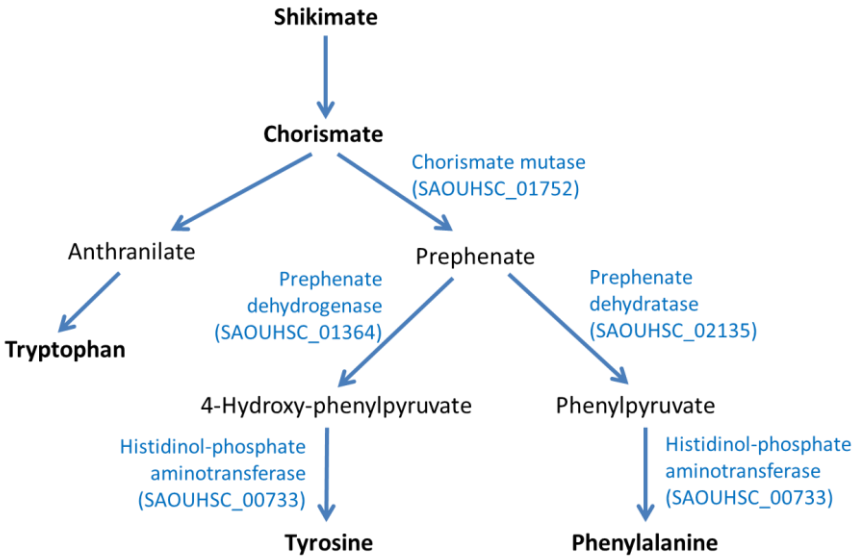

Figure 7.8: Schematic outline of aromatic amino acid biosynthetic pathways modified from KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway: phenylalanine, tyrosine and tryptophan biosynthesis, http://www.genome.jp/kegg/pathway/map/map00400.html. The enzymes catalysing tyrosine and phenylalanine biosynthesis are shown in blue characters, and their corresponding gene tags in *S. aureus* NCTC 8325 (GenBank accession CP000253) are included in brackets.

Table 7.2: Nucleotide differences in *tyrA* from *S. aureus* LS-1 SCV445 and 8325-4. The mutations in genetic codes are highlighted as bold characters.

Nucleotide position	**8325-4**	**LS-1**	**SCV445**	**Genetic code change 8325-4 → LS-1**
68	A	G	G	C**A**T (histidine^{23}) → C**G**T (arginine^{23})
107	T	C	C	A**T**T (isoleucine^{36}) → A**C**T (threonine^{36})
525	T	C	C	GC**T** (alanine^{175}) → GC**C** (alanine^{175})
673	G	T	T	**G**CT (alanine^{225}) → **T**CT (serine^{225})

Whether the 68th and/or the 673th nucleotide substitution affected the function of the protein was assessed by analysing the theoretical tolerance of these amino acid substitutions using the SIFT program (Sorting Intolerant from tolerant) available online at http://sift.bii.a-star.edu.sg/. This program predicts whether an amino acid substitution affects protein function based on the physical properties of amino acids and sequence homology in protein sequence databases, such as the UniProt-

SwissProt, UniProt-SwissProt/TreMBL and NCBI non-redundant protein databases (Ng and Henikoff, 2003). The protein sequence of TyrA was analysed and the tolerance of these two substitutions $H^{23}R$ and $A^{225}S$ was predicted. Replacing the 23rd histidine with arginine was predicted to be tolerable for maintaining protein function. H^{23} is likely to be unimportant for protein function, as substitution with any one of the other 19 amino acids was predicted to be tolerable. However, A^{225} was more likely to be a highly conserved position, as substitution with no other amino acid was predicted to be tolerable. In fact, very few substitutions were predicted to be tolerable in the region from amino acid 213th to 229th, suggesting that this region may be highly conserved and essential for the protein function. The physical properties of the relevant amino acids suggest that it is reasonable that substitution of $H^{23}R$ does not affect the protein function, as both amino acids are basic hydrophilic molecules. However, the substitution of $A^{225}S$ alters the amino acid at this position from hydrophobic to hydrophilic, which possibly prevents the protein from folding into the functional structure.

7.9 Susceptibility of *S. aureus* SCVs to LL-37

To examine whether a reduced membrane potential in *S. aureus* hemin or menadione auxotrophs confers resistance to the cationic AMP LL-37, the MBCs of LL-37 were determined as described in section 2.6.7 with SCVs auxotrophic for hemin or menadione, including LS-1 Δ*hemB* (Wright and Nair, 2012), 8325-4 Δ*hemB* (vonEiff *et al.*, 1997b), 8325-4 Δ*menD* (Bates *et al.*, 2003) and SCV445. These experiments were performed using a physiological concentration of bicarbonate (50mM $NaHCO_3$) since it has been suggested to increases cell membrane permeability, thereby increasing the susceptibility of bacteria to the LL-37 (Dorschner *et al.*, 2006). To assess whether $NaHCO_3$ effects the susceptibility of *S. aureus* SCVs by acting on cell membrane, the MBCs of LL-37 for each strain were determined in both buffer III and buffer III + C (buffer III + 50mM $NaHCO_3$).

The MBCs for the SCVs and their respective wild-type strains are summarised in table 7.3. When measured in buffer III, all the SCVs showed the same level of susceptibility as their respective wild-type parental strains, demonstrating that the SCV-associated

physiological properties, such as slow metabolism and reduced membrane potential, do not contribute to increased resistance to LL-37 in buffer III. However, altered levels of susceptibility to LL-37 were observed amongst the strains when assayed in buffer III + C. The two LS-1-derived SCVs showed greatly increased resistance to LL-37 (MBCs >128μg/ml) compared to LS-1 (MBC = 16 μg/ml), while the two 8325-4-derived mutants did not exhibit increased resistance in comparison with 8325-4.

Table 7.3: The MBCs of LL-37 against *S. aureus* wild-types and SCVs in buffer III and buffer III + C. The median values from three independent experiments are presented. The MBC ranges from the three experiments are included in brackets if the results were not consistent in the three experiments. MBCs beyond the tested concentration range 0-128 μg/ml are presented as >128 μg/ml.

Strains	MBC of LL-37 (μg/ml) in the absence of 50mM $NaHCO_3$	MBC of LL-37 (μg/ml) in the presence of 50mM $NaHCO_3$
LS-1	8	16 (16 – 32)
LS-1 Δ*hemB*	8	>128
SCV445	8	>128
8325-4	8	8
8325-4 Δ*hemB*	8	8 (8 - 16)
8325-4 Δ*menD*	8	8 (8 – 16)

It was interesting that the SCVs originating from LS-1 and 8325-4 exhibited different levels of susceptibility to LL-37 in the presence of $NaHCO_3$. Given that the two *hemB* mutants originating from LS-1 and 8325-4, exhibited such different levels of resistance to LL-37 in buffer III + C, there must be other genetic factors that are different in the two genetic backgrounds and that accounts for this difference. For example, as presented in section 7.8, LS-1 is defective in *tyrA*, and *S. aureus* 8325-4 is defective in *rsbU* and *tcaR*, which are activators of the alternative sigma factor SigB and protein A, respectively. SigB is an important regulator that responds to environmental changes and stress, the expression of SigB was also found to reduce approximately 10-fold when grown in the presence of $NaHCO_3$ (Dorschner *et al.*, 2006). SCV445 and 8325-4 Δ*menD* are both auxotrophic for menadione, but they are

defective in *aroD* and *menD*, respectively. The different response to $NaHCO_3$ between SCV445 and 8325-4 Δ*menD* may also be due to differences in the genetic backgrounds of LS-1 and 8325-4 or it could be caused by the fact that the mutations are in different metabolic genes. Furthermore, it is uncertain whether SCV445 carries additional mutations other than that in *aroD*, which could also result in the difference in susceptibility to LL-37 between SCV445 and 8325-4 Δ*menD.*

To elucidate the basis of the difference in susceptibilities of the SCVs derived from LS-1 and 8325-4 to LL-37, a SCV with a *menD* mutation in the LS-1 background, and defined *aroD* mutants in strains LS-1, 8325-4, and two other strains SH1000 (Horsburgh *et al.*, 2002) and HG003 (Herbert *et al.*, 2010) were constructed. As shown in figure 7.9, strains 8325-4, SH1000 and HG003 are all derived from strain NCTC 8325, which is defective in *rsbU* and *tcaR*, but carrying three prophages ø11, ø12 and ø13. Strain 8325-4 was generated by curing the three prophages ø11, ø12 and ø13 from NCTC 8325. SH1000 was generated by repairing the defective *rsbU* gene in 8325-4. HG003 was generated by repairing both the *rsbU* and *tcaR* genes in NCTC 8325 (Herbert *et al.*, 2010). Therefore, HG003 contains functional *rsbU* and *tcaR*, as well as prophage ø11, ø12 and ø13. Using these strains it should be possible to ascertain whether defects in *rsbU* or *tcaR* in strain 8325-4 explain the differing responses of the SCVs to LL-37 in the presence of $NaHCO_3$, and whether mutation in *hemB*, *menD* or *aroD* confer different levels of resistance to LL-37 in the presence of $NaHCO_3$.

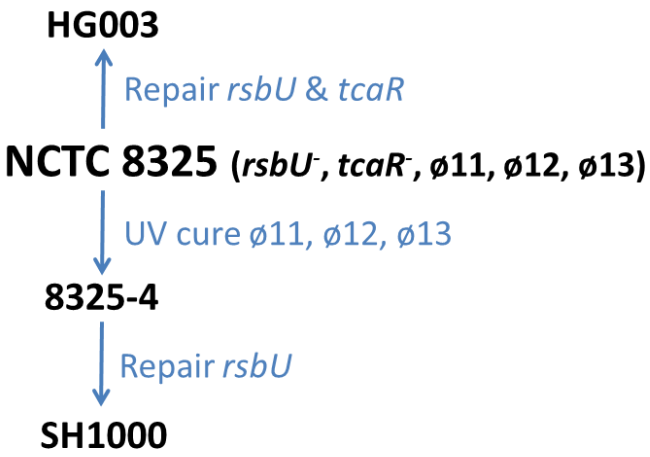

Figure 7.9: Relationship of strains derived from NCTC 8325, which is defective in *rsbU* and *tcaR*, but carries prophages ø11, ø12, ø13 (Herbert *et al.*, 2010).

7.10 Construction of *menD* and *aroD* mutants

A mutant *S. aureus* LS-1 *menD :: erm* was constructed as described in section 2.3.7. Briefly, a ~3.1kb fragment containing *menD-erm-menD* was amplified from *S. aureus* 8325-4 *menD :: erm*, and cloned into pKOR1. The resulting vector was then introduced into *S. aureus* LS-1 to integrate *erm* into *menD* on the chromosome through allelic replacement. The gel electrophoresis images that confirm the success of each cloning steps are shown in figure 7.10. The PCR product containing *menD-erm-menD* was at the expected size ~3.1kb (lane 2) and the digestion of pKOR1 generated two fragments at the expected sizes 7536bp and 2467bp (lane 3). The success of cloning *menD-erm-menD* into pKOR1 was validated by PCR amplification of the insert from the resulting vectors. PCR products at ~3.1kb were generated from the potential vectors pKOR1 :: *menD-erm-menD* (lanes 6 and 7), while no PCR product was generated from vector pKOR1 (lane 5). Finally, the success of constructing *S. aureus* LS-1 Δ*menD* was confirmed by PCR amplification of the mutated region using primers specific to the chromosome. PCR amplification from wild-type genomic DNA resulted in the expected product at 2258b (lane 9), while those from the potential *menD* mutants generated products of ~4.2kb, indicating the insertion of a ~2kb fragment into *menD*. The two mutants were also confirmed to be free of the pKOR1 derived plasmid and to be menadione auxotrophs (data not shown).

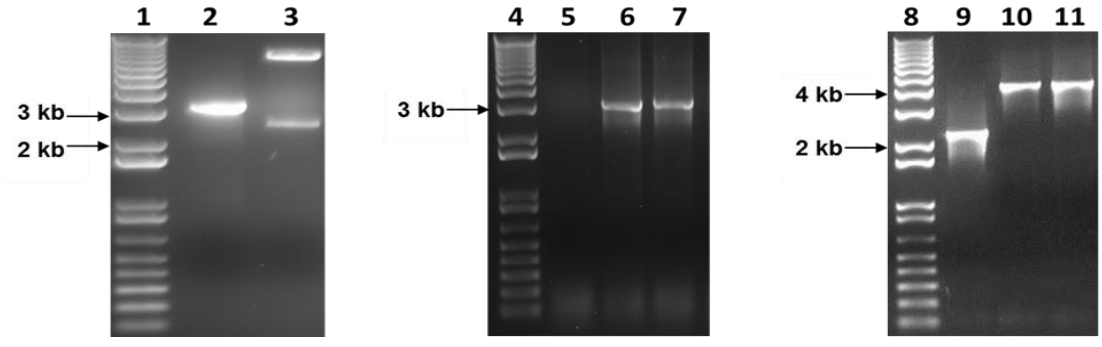

Figure 7.10: Gel electrophoresis images of PCRs and digests involved in construction of *S. aureus* LS-1 *menD* :: *erm*. Lanes 1/4/8: 1kb plus DNA ladder (Invitrogen). Lane 2: PCR product of a fragment containing *menD-erm-menD*, expected size: ~3.1kb. Lane 3: EcoR1-EcoRV digested pKOR1, expected pattern: two fragments at sizes 7536bp and 2467bp. Lanes 5/6/7: PCR products of amplification using primers P125 and P126 from pKOR1 (lane 5, no product was expected) and potential pKOR1 :: *menD-erm-menD* (lanes 6/7, expected product size: ~3.1kb). Lanes 9/10/11: PCR products of amplification using primers P125 and P128 from genomic DNA of strains LS-1 (lane 9, expected product size: ~2.2kb) and two potential LS-1 *menD :: erm* (lanes 10/11, expected product size: 4.2kb).

S. aureus aroD mutants were constructed to mimic the mutation site in SCV445. A stop codon was placed at the 150th codon of the *aroD* gene and the remaining downstream sequence of this gene was deleted. Construction of the desired mutant was achieved by cloning upstream and downstream regions, of the target region for deletion, into pKOR1, followed by allelic replacement between the resulting plasmid and chromosome. The detailed method is described in section 2.3.8. The gel electrophoresis images that confirm the success of each cloning steps are shown in figure 7.11. PCR amplification of the upstream and downstream fragments generated products corresponding to the expected sizes of 806bp and 851bp for upstream (lanes 2) and downstream (lane 3) regions, respectively. Cloning of these two fragments into pKOR1 was confirmed by digestion of the vectors with XhoI. As expected, digestion of pKOR1 with XhoI produced a single fragment (lane 5), whereas digestion of pKOR1 containing the *aroD* upstream and downstream fragments generated two fragments corresponding to the expected sizes 6279bp and 3139bp (lanes 6/7/8). The success of constructing the *aroD* mutants was validated by PCR amplification covering the deleted region. As shown in lane 10, a 1252bp product was amplified from the wild-type *S. aureus* LS-1, while expected smaller PCR products of 983bp were generated from the potential *aroD* mutants. Lanes 11-13, 14-16, 17-18 and 19-20 represent putative *aroD* mutants from LS-1, 8325-4, SH1000 and HG003, respectively.

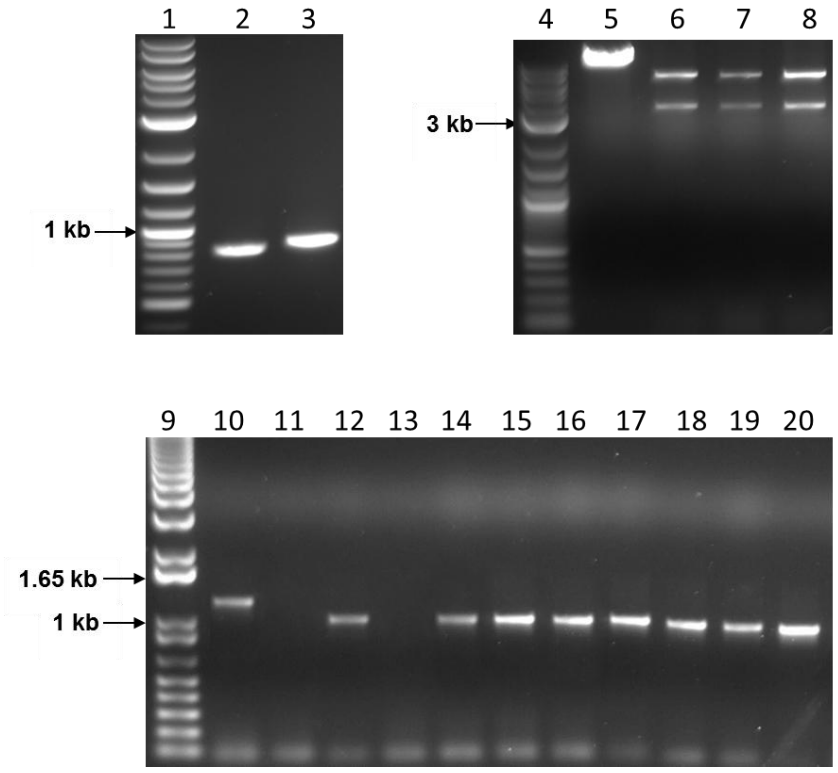

Figure 7.11: Gel electrophoresis images of PCR products and digests involved in the construction of *S. aureus* LS-1 Δ*aroD* and 8325-4 Δ*aroD*. Lanes 1/4/9: 2-log DNA ladder (NEB). Lanes 2 & 3: PCR product of *aroD* upstream and downstream fragments, expected sizes: 806bp and 851bp, respectively. Lane 5: XhoI digested pKOR1, expected pattern: one fragment at 10003bp. Lanes 6/7/8: XhoI digested potential pKOR1 containing *aroD* upstream and downstream fragments, expected digestion pattern: two fragments at 6279bp and 3139bp. Lane 10-20: PCR products of amplifications using primers P172 and P173 which flank the deletion region in *aroD*. Lane 10: from wild-type LS-1, expected product size: 1252bp. Lanes 11-13, 14-16, 17-18 and 19-20: from putative *aroD* mutants in LS-1, 8325-4, SH1000 and HG003, respectively, expected product size: 983bp.

7.11 Complementation of the *aroD* mutant strains

To complement the *aroD* mutation, a *Pspac* promoter and the *aroD* gene were cloned into pSK236 generating plasmid pPZ137-3. The cloning details are described in section 2.3.9. The gel electrophoresis images that confirmed the success of cloning are shown in figure 7.12. The PCR product of the *aroD* gene was of the expected size of 734bp (lane 2). The success of cloning *Pspac-aroD* into pSK236 was confirmed by digesting potential pSK236 :: *Pspac-aroD* with HindIII. Lane 4 displays HindIII-digested pSK236, which gives a band of ~3kb. When exposing the gel to UV light for shorter periods, this band appeared as two separated bands with the expected sizes of 2689bp and 2910bp (lane 12). HindIII digestion of putative complementation plasmids are shown in lanes 5-10, with only those in lane 7-10 showing the expected

digestion pattern of three fragments at 4297bp, 2910bp and 780bp. These four vectors were thereafter designated pPZ137-3/4/5/6. Plasmid pPZ137-3 was sequenced at the cloning region to confirm that no mutation was present in the *Pspac* promoter and the *aroD* gene, and was used for the complementation of *aroD* mutants.

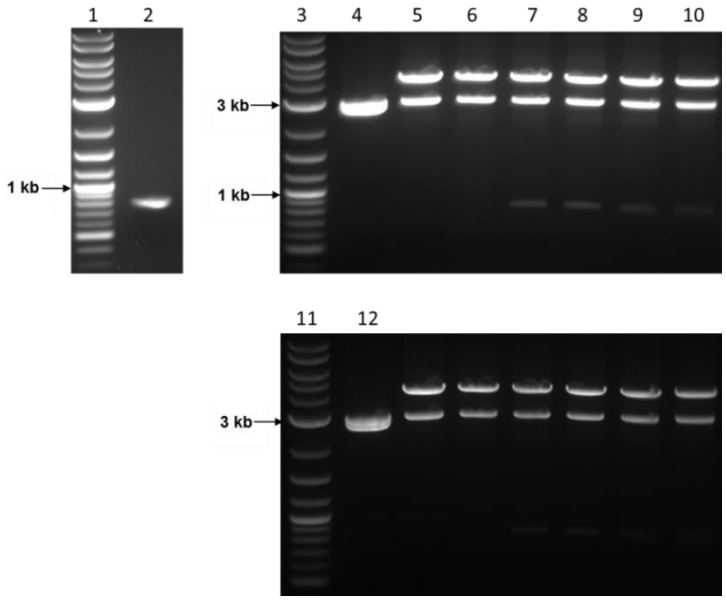

Figure 7.12: Gel electrophoresis images of PCR products and digests involved in construction of the complementation plasmid for the *aroD* mutants. Lane 1/3/11: 2-log DNA ladder (NEB). Lane 2 PCR product of *aroD* gene, expected size: 734bp. Lanes 4/12: HindIII digested pSK236, expected pattern: two fragments at 2689bp and 2910bp. Lanes 5-10: HindIII digested putative complement plasmids, expected digestion pattern: three fragments at 4297bp, 2910bp and 780bp.

The plasmid pPZ137-3 was introduced into LS-1 Δ*aroD*, 8325-4 Δ*aroD*, SH1000 Δ*aroD* and HG003 Δ*aroD*. The wild-types, *aroD* mutants and *aroD* mutants with pSK236 or pPZ137-3 were streaked onto TSA and incubated at 37˚C for 24 hours. The resulting plates were photographed and are displayed in figure 7.13. The wild-types LS-1 and 8325-4 grew to large colonies with diameters approximately 1.5mm, while the colonies of the *aroD* mutants were approximately 1/10th of the wild-type size. The *aroD* mutants harbouring pSK236 did not have restored growth, while those containing pPZ137-3 appeared as colonies resembling the wild-type size. This demonstrates that truncation of the C-terminal of AroD by introduction of a stop codon in the gene, from the 150th codon, inactivates protein function, which in turn causes the slow growth rate in these LS-1 and 8325-4 mutants. Complementation of the *aroD* gene using a plasmid was able to restore the growth to wild-type level.

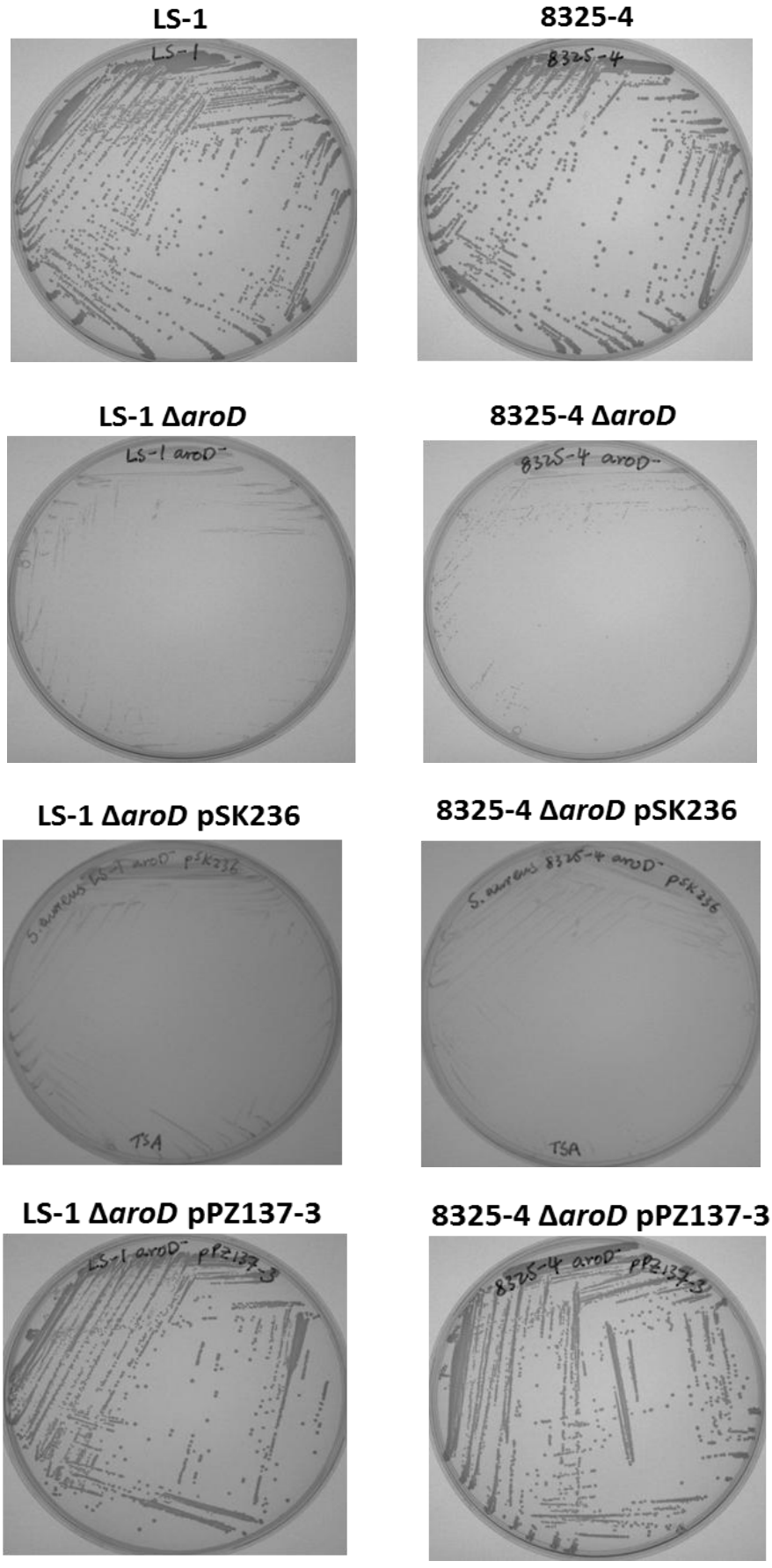

Figure 7.13: Photographs of TSA plates streaked with *S. aureus* wild-types, *aroD* mutants and *aroD* mutants with pSK236 and pPZ137-3 grown at 37˚C for 24 hours.

The growth dynamics of the defined *aroD* mutants and their complemented strains were characterised in TSB as described in section 2.1.6. Viable counts of the cultures at various time intervals were determined, and are presented in figure 7.14 for LS-1 and its derivatives, and figure 7.15 for 8325-4 and its derivatives. In comparison with the wild-types, SCV445 and the constructed *aroD* mutant replicated at a reduced rate and entered into stationary phase at a lower cell density. The presence of plasmid pSK236 in the *aroD* mutants did not alter their growth dynamics. However, the constructed *aroD* mutant containing pPZ137-3 had its growth restored to that of the wild-type. This confirms that AroD is important for *S. aureus* growth, and a truncated AroD containing the first 149 amino acids is unable to maintain rapid growth in *S. aureus*.

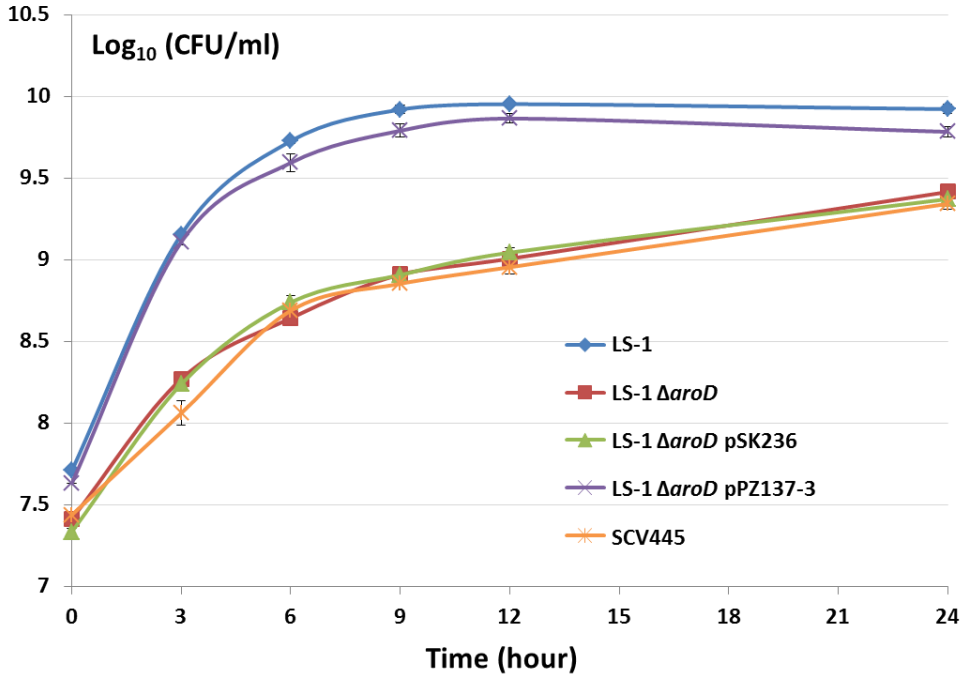

Figure 7.14 Growth curves of strains LS-1, SCV445, LS-1 Δ*aroD*, LS-1 Δ*aroD* pSK236 and LS-1 Δ*aroD* pPZ137-3. Presented data are the means of three independent experiments with error bars showing the standard deviations.

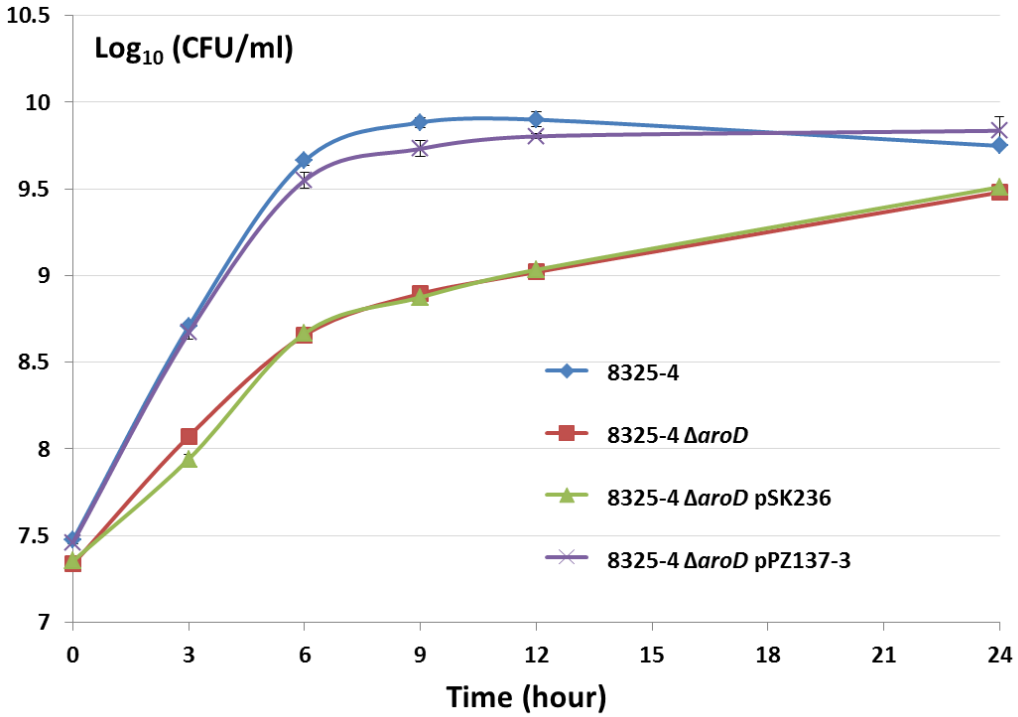

Figure 7.15: Growth curves of strains 8325-4, 8325-4 Δ*aroD*, 8325-4 Δ*aroD* pSK236 and 8325-4 Δ*aroD* pPZ137-3. Presented data are the means of three independent experiments with error bars showing the standard deviations.

7.12 Susceptibility of *aroD* and *menD* mutants to LL-37

The MBCs of LL-37 against the defined SCVs derived from LS-1, 8325-4, SH1000 or HG003 were measured in buffer III and buffer III + C as described in section 2.6.7, and summarised in table 7.4. As observed previously, all the strains tested showed a similar level of resistance to LL-37 in buffer III, suggesting that none of these genes, *hemB*, *menD* or *aroD*, plays a role in susceptibility to LL-37 in this buffer. However, they demonstrated different levels of susceptibility to LL-37 in buffer III + C. In the presence of $NaHCO_3$, LS-1 Δ*hemB* and LS-1 Δ*menD* showed greatly increased resistance to LL-37 compared to their parental strain, whereas $NaHCO_3$ did not alter the susceptibility of 8325-4 *hemB* and *menD* mutants to LL-37. It is likely that the difference of genetic background between LS-1 and 8325-4 results in the differing phenotypes of *hemB* and *menD* mutants derived from them. These data suggest that differences in the genetic background of 8325-4 and LS-1, such as defects in *rsbU* and *tcaR*, may be responsible for the difference in LL-37 resistance phenotypes of the *hemB* and *menD* mutants.

Interestingly 8325-4 Δ*aroD* had greatly increased resistance to LL-37 in the presence of $NaHCO_3$. In addition, all the other *aroD* mutants derived from LS-1, SH1000 or HG003 also showed $NaHCO_3$-dependent resistance to LL-37. The increased resistance to LL-37 in the presence of $NaHCO_3$ in all of the *aroD* mutants was abolished when an intact *aroD* gene was introduced into these mutants. This confirms that deletion of *aroD* causes increased resistance to LL-37 in the presence of $NaHCO_3$.

Table 7.4: The MBCs of LL-37 against *S. aureus* wild-types, SCVs and complement strains in buffer III and buffer III + C. The median values from three independent experiments are presented. The MBC ranges from the three experiments are included in brackets if the results were not consistent in the three experiments. MBCs beyond the tested concentration range 0-128 μg/ml are presented as >128 μg/ml.

Strains	MBCs of LL-37 (μg/ml)	
	Buffer III	Buffer III + C
LS-1	8	16 (16-32)
LS-1 Δ*hemB*	8	>128
LS-1 Δ*menD*	8	>128
LS-1 Δ*aroD*	8	>128
LS-1 Δ*aroD* pSK236	8	>128
LS-1 Δ*aroD* pPZ137-3	8	16 (8-16)
8325-4	8 (4-8)	16 (8 – 16)
8325-4 Δ*hemB*	8	8 (8 – 16)
8325-4 Δ*menD*	8	8 (8 – 16)
8325-4 Δ*aroD*	8	128 (64 – 128)
8325-4 Δ*aroD* pSK236	8	128 (64 – 128)
8325-4 Δ*aroD* pPZ137-3	8 (4-8)	16 (16 – 32)
SH1000	16 (8-16)	128
SH1000 Δ*aroD*	8	>128
SH1000 Δ*aroD* pSK236	8	>128 (128 - >128)
SH1000 Δ*aroD* pPZ137-3	8	64 (64-128)
HG003	16 (8-16)	32
HG003 Δ*aroD*	8	>128 (128 - >128)
HG003 Δ*aroD* pSK236	8	128 (128 - >128)
HG003 Δ*aroD* pPZ137-3	8	32 (16 – 64)

Unexpectedly, the *rsbU* repaired strain of *S. aureus* strain 8325-4, SH1000, had a high level of resistance to LL-37 in the presence of $NaHCO_3$, as shown in table 7.4. Given that *rsbU* positively regulates *sigB*, it is likely that SigB plays an important role in response to the presence of $NaHCO_3$. However, the strains LS-1 and HG003 also possess functional *rsbU* (Nair *et al.*, 2003; Herbert *et al.*, 2010), but they did not have such a high level of resistance to LL-37 in the presence of $NaHCO_3$. The genetic difference between HG003 and SH1000 is that HG003 contains three prophages, ø11, ø12 and ø13, and a repaired *tcaR*. Therefore, it is likely that the three prophages, ø11, ø12 and ø13, and/or *tcaR* also have an effect on the susceptibility to LL-37. In order to examine the roles SigB, TcaR and the prophages play in resistance to LL-37 in the presence of $NaHCO_3$, the decision was made to first determine if the elevated resistance to LL-37 in SH1000, compared to 8325-4 was due to *sigB*.

7.13 Construction of SH1000 *rsbUVWsigB* mutant

A SH1000 *rsbUVWsigB* mutant was constructed by transduction of the *rsbUVWsigB :: ermB* mutation from *S. aureus* LS-1 *rsbUVWsigB :: ermB* (Nair *et al.*, 2003) using ø85 as described in section 2.3.6. Potential mutants were verified by PCR using primers P226 and P105. P226 was designed to anneal to the downstream of *sigB* and P105 was designed to anneal within *ermB*. These two primers should amplify a PCR product of around 1.5kb if the mutation was successfully transduced, while no product should be produced from the wild-type strain due to the absence of the *ermB* gene. The gel image of the PCR products amplified from genomic DNA from each strain is shown in figure 7.16. As expected, *S. aureus* LS-1 *rsbUVWsigB :: ermB* (lane 2) and the three potential *rsbUVWsigB :: ermB* mutants of SH1000 (lanes 4-6) showed a PCR product at approximately 1.5kb, while the wild-type SH1000 (lane 3) did not generate a PCR product, confirming that the three potential mutants harboured *ermB* genes at the expected site to replace the *rsbUVWsigB* operon.

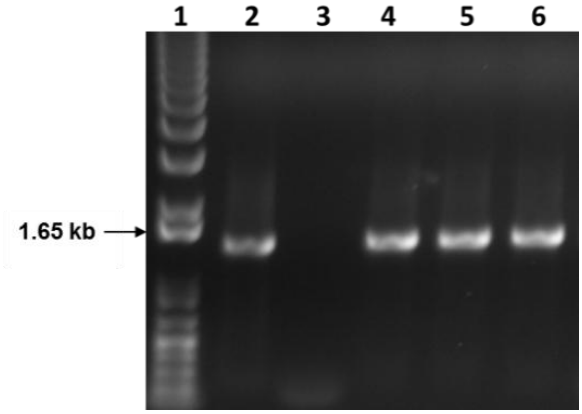

Figure 7.16: Gel electrophoresis image of PCR products amplified from *S. aureus* LS-1 *rsbUVWsigB* :: *ermB* (lane 2), SH1000 (lane 3) and potential SH1000 *rsbUVWsigB* :: *ermB* mutants (lanes 4-6). Lanes 1: 1kb plus DNA ladder (Invitrogen).

7.14 Susceptibility of the SH1000 Δ*rsbUVWsigB* to LL-37

The MBCs of LL-37 against *rsbUVWsigB* mutants of LS-1 and SH1000, and the *rsbU*$^-$ strain 8325-4 in buffer III and buffer III + C were measured as described in section 2.6.7. The MBCs from three independent experiments are summarised in table 7.5. Interestingly, deletion of *rsbUVWsigB* in SH1000 reduced the resistance to LL-37 in buffer III + C to the same level as observed in 8325-4 (8μg/ml). It suggests that SigB contributes to $NaHCO_3$-induced resistance to LL-37 in SH1000. However, *rsbUVWsigB* mutation in strain LS-1 did not change the susceptibility to LL-37 in buffer III + C.

Table 7.5: The MBCs of LL-37 against *S. aureus* wild-types and mutants in buffer III and buffer III + C. The median values from three independent experiments are presented. The MBC ranges from the three experiments are included in brackets if the results were not consistent in the three experiments. MBC beyond the tested concentration range 0-128 μg/ml are presented as >128 μg/ml.

Strains	MBC of LL-37 (μg/ml)	
	Buffer III	Buffer III + C
LS-1	8	16 (16-32)
LS-1 Δ*rsbUVWsigB*	4 (4-8)	8 (4-16)
8325-4 (*rsbU*$^-$)	8 (4-8)	8
SH1000 (*rsbU*$^+$)	8	>128 (128->128)
SH1000 Δ*rsbUVWsigB*	8	8

The MBC data for SH1000 and SH1000 Δ*rsbUVWsigB* suggests that the *sigB* operon is important for $NaHCO_3$-induced resistance to LL-37. However, both LS-1 and HG003 contain functional *sigB* operons, and they do not demonstrate $NaHCO_3$-induced resistance to LL-37 (table 7.4). Therefore factors other than SigB must play a role in the susceptibility of the wild type *S. aureus* strains to LL-37 in the presence of $NaHCO_3$. To examine this further, another two NCTC8325-4-derived strains, HG001 and HG002, were obtained from Professor Gotz (Herbert *et al.*, 2010). Figure 7.17 shows a schematic map of the relationship between NCTC 8325-derived strains. HG003, in comparison with SH1000, has *tcaR* repaired and three extra prophages, any of these differences could be responsible for the reduced resistance to LL-37 observed in HG003 compared to SH1000 in buffer III + C. HG001 and HG002 are directly modified from strain NCTC 8325, with *rsbU* and *tcaR* repaired, respectively. Thus, these two strains were tested to elucidate the roles RsbU, TcaR and and the prophages play in susceptibility to LL-37 in the presence or absence of $NaHCO_3$.

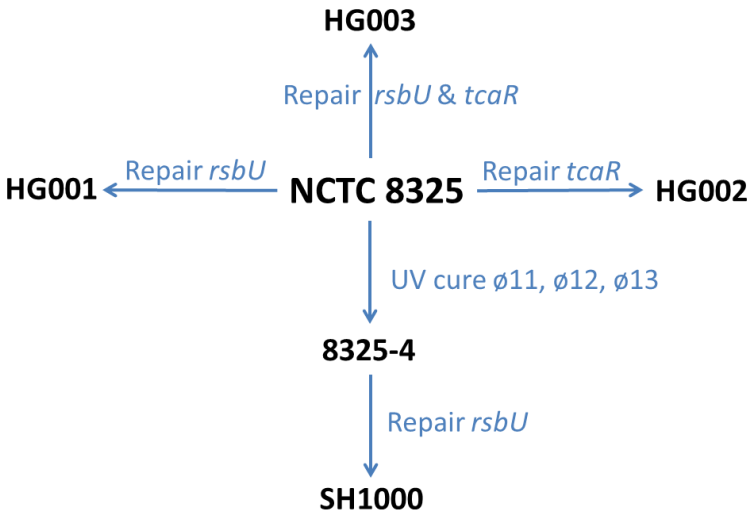

Figure 7.17: Relationship of strains derived from NCTC 8325 (*rsbU*$^-$, *tcaR*$^-$, ø11, ø12, ø13) (Herbert *et al.*, 2010).

7.15 Susceptibility of NCTC 8325-derived strains to LL-37

The MBCs of LL-37 against NCTC 8325-derived strains were determined in buffer III and buffer III + C as described in section 2.6.7. The MBC values from three independent experiments are summarised in table 7.6. HG001 showed a similar level of susceptibility to LL-37 as SH1000, while HG002 was as susceptible to LL-37 as HG003, either in the presence or absence of $NaHCO_3$. Therefore it seems that only

strains with the genotype *rsbU*$^{+}$*tcaR*$^{-}$ exhibit $NaHCO_3$-dependent resistance to LL-37, while strains with genotype *tcaR*$^{+}$ failed to show this phenotype.

Table 7.6: The MBCs of LL-37 against *S. aureus* strains derived from NCTC 8325 in buffer III and buffer III + C. The median values from three independent experiments are presented. The MBC ranges from the three experiments are included in brackets if the results were not consistent in the three experiments. MBC beyond the tested concentration range 0-128 μg/ml are presented as >128 μg/ml.

Strains	MBC of LL-37 (μg/ml)	
	Buffer III	Buffer III + C
8325-4	8 (4-8)	8
SH1000 (*rsbU*$^{+}$)	8	>128 (128->128)
SH1000 Δ*rsbUVWsigB*	8	8
HG001 (*rsbU*$^{+}$, ø11, ø12, ø13)	8	128
HG002 (*tcaR*$^{+}$, ø11, ø12, ø13)	8	16 (16-32)
HG003 (*rsbU*$^{+}$ *tcaR*$^{+}$, ø11, ø12, ø13)	8	16 (16-32)

7.16 The capacity of cytochrome c to bind to SCVs

Cell surface negative charge has been demonstrated to be an important factor that influences *S. aureus* susceptibility to cationic AMPs (Kraus *et al.*, 2008; Matsuo *et al.*, 2011), and as reported in chapter 6, reduced cell surface negative charge may be linked with increased resistance to LL-37 in the *yycHI* and *yycI* mutants. In order to examine whether the increased resistance to LL-37, in the presence of $NaHCO_3$, of the SCVs derived from LS-1 and 8325-4 are due to different capacities to bind to LL-37, cytochrome c was used to assess if there was an alteration in bacterial surface charge. The cytochrome c binding assays were performed in buffer III and buffer III + C as described in section 2.9. The binding of cytochrome c by the wild-types LS-1 or 8325-4 in buffer III was considered as 100%, and the binding by the mutants in buffer III, and all of the strains in buffer III + C was calculated and presented as a percentage relative to this. The relative binding of cytochrome c by LS-1 and its derivative SCVs is plotted in figure 7.18 and that by 8325-4 and its derivatives is plotted in figure 7.19.

When the cells were treated and assayed in buffer III, there was no significant difference of cytochrome c binding between the LS-1 *hemB* mutant and the wild-type

LS-1. However significantly less cytochrome c bound to the LS-1 *menD* and *aroD* mutants compared to the wild-type, demonstrating reduced cell surface charge in these two mutants. In buffer III + C none of the three mutants demonstrated significantly different capacity to bind to cytochrome c compared to the wild type. A comparison of the capacity of each strain to bind to cytochrome c in buffer III with that in buffer III+C shows that LS-1 and the *hemB* mutant have significantly reduced binding capacity in the latter buffer, whilst there was no significant difference in the binding capacity of the *menD* or *aroD* mutant. LS-1 and all three of the SCVs demonstrated the same level of susceptibility to LL-37 in buffer III, but apparently they have very different surface charges, at least as measured by cytochrome c binding, suggesting that this level of change in apparent surface charge does not affect bacterial susceptibility to LL-37 in this buffer. All three SCVs showed increased resistance to LL-37 compared to LS-1 in buffer III + C. However none of them had a significantly reduced capacity to bind to cytochrome c compared to LS-1. These data suggest that the change in net cell surface charge or the capacity to bind to positively charged molecules may not be important for the resistance to LL-37 in these SCVs.

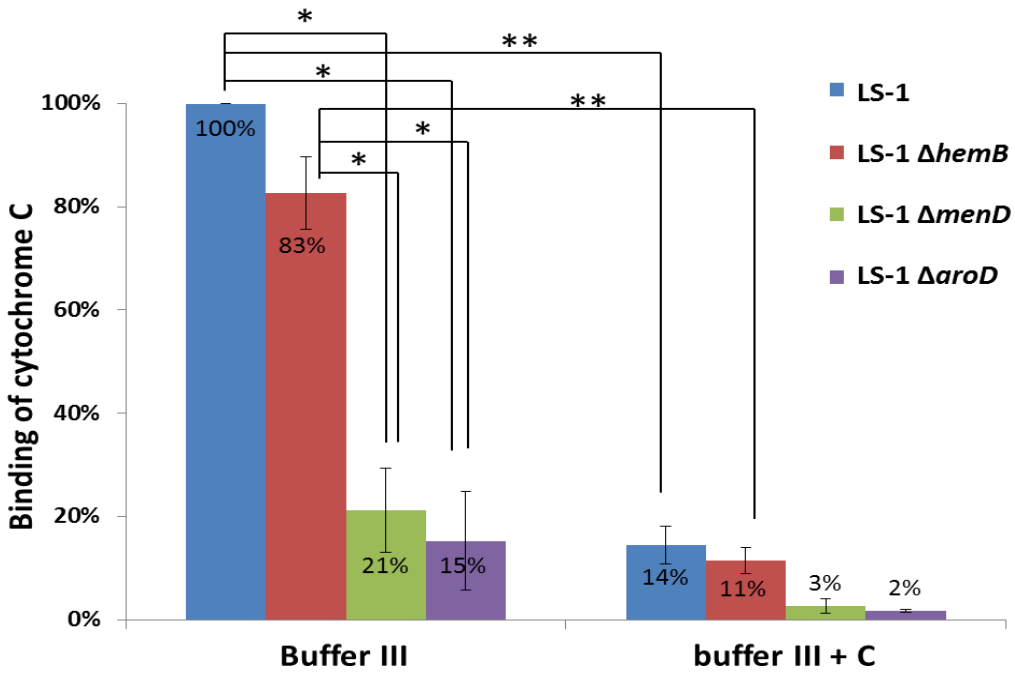

Figure 7.18: The capacity to bind to cytochrome c in LS-1 and LS-1-derived SCVs in buffer III (+/-C). Capacity is plotted as percentages relative to that of LS-1 in buffer III. Data presented are the means of three independent experiments with error bars showing the standard deviations. Statistical significance was analysed using ANOVA, *: $p < 0.05$, **$p < 0.01$.

For the SCVs in the 8325-4 strain background, when the cells were treated and assayed in buffer III, no significant difference of cytochrome c binding between any of the three SCVs and the wild-type, suggesting that the SCVs have similar surface charge to 8325-4. In buffer III + C, only the *aroD* mutant demonstrated significantly reduced capacity to bind to cytochrome c compared to 8325-4. In comparison with cytochrome c binding in buffer III, 8325-4, the *hemB* mutant and the *aroD* mutant, but not the *menD* mutants, showed significantly reduced capacity to bind to cytochrome c in buffer III + C. Since all the strains were as susceptible to LL-37 in buffer III, and only the *aroD* mutants demonstrated increased resistance to LL-37 in the presence of $NaHCO_3$, it suggests that a reduction of even 81% (*menD* mutant in buffer III + C) is not sufficient to increase resistance to LL-37. These findings suggest that either a change in surface charge is not involved in resistance to LL-37 or that there is a threshold value at which it becomes an important factor for resistance, and that this threshold must be below a reduction in net negative surface charge that would result in a reduction in cytochrome c binding by more than 81%.

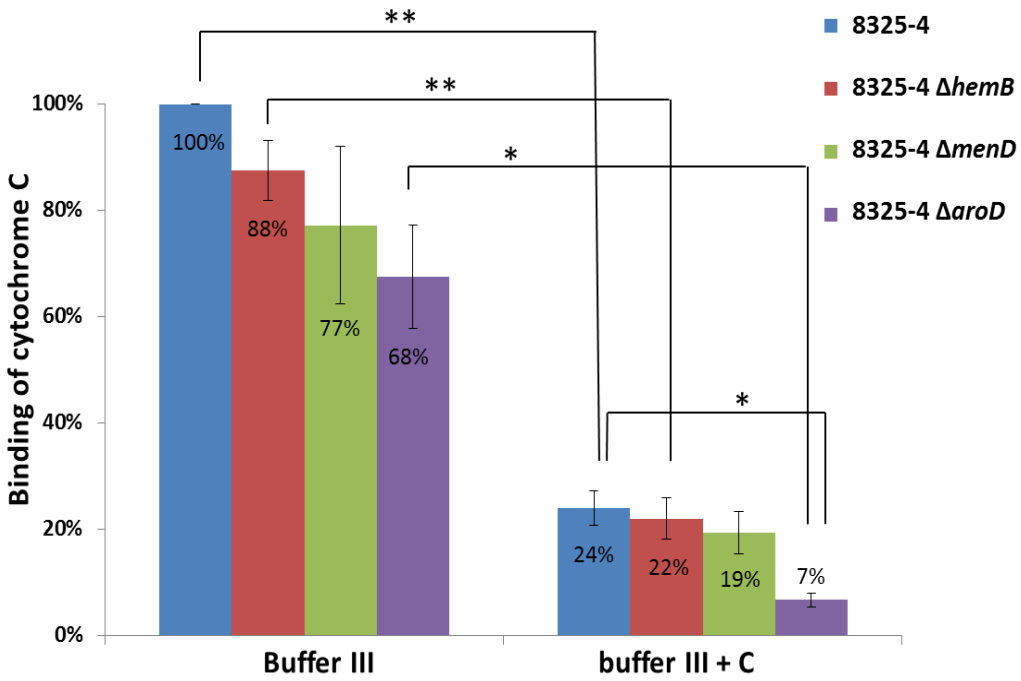

Figure 7.19: The capacity to bind to cytochrome c in 8325-4 and 8325-4-derived SCVs in buffer III (+/-C). Capacity is plotted as percentages relative to that of 8325-4 in buffer III. Data presented are the means of three independent experiments with error bars showing the standard deviations. Statistical significance was analysed using ANOVA. *: $p < 0.05$, **: $p < 0.01$.

7.17 Ionic factors causing $NaHCO_3$-induced resistance to LL-37

The *aroD* mutants originating from LS-1, 8325-4, SH1000 and HG003, and the *hemB* and *menD* mutants derived from LS-1 demonstrated increased resistance to LL-37 compared to their respective parental strains in the presence of $NaHCO_3$. In order to identify the ionic factors, Na^+ or HCO_3^-, that caused $NaHCO_3$-induced resistance to LL-37 in these SCVs, MBCs of LL-37 against LS-1 and LS-1 Δ*aroD* were measured in different assay buffers. Buffer III and this buffer supplemented with either $NaHCO_3$, or NaCl or NaH_2PO_4-Na_2HPO_4 were used for MBC assays. All the buffers were adjusted to pH = 7.4, and the supplemented Na^+ from $NaHCO_3$, NaCl or NaH_2PO_4-Na_2HPO_4 was normalised to 50mM. The MBCs of LL-37 were determined as described in section 2.6.7 and the results from three independent experiments are summarised in table 7.7.

LS-1 and the LS-1 *aroD* mutant had the same susceptibility to LL-37 in buffer III, and supplementation of $NaHCO_3$ into buffer III increased the MBCs of LL-37 by 8-fold for LS-1, and by more than 32-fold for the *aroD* mutant. When supplemented with NaCl, the MBCs of LL-37 were increased 4-fold for LS-1 and 8-fold for the *aroD* mutant. Taking into account the MBC range generated in the three experiments, the susceptibility of LS-1 to LL-37 is similar in buffer III supplemented with $NaHCO_3$ or NaCl, while the *aroD* mutant demonstrated increased resistance to LL-37 in buffer III + $NaHCO_3$ compared to that in buffer III + NaCl. These data suggest that the anion HCO_3^- contributes to the resistance of the *aroD* mutant to LL-37. As presented in chapter 6, both LS-1 and the *yycHI* mutant demonstrated highly increased resistance to LL-37 in the presence of NaH_2PO_4-Na_2HPO_4 (section 6.7), the *aroD* mutant also showed substantially increased resistance to LL-37 in buffer III supplemented with NaH_2PO_4-Na_2HPO_4. This suggests that the phosphate may contribute to *S. aureus* resistance to LL-37.

Table 7.7: The MBCs of LL-37 against LS-1 and LS-1 Δ*aroD* in different assay buffers. The median values from three independent experiments are presented. The MBC ranges from the three experiments are included in brackets if the results were not consistent in the three experiments. MBCs beyond the tested concentration range 0-128 μg/ml are presented as >128 μg/ml.

Assay buffer (pH = 7.4)	**Strain**	**MBC of LL-37 (μg/ml)**
1mM NaH_2PO_4 (Buffer III)	LS-1	4 (4-8)
	LS-1 Δ*aroD*	4 (4-8)
Buffer III + 50mM $NaHCO_3$	LS-1	32 (16-32)
	LS-1 Δ*aroD*	>128
Buffer III + 50mM NaCl	LS-1	16 (16-32)
	LS-1 Δ*aroD*	32 (32-64)
Buffer III + NaH_2PO_4-Na_2HPO_4 (Na^+ supplemented at 50Mm)	LS-1	>128
	LS-1 Δ*aroD*	>128

7.18 Reversion rate of SCV445

The rate of SCV445 reversion to a wild-type growth phenotype was measured using fluctuation tests (Luria and Delbruck, 1943) as described in section 2.13. An overnight culture of SCV445 in TSB + 50 μg/ml kanamycin was plated on TSA to screen for revertants. After 24 hours of incubation, none of the colonies appeared to be distinguishably larger than each other. However, some potential revertants were identified after 48 hours of incubation. There were six colonies from three individual cultures, which formed substantially larger colonies. Streaking these six colonies on TSA confirmed that they formed similar size colonies to the wild-type LS-1 after 24 hours of incubation. An average reversion rate of 6.79×10^{-8} per cell per generation was calculated from the three cultures.

7.19 Genetic basis of reversion

To characterise the genetic basis of reversion, *aroD* from the 6 revertants was amplified and sequenced as described in section 2.12. The sequencing results revealed that four of the revertants had reverted to the wild-type *aroD*, while the

remaining two revertants still retained the mutation resulting in a stop codon at the 150th codon. In an attempt to elucidate the genetic basis of the restored growth rate in these two revertants, it was reasoned that they may carry a compensatory mutation in an appropriate tRNA anticodon. As a result, the altered tRNA may recognise the stop codon UAA and incorporate an amino acid, allowing the continuation of translation of the *aroD* mRNA past this point (Murgola, 1990). There are 7 tRNA molecules with a complementary anticodon that, with a single base change, could recognize a UAA stop codon. These include tRNA-tyrosine (UAU), tRNA-tyrosine (UAC), tRNA-leucine (UUA), tRNA-serine (UCA), tRNA-lysine (AAA), tRNA-glutamine (CAA) and tRNA-glutamic acid (GAA). These tRNA genes were amplified from the two revertants and sequenced as described in section 2.12. Both revertants were found to carry a mutation in tyr-tRNA, which causes the anticodon to change from AUG to AUU, thus recognising stop codon UAA. The mutation was found in different copies of the tyr-tRNA, with locus tags SAOUHSC_T00057 and SAOUHSC_T00058, in the two revertants, which were designated *S. aureus* PZ164 and *S. aureus* PZ165 thereafter.

The mechanism of how these compensatory mutations in tyr-tRNAs suppress the translation termination of *aroD* in *S. aureus* PZ164 and PZ165 is graphically illustrated in figure 7.20 based on the scheme described by Murgola, (1990). In SCV445, none of the tRNAs can recognise the stop codon UAA, therefore translation termination is mediated by release factors when it proceeds to the 150th stop codon in *aroD* (Bertram *et al.*, 2001; Korostelev, 2011). As a consequence, the *aroD* transcript is not fully translated, resulting in the lack of functional AroD in SCV445. However, when a mutation G to U occurs in tyr-tRNA anticodon in PZ164 and PZ165, the anticodon is able to recognise the UAA stop codon, resulting in the incorporation of the amino acid tyrosine and the translation of the rest of *aroD* mRNA generating a full length of AroD protein. The difference between AroD generated in PZ164/PZ165 and that in LS-1 is the substitution of lysine^{150} with tyrosine^{150}. Apparently, this substitution does not seem to influence the function of AroD, or at least not enough to result in an observable effect on *S. aureus* LS-1 growth.

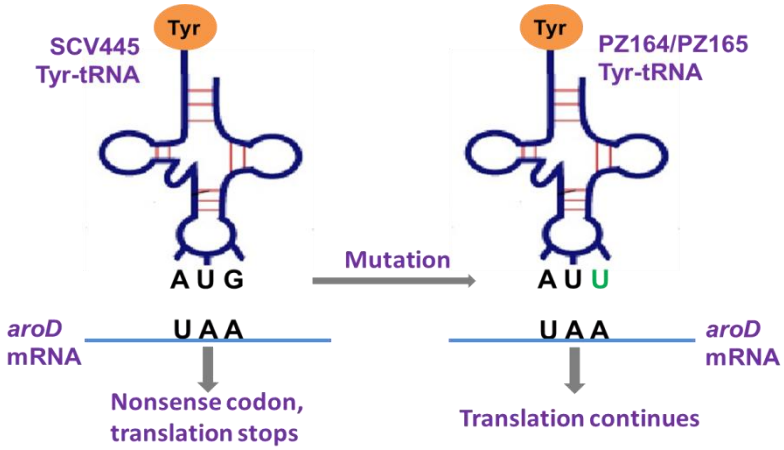

Figure 7.20: Graphic illustration of the mechanism whereby a compensatory mutation in tyr-tRNA restores a functional AroD in SCV445 revertants PZ164/PZ165 (Murgola, 1990).

7.20 Growth dynamics of PZ164 and PZ165

The suppressor tyr-tRNAs in PZ164 and PZ165 should repress the translation termination of all genes with TAA stop codons to some extent. This may disturb protein translation and global gene expression and confer altered phenotypes to the strains. To examine whether this affects the growth rate of the strains, the growth dynamics of these two revertants were characterised alongside LS-1. The viable counts of strains growing in TSB were determined at various time intervals as described in section 2.1.6 and are plotted against time in figure 7.21. The two revertants grew at a similar rate to the wild-type, suggesting that the suppression of TAA-associated translation termination does not influence the growth rate of PZ164 and PZ165.

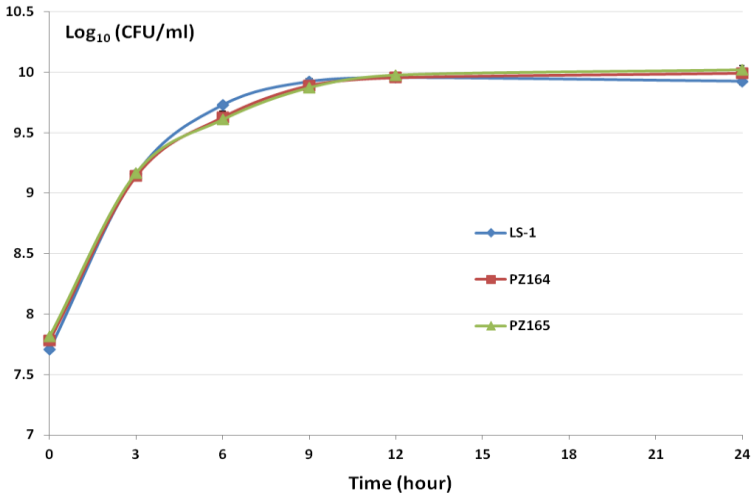

Figure 7.21: Growth curves of strains LS-1, PZ164 and PZ165. Presented data are the means of three independent experiments with error bars showing the standard deviations.

7.21 Susceptibility of PZ164 and PZ165 to LL-37

The susceptibility of PZ164 and PZ165 to LL-37 was measured alongside LS-1 to assess whether the suppression of TAA-associated translation termination affects the susceptibility to LL-37. The MBCs of LL-37 were measured in buffer III and buffer III + C, and are summarised in table 7.8. PZ164 was slightly more susceptible to LL-37 compared to the wild-type LS-1 in both buffer III and buffer III + C, and PZ165 showed the same level of susceptibility to LL-37 as the wild-type. Global suppression of TAA-associated translation termination also does not affect the susceptibility of the strains to antimicrobial peptide LL-37 in the presence of absence of $NaHCO_3$.

Table 7.8: The MBCs of LL-37 against *S. aureus* LS-1, PZ164 and PZ165 in buffer III and buffer III + C. The median values from three independent experiments are presented. The MBC ranges from the three experiments are included in brackets if the results were not consistent in the three experiments.

Strains	MIC of LL-37 (μg/ml)	
	Buffer III	Buffer III + C
LS-1	8	16 (8-16)
PZ164	4	8 (8-16)
PZ165	8	16 (8-16)

7.22 *Galleria mellonella* virulence assay

In order to assess the virulence of the novel *S. aureus* SCV containing an *aroD* mutation, the *aroD* mutants, *aroD* complement strains, and the wild-type strains LS-1 and 8325-4 were used to infect larval-stage *G. mellonella* as described in section 2.10. Each strain was used to infect 10 worms on three different occasions. The percentage survival of the 30 worms, 10 from each of three independent experiments, during the five-day incubations was presented in figure 7.22. In comparison to their parental strains, LS-1 Δ*aroD*, 8325-4 Δ*aroD* and SCV445 demonstrated a highly significant reduction in their virulence ($P < 0.001$). The virulence of *aroD* mutants containing an intact *aroD* gene introduced using plasmid pPZ137-3 (pSK236 :: *aroD*), was similar to the wild type strains. The virulence of the two revertants PZ164 and PZ165 was partially restored but was still significantly

attenuated compared to the wild-type LS-1 ($P < 0.001$), although it was significantly increased compared to SCV445 ($P < 0.001$). There was no significant difference between the two revertants ($P = 0.479$). These data demonstrated that mutation in *aroD* results in significantly attenuated *S. aureus* virulence in *G. mellonella* model, and suppression of TAA-associated translation termination (PZ164 and PZ165) partially restores the virulence of *S. aureus* in this model.

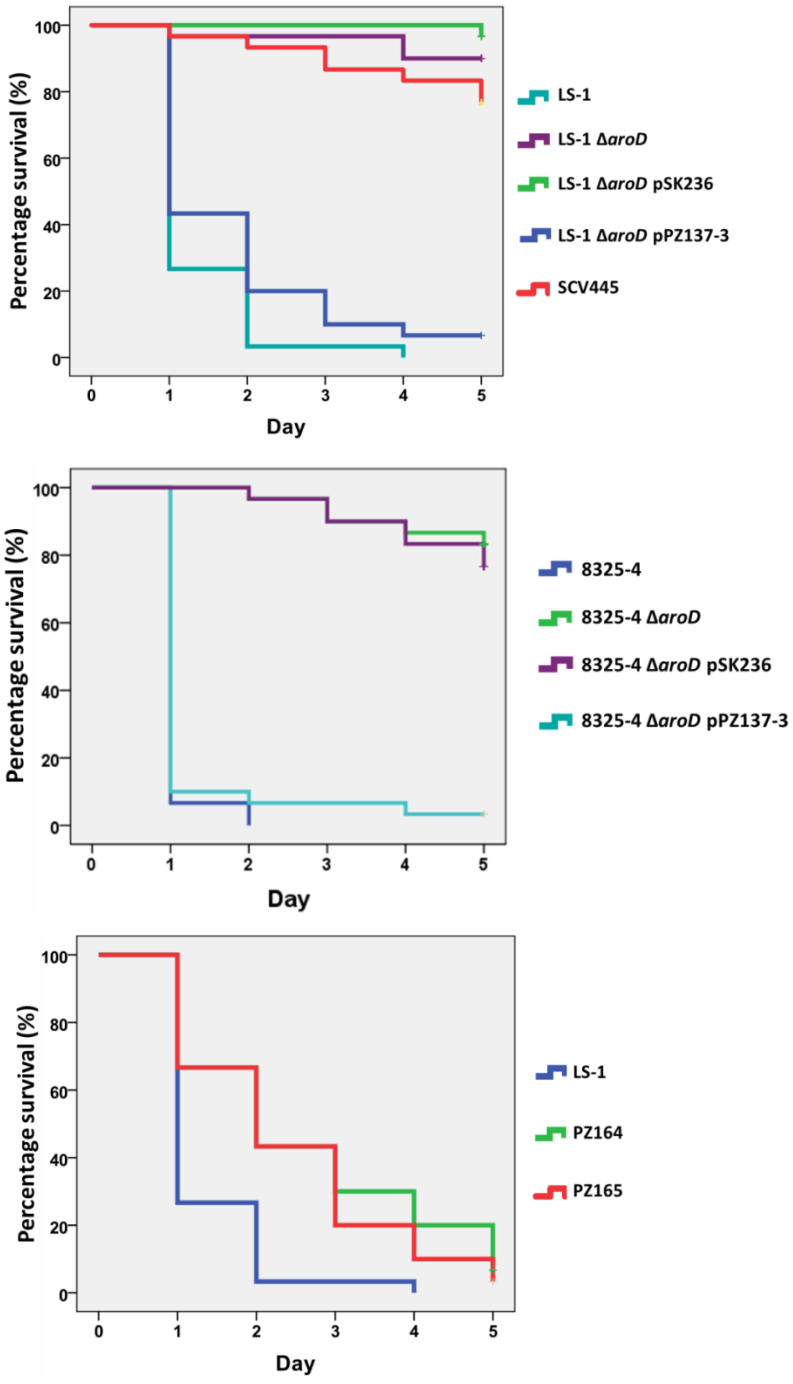

Figure 7.22: Percentage survival of *G. mellonella* infected with *S. aureus* strains during a 5-day incubation period. Data presented are the percentages of 30 worms which were assayed in groups of 10 on three different days.

7.23 Discussion

S. aureus small colony variants are associated with persistent and reoccurring infections (von Eiff *et al.*, 2006b). They are frequently isolated from patients, but the genetic basis causing SCV phenotypes has rarely been characterised and still not completely understood (Kahl *et al.*, 2003; Sadowska *et al.*, 2002). Over recent years, studies on small colony variants of *S. aureus* have focused on hemin or menadione auxotrophs, mainly using defined *hemB* and *menD* mutants constructed in the laboratory (Bates *et al.*, 2003; von Eiff *et al.*, 2004, 2006a; Sifri *et al.*, 2006). However, a number of other mutations have been found in clinical SCVs. For example in *menB*, another gene involved in menadione synthesis, were identified as being responsible for three clinical isolated *S. aureus* menadione auxotrophs (Lannergard *et al.*, 2008). In addition, a novel genetic basis for the SCV phenotype was found to be a mutation in *relA*, which encodes guanosine polyphosphate pyrophosphohydrolase/synthetase (ppGpp synthetase), which is involved in the stringent response in *S. aureus* (Gao *et al.*, 2010). In the study reported in this chapter, a spontaneous *S. aureus* menadione auxotroph, which carries a stop codon mutation in the *aroD* gene, was isolated *in vitro* by exposing LS-1 to kanamycin. The study reported herein and those published by others demonstrate that there is diversity in the genetic basis that can confer the SCV phenotype. Therefore a wider range of SCVs than the *hemB* and *menD* defined mutants need to be studied in order to better understand how SCVs cause persistent infections and to identify new treatment methods.

Natural SCVs are not very often studied, as they are thought to be unstable and can easily revert to fast growing cells, which would rapidly reproduce in cultures (Becker *et al.*, 2006). In this study, a reversion rate of 6.79×10^{-8} per cell per generation was determined for a spontaneous small colony variant SCV445. This rate is close to the reversion rate of 1.8×10^{-8} per cell per generation for a clinically isolated *S. aureus* menadione auxotrophic SCV (Lannergard *et al.*, 2008). It is also similar to the natural mutation rate in many bacteria (Martinez and Baquero, 2000), suggesting that reversion was due to spontaneous mutation under standard laboratory condition, rather than the stability of the mutation in spontaneous SCVs.

S. aureus SCVs auxotrophic for hemin or menadione are defective in the electron transport chain, which results in decreased membrane potential (Proctor *et al.*, 2006). To investigate whether this phenotype confers increased resistance to cationic AMPs, The susceptibility to LL-37 of a range of *S. aureus* hemin or menadione auxotrophs was determined. These SCVs, which have mutations in *hemB*, *menD* or *aroD* and were found to be as susceptible to LL-37 as their parental strains in buffer III, suggesting that reduced membrane potential in these SCVs does not contribute to altered resistance to LL-37. In agreement with this finding there are a few studies showing that resistance to AMPs is not due to reduced membrane potential in *S. aureus* SCVs. A menadione auxotroph strain JB-1 is more resistant to tPMP compared to the wild-type strain 6850, and the membrane potential of JB-1 is 67.8% of that in 6850. However, normalisation of the membrane potential in JB-1 by supplementation with menadione did not restore the susceptibility to tPMP (Koo *et al.*, 1996). In addition, Samuelsen *et al.* (2005) have found that *S. aureus* hemin and menadione auxotrophs are more resistant to lactoferricin B than the wild-types, but characterising the uptake of this AMP revealed that the *hemB* mutant actually takes up more lactoferricin than the wild-type (Samuelsen *et al.*, 2005). These findings suggest that susceptibility to AMPs in *S. aureus* SCVs does not only involve membrane potential or the uptake of AMPs. For a better understanding of how SCV phenotypes might confer increased resistance to AMPs, a more comprehensive examination is necessary, such as characterisation of cell surface negative charge (Kraus *et al.*, 2008), membrane permeability (Dorschner *et al.*, 2006) and global gene expression that may influence the expression of AMP-sensing and regulation systems (Gebhard and Mascher, 2011).

Interestingly, in comparison to LS-1, increased resistance to LL-37 was observed in *hemB*, *menD* and *aroD* mutants of LS-1 in the presence of $NaHCO_3$. Mutation of *aroD* in 8325-4, SH1000 and HG003 also resulted in increased resistance to LL-37 in the presence of $NaHCO_3$. The fact that these SCVs only demonstrated $NaHCO_3$-dependent resistance to LL-37 indicates that ionic conditions in the mammalian body may play an important role in mediating SCV resistance to human AMPs. In an attempt to determine the ionic factor contributing to the $NaHCO_3$-induced resistance

to LL-37 in these SCVs, the susceptibility of *S. aureus* LS-1 Δ*aroD* to LL-37 was determined in a range of buffers and found to specifically respond to HCO_3^-. The mechanism of $NaHCO_3$-induced resistance in these SCVs is still not known. Given that the *hemB* and *menD* mutants derived from 8325-4 did not exhibit altered susceptibility to LL-37 in the presence of bicarbonate, it is likely that the difference between the genetic backgrounds of strain LS-1 and 8325-4, i.e. *rsbU*, *tcaR* and / or *tyrA*, is somehow involved in the bicarbonate-induced resistance to LL-37 in these SCVs. In addition, the *aroD* mutant derived from 8325-4 is more resistant to LL-37 in buffer III + C than the 8325-4 *hemB* and *menD* mutants, which indicates that different mutations also confers different levels of susceptibility to LL-37 despite the fact that they are all SCVs and share a range of physiological characteristics. *S. aureus* SCVs with mutations in *hemB* or *menD* have also been reported to differ in a number of ways. For example, *S. aureus* 8325-4 Δ*menD* is less susceptible to tPMP-1 compared to 8325-4 Δ*hemB*, and is also significantly more capable of surviving in the kidneys and spleen of rabbits than the *hemB* mutant when subjected to oxacillin therapy (Bates *et al.*, 2003). Additionally, phenotype microarray analysis of *hemB* and *menD* mutants revealed that defects in metabolism of carbones, such as mannitol, maltotriose and dextrin, were more severe in the *menD* mutant than in the *hemB* mutant (von Eiff *et al.*, 2006a).

In an attempt to ascertain whether the increased resistance to LL-37 in the SCVs was associated with reduced cell surface charge, the capacity to bind to cytochrome c was characterised for the wild-type strains and the derived SCVs in the presence or absence of $NaHCO_3$. No coherent relationship between cell surface net charge and level of resistance to LL-37 could be drawn for LS-1 and LS-1-derived SCVs (figure 7.18), suggesting that cell surface net charge is not involved in resistance to LL-37 in these SCVs. In addition, for 8325-4-derived strains, increased resistance to LL-37 was only observed with the *aroD* mutant in buffer III + C, which has only 7% of wild-type capacity to bind to cytochrome c in buffer III (figure 7.19). While the wild-type, *hemB* and *menD* mutants in buffer III + C, although their capacity of binding to cytochrome c was reduced to 24%, 22% and 19%, respectively, of the wild-type in buffer III, the resistance to LL-37 was not enhanced. As discussed in chapter 6, either a reduction

threshold of more than 81% of cell surface net negative charge is required for conferring resistance to LL-37, or the reduction of cell surface charge may not be important in resistance to LL-37 in the SCVs.

Strain SH1000 which is derived from 8325-4, with *rsbU* repaired also demonstrated a $NaHCO_3$-induced resistance to LL-37, and deletion of the *sigB* operon in SH1000 abolished the $NaHCO_3$-dependent resistance to LL-37. This suggests that *sigB*, positively regulated by *rsbU*, is important in resistance to LL-37 in the presence of $NaHCO_3$. Strain HG001, derived from NCTC 8325 with *rsbU* repaired, also showed similar $NaHCO_3$-induced resistance to LL-37 as observed in SH1000, confirming the important role that *sigB* plays in $NaHCO_3$-associated resistance to LL-37. However, it was also found that restoring both *rsbU* and *tcaR* in NCTC 8325 abolished the $NaHCO_3$-induced resistance to LL-37. In addition, repairing only *tcaR* in NCTC 8325 also failed to result in $NaHCO_3$-induced resistance to LL-37. Since *rsbU* positively regulates the global regulator SigB, it is possible that *tcaR* may also have an effect on SigB or SigB-dependent genes involved in resistance to LL-37 in the presence of $NaHCO_3$. In addition to being an activator of *spa*, TcaR is also an activator of the global regulator *sarS* (a homologue of the staphylococcal accessory regulator SarA), and attenuates full-length transcription of sasF, resulting in a truncated transcript lacking the 3' terminus (McCallum *et al.*, 2004). The gene *sasF* encodes a cell surface associated protein, which is important for the resistance to linoleic acid (Kenny *et al.*, 2009). We have tested the susceptibility to LL-37 of a SH1000 *sasF* mutant, obtained from Dr. Malcolm Horsburgh (Kenny *et al.*, 2009), and no evident change in susceptibility to LL-37 was found in the presence or absence of $NaHCO_3$ when compared to the wild-type. Given that *sigB* has been suggested to be a positive regulator of *sarS* (Tegmark *et al.*, 2000; Bischoff *et al.*, 2004), it is unlikely that *sarS* is directly involved in $NaHCO_3$-dependent resistance, as both *tcaR* and *sigB* up-regulate *sarS*, and increased resistance to LL-37 in the presence of $NaHCO_3$ was only seen in *S. aureus* strains that had a $TcaR^-$ $RsbU^+$ background.

In this study, we have demonstrated that *aroD* mutants of *S. aureus*, including the naturally formed mutant SCV445, are significantly attenuated in a *G. mellonella*

infection model. A spontanous *S. aureus* SCV formed under triclosan treatment was also recently reported to have reduced virulence in *G. mellonella* model (Latimer *et al*., 2012). The enzyme encoded by *aroD*, 3-dehydroquinate dehydratase, is part of the shikimate pathway and is responsible for the synthesis of chorismate, a branching point for the biosynthesis of menaquinone, aromatic amino acids, and several other metabolites (figure 7.4). The shikimate pathway in a number of bacterial pathogens has been studied and many *aro* mutants with mutations affecting shikimate pathway have been shown to be attenuated in virulence models. The most extensive characterisation of *aro* mutants has been in *Salmonella* where their potential to be live vaccines has been evaluated (Hoiseth and Stocker, 1981). The *aro* mutants of *S.* Typhimurium have been shown to be auxotrophic for para-aminobenzoic acid (PABA) (for folate biosynthesis) and dihydroxybenzoate (DHB) (for the synthesis of enterochelin, an iron-acquisition compound). These two compounds are not sufficiently available in mammalian tissues to maintain the growth of *aro* mutants, conferring greatly reduced virulence to these mutants in mice and cattle (Hoiseth and Stocker, 1981; Smith *et al.*, 1984). *Salmonella enterica* serovar Typhi mutants with deletions of *aroD* and *htrA* (encodes a heat shock protein) have been proven to be promising vaccines against human typhoid, by stimulating the production of anti-lipopolysaccharide (LPS) immunoglobulin A (IgA), lymphocyte proliferation, gamma interferon production, and the elimination of the vaccine organism from the blood (Tacket *et al.*, 1997, 2000). The potential of using *S. aureus aro* mutants as vaccine against *S. aureus* infections has also been examined. A *S. aureus* mutant carrying a Tn*917*-insertion in *aroA* demonstrated attenuated virulence and a decreased ability to persist in the lungs, spleens and mammary glands of mice (Buzzola *et al.*, 2006). This research group has also recently shown that intranasal administration of the attenuated *S. aureus aroA* mutant was able to reduce nasal colonization by more virulent strains, and largely reduced the inflammatory response to *S. aureus* (Barbagelata *et al.*, 2011).

In spite of a large amount of data showing attenuation of virulence in *aro* mutants, the potential of using these mutants as live vaccines is still questionable. As described in section 7.1, SCVs are associated with persistent and recurrent infections

probably due to their enhanced persistence in non-phagocytic host cells such as epithelial cells and endothelial cells (Schroder *et al.*, 2006a; Atalla *et al.*, 2010; vonEiff *et al.*, 1997b). An increased ability to persist in non-phagocytic cells has also been found in some *aro* mutants. For example, a spontaneous *aroD* mutant of *S.* Typhimurium SL1344 was isolated from NRK-49F fibroblasts infected with this strain. Although this mutant was attenuated in the BALB/c murine typhoid model, it was also found to persist in intracellular fibroblasts 3 to 4 logs higher than its parental strain (Cano *et al.*, 2003). In addition, the data generated from this study showed that *S. aureus aroD* mutants are highly more resistant to the human antimicrobial peptide LL-37 in the presence of a physiological concentration of bicarbonate than their parental strains. This finding indicates that *S. aureus aro* mutants may be better able to survive from killing by AMPs in the human body, which may contribute to their persistence or proliferation in the host. Therefore, whether it is safe to use *aro* mutants as vaccines still requires further investigation.

Chapter 8: Discussion and Future Work

8.1 The significance of this study

8.1.1 Tools useful for the study of *S. aureus*

The understanding of the molecular mechanisms by which *S. aureus* causes pathogenesis largely rely on the availability of genetic tools which can be used to identify genes important for survival, growth and virulence. Such genes represent potential targets for developing drugs or vaccines against *S. aureus* infections. This study describes the construction and characterisation of two valuable tools, a transposon mutagenesis system and a R-IVET system. Both systems allow genome-wide screening of *S. aureus* to identify important genes, and may also be readily applied to other Gram-positive bacteria.

Transposon mutagenesis has previously been shown to be a useful approach for the identification of *S. aureus* genes that play roles in different aspects of the bacterium's biology, such as virulence in the nematode, *Caenorhabditis elegans*, infection model (Bae *et al.*, 2004; Begun *et al.*, 2005), resistance to the human AMP dermcidin (Li *et al.*, 2009), and polysaccharide-independent biofilm formation (Boles *et al.*, 2010). By modifying a *Himar1* mariner transposon delivery system designed for the use in *L. monocytogenes* (Cao *et al*., 2007), we have developed a transposon mutagenesis system based on the newly constructed transposon delivery vector pPZ4, which has been demonstrated to be efficient in construction of genome-wide mutant libraries in *S. aureus*. The use of an inducible promoter *Pxyl/tetO* for the control of transposase expression in pPZ4 enables the generation of a mutant library in which only 5% of the mutants have multiple insertions of the transposon. A mutant library with a low proportion of mutants with multiple insertion of the transposon allows efficient screening for individual genes that are involved in a function. This development reduces the time, workload and potential costs involved in screening. The mutant library generated using pPZ4 could literally be used for screening to identify *S. aureus* genes that are important under any condition, *in vitro* or *in vivo*.

The other useful tool that we have constructed is the *cre-loxP* R-IVET system described in chapter 4 for the screening of conditionally induced genes. This system has been preliminarily characterised, the *loxP* cassette was confirmed to be stable in the *S. aureus* LS-1 chromosome in the presence or absence of a promoter-less *cre*. Resolution of *cre-loxP* occurred at a high frequency when a *S. aureus* promoter *saeP1* was cloned upstream of *cre*, indicating the high sensitivity of the system. In comparison with older R-IVET systems, such as those employing a *res-kan-res* resolution cassette (Lowe *et al.*, 1998), our system has an advantage of simplicity. By using two reporter genes, *ermB* and *lacZ*, in the *loxP* cassette that integrated in the chromosome, this system allows the identification of cells that have lost the *loxP* cassette by direct visualisation of the colour of the colonies, rather than by replica plating on plates with and without antibiotics. This system can be widely used for the screening of genes that are activated under various conditions, such as genes that specifically respond to environment conditions (e.g. pH, temperature, O_2 and CO_2), stresses (e.g. oxidant, salts, nutrient starvation, toxic chemicals), antimicrobial agents, or specific mammalian cells (e.g. neutrophils, macrophages, epithelial cells). Furthermore, there is an increasingly recognised difference between virulence gene expression observed in animal models and that detected *in vitro*, and there is a recognition that more attention should be focused on the study of bacterial pathogenesis *in vivo* (Smith, 1998; Pragman and Schlievert, 2004). This system is also useful for the identification of *in vivo*-induced genes in animal models, which may be important virulence genes that mediate infection.

8.1.2 The importance of YycFG in *S. aureus*

The two component system YycFG is essential in low-GC content Gram-positive bacteria, which has rendered this TCS an attractive target for novel antimicrobial compound design (Ng and Winkler, 2004). The primary function of YycFG has been suggested to be in regulating cell wall metabolism and cell division in *S. aureus* (Dubrac *et al.*, 2007; Delaune *et al.*, 2011). This TCS has also been demonstrated to be involved in resistance to a range of antibiotics in *S. aureus*, including MLS_B antibiotics (Martin *et al.*, 1999), daptomycin (Friedman *et al.*, 2006; Howden *et al.*, 2011) and vancomycin (Jansen *et al.*, 2007; Howden *et al.*, 2011). A recent

publication also reported the important role *yycFG* plays in *S. aureus* virulence (Delaune *et al.*, 2012). It positively regulates virulence genes or virulence regulators such as *fnbA*, *fnbB*, *hla*, *hlb* and SaeSR. When the regulator YycF is artificially modified to be constitutively active it confers on *S. aureus* increased ability to form biofilm, triggers a greater inflammatory response in mice, and results in reduced virulence in a murine sepsis model (Delaune *et al.*, 2012).

In this study, we have identified two transposon mutants LS-1 Δ*yycHI* and LS-1 Δ*yycI* that demonstrated increased resistance to LL-37 in the presence of bicarbonate. Since both *yycH* and *yycI* encode suppressors of YycFG, our data suggests that this TCS is also involved in resistance to the human cationic AMP LL-37. However, the increased resistance to LL-37 in these two mutants was only observed in the presence of a physiological concentration of bicarbonate. Given that bicarbonate is an important substance abundantly present in all human body fluids and organs (Cordat and Casey, 2009), this data may also suggest a role for YycFG in adapting to the host environment enabling the bacterium to survive killing by host immune effectors such as AMPs. However, we have also found that the *yycHI* mutant of *S. aureus* LS-1 is significantly attenuated in the *G. mellonella* virulence model. Since YycH and YycI inhibit YycFG, this finding is in accordance with that reported by Delaune *et al*. (2012), that constitutively active YycF resulted in attenuated virulence of *S. aureus* in a murine sepsis infection model. It is apparent that the role YycFG plays in resistance to AMPs *in vivo* requires further examination under conditions that more closely resemble the host environment. Nevertheless, the data generated from this study has confirmed the importance of YycFG in *S. aureus* virulence, and also for the first time identified that YycFG may be important for resistance to human AMPs in the presence of the human body fluid component, bicarbonate, and has thus contributed to our understanding of this important TCS in *S. aureus*.

8.1.3 The significance of the SCV phenotype in resistance to AMPs

S. aureus SCVs have been recognised as a subpopulation of *S. aureus* that is able to survive and persistent in a host (von Eiff *et al.*, 2006b). One of their important features is an increased resistance to antibiotics, particularly aminoglycosides

(Massey *et al.*, 2001). The increased resistance to aminoglycosides is a result of reduced membrane potential in the SCVs which are defective in the electron transport chain (von Eiff *et al.*, 2006b). We have examined whether the reduced membrane potential in SCVs also leads to reduced susceptibility to cationic AMPs. The susceptibility to the human cationic AMP LL-37 was determined in *S. aureus* hemin or menadione auxotrophs with mutation in *hemB*, *menD* or *aroD*. None of these mutants was found to be more resistant to LL-37 than the wild-type in a phosphate buffer (buffer III), suggesting that reduced membrane potential does not confer resistance to LL-37 in these SCVs. However, increased resistance to LL-37, compared to the wild-type, was observed in these SCVs in the presence of $NaHCO_3$. The fact that these SCVs exhibited $NaHCO_3$-dependent resistance to LL-37 indicates that the ionic conditions in the mammalian body may contribute to the survival of SCVs in the human body at least with respect to killing by AMPs. These data also indicate that it is important to investigate the susceptibility of SCVs to AMPs under conditions representing the host environment, which may contribute to our understanding of the mechanisms by which SCVs are more capable of persistence in the host than the parental strains that they arose from.

8.1.4 The development of AMPs as potential antibiotics

The increasing resistance of bacteria to conventional antibiotics is a growing challenge in the clinic (Rossolini and Mantengoli, 2008), and infections due to resistant bacteria have a great impact on the whole of society (Gould *et al.*, 2010). In attempts to search for novel antibiotics, more and more attention is focusing on antimicrobial agents that have a low potential for bacteria to develop resistance to. This has stimulated interest in AMPs as promising drugs against pathogenic species, because AMPs possess potent antimicrobial activity and typically employ a receptor-independent mode of action by disruption of bacterial cell membranes, which is thought to have a low possibility of inducing bacterial resistance (Hancock and Sahl, 2006; Giuliani *et al.*, 2007; Afacan *et al.*, 2012).

This study describes the susceptibility of *S. aureus* to LL-37, and the data generated may be important for the design of effective AMP drugs against *S. aureus*. First, the human body component, bicarbonate, was found to generally increase *S. aureus* resistance to LL-37, slightly in the wild-types and substantially in a number of mutants. This information suggests the importance of screening or analysing antimicrobial compounds, either AMPs or other types of compounds, in an environment corresponding to the mammalian host. In my study, a range of SCVs, including a spontaneous SCV, were found to have considerably higher levels of resistance to LL-37 in the presence of bicarbonate compared to the wildtype strains. This poses a potential problem for the development of AMP antibiotics, as selection of such mutants, or down-regulation of the corresponding genes that are mutated in the SCVs, could be a way for *S. aureus* to overcome AMP killing in bicarbonate-containing host environments. The SCV subpopulation is therefore worthy of consideration, when designing effective AMP drugs against all forms of *S. aureus*.

8.2 Future work

8.2.1 Further identification of genes involved in resistance to LL-37

In order to comprehensively identify genes responsible for resistance to LL-37, mutants with reduced resistance to LL-37 can be screened using the transposon mutant library of *S. aureus* LS-1. The genes disrupted in mutants with reduced resistance to AMPs represent potential drug targets. For example, inhibitors of these genes could help the host response be more effective by making the bacteria more sensitive to AMPs or the inhibitors could be used to enhance the sensitivity of bacteria to exogenous AMPs to achieve effective antimicrobial activity against *S. aureus*.

A potential high throughput strategy for screening mutants with reduced resistance is to use the Illumina sequencing technology, which allows the sequencing of a large number of DNA fragments simultaneously through synthesis of DNA fragments using fluorescently labelled nucleotides (Hernandez *et al.*, 2008; Holt *et al.*, 2008). The *S. aureus* LS-1 transposon mutant libraries could be incubated with or without LL-37, and genomic DNA of the mutants recovered from the samples with or without LL-37

treatment can be prepared and processed for Illumina sequencing. In brief, the genomic DNA is randomly sheared to an average size of approximately 300bp, followed by ligation with designed short oligonucleotide linkers. This pool of DNA is then PCR amplified using a transposon specific forward primer and a custom Illumina reverse primer that is specific to the short oligonucleotide linker (Langridge *et al.*, 2009). The resulting DNA is cleaned and subjected to Illumina sequencing using the transposon specific primer and the Illumina primer. The sequence data generated from each sample can be analysed to identify the mutants that are absent from the sample with LL-37 treatment compared to that without LL-37.

The activity of AMPs has been found to be repressed by mammalian serum salts, such as NaCl, KCl, $ZnCl_2$, $MgCl_2$ and $CaCl_2$ (Maisetta *et al.*, 2008). Potential future work may also involve identification of *S. aureus* genes that are specifically responsible for the enhanced resistance to LL-37 in the presence of mammalian serum salts. These genes could serve as promising drug targets.

As described in chapter 7, in comparison to the wild-type LS-1, increased resistance to LL-37 was observed for *S. aureus* LS-1 *hemB*, *menD*, and *aroD* mutants in the presence of $NaHCO_3$. However, mutants defective in these genes were not detected when screening the genome-wide mutant library of LS-1 for increased resistance in the presence of $NaHCO_3$ (chapter 5). A possible reason is that these SCVs are present in the mutant library in relatively low copies, due to their slow growth rate. Given that three SCVs derived from LS-1, with disruptions in *hemB*, *menD*, and *aroD*, were found to be more resistant to LL-37 than the wild-type in the presence of $NaHCO_3$ (described in chapter 7), it suggests that SCVs with other mutations may be important organisms to examine for resistance to AMPs. Therefore, it would be worth growing the transposon mutant library on agar and isolating a range of SCVs. The susceptibility of these SCVs to LL-37 can then be characterised to gain a comprehensive picture of SCVs resistance to LL-37.

8.2.2 Screening for genes involved in sensing and resistance to LL-37

As described in chapter 1, two AMP-sensing and regulatory systems have been described in *S. aureus*, the GraXRS-VraFG system which is responsible for the resistance to a range of cationic AMPs, such as tPMPs, HNP-1 and polymyxin B (Yang *et al.*, 2012), and the BraRS-BraDE-VraDE system that mediates resistance to bacitracin and nisin (Hiron *et al.*, 2011). These systems are able to sense the presence of AMPs and regulate resistance to the AMPs by up-regulating resistance effectors. For a full understanding of the molecular mechanism that *S. aureus* uses to sense and regulate resistance to LL-37, screening of genes that are induced in the presence of LL-37 on a genome-wide scale could be worthwhile. These genes may be involved in sensing the presence of LL-37, and / or may be important genes that are responsible for resistance.

The R-IVET system we have constructed is a promising tool that could be used to achieve this goal. *S. aureus* LS-1 genomic DNA can be digested by restriction endonucleases that generate blunt-end DNA fragments with an average size of approximately 500bp. To maximise the coverage of *S. aureus* promoters, genomic DNA can be separately digested using several different endonucleases and pooled. The fragments from all the digests can then be fractionated by agarose gel electrophoresis. Fragments with sizes of 0.4-2kb can be extracted and ligated into pNZ5520 upstream of *cre*. The ligation mix can then be transformed into *L. lactis* to allow propagation. The plasmids harvested from *L. lactis* would be introduced into strain RN4220 and then strain PZ134-2. Given that a plasmid library containing genome-wide promoters is important for comprehensive screening of promoters in the whole genome of *S. aureus*, the transformation efficiency of each step is obviously particularly important. It may be necessary to optimise the transformation efficiency in both *L. lactis* and *S. aureus* prior to this step. The resulting transformants should carry a library of pNZ5520-derived plasmids containing promoter-*cre* fusions. This pool of transformants can then be cultured with or without LL-37 at a sub-inhibitory concentration, and the cells will be plated onto TSA + X-gal. The white colonies grown on the agar from each sample can be isolated and further characterised to identify the promoters that were activated in the presence of LL-37.

8.2.3 Investigation of the mechanisms by which YycFG regulates resistance to LL-37

Future work could also include identification of the mechanism by which the *yycHI* mutant has increased resistance to LL-37 in the presence of $NaHCO_3$. As described in chapter 6, it is not known whether the decrease in cell surface negative charge observed was involved in resistance to LL-37. However, the *yycHI* mutant was found to be less capable of binding to cytochrome c in the presence or absence of $NaHCO_3$ compared to the wild-type, demonstrating that the *yycHI* mutant has reduced surface negative charge. Given that YycFG and PhoPR TCSs have been found to act together to repress the teichoic acid biosynthetic genes *tagAB/DEF* in *B. subtilis* in response to phosphate limitation (Howell *et al.*, 2006), and YycHI represses YycFG, it is possible that teichoic acid biosynthesis is inhibited in the *yycHI* mutant. Therefore, the reduced cell surface negative charge in the *yycHI* mutant may be a consequence of reduced teichoic acid production. It would therefore be interesting to measure the expression of teichoic acid biosynthetic genes by quantitative PCR (qPCR) (Riedy *et al.*, 1995) and to assess the teichoic acid content of the wild-type and the *yycHI* mutant in the presence or absence of bicarbonate.

Another property of the wild-type and the *yycHI* mutant that would be interesting to examine would be membrane permeability. As also discussed in chapter 6, YycFG has been reported to regulate fatty acid biosynthesis pathways in *Streptococcus pneumoniae*, thereby effecting the cell membrane composition (Mohedano *et al.*, 2005). Over-expression of YycF was shown to result in an increased C_{18} to C_{16} fatty acid ratio, which in turn leads to reduced membrane permeability (Mohedano *et al.*, 2005; Zhang and Rock, 2008). This finding suggests that the *S. aureus yycHI* mutant may have reduced membrane permeability, due to increased YycF activity. However, the *yycHI* mutation only caused increased resistance to LL-37 in the presence of bicarbonate, and interestingly, bicarbonate was suggested to increase cell membrane permeability in *S. aureus*, thus increasing their susceptibility to LL-37 (Dorschner *et al.*, 2006). This contradiction between our data and those generated by Dorschner *et al*. (2006) suggests that the effect of bicarbonate on membrane permeability in both the wild-type and the *yycHI* mutant warrants further investigation.

8.2.4 Investigation of the roles played by SigB and TcaR in resistance to LL-37

S. aureus 8325-4 is a strain which produces low levels of SigB due to a natural deletion in *rsbU*, which encodes an important activator of SigB (Giachino *et al.*, 2001; Horsburgh *et al.*, 2002). Strain SH1000 was derived from 8325-4 by repairing *rsbU* (Horsburgh *et al.*, 2002). It was found that SH1000 showed increased $NaHCO_3$-dependent resistance to LL-37 compared to 8325-4, and that deletion of the *rsbUVWsigB* operon in SH1000 restored the susceptibility to the same level as strain 8325-4 (described in section 7.14). In addition, $TcaR^-$ $RsbU^+$ strains (SH1000, HG001) demonstrated substantially higher $NaHCO_3$-dependent resistance to LL-37 than the $TcaR^+$ $RsbU^+$ (LS-1, HG003), $TcaR^-$ $RsbU^-$ (8325-4, SH1000 Δ*rsbUVWsigB*) and $TcaR^+$ $RsbU^-$ (HG002) strains, which had similar susceptibility to LL-37 (described in section 7.15). These data suggest that SigB and TcaR are important regulators of resistance to LL-37 in *S. aureus* at least in the presence of bicarbonate. The interplay between SigB and TcaR in resistance to LL-37 in the presence of $NaHCO_3$ would be worthy of further investigation.

In summary, the data generated in the work described in this thesis has improved our understanding of AMP resistance mechanisms in *S. aureus*, which may be useful for the development of effective AMPs as antibiotics against *S. aureus* infections. The bicarbonate-dependent resistance to LL-37 that we have found for a range of *S. aureus* mutants highlights the importance of examining the susceptibility to AMPs under conditions that mimic the host environment. Furthermore, the data from this PhD study also emphasised the importance of the SCV subpopulation in resistance to antimicrobial peptides, which warrants further investigation given the increased capacity of SCVs to persist in the host and the significance of this to relapsing and persistent infections.

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