

Novel inhibitors of the
Staphylococcus aureus quorum
sensing system

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Declaration

Unless otherwise stated, all work presented here is my own and has not been submitted for any other degree at the University of Nottingham or any other institute of learning.

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Abstract

The recent rise of antibiotic resistant micro-organisms has led to an increased demand for the production of novel and effective antimicrobial therapies. *Staphylococcus aureus* is a Gram positive human pathogen that some clinical isolate has evolved resistance to all currently available ‘front line’ antibiotics. In this study a series of small molecules, both natural and synthetic, were assessed for their ability to inhibit *S. aureus* virulence factor production, in the hope to identify novel molecules that inhibit *S. aureus* pathogenicity. Such molecules have the advantage over classical antibiotics in that they do not generate a selective pressure that can cause the evolution of drug resistant strains.

IQS (Integrated Quorum Sensing Signal, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde), is a secondary metabolite produced by *Pseudomonas aeruginosa* that has been suggested to function as a quorum sensing signal molecule. Here, IQS was found to inhibit *S. aureus* exotoxin production. In an attempt to improve potency, a small library of structurally diverse IQS analogues was synthesised. Two analogues were shown to have increased potency relative to natural IQS. Interestingly, data suggest that the active analogues, which are structurally distinct, have a different mechanism of action. The use of both *in vivo* reporters and biochemical analysis with purified AgrA, the response regulator that transduces the *S. aureus* QS signal, suggested that the lead analogue (IQS3) binds to and disrupts the DNA binding of AgrA, thus inhibiting QS by *S. aureus*. RNAseq data support the biochemical analysis in that the expression of virulence genes by *S. aureus* treated with the lead analogue mirrors that an *S. aureus* QS mutant. Finally the cytotoxicity of our lead analogues were assessed. Overall, data suggest that our lead IQS analogue is a good ‘starter’ molecule for further modification in the hope to increase anti-virulence properties whilst reducing cytotoxicity.

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Abbreviations

Å	Angstrom
°C	degree centigrade
Ω	ohm
¹ H NMR	proton nuclear magnetic resonance
7-AAD	7-aminoactinomycin D
<i>agr</i>	accessory gene regulator
AgrAc	AgrA DNA-binding domain
AIP	autoinducing peptide
BHI	brain heart infusion
bp	base pair
BPROM	bacterial promoter predictor
CA-MRSA	community associated methicillin resistant <i>Staphylococcus aureus</i>
CAS	Chrome Azurol S
cDNA	complementary deoxyribonucleic acid
Ct	cycle threshold
dATP	deoxyadenosine triphosphate
dH ₂ O	deionised water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
DSF	differential scanning fluorimetry
EC ₅₀	median effective concentration (required to induce a 50% effect)
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
Fe	iron
g	gram
GFP	green fluorescent protein
h	hour
HA-MRSA	hospital associated methicillin resistant <i>Staphylococcus aureus</i>
Hla	α-haemolysin

HPLC	high-performance liquid chromatography
HTH	helix-turn-helix
IC ₅₀	median inhibition concentration (required to reduce the effect by 50 %)
Ig	immunoglobulin
IPTG	isopropyl β-D-1-thiogalactopyranoside
IQS	integrated quorum sensing signal, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde
kb	kilobases
kDa	kiloDalton
kV	kilovolt
L	liter
LB	Luria Bertani
LD50	median lethal dose (concentration required to kill by 50 % of a test sample)
<i>lux</i>	cluster of luminescence genes
M	molar
mA	milliampere
mg	milligram
min	minutes
ml	milliliters
mm	millimeters
mM	millimolar
MSSA	methicillin susceptible <i>Staphylococcus aureus</i>
mRNA	messenger ribonucleic acid
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
NanoDSF	nanodifferential scanning fluorimetry
ng	nanogram
OD ₆₀₀	optical density at a wavelength of 600 nanometers
ORF	open reading frame
PAGE	polyacrylamide gel electrophoreses
PBP2a	penicillin binding protein 2A
PBS	phosphate buffer saline
PBST	phosphate buffer saline tween20

PCR	polymerase chain reaction
PFGE	pulse-field gel electrophoresis
PVL	Panton Valentine leukocidin
qRT-PCR	qualitative reverse transcriptase polymerase chain reaction
QS	quorum sensing
RLU	relative light units
RNA	ribonucleic acid
RNA-Seq	ribonucleic acid sequencing
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
s	seconds
SarA	staphylococcal accessory regulator A
SCAM	substituted cysteine accessibility method
SCCmec	staphylococcal cassette chromosome mec
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
TSB	tryptic soy broth
TSST-1	toxic shock syndrome toxin
U	units
V	Volt
VRSA	vancomycin-resistance <i>S. aureus</i>
WHO	World Health Organization
7-AAD	7-aminoactinomycin D
% (v/v)	percent volume per volume
% (w/v)	percent weight pr volume
μF	microFarad
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar

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Chapter 1 Introduction

1.1 General information of *Staphylococcus aureus*

Staphylococcus aureus is a member of the family Staphylococcaceae, order Bacillales, class Bacilli, phylum Firmicutes. It is a Gram-positive coccus that forms clusters that look like a bunch of grapes, a facultative anaerobe, non-motile and non-spore forming (Freney et al., 1999). Among *Staphylococcus spp.* that produce coagulase, *S. aureus* is the only organism that frequently causes infections in humans (Freney et al., 1999, Wang et al., 2013, Stull et al., 2014)

The *S. aureus* chromosome is circular with a low G + C content, around 30 % (Freney et al., 1999) the length varies according to strain, ranging from 2.6 to 2.9 million bp encoding approximately 2,600 to 2,700 proteins (Mlynarczyk et al., 1998, Hecker et al., 2010, Plata et al., 2009). In addition, *S. aureus* usually harbours one or more plasmids, ranging from 1 to 60 kb that carry antibiotic resistance gene(s) (Mlynarczyk et al., 1998).

1.2 Pathogenicity and colonisation of *S. aureus*

Staphylococcus aureus was first identified in pus from a surgical abscesses by Alexandra Ogston in 1880 (Ogston, 1984). It can cause a wide range of symptoms from mild skin infections like pimples, impetigo and cellulitis to invasive life-threatening infections, such as toxic shock syndrome, sepsis and endocarditis (Powers et al., 2015, Plano, 2004).

Approximately 14 - 50 % of healthy adults are nasal carriers of *S. aureus* as a commensal organism (Eriksen et al., 1995, Mertz et al., 2007). Nasal colonization is identified as one of the risk factors for *S. aureus* infections (Luzar

et al., 1990, Kluytmans et al., 1995). Despite the introduction of nasal swabbing and decontamination methods to reduce *S. aureus* infections especially antibiotic resistance strains (<http://www.nhs.uk/conditions/MRSA/Pages/Introduction.aspx>), there are still nosocomial cases of *S. aureus* infections. *S. aureus* can also colonise the throat, groin, axilla and rectum, therefore screening and control methods can be both difficult and time consuming (Mertz et al., 2007, Marshall and McBryde, 2014, Ridder-Schaphorn et al., 2007). Community acquired-MRSA (CA-MRSA, will be reviewed in more detail in section 1.3.4) difficult to screen for and control as the source of the infection is often difficult to determine due to the size and complexity of social interaction (Kazakova et al., 2005, Albrecht et al., 2015).

1.3 Antibiotic resistance

1.3.1 Historic and current situation

Penicillin which targets bacterial cell wall biosynthesis was introduced for use against *S. aureus* infections in the early 1940s and soon after that penicillin-resistant *S. aureus* was discovered (Rammelkamp and Maxon, 1942). Penicillinase or β -lactamase cleaves the β -lactam ring of penicillin leading to inactivation of the antibiotic (Spink and Ferris, 1947, Pollock, 1967). Penicillinase is encoded by the *blaZ* gene on a plasmid and is regulated by two genes located upstream, *blaR1* encodes a signal-transducing membrane protein and *blaI* encodes a repressor protein (Novick and Richmond, 1965, Hackbarth and Chambers, 1993). Currently, most *S. aureus* clinical isolates are resistant to penicillin (Lowy, 2003, Otto, 2012).

Methicillin which is a semisynthetic agent derived from penicillin was introduced for curing penicillin-resistant *S. aureus* infections. About one year later methicillin-resistant *S. aureus* (MRSA) was found in the UK (Jevons, 1961). The mechanism by which *S. aureus* employs to inactivate methicillin also protects against other antibiotics of the entire class of β -lactam (Otto, 2012). MRSA became epidemic in Europe in the 1980s before lineages of MRSA spread causing pandemic MRSA infections (Otto, 2012). In 2014 the World Health Organization (WHO) reported data collected from 2008 – 2013 that showed the proportion of MRSA among *S. aureus* infections was approximately 20 % in all WHO regions (WHO, 2014). MRSA infections that cause morbidity and mortality have a massive socio-economic impact.

1.3.2 Penicillin binding protein 2A

Penicillin binding protein 2A (PBP2a) is a key determinant of resistance to methicillin and other antibiotics in class β -lactam. MRSA strains display low affinity for methicillin (Hartman and Tomasz, 1984). Crystallography revealed that PBP2a structure provides methicillin resistance as shown in Figure 1.1 (Lim and Strynadka, 2002). Briefly, PBP2a is comprised of 4 domains. (i) N-terminal transmembrane anchor (ii) N-terminal extension which is a unique part of PBP2a. (iii) Non-penicillin binding protein domain that plays a key role in sending the transpeptidase domain to cell membrane and lastly (iv) the transpeptidase domain which has a similar structure in both Gram positive and negative bacteria (Lim and Strynadka, 2002). PBP2a reduces the efficacy of β -lactam drugs, leading to the failure of a covalent acyl-penicillin binding protein complex and normal formation of peptide crosslinks transpeptidation between

glycan chains of the cell wall (Lim and Strynadka, 2002, Peacock and Paterson, 2015).

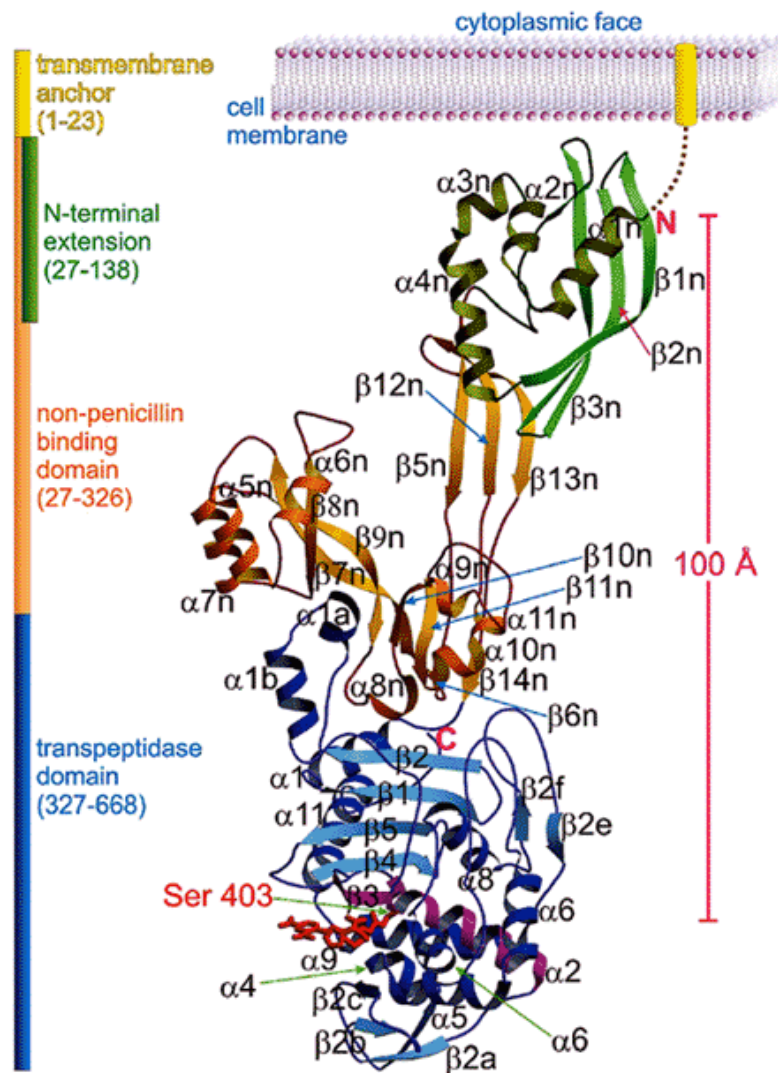


Figure 1.1 Structure of *S. aureus* PBP2a. The transmembrane anchor is coloured yellow, N-terminal extension is coloured green, bilobed non-penicillin binding domain is coloured orange and transpeptidase domain is coloured in blue with the position of active site in red. Reproduced from (Lim and Strynadka, 2002).

1.3.3 The *mecA* gene encodes PBP2a

The emergence of MRSA is due to the *mecA* gene, which encodes PBP2a and is located on a distinct mobile genetic element on the staphylococcal chromosome (SCCmec) (Katayama et al., 2000). SCCmec is comprised of the *mec* gene complex (*mecA*, and its regulator genes *mecI* and *mecR*), a cassette chromosome recombinase (*ccr*) complex containing one or two site-specific recombinase genes and joining regions which usually contains antibiotic and heavy metal-resistance genes (Ito, 2009). The SCCmec element is highly diverse in both structural organisation and genetics, it is therefore used for MRSA typing and subtyping (Ito, 2009). Currently, there are twelve types of SCCmec that have been identified (Wu et al., 2015).

mecA is extensively distributed among *Staphylococcal spp.* (Suzuki et al., 1992). *Staphylococcus sciuri* (Wu et al., 1996) and *Staphylococcus fleuretti* (Tsubakishita et al., 2010) contain *mecA* homologues. Horizontal gene transfer is considered to play a key role in *mecA* interspecies transfer. Strong evidence that suggests such a mechanism of transfer can be gathered from a clinical case report which showed that an infant patient who had a respiratory infection before developing bacteraemia had MSSA that responded well to antibiotic treatment. Later, MRSA was found from a nasal swab along with many *mecA*⁺ coagulase-negative *Staphylococcus spp* (Wielders et al., 2001). The pulse-field gel electrophoresis (PFGE) showed that the MSSA and MRSA isolates gave a similar pattern of bands except for one which was subsequently confirmed by sequencing the *mecA* gene (Wielders et al., 2001, Bloemendaal et al., 2010). In addition, the *mecA* gene as analysed using PFGE had a similar banding pattern for the MRSA and an isolated *Staphylococcus epidermis* (Wielders et al., 2001).

Therefore, it is important to understand *SCCmec* and other *mecA*⁺ staphylococci which can act as a *mecA* reservoir for *S. aureus*.

1.3.4 Hospital-acquired MRSA vs community-acquired MRSA

Currently, MRSA can be divided into two groups, hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) which was first recognised around 1990 and is becoming increasingly widely spread.

HA-MRSA harbouring a large *SCCmec* type I, II or III that carry multiple drugs resistance genes (Hiramatsu et al., 2002, Deurenberg and Stobberingh, 2008) are problematic in the treatment of hospital patients. Meanwhile, most CA-MRSA carry the smaller *SCCmec* type elements IV, V and VII that encode resistance to antibiotics, mainly in the β -lactam class (Valsesia et al., 2010, Deurenberg and Stobberingh, 2008). Many CA-MRSA encode the *SCCmec* IV and Panton-Valentine leukocidin (*PVL*) locus (Vandenesch et al., 2003). *PVL* is a toxin that causes skin and soft tissue destruction, however, not every CA-MRSA isolate produces this toxin (Otto, 2013). HA-MRSA, particularly carrying *SCCmec* type II, is considered less virulent than CA-MRSA carrying *SCCmec* type IV as the infection is limited to only healthcare setting and is less toxic to T cells *in vitro* (Collins et al., 2010).

1.4 *S. aureus* virulence factors

S. aureus produces numerous virulence factors to both invade a host and protect itself from host immunity as shown in Table 1.1 (Foster, 2005, Zecconi and Scali, 2013).

Table 1.1 Examples of *S. aureus* virulence factors and its function. Adapted from (Zecconi and Scali, 2013)

Name	Function
Invasion	
α -haemolysin	Cytolytic pore-forming protein
PSM peptides	Pore-forming peptides
TSST-1	Endothelial toxicity (direct and cytokine-mediated); superantigen
Fibronectin binding protein (FnBP) A, B	Adhesins for fibrinogen (only FnBPA), fibrinogen and elastin
Coagulase	Binds and activates prothrombin; promote conversion of fibrinogen to fibrin
Evasion	
γ toxin	Bicomponent leukocidin; haemolysis
δ toxin	Cytolytic toxin; binds neutrophils and monocytes
PVL	Bicomponent leukocidin; pore-forming toxin; kills leukocytes
Protein A	Anti-opsonophagocytosis molecule

Alpha-haemolysin encoded by the *hla* gene, located on a mobile genetic element and is found in 80 % of *S. aureus* infections worldwide in a key virulence determinant (Tabor et al., 2016). Alpha-haemolysin is a 30 kDa protein that forms pores in the host cell membrane causing cell lysis (Bubeck Wardenburg et al., 2007), it also has been shown to inhibit platelet aggregation and promote the host inflammation response and is a key causative agent in the development of sepsis (Powers et al., 2015).

Toxic shock syndrome toxin (TSST-1) is a superantigen exclusively produced by *S. aureus* and is encoded from the *tst* gene (Lindsay et al., 1998). Approximately 20 % of all *S. aureus* isolates produce this toxin that causes toxic shock syndrome (Lindsay et al., 1998). TSST-1 was the cause of the 1928 Bundaberg disaster; twelve children died within 48 h after receiving vaccination contaminated with *S. aureus* (Mueller et al., 2015). Multiple logistic regression analysis indicates that TSST-1 production increased the death rate 4 fold in a patient who was infected by TSST-1 positive strains (Maeda et al., 2016).

Protein A encoded from *spa* gene is anchored to the bacterial cell wall and is also secreted to the surroundings (Falugi et al., 2013, O'Halloran et al., 2015). Protein A is an important virulence factor for evading the host immune system. Protein A binds to the Fc γ domain of immunoglobulin (Ig) G and Fab domains of IgG and IgM protecting the bacteria from opsonophagocytosis (Graille et al., 2000, Falugi et al., 2013). *S. aureus* mutants deficient for Protein A were found to have a reduced bacterial load and less severe pathogenesis in a mouse infection model (Falugi et al., 2013).

1.5 Regulation of Virulence factor production by *S. aureus* is controlled by global transcriptional regulators

Virulence factor production by bacteria exerts a high metabolic demand, therefore the expression and production of virulence factors is tightly regulated. The well-studied *S. aureus* global regulator, *agr* (accessory gene regulator) is a quorum sensing system that along with the staphylococcal accessory regulator A (SarA) and MgrA coordinate gene expression so that the correct genes are switched on or off at the appropriate time.

1.5.1 The *S. aureus* quorum sensing system, *agr*

A number of bacterial species employ quorum sensing (QS) to communicate to others by detecting a signal molecule that accumulates in the surrounding environment (Thoendel et al., 2011). The accessory gene regulator (*agr*) is utilised by *S. aureus* to determine local cell numbers. Once signal peptide concentration reaches a threshold limit the activation of *agr* is utilised to switch on the expression of several genes including virulence factors. The *agr* locus

consists of two divergent transcripts, RNAII and RNAIII under the regulation of the P2 and P3 promoters, respectively (Thoendel et al., 2011). The RNAII transcript is an operon of four genes, *agrBDCA* that encodes proteins that play a key role in synthesis of the autoinducing peptide (AIP) signal molecule and RNAIII, a regulatory RNA that is essential to expression of several genes (Thoendel et al., 2011). The *agrBDCA* operon consists of two systems, (i) production of autoinducing peptide (AIP) by AgrB and AgrD (Ji et al., 1995) and (ii) two-component system which senses AIP signalling molecules (Novick et al., 1995).

1.5.1.1 Biosynthesis of AIP

AgrB is an integral membrane endopeptidase. Residues essential for AIP biosynthesis were identified using random mutagenesis. A substituted cysteine accessibility study (SCAM), as shown in Figure 1.2a is predicted AgrB to have five transmembrane regions, and forms a homo-oligomeric structure within the cytoplasmic membrane (Thoendel and Horswill, 2013).

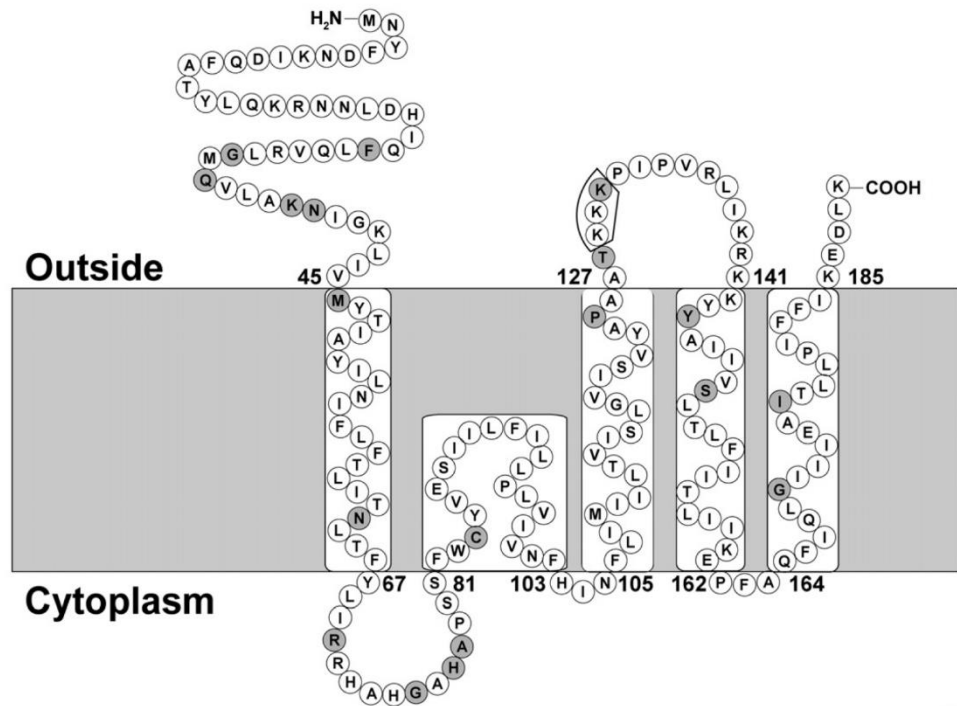


Figure 1.2 Topology model of AgrB. The AgrB group I model is created from the data assessed by substituted cysteine accessibility method. The amino acid residues playing a key role in peptidase activity during AIP production are shaded grey. K129 – K131 residues are essential in AIP production after step of AgrD cleavage. Reproduced from (Thoendel and Horswill, 2013)

AIP production in short includes the following steps; (Figure 1.3), (i) AgrD, a peptide precursor of AIP, localises and anchors to the cell membrane by its N-terminal amphipathic leader (Wang et al., 2015, Thoendel and Horswill, 2009). (ii) The C-terminal tail of AgrD is then cleaved by AgrB (Thoendel and Horswill, 2009, Thoendel and Horswill, 2013, Wang et al., 2015). (iii) AgrB also catalyse the cyclisation, by forming a thio-lactone bond between the cysteine and methionine of the immature peptide (iv) the intermediate is transported to the outer face of the membrane by as yet unidentified mechanism (Thoendel and Horswill, 2009, Wang et al., 2015). Moreover, the function of AgrB residues 129 – 131 have been assessed and found to play a key role in cyclisation (Thoendel and Horswill, 2013). Lastly, the signal peptidase SpsB is thought to cleave the N-terminal of intermediate and release mature AIP (Kavanaugh et al., 2007).

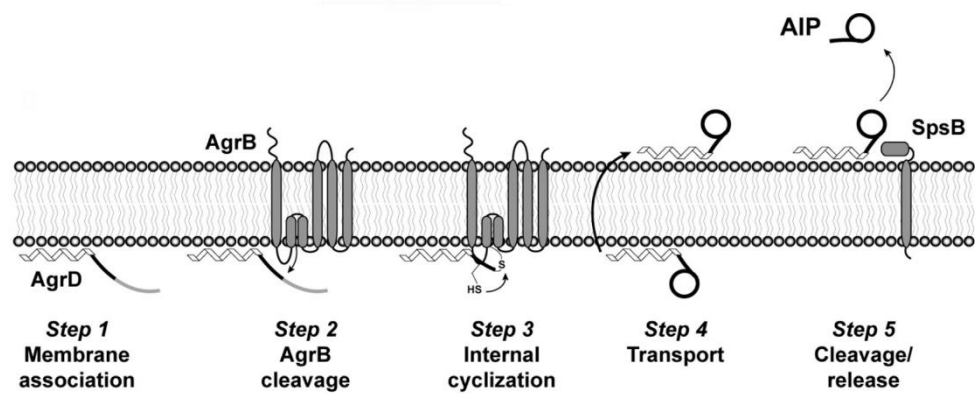


Figure 1.3 A model for AIP biosynthesis. The process is concluded into 5 steps. Step 1, N-terminal of AgrD associates with the cell membrane. Step 2, AgrB cleaves the C-terminal of AgrD. Step 3, the remaining AgrD fragment (AIP precursor) is cyclised and forms thiolactone ring. Step 4, the AIP precursor is transported to the outer face of the cell membrane. Lastly, SpsB cleaves the N-terminal amphipathic leader and the AIP is released from the membrane to the environment. Reproduced from (Thoendel and Horswill, 2013).

1.5.1.2 AgrC and AgrA form a two-component signalling system

Two-component signalling system enables bacterium to sense and responding to the environment. Typical components of the system are a sensor histidine kinase and a response regulator (Podgornaia and Laub, 2013). AgrC and AgrA proteins belong to this system.

Numerous groups have proposed the predicted topology of AgrC in the cytoplasmic membrane (Lina et al., 1998, Geisinger et al., 2008). Recently, GFP fusion and SCAM analysis was employed to generate a topological model for AgrC and it suggested that AgrC is composed of seven transmembrane regions, with an N-terminal protruding to outside of the cell, whereas the C-terminal is in the cytoplasm (Wang et al., 2014).

Interaction between histidine kinase AgrC and cognate AIP activates AgrC to phosphorylate the response regulator AgrA leading to binding of the AgrA LytTR domain to the targeted gene promoter facilitating the control of gene expression. Figure 1.4a shows a schematic of the AgrA DNA binding domain (AgrAc, residue 137 – 238) which reveals the novel topology of this domain. AgrAc contains 10 β -strands arranged into three antiparallel β -sheets. Sheet 2, β 3- β 7, is hydrophobic and is sandwiched between two small amphipathic β -sheets 1 and 3. AgrAc also has a small two-turn α -helix that is located between β 5 and β 6. The α -helix does not play a role in DNA-binding but is important for the formation of a salt bridge that stabilises the connection between β -sheet 2 and 3 (Sidote et al., 2008). Most DNA-binding proteins interact with the DNA major groove via a recognition helix, however, AgrAc interacts with DNA by sitting on DNA and inserts a loop into successive major grooves directly interacting with each DNA base in the major groove (Sidote et al., 2008). The

DNA-binding of AgrAc is specific to a conserved 9-bp consensus sequence as shown in Figure 1.4b. The interactions in this 9 bp consensus region are non-specific and mediated by water, which is crucial to the stability of the complex. H169 and R233 are involved in direct base-specific interactions with DNA in the major groove (Sidote et al., 2008). These residues make hydrogen bonds and also water-mediated hydrogen bonds to the DNA. N201 interacts with the DNA in the minor groove (Sidote et al., 2008). The structure of AgrAc does not reveal the interaction of the AgrA dimer when it binds to the adjacent target DNA such as the P2 P3 promoters (Figure 1.4c). This suggests that dimerization, occurs at the unresolved N-terminal domains.

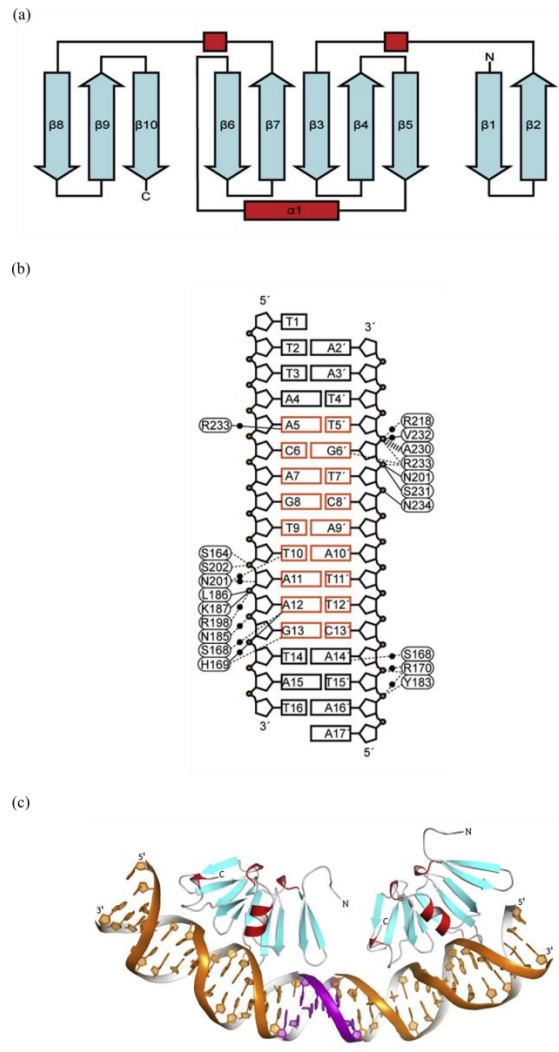


Figure 1.4 Topology of AgrA DNA-binding domain and DNA interaction.

(a) Topology diagram of the AgrA DNA-binding domain. Beta-sheets are coloured in blue and helices are in red. (b) Schematic diagram of the interactions between AgrA DNA-binding domain and DNA. Amino acid residues are presented as single letter code in ovals, whereas DNA bases are in rectangles and the consensus DNA-binding sequence is in red. Solid lines and dash line indicate contact between the backbone atoms of amino acid to DNA and contact between the sidechain of amino acid residue to DNA, respectively. Black circles indicate water-mediated interactions. The wide dash line indicates a van der Waals interaction. (c) Model of two AgrA DNA-binding domains directly bind to repeat DNA recognition element with the intervening 12 bp spacer modelled as B-form DNA. Reproduced from (Sidote et al., 2008).

Binding of AgrA to the P2 promoter activates transcription of RNAII encoding AgrB, AgrD, AgrC and AgrA generating a positive feedback of the system. Phosphorylated AgrA also binds to the P3 promoter resulting in enhanced transcription of RNAIII. This *agr* signalling pathway is summarised in Figure 1.5.

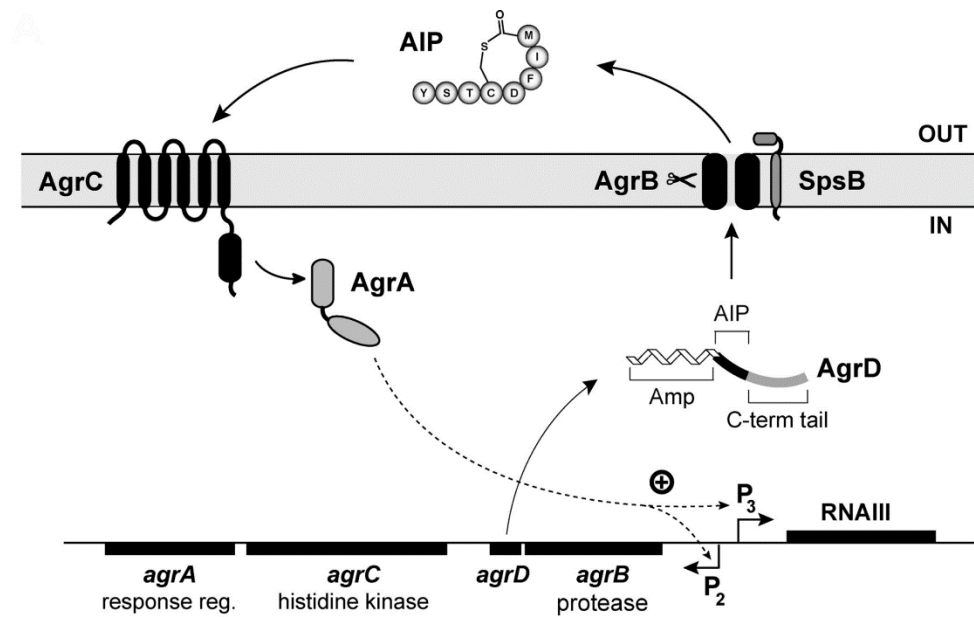


Figure 1.5 Schematic of *S. aureus* *agr* system. The system contains two operons controlled by different promoters, P2 and P3. AgrD is matured and translocalised as AIP signalling molecule by the AgrB and SpsB. AIP binds to AgrC leads to activation of AgrA and activation of operon *agrBDCA* like positive feedback loop. Activated AgrA also binds to P3 resulting in activation of RNAIII. Reproduced from (Thoendel and Horswill, 2009).

1.5.1.3 RNAIII

RNAIII is a regulatory RNA, the first section of which encodes the mRNA for the lytic enzyme δ -haemolysin. The RNA effector which is 514 nucleotides in length acts in an anti-sense manner (Boisset et al., 2007). The secondary structure of RNAIII which is shown in Figure 1.6, contains fourteen hairpin loops connected by unpaired nucleotides the structure of which is essential its regulatory functions (Benito et al., 2000). The *agr* system controls gene expression, enhancing expression of the RNAIII transcript that leads to promotion or inhibition of the transcription or translation of many genes that are regulated by RNAIII. Increased levels of RNAIII increases the production of α -haemolysin by directly binding to the *hla* mRNA transcript resulting in an increase of *hla* translation (Morfeldt et al., 1995). On the other hand, translation of Protein A mRNA is inhibited by the binding of RNAIII. RNAIII binds to the Protein A transcript at the ribosome binding site blocking translation and subsequently enhancing degradation of the transcript (Huntzinger et al., 2005). Microarray studies have revealed that there are 104 *agr*-upregulated genes including secreted virulence factor genes, transporter genes and capsular genes and 34 genes that are down-regulated in an *agr*-dependent manner, such as cell surface virulence factor genes, DNA replication genes and transporter genes, etc (Dunman et al., 2001). TSST-1, α -haemolysin, protease, lipase are up-regulated by the *agr* system during the post-exponential phase of growth whereas this system down-regulated surface virulence factors involved in cell adhesion, biofilm formation and antibody binding-protein A at the same time (Thoendel et al., 2011, Dunman et al., 2001).

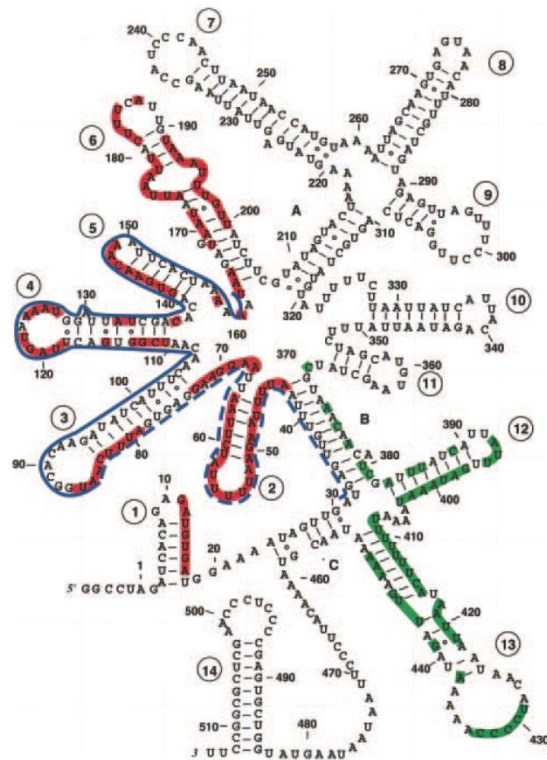
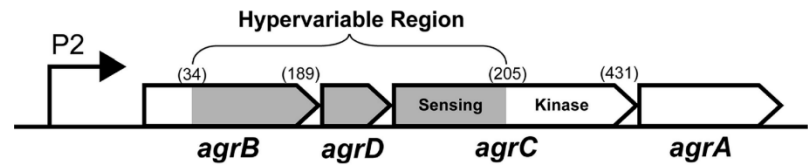


Figure 1.6 Secondary structure of RNAIII. Number 1 – 14 refers to hairpins. The *hld* coding region and potentially translatable region are outlined in solid and dashed blue, respectively. The regions that complementary with the *hla* and *spa* mRNA is highlighted in red and green, respectively. Reproduced from (Novick, 2003).

S. aureus can be divided into four groups according to the sequence of the *agrD* peptide. A hypervariable region in the *agr* operon is located at the 3' end of *agrB* that reaches to the 5' end of the *agrC* gene. *agrA* is conserved among all *agr* groups (Thoendel et al., 2011), see Figure 1.7a. The amino acid sequences of AgrD in each group are shown in Figure 1.7b. Activation of the *agr* system requires the interaction between cognate AgrD and AgrB in order to produce AIP. Binding of non-cognate AgrD leads to failure of AIP production, e. g. AgrB-I cannot cleave and transport AgrD-II (Ji et al., 1997). However, selectivity is not strict as AIP-III and -IV can activate *agr* group I and *vice versa* (Ji et al., 1997). In general, the structures of AIP in each *agr* group are similar, composed of a five-residue thiolactone ring based on linkage of the cysteine sulphhydryl to the C-terminal carboxylate and 2 – 4 residue extension at N-terminus, shown in Figure 1.7 c (Thoendel et al., 2011). Specificity of activation between cognate AIP and AgrC is similar to the interaction between AgrD and AgrB. AIP-II inhibits *agr*-I and AIP-IV can activate *agr*-I system (Geisinger et al., 2009).

(a)



(b)

Group I	MNTLFNLFDFFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE
Group II	MNTLVNMFDFFIKLAKAIGIVGGVN ACSSLF DEPKVPAELTNLYDK
Group III	MKKLLNKVIELLVDFNFNSIGYRAAY INCD FLLEAEVPKELTQLHE
Group IV	MNTLYKSFFDFITGVLKNIGNVAS YSTCYFIM DEVEIPKELTQLHE

(c)

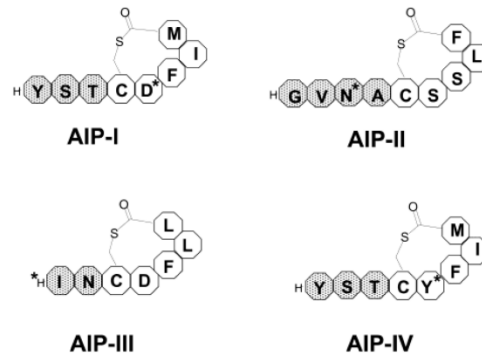


Figure 1.7 Hypervariable regions of the *agrBDCA* operon and variable of AgrD in each *agr* groups. (a) The regions shaded with grey are hypervariable regions, whereas white area is highly conserved area among four *agr* groups. (b) Amino acid sequence of AgrD from each *agr* group. (**Bold**), AIP region; N-terminal, amphipatic leader; C-terminal, C-tail. (c) Structure of AIPs. Tails are represented in shaded grey circles. The residues that play a role in activation are marked with asterisk (*). Reproduced from (Thoendel et al., 2011, Lyon et al., 2002, Zhang et al., 2004).

1.5.2 SarA family

The SarA family contains eleven members which are all transcription factors that respond to a change in the local microenvironment (Cheung et al., 2008). In this report SarA and MrgA will be focused on as they are global regulators that function to positively control the expression of several exotoxin genes, similar to *agr*.

1.5.2.1 SarA protein

SarA is an important global regulatory protein in *S. aureus*. The *sarA* locus contains three overlapping transcripts which are under the control of three distinct promoters, but share the same poly(T) terminal signal. The three transcripts of *sarA* contain an ORF that encodes the 14k Da SarA protein (Bayer et al., 1996). The SarA protein can be found from early through to post-exponential phase after which expression declines in post-exponential growth (Ballal and Manna, 2009).

The crystal structure of the SarA protein was solved and further studies using single isomorphous replacement and Difference Fourier's method revealed that SarA is a winged helix protein, with a similar structure to other proteins in the SarA protein family, Figure 1.8 (Liu et al., 2006). A SarA monomer consists of five α -helices and three β -strands. The structure of SarA indicates that the protein is a homodimer as the $\alpha 1$ and $\alpha 1'$ of each monomer brings two monomers together with the help of a Ca^{2+} atom (Liu et al., 2006). Independent studies also reported that SarA is a dimer (Rechlin et al., 1999, Schumacher et al., 2001). A helix-turn-helix (HTH) domain is formed by the $\alpha 3$ and $\alpha 4$ helices whilst the

winged region that is expected to interact with the minor groove of target DNA is formed by $\beta 2$ and $\beta 3$ (Liu et al., 2006).

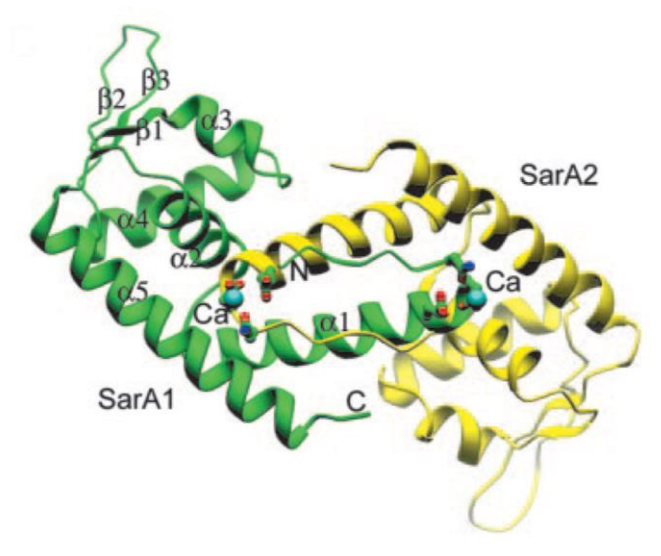


Figure 1.8. Structure of the SarA dimer. Two β -hairpins, $\beta 2$ and $\beta 3$ of each monomer and two helix-turn-helix motifs ($\alpha 3$ and $\alpha 4$ of each monomer) build up the putative DNA-binding surface on the concave side of SarA, Ca^{2+} -binds to the opposite side. Reproduced from (Liu et al., 2006).

Microarray studies have shown that SarA positively regulates 76 genes involved in both virulence factor production, cell attachment and signal transduction (*agr* system), whereas it down-regulates 44 genes, some of which encode for virulence factors such as protein A (Dunman et al., 2001). The SarA protein is able to regulate target gene expression directly by binding to the promoters of target genes to up-regulate their expression, such as *hla* (Chien et al., 1999), *tst* (Andrey et al., 2010) and *agr* (Chien and Cheung, 1998). SarA also represses target gene expression by binding to the promoter of target genes such as collagen adhesin gene (Blevins et al., 1999) and *spa* gene (Chien et al., 1999). SarA indirectly up-regulates and down-regulates targeted genes via other regulatory proteins or systems. For example, SarA can increase *hla* expression in an *agr*-dependent manner (Dunman et al., 2001), whereas it down-regulates the expression of *spa* by repressing SarS, another regulatory protein in the SarA family (Cheung et al., 2001).

1.5.2.2 MgrA

MgrA is a 17.6-kDa protein and is a homologue of SarA and belongs to the MarR subfamily (Cheung et al., 2008, Truong-Bolduc et al., 2003). Figure 1.9 shows the crystal structure of MgrA revealing that it is a winged-helix protein that forms a dimer similar to SarA (Chen et al., 2006). Each monomer consists of seven α -helices and three β -sheets. Dimerisation occurs by the interaction of each monomer at $\alpha 1$, $\alpha 6$ and $\alpha 7$ which are hydrophobic residues (Chen et al., 2006). The DNA binding domain consists of $\alpha 1$, $\alpha 5$ and $\alpha 6$ of each monomer with the space between $\alpha 4$ - $\alpha 4'$ of the dimer is 34 Å which is large enough to fit the DNA major groove (Chen et al., 2006).

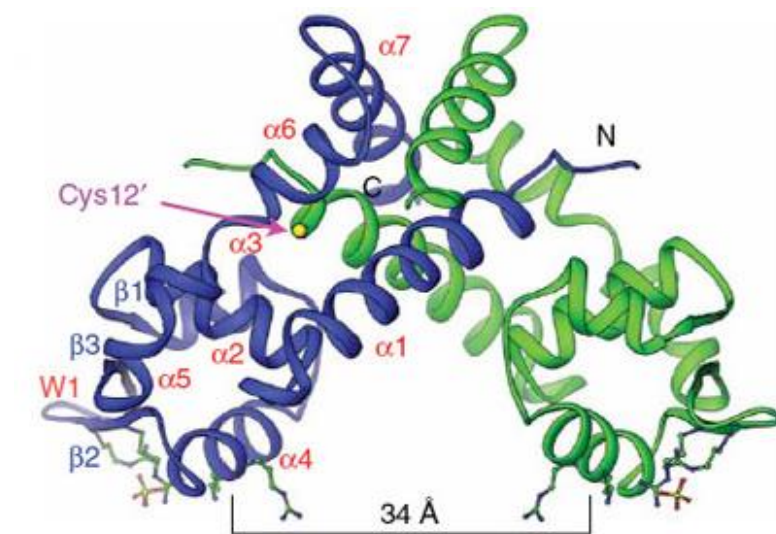


Figure 1.9 Structure of MgrA. Ribbon representation of the MgrA dimer. One subunit is coloured blue and the other in green. Each subunit is composed of seven α -helices, three β -sheets and W1 which is the wing region (Cheung et al., 2008).

MgrA is a pleiotropic regulator that positively regulates 175 genes and negatively regulates 180 genes during exponential and early stationary phase growth as assessed by Microarray analysis (Luong et al., 2006). In addition, there are more genes regulated during exponential phase than stationary phase of growth, suggesting MgrA is an early-growth-phase regulator (Luong et al., 2006). MgrA plays a key role in autolysis by repressing autolytic genes and positively regulating anti-autolytic genes (Ingavale et al., 2003, Ingavale et al., 2005, Luong et al., 2006).

MgrA positively regulates *hla* by binding to the *hla* promoter via an *agr*-dependent pathway (Ingavale et al., 2005), however, there are many studies that contradict those results (Luong et al., 2003, Crosby et al., 2016). MgrA indirectly represses protein A production through SarS which is a SarA homolog and an activator of *spa* (Ingavale et al., 2005).

MgrA is involved in pathogenesis of *S. aureus* infections. The virulence of a *mgrA* mutant is attenuated and the survival rate of the infected mouse significantly increases, weight loss is reduced, bacterial load is less and there is less destruction of cartilage and bone compared to a wild-type infected mouse using the septic arthritis model (Jonsson et al., 2008).

Noticeably, the *agr* system, SarA and MgrA share many target genes which regulate similarly or inversely as shown in Table 1.2.

Table 1.2 Type of regulation of global regulator on interested targets
(Peacock et al., 2002, Dunman et al., 2001, Crosby et al., 2016, Thoendel et al., 2011, Atwood et al., 2015).

Name	Putative function	<i>agr</i>	SarA	MgrA
Gene regulator				
RNAIII	up-regulates exotoxin and down-regulates adhesin genes	+	+	+
Exotoxins				
α -haemolysin	cytolytic pore-forming toxin	+	+	?
TSST-1	exotoxin with superantigen activity	+	+	N/D
Adhesion				
Protein A	anti-opsonophagocytosis molecule	-	-	-
Biofilm	protects the bacteria from host immune system and antibiotic	-	+	-

+, up-regulated;
-, down-regulated;
?, unclear;
N/D, not determined

1.6 Alternative therapeutic strategies to treat *S. aureus* infections

Nowadays, MRSA is widely spread in many countries. New therapies for curing MRSA infection are urgently needed. Since, *S. aureus* developed resistance to conventional antibiotics, especially the β -lactam group quickly. This suggests that therapeutics that targets essential cellular processes may trigger the development of resistance mechanism. Inhibiting virulence factor production by inhibiting global regulators is one option for a novel therapeutic (Alksne and Projan, 2000, Otto, 2004, Bhardwaj et al., 2013, Defoirdt et al., 2013, Gordon et al., 2013).

The *S. aureus* QS system, *agr*, and SarA are possible targets for developing new therapies for treating *S. aureus* infections as the virulence of a single *agr* or *sarA* mutant is reduced in multiple animal infection studies (Cheung et al., 1994, Blevins et al., 2003). Inhibition of these global regulators reduce expression of virulence factors and allows the host immune system to eliminate the infection (Gordon et al., 2013). Furthermore, such inhibitors would not affect bacterial fitness as both *agr* and SarA do not play a role in bacterial fitness, and therefore such therapy may exert less selective pressure on the bacteria and reduce the risk of resistance evolving (Gordon et al., 2013).

1.6.1 Examples of anti-*S. aureus* virulence agents

Several compounds, natural and synthetic, have shown activity in reducing virulence factor production by *S. aureus*.

1.6.1.1 AgrA inhibitors

Savirin

S. aureus virulence inhibitor (Savirin), Figure 1.10, was a lead compound from high throughput screening of 24,087 compounds from the Molecular Libraries Small Molecule Repository of the NIH Molecular Libraries Screening Center Network. It was found to inhibit RNAIII expression leading to a reduction of *hla*, phenol-soluble modulins (psm) gene and *PVL* expression without killing the bacteria. Its mechanism of action was shown to be prevention of AgrA binding to DNA. Savirin has also showed protective activity in a mouse skin infection model (Sully et al., 2014).

ω -hydroxyemodin

A metabolite from solid phase culture of *Penicillium restrictum*, polyhydroxyanthraquinone ω -hydroxyemodin (OHM) which the chemical structure is shown in Figure 1.10 directly binds to AgrA preventing the protein binding to the *S. aureus* P2 and P3 promoters, leading to a reduction of *hla* and *psm* expression. In addition, OHM enhances innate immune cells to kill bacteria but limits inflammation resulting in attenuation of *S. aureus* in a mouse skin and soft tissue infection model (Daly et al., 2015).

Diflunisal

Anti-inflammatory drug diflunisal (2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid, Figure 1.10) which is approved by US Food and Drug Administration is a lead compound from 90,000 compounds in the National Cancer Institute Library high throughput screening. It shows activity in reducing *hla*, *psma* and *RNAIII* but also increases *spa* expression (Khodaverdian et al., 2013). At the translational level, it reduces α -haemolysin production (Khodaverdian et al., 2013). It was shown by EMSA that diflunisal can inhibit AGRA binding to DNA. Recently, a study showed that diflunisal protects primary murine and human osteoblast cells from *S. aureus* infection in an anti-inflammatory-independent manner (Hendrix et al., 2016). Applying diflunisal at the osteomyelitis infection site also reduced bone destruction in a mouse infection model (Hendrix et al., 2016).

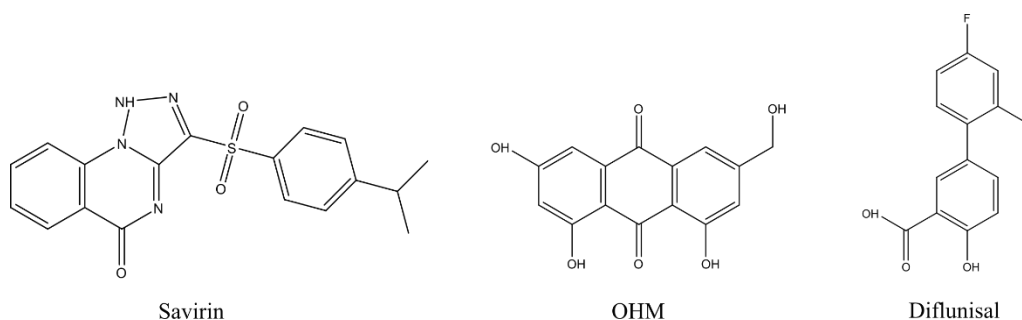


Figure 1.10 Structure of AgrA inhibitors, savirin, OHM and diflunisal (Sully et al., 2014, Daly et al., 2015, Khodaverdian et al., 2013)

1.6.1.2 AgrC inhibitors

As it has been known that there is cross-talk inhibition between AgrC and non-cognate AIP (Geisinger et al., 2009, Lyon et al., 2000). Several studies have modified AIP molecules to become AgrC inhibitor (Lyon et al., 2002, Tal-

Gan et al., 2013, Dowell et al., 2001, Jensen et al., 2008). Recent study showed that D4A mutation of AIP-III (amino acid sequence, I-N-(C-A-F-L-L)) is a potent AgrC inhibitor as it has inhibition activity across all four *agr* systems and it has activity in reduction of haemolysin, TSST-1 at nanomolar level (Tal-Gan et al., 2013).

Figure 1.11, a QS signalling molecule N-(3-oxododecanoyl-L-homoserine lactone (3-oxo-C₁₂-HSL) from *Pseudomonas aeruginosa* reduces α -haemolysin, δ -haemolysin and TSST-1 and up-regulated protein A production by inhibiting AgrC in non-competitive manner (Murray et al., 2014, Qazi et al., 2006).

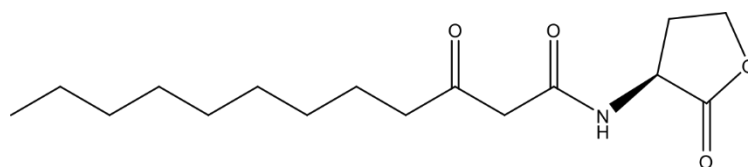


Figure 1.11 Structure of 3-oxo-C₁₂-HSL. (Murray et al., 2014).

1.6.1.3 SarA inhibitor

4-[(2,4-difluorobenzyl)amino]cyclohexanol (Figure 1.12) is a potential compound that shows activity against SarA. Using computational docking analysis (Arya and Princy, 2013) the compound is thought to bind to amino acid residues (D88 and E89) of SarA. 4-[(2,4-difluorobenzyl)amino]cyclohexanol does not affect growth of *S. aureus*. It was shown to reduce haemolysin activity and also reduces cell adherence and biofilm formation, had also protected in a vascular graft infection study (Arya et al., 2015).

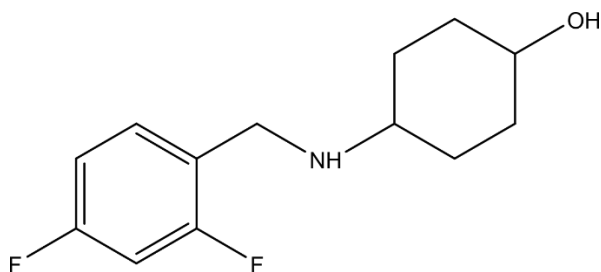


Figure 1.12 Structure of 4-[(2,4-difluorobenzyl)amino]cyclohexanol. (Arya et al., 2015)

1.6.1.3 MgrA inhibitor

The small molecule MDSA (5, 5-methylenedisalicylic acid) was a compound in the National Screening Laboratory for the Regional Center of Excellence in Biodefense and Emerging Infectious Disease at Harvard Medical School, USA (Sun et al., 2011). MDSA is able to bind to MgrA leading to a change of the MgrA tertiary structure, leading to disruption of MgrA DNA-binding activity and reduction of *hla* mRNA (Sun et al., 2011). In addition, the growth of *S. aureus* was not affected by MDSA even at high concentration up to 10 mM (Sun et al., 2011). MDSA showed its property in attenuation of *S. aureus* infections in the mice. It was found that abscess formation in mice that had MDSA co-administered with the bacteria was reduced with bacterial load also reduced by up to ~90% in some tissues compared with the untreated control group (Sun et al., 2011).

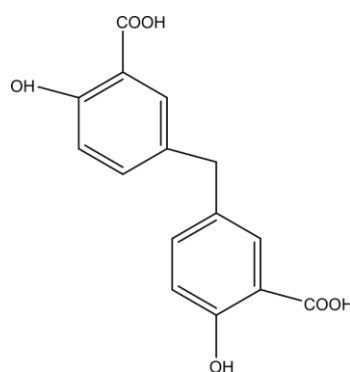


Figure 1.13 Structure of MDSA (Sun et al., 2011).

1.6.2 Vaccines

Vaccination is an alternative method for tackling infection caused by antibiotic-resistance bacteria. There are many advantages of using vaccination over antibiotics. (i) The existing antibiotic-resistance bacteria will not affect the efficacy of the vaccine as the mode of action of a vaccine and antibiotic are different. (ii) It is unlikely to generate selective pressure that results in the evolution of a resistance mechanism. (iii) Immune response elicited by a vaccination is specific to only one species of bacteria (or a few), therefore it will not disturb normal flora (Garcia-Quintanilla et al., 2016).

There are many active and passive anti-*S. aureus* vaccines approaches that have been developed, however, none of them have been licensed for use. Among the vaccines that have been developed to prevent *S. aureus* infections, StaphVax™ (Fattom et al., 2015) which contains type 5 and 8 *S. aureus* capsular polysacharrides and may be the most successful to date and is currently in Phase III clinical trials. StaphVax™, however failed to show a protective effect against *S. aureus* bacteremia in end-stage renal disease patients (Fattom et al., 2015). Aurograb, a single chain antibody against an ATP-binding cassette transporter together with vancomycin, was tested in a Phase III trial. The study has been completed; however, no results have been reported (<https://clinicaltrials.gov/ct2/show/NCT00217841>).

1.7 Study aim

As the number of MRSA and vancomycin-resistance isolate are increasing, finding a new therapeutic agent for treating *S. aureus* infections is an urgent issue.

The aims of this study are as follows:

- To screen analogues in class of quinone, rhodanine and IQS for anti-*S. aureus* virulence activity
- To identify the mechanism of action of any lead compound
- To determine cytotoxicity of lead compounds

Chapter2: Materials and Methods

2.1 Materials

2.1.1 Quinones and rhodanines

The synthetic compounds used in this study can be divided into two groups according to their core structures, quinone or rhodanine. All of these compounds were synthesised and generously supplied by Dr. Fabio Rui (School of Pharmacy, University of Nottingham). The structures of these compounds are shown in Chapter 3, Table 3.1. All synthesised compounds were dissolved and diluted in DMSO before use.

2.1.2 IQS analogues

IQS analogues were synthesised by Alex Truman (School of Life Sciences, University of Nottingham) and Dr. Siri Ram Chhabra (School of Life Sciences, University of Nottingham). The structures of the IQS analogues are shown in Chapter 4, Table 4.1. All were dissolved and diluted in DMSO before use.

2.1.3 Dimedone, MDSA and AIPs

Dimedone purchased from Sigma specifically binds to cysteine residues of proteins (Charles et al., 2007) and 5, 5-methylenedisalicylic acid (MDSA), an inhibitor of MgrA, a global regulator of *S. aureus* virulence factor expression (Sun et al., 2011) was purchased from Acros Organics.

The AIP analogue, AIP84 also called tAIP-III D2A, a truncated AIP-III that has inhibitory activity against all four *agr* groups (Tal-Gan et al., 2013) was synthesised by Dr Fabio Rui.

2.2 Bacterial strains and growth conditions

S. aureus was maintained on brain-heart infusion (BHI; Oxoid) agar and was cultured in tryptic soy broth (TSB; Oxoid). The cultures were grown at 37 °C with shaking at 200 rpm. 5 µg/ml erythromycin, 25 µg/ml lincomycin, 100 µg/ml kanamycin, 10 µg/ml chloramphenicol and 10 µg/ml tetracyclin were used when required.

E. coli was cultured in Luria-Bertani (LB; Oxoid) broth or LB agar at 37 °C, 200 rpm. 100 µg/ml ampicillin, 50 µg/ml carbenicillin or 150 µg/ml erythromycin were added when required. All strains of *S. aureus* and *E. coli* used are listed in Table 2.3.

Table 2.3 *S. aureus* strains used in this study

Strain	Genotype/note	Source/reference
KH1187A	TSST-1 producing strain, <i>agr</i> -I	Keith Holland, University of Leeds, UK
MN8	TSST-1 producing strain, <i>agr</i> -III	(Li et al., 2011)
RN4220	Cloning intermediate	(Nair et al., 2011)
RN6390B	<i>rsbU</i> , standard laboratory strain	(Novick et al., 1993)
Δ <i>sarA</i> RN6390B	<i>sarA::ermC^R</i>	This study
Δ <i>agr</i> RN6390B	<i>agr::ermC^R</i>	This study
SH1000	<i>agr</i> -I	(Horsburgh et al., 2002)
Δ <i>sarA</i> SH1000	<i>sarA::ermC^R</i>	This study
Δ <i>agr</i> SH1000	<i>agr::tetM</i>	This laboratory
SH1000 <i>P3:lux</i>	<i>attB2::P3-luxCDABE:kan^R</i>	This laboratory
Δ <i>sarA</i> SH1000 <i>P3:lux</i>	<i>sarA::ermC^R</i>	This study
Δ <i>agr</i> SH1000 <i>P3:lux</i>	<i>agr::tetM</i>	This laboratory
TS2	Clinical isolate, <i>agr</i> -II	(Sloan, 2014)
TS3	Clinical isolate, <i>agr</i> -IV	(Sloan, 2014)
TS5	Clinical isolate, <i>agr</i> -II	(Sloan, 2014)
USA300 JE2	CA-MRSA, <i>agr</i> -I	NARSA
USA300 LAC	CA-MRSA, <i>agr</i> -I	Ruth C. Massey, University of Bath, UK
ROJ143	RN4220 <i>agr::tetM+P3:luxCDABE pSKermP2:agrCA-1</i>	(Jensen, 2008)
EM28	RN4220 <i>agr::tetM+P3:luxCDABE pSKermP2:agrCA-1I</i>	This laboratory
ROJ154	RN4220 <i>agr::tetM+P3:luxCDABE pSKermP2:agrCA-1II</i>	(Jensen, 2008)
ROJ145	RN4220 <i>agr::tetM+P3:luxCDABE pSKermP2:agrCA-1V</i>	(Jensen, 2008)
S116K	ROJ143 <i>pSKermP2:agrC-I(S116K):agrA-I, cml^R</i>	This laboratory
T197K	ROJ143 <i>pSKermP2:agrC-I(T197K):agrA-I, cml^R</i>	This laboratory
L205R	ROJ143 <i>pSKermP2:agrC-I(L205R):agrA-I, cml^R</i>	This laboratory

Table 2.4 *E. coli* strains that were used in this study

Strain	Genotype/note	Source/reference
TOP10	laboratory cloning strain	Thermo Fisher Scientific
BL21	recombinant protein expression strain	NEB

2.3 Phage transduction.

Mutants generated in this study by transduction using phage phi11 (Φ 11) as described below.

2.3.1 Preparation of phage lysate

Donor *S. aureus* was grown overnight at 37 °C, 200 rpm in 5 ml LK broth with suitable antibiotics. 0.5 ml of an overnight culture was mixed with 5 ml phage buffer and 5 ml LK broth without antibiotics in a 250 ml flask and subsequently various volumes of phage Φ 11 ranging from 10 - 100 μ l added. The mixture was incubated at 30 °C, on a rotary shaker at 30 rpm until the mixture became clear, usually from 4 h to overnight. The phage lysate was collected and filtered through 0.2 μ m syringe filter and stored at 4 °C until required.

LK broth

Tryptone	10 g
Yeast extract	5 g
KCl	7 g
Dissolve in molecular biology grade water and adjusted to	1 l
Autoclave at 121 °C for 15 min	

Phage buffer

MgSO ₄	1 mM
CaCl ₄	4 mM
Tris-base pH7.8	50 mM
NaCl	0.6 % (w/v)
Gelatin	0.1 % (w/v)

Dissolve in distilled water

Autoclave at 121 °C for 15 min

2.3.2 Phage transduction

A recipient *S. aureus* strain was cultured overnight in LK broth. 0.5 ml of overnight culture was mixed with 1.5 ml LK broth, 10 mM CaCl₂ and various volumes of phage lysate ranging from 0 to 200 µl. The mixture was incubated without shaking at 37 °C for 25 min and followed by incubation at 37 °C, with shaking at 200 rpm for 15 min. 1 ml of ice-cold 20 mM trisodium citrate was added and the mixture was placed on ice for 5 min. Subsequently, the mixture was centrifuged at 7,600 x g. The supernatant was discarded and the cell pellet was suspended with 1 ml of 20 mM trisodium citrate. The cell suspension was incubated on ice for 4 - 5 h before plating on TSB agar (Oxoid) containing 20 mM trisodium citrate and suitable antibiotics for selection. The colonies obtained were streaked on to TSB agar containing 20 mM trisodium citrate and the antibiotic(s) required for selection.

LK agar

Tryptone	10 g
Yeast extract	5 g
KCl	7 g
Oxoid agar No.1	15 g
Dissolve in distilled water and adjust to	1 l

Autoclave at 121 °C for 15 min

Before use, add sterile 20 mM trisodium citrate and antibiotics as required

2.3.3 Determination of phage titre

This method was used to determine the activity of the phage lysate and susceptibility of a recipient strain to phage. A recipient *S. aureus* strain was grown in 5 ml BHI broth to OD₆₀₀ of 0.5. The phage lysate was serially diluted in phage buffer. 100 µl of diluted phage lysate was mixed with 400 µl of the recipient culture and 50 µl of 1 M CaCl₂. The mixture was incubated at room temperature for 10 min before adding 5 ml phage top agar and overlaying on a phage bottom agar plate. A successful phage lysate should make 10⁻⁷ to 10⁻¹⁰ plaque forming units per ml.

Phage agar

Casamino acids (Becton BD)	3.0 g
Yeast extract	3.0 g
NaCl	5.9 g
Oxoid agar No.1	10.0 g for phage bottom agar
Oxoid agar No.1	3.3 g for phage top agar

Dissolve and adjust the volume to 1 l

Autoclave at 121 °C for 15 min

2.3.4 Confirmation of Transduction

Transductants were validated by testing their antibiotic resistance and by PCR utilising primers that annealed at a distance up and downstream of the target gene deletion. The primers used are listed in Table 2.5. PCR reactions were set-up as described in section 2.5.1

2.4 Blood agar plate

To investigate their haemolytic activity, bacteria were cultured at 37 °C overnight on tryptic soy broth agar plate supplemented with 5 % defibrinated rabbit blood (TCS BioScience) or 5 % defibrinated sheep blood (TCS BioScience) as indicated in the Results. Haemolysin was visualized as a clear zone around the bacterial colonies.

2.5 General cloning techniques

2.5.1 Polymerase chain reaction (PCR)

PCR was performed for amplifying target genes or validating transductants. Phusion DNA polymerase (Life Technologies) a high-fidelity enzyme was used in all reactions. A 25 µl total reaction volume was composed of 1 x Phusion HF buffer, 200 µM dNTPs, 1 µl of each 10 µM forward and reverse primer, 0.5 units of Phusion DNA polymerase and 1 - 100 ng DNA

template. All primers were synthesised by Sigma-Aldrich. A list of primers is shown in Tabel 2.5.

Table 2.5 Primers

Primer	Sequence	Note
YJ1	ACA CTT TAA CGT GCT ATA GAT TG	forward primer for validating <i>sarA</i> mutant; this study
VF173	ATT ATA GTT CAA TTT CGT TGT TTT C	reverse primer for validating <i>sarA</i> mutant; Victoria Steele's unpublished's data
YJ2	AGC ACT GAG TCC AAG GAA AC	forward primer for validating <i>agr</i> mutant; this study
YJ3	GAT AGT CCG ATG AGA GAT GC	reverse primer for validating <i>agr</i> mutant; this study
YJ10	AAA AAG <u>AGC TCA</u> TGA AAA TTT TCA TTT GCG AAG ACG ATC C	forward primer for amplifying <i>agrA</i> gene for protein expression; underlined, <i>SacI</i> restriction site; this study
YJ12	AAA AAT <u>CTA GAT</u> TAT ATT TTT TTA ACG TTT CTC ACC GAT GCA TAG C	reverse primer for amplifying <i>agrA</i> for protein expression; underlined, <i>XbaI</i> restriction site; this study
YJ44	AAA AAT <u>GTA CA</u> a aaa agc agg ctA GGA GGA a ata aaa aAT GAA AAT TTT CAT TTG CGA AGA CG	forward primer for amplifying <i>agrA</i> gene for constructing inducible AgrA reporter strain; underlined, <i>BsrGI</i> restriction site; bold, Shine-Dalgarno sequence
YJ45	AAA AAT <u>GTA CAT</u> TAT ATT TTT TTA ACG TTT CTC ACC G	reverse primer for amplifying <i>agrA</i> gene for protein expression; underlined, <i>BsrGI</i> restriction site; this study
YJ46	CCT CAT TAA GCA GCT CTA ATG	forward primer for sequencing insert in pUNK plasmid; this study
YJ47	GCG CTA CGG CGT TTC AC	reverse primer for sequencing insert in pUNK plasmid; this study

PCR reactions were performed using T3000 Thermocycler, Biometra. The routine thermocycling conditions were

1 cycle	Initial denaturation	98 °C for 30 s
30 - 35 cycle	Denaturation	98 °C for 10 s
	Annealing	55 - 60 °C for 20 s
	Extension	72 °C for 15 - 30 s/kb
1 cycle	Final extension	72 °C for 10 min

PCR products were analysed via agarose gel electrophoresis with SYBR safe in 1 x TAE buffer to visualize the DNA

50 x TAE electrophoretic buffer

Tris-base	242.00 g
Disodium EDTA	18.61 g
Glacial acetic acid	57.10 ml
Adjust the volume to	1 l

Before use, dilute to 1 x with distilled water

2.5.2 A-tailing

As a high-fidelity DNA Taq Polymerase was used for PCR, PCR products were not routinely A-tailed as standard. In order to ligate the PCR product into a TA cloning vector, A-tailing was required. A 70 µl A-tailing reaction contained 50 µl of purified PCR product, 1 x Gotaq[®] buffer, 1.5 mM MgCl₂, 0.2 mM dATP and 5 U of Gotaq[®]. The reaction was set at 95 °C for 2 min, 72 °C for 10 min, reactions were stored at -20 °C until use.

2.5.3 Ligations

A-tailed PCR products were purified using Wizard[™] SV Gel and PCR Clean-Up System (Promega) before ligating into either pGEM-T or pGEM-T easy vector (Promega). Ligation mixtures contained 1 x ligation buffer, 50 ng of vector, 3 Weiss units of T4 DNA ligase and the PCR product. The process was undertaken at 16 °C for 16 h. The ligation products were transformed into *E. coli* TOP10 competent cells as describe below. Plasmids from transformants were

isolated using a GenElute Plasmid Mini-Prep (Sigma). To confirm plasmids were as expected, vectors were treated with restriction enzymes by incubating at 37 °C for at least 4 h to overnight, separating the insert from the vector by running on suitable percentage of agarose gel and purified using Wizard™ SV Gel and PCR Clean-Up System (Promega). Inserts were ligated into suitable vectors before the ligation products were transformed into appropriate *E. coli* TOP10. The list of plasmids used is shown in Table 2.6.

Table 2.6 Plasmids

Plasmid	Genotype/note	Ref/source
pGEM-T and pGEM-T easy	cloning vector; <i>amp</i> ^R	Promega
<i>Pxyl/tet:gfp:luxCDABE</i> -pUNK	<i>ermC</i> ^R	Beth Mill's thesis
<i>Pxyl/tet</i> -pUNK	<i>ermC</i> ^R	This study
<i>Pxyl/tet:agrA-I</i> -pUNK	<i>ermC</i> ^R	This study
pCOLD I	Expression vector; <i>amp</i> ^R	Takara
<i>agrA-III</i> -pCOLD I	<i>agrA</i> was amplified from <i>S. aureus</i> MN8	This study

2.5.4 Preparation of *E. coli* competent cells

5 ml of overnight culture of *E. coli* TOP10 in LB broth were inoculated into 500 ml fresh LB broth and cultured at 37 °C, 200 rpm until the culture reached an OD₆₀₀ 0.4 - 0.6. The culture was cooled rapidly by swirling on ice for 30 min. Cells were harvested by centrifugation at 6,000 rpm for 5 min. The cell pellet was washed with ice-cold sterilised distilled water three times before resuspending the cells in 10 % (v/v) glycerol. Cells were mixed gently by repeated pipetting up and down. 50 µl of cell suspension was aliquot into sterile

microcentrifuge tubes and the tubes were snap frozen in liquid nitrogen. The competent cells were stored at -80 °C.

2.5.5 Transformation of *E. coli* by electroporation

Frozen *E. coli* competent cells were thawed on ice before gently mixing with plasmid or ligation product. The mixture of cells and plasmid was transferred to a 1 mm electroporation cuvette (Bio-Rad). Electroporation was carried out at 1.75 kV, 25 µF, 200 Ω. 1 ml of LB broth was immediately added after electroporation. Cells were transferred into a universal tube and incubated at 37 °C, 200 rpm for 1 h before plating on LB agar containing the appropriate antibiotic.

2.5.6 Preparation of competent *S. aureus* cells

5 ml of an overnight *S. aureus* culture grown in BHI broth was inoculated into 500 ml of fresh BHI to give a starting OD₆₀₀ of 0.1. Cells were grown at 37 °C, 200 rpm until OD₆₀₀ reached 0.4 - 0.6. Bacteria were harvested by centrifugation at 6,000 rpm for 5 min at room temperature. The cell pellet was washed with sterile distilled water three times before a further wash with 10 % (v/v) glycerol. The cell pellet was re-suspended in 10 % (v/v) glycerol and left at room temperature for 30 min. Cell suspension was centrifuged, the supernatant discarded and the volume adjusted to approximately 400 µl. 60 µl of cell suspension was aliquoted into sterile microcentrifuge tubes. Competent *S. aureus* cells were snap frozen in liquid nitrogen and stored at -80 °C until required.

2.5.7 Transformation of *S. aureus* by electroporation

Frozen *S. aureus* cells were thawed at room temperature before gently mixing with a plasmid or ligation product, and transferred moved to a 1 mm electroporation cuvette. Electroporation was carried out at 100 Ω , 2.3 kV, 25 μ F. 1 ml of BHI broth was added immediately after electroporation, the cell suspension transferred into a universal tube and incubated at 37 °C, 200 rpm for at least 3 h before plating on BHI agar with the required antibiotic(s).

2.5.8 Plasmid extraction

In this study, GeneElute™ Plasmid Miniprep (Sigma) was used to isolate plasmids from *E. coli* and *S. aureus*. In addition, in order to extract plasmids from *S. aureus*, there was an extra-step to degrade the *S. aureus* cell wall peptidoglycan. 2 ml of overnight culture was collected and re-suspended with 50 μ l sterilised molecular biology-grade water containing 25 μ g of lysostaphin (Sigma), the cell mixture was incubated at 37 °C for 45 min before following the GeneElute™ Plasmid Miniprep manufacturer's protocol. Briefly, 3 - 10 ml of the overnight culture was collected by centrifugation. The cell pellet was re-suspended with 200 μ l resuspension buffer. Cells were lysed by adding 200 μ l lysis buffer and inverting gently 5-7 times and incubated for 5 min. The lysis solution was neutralised by adding 350 μ l neutralization buffer, inverting gently 4-6 times. The mixture was centrifuged at 20,000 x g for 15 min. The supernatant was loaded into a pre-treated binding column before centrifugation at 20,000 x g. The flow-through was discarded and the plasmid attached to the binding column matrix was washed with wash solution. To remove ethanol, the column was dried by centrifugation for 1 min. Plasmid was eluted from the column with

50 µl molecular biology-grade water. Plasmid concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific)

2.6 Construction of an inducible *agrA* reporter

The *agrA* gene was amplified from genomic DNA of *S. aureus* strain RN6390B by PCR using the primer pair YJ44 and YJ45 (Table 2.5) following the protocol described above. The PCR product was purified using Wizard® SV Gel and PCR Clean-up System (Promega) and was A-tailed added prior to ligation into the pGEM-T easy vector (Promega) and finally transformed into *E. coli* TOP10. Plasmid was extracted and the *agrA* insert was digested by incubating with the restriction enzyme *BsrGI* at 37 °C for 4 h. The insert was purified using the gel extraction kit Wizard® SV Gel and PCR Clean-up System (Promega).

The destination vector was prepared by digesting *Pxyl/tet:gfp:luxCDABE*-pUNK (Mills, 2014) with *BsrGI* at 37 °C for 4 h to remove the *gfp:luxCDABE* cassette. Digested plasmid was purified by gel extraction. Subsequently, the digested plasmid was dephosphorylated using Antarctic phosphatase (NEB) and purified using Wizard® SV Gel and PCR Clean-up System (Promega).

The *agrA* insert was ligated into *Pxyl/tet*-pUNK using T4 DNA ligase prior to transformation into *E. coli* TOP10. The presence of the *agrA* insert was initially checked by PCR using primer pair YJ46 and YJ47 (Table 2.5) before being sent for sequencing by Source Bioscience. After DNA sequencing had confirmed the construct was correct, the plasmid was transformed into *S. aureus*

RN4220, an intermediate strain, and subsequently extracted and transformed into the SH1000 Δagr $P3:lux$ reporter strain.

2.7 Measuring *agrP3* promoter activity by luminescence

2.7.1 AgrCA reporter strains: ROJ143, EM28, ROJ154 and ROJ145

ROJ143, EM28, ROJ154 and ROJ145 are RN4220 $\Delta agr::P3:luxCDABE$ derivatives harbouring a plasmid containing $P2:agrCA$ group I to IV, respectively (Table 2.3). These reporter strains require cognate AIPs to activate *agr* to generate bioluminescence. The concentrations of AIPs used in specific experiments are indicated in the relevant results section.

To evaluate the fluorescence of reporter strains, cells were cultured in a black polystyrene 96 - well flat bottom plate (Greiner) at 37 °C under static conditions in a microplate reader (Infinite 200, Tecan). Growth of reporter strains was measured at OD₆₀₀. Luminescence was evaluated at an integration time of 2,000 ms at every 30 min for over 16 h. The activity of $P3$ promoter was determined from the maximal luminescence signal divided by OD₆₀₀.

2.7.2 Constitutive AgrC reporter strains: S116K, T197K, L205R

These reporter strains were pAgrCA *agr*-I containing the indicated *agrC* mutation (Table 2.3) that constitutively activate AgrC-dependent *agrP3* promoter activity in the absence of cognate AIP. Therefore, no AIP-1 was added to these cultures unless indicated. Bacterial growth conditions and plate reader settings were as used with the AgrCA reporter strain (subsection 2.7.1)

2.7.3 Inducible AgrA reporter strain

This reporter strain was constructed as described in section 2.6. 250 ng/ml of anhydrotetracyclin was added to activate the expression of *agrA* to induce the *P3* promoter and hence bioluminescence. As the bioluminescence signal of this reporter strain was low, the integration time for luminescence was increased to 4,000 milliseconds. Data was evaluated as area under the curve divided by optical density.

2.8 Construction and purification of recombinant AgrA protein

The open reading frame of *agrA* was amplified from *S. aureus* MN8 genomic DNA using primers YJ10 and YJ12 (Table 2.5). The PCR product was A-tailed and purified using Wizard® SV Gel and PCR Clean-up System (Promega) before ligating into pGEM-T easy vector and transforming into *E. coli* TOP10. The plasmid was isolated using Sigma GenElute™ Plasmid Miniprep. Inserts and pCOLD I (Takara) were digested with *SacI* and *XbaI* (Promega) at 37 °C for 4 h. Insert and vector were separated from unwanted DNA by running in a 2 % and 0.8 % agarose gel respectively, before purification using Wizard® SV Gel and PCR Clean-up System (Promega). Inserts were ligated into digested pCOLD-I at 16 °C for 16 h before transformation into *E. coli* TOP10. Plasmid was isolated from putative transformants using the GenElute™ Miniprep kit (Sigma) and sent to Source Bioscience for sequencing.

A plasmid with the correct *agrA* DNA sequence was transformed into *E. coli* BL21 and cultured on LB agar containing 100 µg/ml ampicillin at 37 °C. A single colony was picked and grown in LB broth containing 50 µg/ml carbenicillin at 37 °C, 200 rpm. The overnight culture was adjusted to an OD₆₀₀ of 0.01 in 500 ml Terrific broth with 50 µg/ml carbencillin. To overcome the

insolubility of recombinant AgrA protein, the medium was supplemented with 1 M sorbitol. The culture was incubated until OD₆₀₀ 1.0 - 1.2 and chilled at 15 °C for at least 30 min. Expression was induced by adding 0.5 mM IPTG and the culture was incubated for 18 h at 15 °C, 200 rpm.

E. coli BL21 cells that expressed recombinant AgrA were collected and washed with PBS three times before re-suspending in 35 ml sonication buffer (20 mM sodium phosphate pH 7.4, 50 mM NaCl, 20 mM imidazole). To degrade the cell membrane, cells were freeze – thawed by immersing in liquid nitrogen for 15 min before slowly thawing at -80 °C for 10 min and then completely thawed at room temperature. In order to inhibit protease activity, one cOmplete™, mini, EDTA-free Protease Inhibitor Cocktail Tablet (Roche) was added to the solution. Cells were sonicated at 12 amplitude microns for 10 min (15 s on; 15 s off). Sodium chloride was added and adjusted to 500 mM in order to stabilise the recombinant protein. Cell debris was removed by centrifugation at 5,000 x g at 4 °C for 10 min. The insoluble fraction was isolated by centrifugation at 34,000 x g at 4 °C for 30 min.

Expression vector pCOLD-I system (Takara) contains a hexa-histidine tag at the 5' end of the multiple cloning site allowing purification by nickel affinity chromatography. HiTrap HP column (GE Healthcare) charged with 0.1 M NiSO₄ was used. After loading the sample, the column was washed with 20 ml of binding buffer pH 7.5 (20 mM sodium phosphate pH 7.4, 0.5 M sodium chloride, 20 mM imidazole). Recombinant protein was eluted with 40 ml of 10 – 100 % elution buffer pH 7.5 (20 mM sodium phosphate pH 7.4, 0.5 M sodium chloride, 500 mM imidazole) using an ÄKTA system (GE Healthcare Life Sciences) and 2 ml fractions collected. To investigate expression and protein

purification, each fraction was mixed with SDS-PAGE loading dye and heated at 95 °C for 5 min before run on a 12 % SDS-PAGE and stained with Coomassie blue. Fractions that contained soluble recombinant AgrA were added to a Vivaspin6 MWCO 10000 system column (GE Healthcare) and centrifuged at 5,000 x g for 30 min at 4 °C to remove excess NaCl and imidazole. Desalted recombinant AgrA was supplemented with 10 mM Tris-HCl pH 7.5 and 10 % glycerol and stored at -80 °C until required. Protein was thawed once and kept at 4 °C or on ice. After thawing, protein was used within 2 weeks.

Terrific broth

Tryptone	6 g
Yeast extract	12 g
Glycerol	2 ml
Distilled water	450 ml
Autoclave at 121 °C for 15 min	
Then, add	50 ml terrific buffer

Terrific buffer

KH ₂ PO ₄	4.63 g
K ₂ HPO ₄	25.08 g
Distilled water	200 ml
Autoclave at 121 °C for 15 min	

Sonication buffer

0.1 M sodium phosphate pH 7.4	10.0 ml
5 M NaCl	0.5 ml
5 M imidazole	0.5 ml
Distilled water	39.0 ml

Binding buffer

0.1 M sodium phosphate pH 7.4	100 ml
5 M NaCl	50 ml
5 M imidazole	5 ml
Distilled water	300 ml

Adjust the pH with HCl to pH 7.4 - 7.6

Adjust the volume to 500 ml with distilled water

Filter the buffer using a 0.4 μ m filter before use

Elution buffer

0.1 M sodium phosphate pH 7.4	100 ml
5 M NaCl	50 ml
5 M imidazole	50 ml
Distilled water	300 ml

Adjust pH with HCl to 7.4 - 7.6

The volume was adjusted to 500 ml with distilled water

Buffer was filtered using a 0.4 μ m filter

2.9 Protein sample preparation

To investigate the protein profile and expression of a specific protein in a sample, bacterial cultures were centrifuged at 8,000 x g for 2 min. The supernatant containing the secreted proteins was harvested from cultures grown to an OD₆₀₀ 4.0. For cellular protein analysis, cultures were diluted to OD₆₀₀ 2.5, centrifuged and the cell pellet collected.

Secreted proteins from supernatant samples were precipitated using 20 % (w/v) trichloroacetic acid (Sigma) at -20 °C overnight. The precipitated solution was thawed at room temperature and centrifuged at 20,000 x g for 15 min. The protein pellet was washed with ice-cold acetone three times. The protein pellet was air-dried at room temperature for 5 min before adding 1 x SDS-PAGE loading dye and heating at 95 °C for 5 min.

Intracellular and membrane-bound proteins were prepared by washing the cell pellet with PBS pH 7.4 three times before lysing the cells with 25 µg lysostaphin in 50 µl molecular biology grade water at 37 °C for 1 h. The sample was mixed with an equal amount of 2 x SDS-PAGE loading dye and heated at 95 °C for 5 min.

Purified recombinant protein was mixed with 2 x SDS-PAGE loading dye before heating at 95 °C for 5 min.

2 x SDS-PAGE sample loading dye

0.5 M Tris-base pH6.8	2.5 ml
Glycerol	2.0 ml
10 % (w/v) SDS	4.0 ml
1 % (w/v) bromophenol blue	50.0 µl

Distilled water	1.5 ml
β -mercaptoethanol	1.0 ml
Ready to use with supernatant samples and recombinant protein samples	
Dilute to 1 x before used with cell lysate samples	

2.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie blue staining and Western blot

Twelve percent acrylamide SDS-PAGE was used routinely to investigate the protein profiles and expression of recombinant AgrA. 12 % SDS-PAGE gels were made as described below. SDS-PAGE was run at 200 V until the loading dye reached the bottom of the gel. SDS-PAGE was stained with Coomassie blue staining (GelCode™ Blue Safe Protein Stain; Thermo Scientific).

SDS-PAGE gel recipe

Separating gel - 1 gel

Reagent	12%
Distilled water	1.60 ml
30 % acrylamide	2.00 ml
1.5 M Tris-base pH 8.8	1.25 ml
10 % SDS	50.00 μ l
10 % (w/v) ammonium persulfate	50.00 μ l
TEMED	7.50 μ l

After adding the APS and TEMED, the mixture was poured immediately and a layer of water was gently added to the top of the gel.
Once the separating gel set, water was removed and the stacking gel added.

Stacking gel for 1 – 3 gels

Reagent	
Distilled water	2.50 ml
30 % acrylamide	0.83 ml
0.5 M Tris-base pH6.8	0.62 ml
10 % SDS	50.00 µl
10 % (w/v) ammonium persulfate	50.00 µl
TEMED	5.00 µl

After mixing APS and TEMED, the mixture was poured immediately and a comb inserted. When the gel became solid, the comb was gently removed.

10 x SDS-PAGE running buffer

Glycine	144.0 g
Tris-base	30.3 g
SDS	10.0 g
Dissolve in distilled water and adjust the volume to	1 l

Dilute 1 x with distilled water before use

Western blotting was carried out to determine the presence of a specific protein. After running samples on an SDS-PAGE gel, proteins were transferred to 0.4 µm nitrocellulose membrane (Thermo Scientific) at 100 V for 60 min using electrophoresis power supply (Bio-Rad).

To detect toxic shock syndrome toxin (TSST-1) a purified sheep anti-TSST-1 polyclonal antibody as described below in 5 % (w/v) skim milk (Oxiod) in Tris-buffered saline-tween (TBS-T) as primary antibody by incubating at room temperature for 1 h. Subsequently, 1:5,000 Protein A conjugated with horse radish peroxidase (Sigma) in 5 % skim milk in TBS-T was used as a substitute ‘secondary antibody’ and incubated at the room temperature for 1 h.

Alpha-haemolysin was detected by incubation with 1:5,000 anti α -haemolysin monoclonal antibody (generous gift from Prof. Lindy Durrant, School of Medicine, University of Nottingham) in 5% (w/v) skim milk (Oxoid) in Tris-buffered saline-tween (TBS-T) as primary antibody by incubating at the room temperature for 1 h and 4 °C overnight before incubating with secondary antibody, 1:5,000 Protein A conjugated with horseradish peroxidase (Sigma) in 5 % skim milk in TBS-T at the room temperature for 1 h..

Protein A was detected by incubation with 1:5,000 goat anti-rabbit IgG (whole molecule) monoclonal antibody conjugated with peroxidase (Sigma) at the room temperature.

Recombinant proteins which were expressed using the pCOLD-I vector containing a histidine tag were detected using 1:5,000 Penta His Antibody (Qiagen) in 3 % BSA. Primary antibodies were incubated overnight at 4 °C, followed by secondary antibody incubation of 1:5,000 anti-mouse immunoglobulin G (whole molecule) conjugated with horseradish peroxidase (Sigma) in 5 % skim milk. The ECL™ Western Blotting Detection Reagent (GE Healthcare) was used as a substrate. Alternatively, ECL™ Prime Western Blotting Detection Reagents (GE Healthcare) was used when appropriate.

Transfer buffer

Tris-base	3 g
Glycine	15 g
Methanol	200 ml

Dissolve and adjust the volume with distilled water to 1 L

20 x TBS-T buffer

Tris-base 60.57 g

NaCl 84.66 g

Adjust pH to 7.6 using HCl

Volume was adjusted to 500 ml using distilled water and 500 µl of Tween-20

2.11 Purification of sheep anti-TSST-1 polyclonal antibody

Purification of sheep anti-TSST-1 polyclonal antibody was undertaken using the following protocol. *S. aureus* RN6390B, a non-producing TSST-1 strain, was cultured until stationary phase (OD₆₀₀ 0.1 - 1.2). The supernatant of the culture was collected by centrifugation at 8,000 x g and filtered using 0.2 µm filter. Secreted proteins in the supernatant were concentrated using a 10K centrifugal filter (Millipore) according to the manufacturer's protocol. Sheep anti-TSST-1 polyclonal antibody was mixed with the concentrated RN6390B supernatant and incubated at 4 °C and shaken slowly at 10 rpm overnight. Finally, the purified antibody was diluted down to 1 in 1,000 in 5 % skim milk dissolved in TBS-T buffer. The antibody was stored at -20 °C until used. The antibody could tolerate up to 10 freeze-thaw cycles. After use the antibody was kept at -20 °C.

2.12 Bradford assay for protein quantification

QuickStart™ Bradford 1 X Dye Reagent (Bio-Rad) was used to quantify purified recombinant proteins. The manufacturers' microassay protocol was followed. Briefly, the Bradford reagent was taken out from the cold room and stood at room temperature for at least 30 min before use. Bovine serum albumin

(BSA) which was used as a standard was dissolved in molecular biology-grade water and serially diluted to generate a standard curve. 150 µl of the reagent was mixed with 150 µl of BSA standard or protein sample. 200 µl of the mixture was added into the well of a transparent flat-bottom 96 - well plate (Thermo Scientific). The reaction was incubated at the room temperature for at least 5 min, but no longer than 1 h. The OD₅₉₅ of each reaction was measured using an InfiniteM200 Pro (Tecan i-Control) plate reader.

2.13 Electrophoretic mobility shift assay (EMSA)

2.13.1 Probes

The *agrP2*-P3 intergenic region (~120 bp) and the *agrP2* promoter (48 bp) probes were constructed using primers and oligonucleotides in Table 2.7 and synthesized by Eurofins.

PCR was performed in order to construct the *agrP2*-P3 intergenic probe. 50 µl of PCR reaction consisting of 1 x Phusion HF buffer, 200 µM dNTPs, 0.5 µM of YJ69 and YJ64, 1 U of Phusion DNA polymerase and ~100 ng of MN8 genomic DNA as template. The thermocycling conditions were composed of initial denaturation at 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 58 °C for 20 s, 72 °C for 15 s; and final extension at 72 °C for 10 min. The probe was purified using Wizard[®] SV Gel and PCR Clean-up System (Promega) before the concentration of the *agrP2*-P3 probe was measured using a Nanodrop machine (Thermo Scientific). The *agrP2*-P3 specific inhibitor was made using the same protocol as the probe but using YJ62 and YJ64.

The *agrP2* probe was constructed by mixing 10 µM of YJ65 and YJ66 and annealed using a PCR thermocycler machine. The annealing conditions were

95 °C for 5 min and the temperature was reduced by 1 °C every minute until 25 °C was reached. The annealed oligonucleotides were loaded onto a 2 % agarose gel to purify oligonucleotides using the Wizard® SV Gel and PCR Clean-up System (Promega). Probe concentration was measured using a Nanodrop (Thermo Scientific). The *agr*P2 specific inhibitor was made using the same protocol but using primer pair YJ70 and YJ66 (Table 2.7).

Table 2.7 Primers for constructing EMSA probes

Primer/ oligomer	Sequence	Note
YJ69	AAT TTT TCT TAA CTA GTC GTT TTT TAT TC	5' ATTO700-labelled forward primer for amplifying P2-P3 intergenic region; adapted from (Koenig et al., 2004)
YJ64	CAT TAT ATT GCC TAA CTG TAG G	Reverse primer for amplifying P2-P3 intergenic region; adapted from (Koenig et al., 2004)
YJ62	AAT TTT TCT TAA CTA GTC GTT TTT TAT TC	Unlabelled YJ69 for constructing competitor; adapted from (Koenig et al., 2004)
YJ65	TACATTTAACAGTTAAGTATTTATTTCTACAGTTAGGCAATATAATG	5' ATTO700-labelled P2 oligomer; (Koenig et al., 2004)
YJ66	CATTATATTGCCTAACTGTAGGAAATAAATACTTAAGTTAAATGTA	Complementary strand of P2; (Koenig et al., 2004)
YJ70	TACATTTAACAGTTAAGTATTTATTTCTACAGTTAGGCAATATAAT	Unlabelled P2 oligomer; (Koenig et al., 2004)

2.13.2 EMSA assays

Native Tris-glycine gels were made and pre-run in Tris-glycine buffer at 120 V in a cold room until the current is invariant. The competitor compound was incubated with the recombinant AgrA protein at the indicated concentration for 5 min at the room temperature prior to adding a probe and incubated for another 10 min at room temperature. The samples were mixed with 6 x EMSA loading dye and immediately loaded into the Tris-glycine gel. Electrophoresis was started at 200 V, 10 mA before reducing the voltage to 120 V and running for 2 h. The results were analysed using an Odyssey® Imaging System (LI-COR) at a wavelength 700 nm.

Native Tris-glycine gel

Distilled water	2.7 ml
30 % acrylamide	2.0 ml
50 % glycerol	0.6 ml
1.5 M Tris-base pH 8.8	1.2 ml
10 % (w/v) ammonium persulfate	60.0 µl
TEMED	6.0 µl

5 x Tris-glycine running buffer

Tris-base	16.3 g
Glycine	72.0 g
EDTA, disodium salt	1.8 g
Distilled water	1 L

Dilute to 1 x with distilled water before use

Reaction buffer, adapted from (Morfeldt et al., 1996)

Sterilised molecular-graded water	28 µl
50 % glycerol	20 µl
100 mM Tris-base pH 7.5	20 µl
10 mM EDTA pH 7.5	20 µl
1 M NaCl	10 µl
100 mM dithiothreitol (DTT)	2 µl

6 x EMSA loading dye

50 % glycerol	1 ml
0.5 % bromophenol blue	1 µl

2.14 Differential scanning fluorimetry

This technique is used to study protein folding and stability by detecting the emission of intrinsic fluorescence of native tryptophan and/or tyrosine residues. Here, the stability of 600 nM recombinant AgrA in the presence and absence of the IQS analogue IQS3 at 0.1 and 1 mM. IQS3 was added directly into the recombinant AgrA in the storage buffer without additional buffer. Protein stability and unfolding were analysed using the Prometheus NT.48 (NanoTemper Technologies) where the protein samples were heated from 15 – 95 °C and fluorescence detected at wavelength of 330 nm at which tryptophans and tyrosines buried in the hydrophobic centre of a protein fluoresce at this wavelength whereas tryptophan and tyrosine residues emit at 350 nm when they are in hydrophilic environment. Untreated and DMSO-treated recombinant AgrA proteins were used as negative controls.

2.15 Chrome Azurol S (CAS) assay

A CAS assay was used to evaluate the ferric iron chelating activity of IQS analogues. The method was adapted from (Schwyn and Neilands, 1987). Briefly, a 40 mM stock of FeCl_3 was diluted to 1 mM in 10 mM HCl. Then, 375 μl of 1 mM FeCl_3 was mixed with 1.875 ml of 2 mM Chrome Azurol S (CAS, Sigma-Aldrich) solution, 12.5 ml of 438 mg/l Hexadecyltrimethyl ammonium bromide (CTAB, Molecular Sigma Biology), 9.2 ml of 117.25 g/l piperazine (pH5.6), 1.05 of distilled water and 500 μl of 0.2 M 5-sulfosalicylic acid. The CAS assay solution was filtered through a 0.2 μm filter and used immediately. 500 μl of test compound was mixed with 500 μl CAS assay buffer and incubated at room temperature for 15 min to equilibrate the reaction. 500 μl of distilled

water was mixed with the buffer as a reference solution. A 50 μ M solution of deferoxamine mesylate salt (Sigma) was used as positive control. Distilled water was used as a blank. The Absorbance (A_{630}) at 630 nm was used to obtain data. To calculate the iron chelation unit, the following calculation was used.

$$\% \text{ Iron chelation unit} = 100 \times (\text{OD}_{\text{reference}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{reference}}$$

2.16 Haemolysin assay

An overnight culture of *S. aureus* was inoculated at the ratio of 1:100 and grown for 2 – 3 h at 37 °C and 200 rpm. Cells were diluted into TSB broth to an OD_{600} of 0.05 in the absence or presence of test compounds at a range of concentrations. The culture was grown at 37 °C, 200 rpm. At 6 h and 16 h, the culture was harvested and centrifuged at 12,000 x g for 30 s. 100 μ l of the supernatant were mixed with 900 μ l PBS and 15 μ l defibrinated rabbit blood (TCS biosciences) and incubated at 37 °C for 15 min. 100 μ l of 10 % SDS was used as a positive control and the activity was adjusted to 100 %. The mixture was centrifuged at 12,000 x g for 20 s. The supernatant was collected and the absorbance at 542 nm was measured using a spectrophotometer.

2.17 Preparation of RNA samples and transcriptomic studies

2.17.1 RNA extraction and preparation

Overnight *S. aureus* USA300 LAC cultured in TSB broth was washed with PBS three times and inoculated into 5 ml fresh TSB broth, adjusting the OD_{600} to 0.05. The culture was grown in the presence and absence of 100 μ M IQS3 or IQS11 at 37 °C, 200 rpm for 4.5 h. The negative control groups were a 0.1 % DMSO-treated wild-type and Δ *agr* USA300 LAC. Cells were collected

and adjusted to an OD₆₀₀ of 0.65. The *S. aureus* cell wall was digested using 10 µg lysostaphin (Sigma) at 37 °C for 15 min. The RNA was subsequently extracted using RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. RLT buffer was supplemented with β-mercaptoethanol to prevent RNase activity according to the manufacturer's protocol. 50 µl of sterilised RNase-free molecular-biology grade water was used to elute the RNA from the column.

To eliminate genomic DNA contamination, the RNA samples were treated with TURBO DNA-free™ DNase (Ambion). The final concentration of the reaction contained 1 x TURBO™ DNase buffer and 2 Units of TURBO™ DNase. The reaction was incubated at 37 °C for 30 min before another 2 Units of TURBO™ DNase were added and the reaction was incubated at 37 °C for another 30 min. To remove the TURBO™ DNase, the reaction products were purified using the RNeasy kit (Qiagen). RLT buffer was supplemented with β-mercaptoethanol following the manufacturer's protocol. The RNeasy column was eluted with 30 µl of molecular biology-grade water. Genomic DNA contamination was assessed by PCR using YJ27 and YJ28 to amplify the *rrsA* gene encoding the 16s rRNA (Table 2.8). The quality of RNA was determined using RNA6000 Pico Kit (Agilent) and 2100 Bioanalyzer System (Agilent). The concentration of RNA was measured using a Nanodrop spectrophotometer. A suitable RNA concentration for RNA-Seq must be at least 200 ng/µl and total amount had to be more than 2 µg. The RNA sample was aliquoted and stored at -80 °C until RNA-Seq was carried out by Deep Sequencing facility, Queen's Medical Centre, University of Nottingham.

2.17.2 Complementary DNA library construction and differential gene expression analysis

Complementary DNA (cDNA) library preparation and sequence analysis was carried out by the Deep Sequencing Facility, Queen's Medical Centre, University of Nottingham. In brief, the quality of RNA was assessed using a RNA6000 Pico Kit (Agilent) and 2100 Bioanalyzer System (Agilent) and quantified using a Qubit RNA BR assay kit (Life technologies). Ribosomal RNA was removed from 1 µg of total RNA using Ribo-Zero rRNA Removal Kit (Illumina). Illumina stranded whole transcriptome sequencing libraries were prepared using NEBNext Ultra Directional RNA library prep kit for Illumina (NEB) that requires NEBNext Multiplex Oligos for Illumina (Index Primers Sets 1 and 2, NEB). Library QC was performed using a bioanalyser HS kit (Agilent biotechnologies) and quantified using qPCR (Kapa Biosystems). The libraries were pooled at the desired concentration, denatured and loaded for sequencing according to manufacturer's instructions. Sequencing was performed on the Illumina NextSeq500 sequencing platform to generate 2 x 75 bp reads. The 3' end-adapters were trimmed by Scythe and poor quality bases were also removed from the reads by Sickle. Reads were aligned with tRNA and rRNA genes from the reference genome using software TopHat2 so that they could be removed from the data set. The RNA reads that did not align to tRNA and rRNA sequences were subsequently aligned to the reference sequence using TopHat2. Only unique paired reads were used in downstream analysis. The reference sequence used was strain USA300 FR3757 as it is closely related to USA300 LAC (Kennedy et al., 2008). MAP20Q was used to generate counts per gene. DESeq2 (Love et al., 2014) was used for differential expression analysis .

The function of differentially expressed genes was categorised according to data in three databases, ncbi (<http://www.ncbi.nlm.nih.gov>), UniProt (<http://www.uniprot.org/>) and GenomeNet (<http://www.genome.jp/>).

2.18 Validation of RNA-Seq analysis

2.18.1 Reverse transcription

RNA samples were reversed transcribed using SuperScript™ III (Invitrogen). The manufacturers' protocol was followed. Briefly, 1 µg of DNase-treated RNA sample was mixed with 1 µl of 100 µM Random Hexamer Primer (Thermo Scientific), 1 µl of 10 mM dNTPs mix (Invitrogen) and sterile RNase-free molecular-biology grade water was added to bring the volume to 13 µl. The mixture was incubated at 65 °C for 5 min after that, samples were immediately chilled on ice for at least 1 min before brief centrifugation. 4 µl of 5 x First-strand buffer, 1 µl of 0.1 M dithiothreitol (DTT) and 1 µl of 200 Units of SuperScript™ III were added to the reaction mix. The reaction mixture was heated to 25 °C for 5 min then at 50 °C for 60 min. Reactions were inactivated at 70 °C for 15 min. 80 µl of sterile molecular-grade water was added to the cDNA mixture to dilute the DTT which is an inhibitor of real-time PCR (Wong and Medrano, 2005).

2.18.2 Real-time reverse transcription-PCR (qRT-PCR)

POWER SYBR® Green PCR Master Mix (Applied Biosystems) was routinely used for qPCR. Each 20 µl reaction was composed of 125 nM of each primer (Table 2.8) and 1 µl of cDNA template. Samples were incubated at 95 °C for 10

min, conditions were 35 cycles of 95 °C for 15 s; 62 °C for 30 s and 72 °C for 30 s, finally, a melt step was carried out at 72 °C to 95 °C by incrementally raising 0.5 °C at each step with a hold for 60 s on the first step and 5 s for each step afterwards, using RG-3000 Real-time PCR System (Corbett Research). Gene expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Wong and Medrano, 2005). The calculation formula is shown below. The housekeeping gene *gyrA* was used as an internal control (Eleaume and Jabbouri, 2004).

$$\Delta\Delta Ct = (Ct_{\text{gene of interest}} - Ct_{\text{internal control}})_{\text{sample A}} - (Ct_{\text{gene of interest}} - Ct_{\text{internal control}})_{\text{sample B}}$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Where sample A is the treated sample and sample B is untreated sample

Table 2.8 Primers used for qRT-PCR

Primer	Sequence	Note
YJ27	TGA GTG CAG AAG AGG AAA GTG	forward primer for amplifying <i>rrsA</i>
YJ28	CAC ATC AGC GTC AGT TAC AG	reverse primer for amplifying <i>rrsA</i>
YJ35	ATG CAA TTG GTA GTC ATC ACG	forward primer for amplifying <i>hla</i>
YJ36	TTT TCA CCA GAC TTC GCT AC	reverse primer for amplifying <i>hla</i>
YJ39	GTA CGT ATG GCT CAA GAT TTC	forward primer for amplifying <i>gyrA</i>
YJ40	ATTTTAGTCATACGCGCTTCAG	reverse primer for amplifying <i>gyrA</i>
YJ41	CAT TTT GTT GAG CTT CAT CGT G	forward primer for amplifying <i>spa</i>
YJ42	GTA AAC TAG GTG TAG GTA TTG C	reverse primer for amplifying <i>spa</i>
YJ50	TTG ACT ACC TCA GCG CCA TC	forward primer for amplifying <i>lukS-PV</i>
YJ51	ACT ATT AGC TGC AAC ATT GTC G	reverse primer for amplifying <i>lukS-PV</i>
YJ56	TAA TGG CGA AAC GAG TGT GG	forward primer for amplifying <i>clfA</i>
YJ57	GCG TTT CTT CCG TAG TTG C	reverse primer for amplifying <i>clfA</i>
YJ76	CCC TTC ATT TAT CGC CAC AG	forward primer for amplifying <i>hlgC</i>
YJ77	TGT TGC CAT AAT GCG TTG ATC	reverse primer for amplifying <i>hlgC</i>
YJ78	ACC ACA ACA TTT CAT TCG ATT TC	forward primer for amplifying <i>SAUSA300_2310</i>
YJ79	AGG TGT AGT GTG CAT CTT TAA G	reverse primer for amplifying <i>SAUSA300_2310</i>
YJ80	TCC ACT GAA CCC ACA ATC AAC	forward primer for amplifying <i>SAUSA300_2311</i>
YJ81	AAG ACG ACG CCT ATG AGT AAC	reverse primer for amplifying <i>SAUSA300_2311</i>
YJ82	AAT AAA CCA GCA GCG AAA ACT G	forward primer for amplifying <i>efb</i>
YJ83	CTA AGT TGA CTG CCT TTT GTG	reverse primer for amplifying <i>efb</i>

2.18 Cytotoxicity assays

2.18.1 Cultivation of Jurkat T cell line

Jurkat T cells are a human T lymphoblastoid cell line. Jurkats were cultured in RPMI-1640 supplemented with L-glutamine and sodium bicarbonate (Sigma) with 10 % heat-inactivated fetal bovine serum (Sigma), 1 x penicillin-streptomycin (Sigma) at 37 °C and 5 % environmental CO₂.

2.18.2 Live/dead 7-aminoactinomycin D (7-AAD) staining

500 µl of 8×10^5 cells/ml of Jurkat T cells were seeded into polystyrene 24 - well flat bottom plates (Costar) and 500 µl of test compound was added at various concentrations. 200 µM potassium dichromate was used as positive control, whereas 0.5% DMSO was used as a vehicle control. The plate was incubated at 37 °C, 5 % CO₂ for 24 h. Cells were collected by centrifugation at 300 x g, 4 °C for 10 min. Pellets were washed with wash buffer (2 % FBS in Dulbecco's phosphate-buffered saline) twice before re-suspending in 150 µl wash buffer. 100 µl of cells were mixed with 0.2 mg/ml 7-AAD and incubated on ice for 10 min before addition of 300 µl PBS. Flow cytometry was carried out on an LSRII, BD Biosciences instrument. The excitation wavelength was set at 546 nm and emission wavelength was 647 nm. Twenty thousand events for each sample were counted.

2.18.3 Resazurin reduction assay

100 µl of Jurkat T cells at 4×10^5 cells/ml were seeded into polystyrene 96 – well flat bottom plates (Costar) and mixed with 100 µl of compound at a

range of concentrations. 200 µM potassium dichromate was used as positive control, whereas 0.5% DMSO was used as a vehicle control. Cell culture medium was used as an experimental blank. Cultures were incubated at 37 °C, 5 % CO₂ for 24 h. Cells were stained with 20 µl of a 10 x resazurin stock solution. Cultures were continuously incubated for another 4 h at 37 °C, 5 % CO₂ before fluorescence was evaluated by an Infinite M2000 microplate reader (Tecan) at an excitation wavelength of 535 nm and emission wavelength of 595 nm; integration time was 20 µs. Readings from treated cells were calculated using the shown equation below.

$$\text{Respiratory rate (\%)} = [(\text{Fluorescence}_{\text{treated}} - \text{Fluorescence}_{\text{blank}}) \times 100] / \text{Fluorescence}_{\text{negative control}}$$

100 x resazurin

Resazurin sodium salt, cell culture tested (Sigma-Aldrich)	200 mg
Methylene blue (Sigma-Aldrich)	40 mg
Potassium ferricyanide (BDH AnalaR)	33 mg
Potassium ferrocyanide (BDH AnalaR)	42 mg

Dissolve in 100 ml of 1 M potassium phosphate buffer

Filter sterilise 10 ml aliquot using a 0.22 µm filter into sterile 15 ml centrifuge tube

Wrap in aluminium foil and store at 4 °C for up to 6 months

Dilute to 10 x using 1 M Dubblecco's modified phosphate buffered saline before use

2.19 Statistical analysis

All statistical tests were performed using IBM SPSS Statistics. The difference between each sample was assessed using one-way ANOVA. The Shapiro-Wilk was used to test normality of each group. If a tested group was normally distributed, LSD Post Hoc multiple comparison was performed. If not, Games-Howell was performed. P values of less than 0.05 were considered as significant.

Chapter 3: Synthetic rhodanines and quinones reduce *S. aureus* virulence determinant production

3.1 Introduction

Staphylococcus aureus is a Gram-positive opportunistic pathogen. It can infect various types of host cells and tissues from superficial skin to deep-organs causing mild to life-threatening symptoms. Furthermore, this bacterium has acquired multiple antibiotic resistance mechanisms resulting in both nosocomial and community acquired infections resistant to many antibiotics. Vancomycin is the drug of last resort for treating methicillin-resistant *S. aureus* (MRSA) infections, however, the number of vancomycin-resistance *S. aureus* (VRSA) isolates is increasing. All vancomycin resistant isolate have arisen from MRSA and can be found in hospital and community-settings (Walters et al., 2015, Limbago et al., 2014). In 2015 there were up to 14 vancomycin resistance isolates have been reported (Walters et al., 2015). Therefore, new therapeutic agent for curing *S. aureus* is urgent needed.

S. aureus produces several virulence factors in form of exotoxins and cell surface proteins. The primary role of virulence factors is invading and evading the host immune system, scavenging for nutrients and attaching to and destroying host cells (Foster, 2005). For example, toxic shock syndrome toxin (TSST-1) is associated with toxic shock syndrome by causing massive activation of T cells resulting in the induction of a cytokine storm (Xu and McCormick, 2012). Alpha-haemolysin is an exotoxin which is secreted from *S. aureus* and forms an oligomeric β -barrel pore in the membrane of many types of host cells (Menestrina et al., 2001). Protein A is an immunoglobulin-binding protein and is essential for *S. aureus* immune system evasion by binding to Fc region of

antibody or Fab regions of the B cell receptor inhibiting opsonophagocytosis and causing B cell death (Kobayashi and DeLeo, 2013). Since virulence factors are not essential for bacterial fitness and are energetically expensive, their expression is tightly regulated by global regulators, such as *agr* system and SarA protein in a temporal and cell-density dependent manner (Reynolds and Wigneshweraraj, 2011, Ballal and Manna, 2009)

Quorum sensing is a phenomenon that many bacterial species use to communicate to others by detecting the concentration of a signalling molecule that accumulates in the surrounding environment (Thoendel et al., 2011). *S. aureus* employs quorum sensing accessory gene regulator or *agr* responsible for controlling expression of numerous genes including metabolic, transporter and virulence factors (Thoendel et al., 2011, Dunman et al., 2001, Cheung et al., 2011). The *agr* system is described intensively in the general introduction section. It has been shown that the virulence of *S. aureus agr* mutant is attenuated in animal model (Cheung et al., 1994, Blevins et al., 2003, Cheung et al., 2011). This suggests that *agr* could be a target for developing novel therapeutic agent for protecting or curing *S. aureus* infections.

The regulatory DNA binding protein, SarA up-regulates the expression of several virulence factors including toxic shock syndrome (TSST-1) (Andrey et al., 2010) and α -haemolysin toxin (Chien et al., 1999). It also down-regulates many proteins involved in host cell/surface attachment such as protein A (Chien et al., 1999). Animal studies have shown that *sarA* mutants were attenuated for virulence in both mouse (Zielinska et al., 2012) and rabbit infection models (Cheung et al., 1994) confirming that SarA can be another target for a novel anti-infective drug strategy (Gordon et al., 2013).

This chapter describes the screening of a small library of rhodanines and quinones which designed and synthesised by Dr. Fabio Rui (School of Pharmacy, University of Nottingham). All the compounds were designed to target the cysteine residue at position 9 of SarA, in which is the phosphorylated site, and is required for biological activity, but DNA binding (Sun et al., 2012, Liu et al., 2006). Targeting this reactive SarA cysteine residue would inhibit the protein function and may reduce virulence factor production.

The compounds evaluated can be divided into 2 classes based on their core structures, quinone and rhodanine (Table 3.1). Quinone compounds used in this study were MH13/FR02, FR05, FR09, FR33, FR37, MH09 and MH10. Quinones are commonly found in plant and animal cells. They carry out various functions, such as electron transport chain and photosynthesis (Kishikawa and Kuroda, 2014). Anthraquinones which is a subgroup of quinone have been widely used in clinic for the treatment of tumours, as immunosuppressants and anti-inflammatory agent (Malik and Muller, 2016). The other class of compound used in this study was rhodanine which were FR41, FR44 and FR49 belong to. Rhodanines are widely used for drug development as they offer high density of interaction sites for polar interactions and hydrogen bonding (Mendgen et al., 2011).

Here FR37, FR41 and FR44 were found that had the activity in reducing exotoxin and increasing protein A production in *S. aureus*. FR41 and FR44 showed broader activity against different *S. aureus* strains. The target of them was proposed and the problem that is addressing.

Table 3.1: Synthetic quinones and rhodanines were synthesized by Dr. Fabio Rui

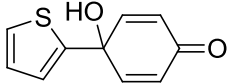
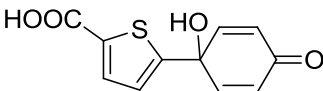
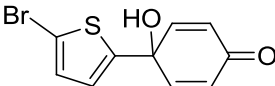
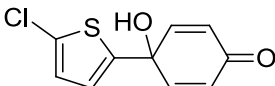
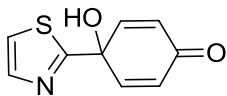
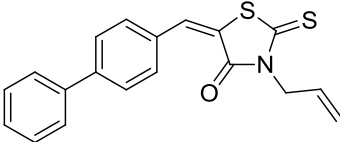
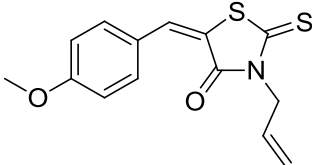
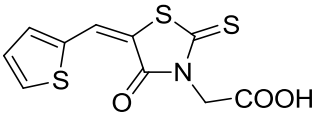
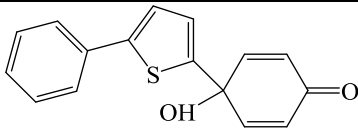
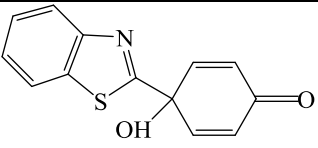
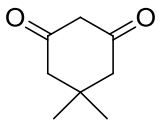
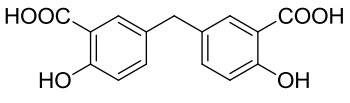
Code	Name	Ref/Note
MH13/FR02	 4-hydroxy-4-(thiophen-2-yl)cyclohexa-2,5-dien-1-one	
FR05	 5-(1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl)thiophen-2-carboxylic acid	
FR09	 4-(5-Bromothiophen-2-yl)-4-hydroxycyclohexa-2,5-dien-1-one	
FR33	 4-(5-Chlorothiophen-2-yl)-4-hydroxycyclohexa-2,5-dien-1-one	
FR37	 4-hydroxy-4-(thiazol-2-yl)cyclohexa-2,5-dien-1-one	
FR41	 (E)-5-([1,1'-biphenyl]-4-ylmethylene)-3-allyl-2-thioxothiazolidin-4-one	
FR44	 (E)-3-allyl-5-(4-methoxybenzylidene)-2-thioxothiazolidin-4-one	

Table 3.1 (continue)

Code	Name	Ref/Note
FR49	 (E)-3-ethyl-5-(thiophen-2-ylmethylene)-2-thioxothiazolidin-4-	
MH09	 4-hydroxy-4-(5-phenylthiophen-2-yl)cyclohexa-2,5-dien-1-one	
MH10	 1-(benzo[d]thiazol-2-yl)-4-methylenecyclohexa-2,5-dien-1-ol	
Dimedone	 5,5-dimethylcyclohexane-1,3-dione	(Charles et al., 2007)
MDSA	 5,5'-methylenebis(2-hydroxybenzoic acid)	(Sun et al., 2011)

3.2 Results

3.2.1 Optimisation of TSST-1 Western blotting

SarA regulates the expression of several virulence factors in conjunction with the *agr* system. SarA is the main activator of TSST-1 expression (Andrey et al., 2010). Therefore, we chose to investigate the effect of our compound library focusing initially on TSST-1 production as an indicator of inhibitory activity.

In order to find a suitable sampling time point for Western blot analysis, *S. aureus* strain KH1187A, a TSST-1 producing strain, was selected for screening. KH1187A was grown in TSB and supernatant samples were collected from early-log to late-stationary phase. Western blot analysis showed that TSST-1 was produced during stationary-phase and the strongest signal was obtained from overnight cultures. As a control, supernatant from the TSST-1 non-producing strain RN6309B were also examined and confirmed to be negative (Figure 3.1). Therefore, samples for all future experiments were collected after overnight growth.

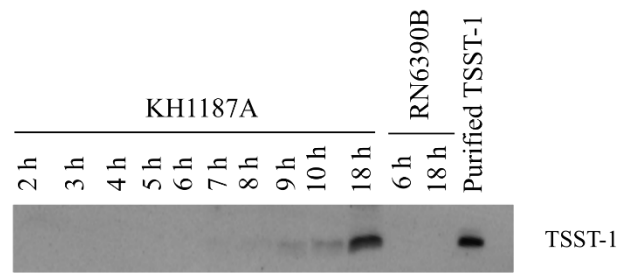


Figure 3.1 Optimisation of sampling time point for TSST-1 Western blot analysis. To observe TSST-1 production of KH1187A, this bacterial strain was cultured in TSB medium and the supernatant collected at the time points shown. TSST-1 was detected using purified sheep antiTSST-1 polyclonal antibody. TSST-1 was initially detected after 9 h of growth. Overnight (18 h) growth gave the strongest TSST-1 signal.

3.2.2 Screening a small compound library for exotoxin inhibitors

Ten rhodanine and quinone compounds (Table 3.1) were screened to determine whether they could affect virulence factor production by *S. aureus* KH1187A. Each compound was added at 100 μ M, except MH09, MH10 and MH13 which were used at 50 μ M due to the low concentration of stock and too high concentration of DMSO may affect the metabolism of *S. aureus*. The cultures were grown for 18 h in the presence of each compound and cell-free supernatants collected. Dimedone specifically binds to cysteine residues (Charles et al., 2007) and may therefore bind to SarA and inhibit exotoxin virulence gene expression. This compound was used as a positive control. MDSA was also used as a positive control as it has been shown to reduce α -haemolysin and increase protein A production by targeting the MgrA regulatory protein (Sun et al., 2011). Untreated and DMSO-treated cultures were used as negative controls.

Figure 3.2a shows that FR33, FR37, FR41 and FR44 all inhibited TSST-1 production whereas the other compounds were inactive. Moreover, FR33, FR41 and FR44 also reduced α -haemolysin production more effectively than MDSA which was inactive in this assay. Interestingly, FR37 inhibited the expression of TSST-1 but not α -haemolysin suggesting a different mode of action. However, in contrast to FR37, FR41 and FR44, FR33 did not up-regulate protein A production (Figure 3.2b). The secreted protein profiles of the cultures treated with FR37, FR41 and FR44 were slightly different from those of the untreated or DMSO-treated cultures as shown in Figure 3.2c. Coomassie blue staining confirmed that the amount of total protein used for each Western blot was equivalent for each sample.

FR37, FR41 and FR44 each down-regulated TSST-1 but up-regulated protein A. In addition, FR41 and FR44 inhibited α -haemolysin. Therefore, FR37 which is only active quinone, FR41 and FR44 which are rhodanines were selected as candidates for further studies.

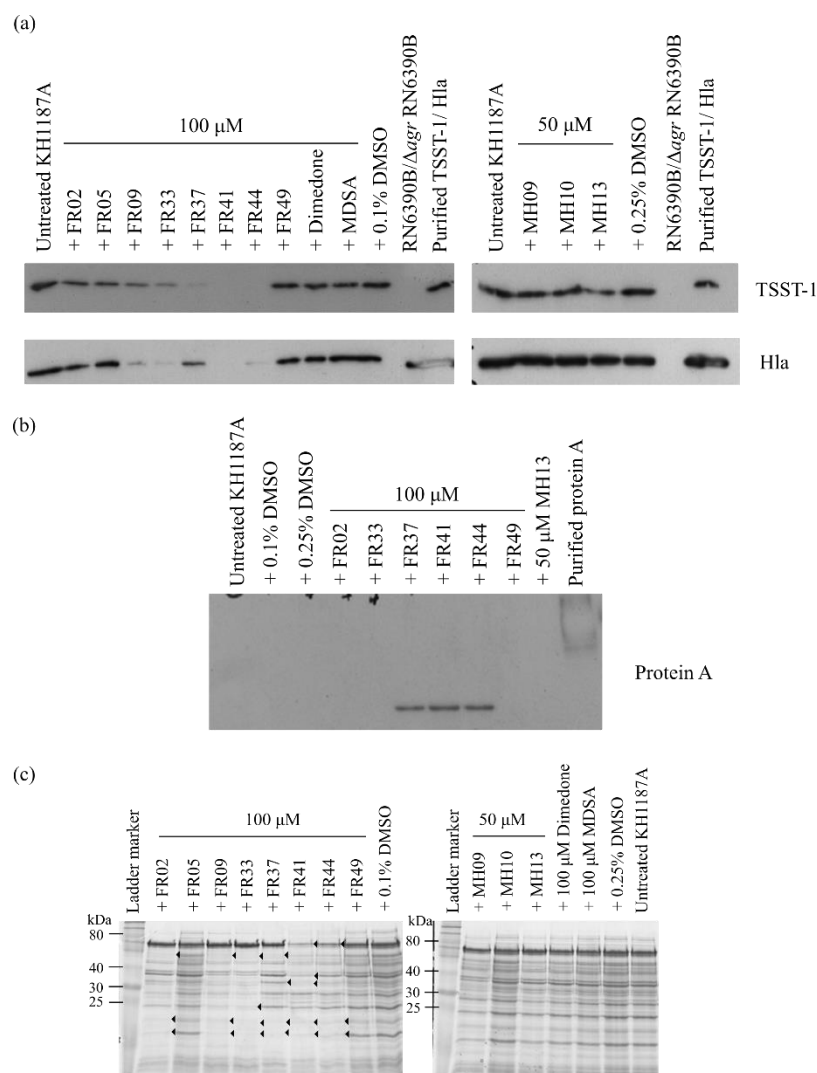


Figure 3.2 Screening for exotoxin inhibitor. *S. aureus* KH1187A was treated with each compound at 100 μ M, except for MH09, MH10 and MH13 which were used at 50 μ M. After 18 h of treatment, the supernatants were collected. (a) Western blot analysis shows TSST-1 and α -haemolysin (Hla) production. Supernatants from the RN6390B Δ agr strain were used as negative controls for TSST-1 and Hla. (b) Protein A Western blot of supernatants from strains treated with each compound where the supernatant from RN6390B Δ agr was used as a positive control, which shows different size due to the variation of the protein between strains. (c) Coomassie blue staining of SDS-polyacrylamide gels shows the total secreted protein profiles. Difference between samples is depicted by an arrowhead. This experiment was repeated independently twice. The data presented is representative.

3.2.3 FR37, FR41 and FR44 do not inhibit the growth of *S. aureus*

Compounds that inhibit the expression of *S. aureus* virulence factors have two main benefits over classical antibiotic treatment. Firstly, anti-virulence treatment specifically targets *S. aureus* and not the commensal microflora. Secondly, anti-virulence treatment is less likely to select for bacterial resistance as bacterial growth is not targeted (Gordon et al., 2013, Bhardwaj et al., 2013, Defoirdt et al., 2013).

To ensure that FR37, FR41 and FR44, do not kill or reduce growth, *S. aureus* was cultured in 96-well plates in the presence and absence of the compounds at a range of concentrations. Figure 3.3 shows that all three compounds do not reduce the growth rate or yield of *S. aureus* even at 100 μ M.

These data show that in conventional laboratory media FR37, FR41 and FR44 do not reduce virulence factor production through growth inhibition.

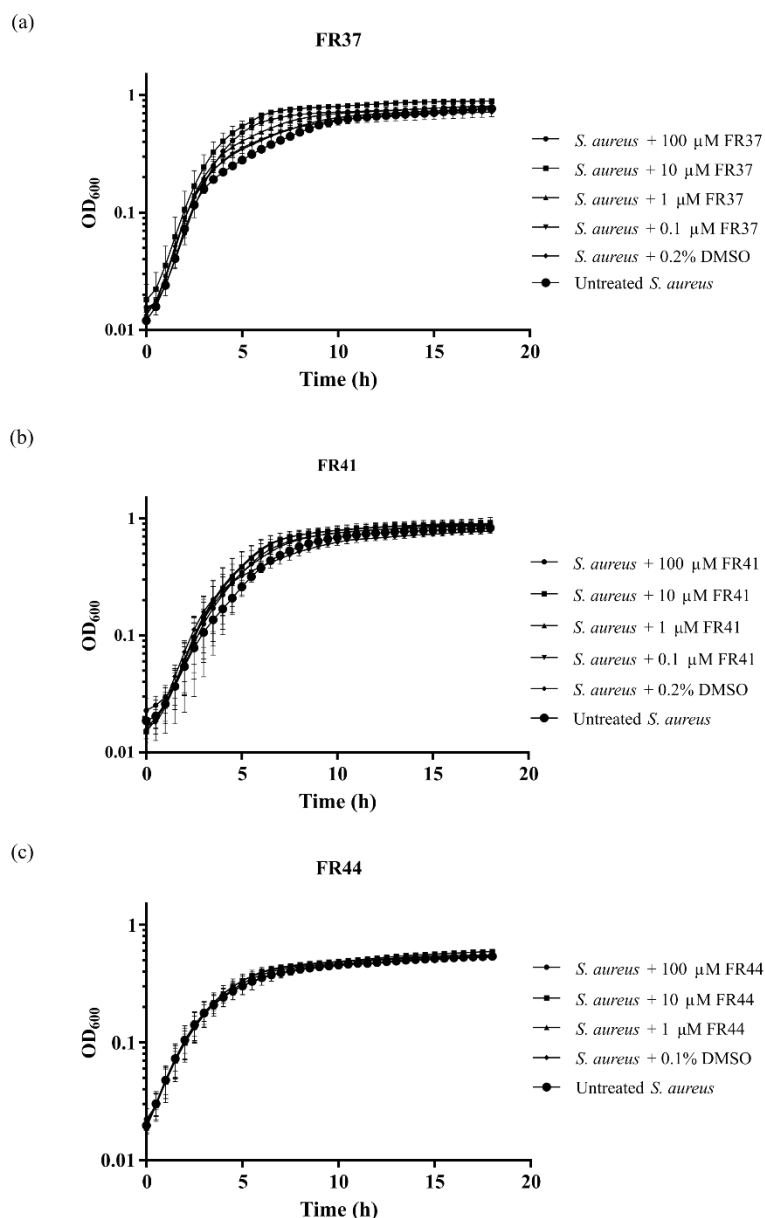


Figure 3.3 Effect of FR37, FR41 or FR44 on the growth of *S. aureus*. To determine the effect of these active compounds on growth of *S. aureus*, RN6390B was cultured in the absence or presence of FR37 (a), FR41 (b) or FR44 (c) at concentrations ranging from 1 - 100 μ M overnight at 37 °C without shaking in a microplate reader (Infinite F200, Tecan). Growth was measured as OD₆₀₀ every 30 min. The negative controls were the untreated and DMSO-treated cultures. The data obtained represent mean of three independently experiments $\pm$ standard deviation. FR37, FR41 and FR44 did not inhibit *S. aureus* even at concentrations up to 100 μ M.

3.2.4 The effects of FR41 and FR44 on exotoxins and protein A in *S. aureus* strain-independent.

FR37, FR41 and FR44 have shown their efficacies in down-regulating TSST-1 and α -haemolysin production while up-regulating protein A in *S. aureus* laboratory strain KH1187A. In order to select a lead compound which is effective in a strain-independent manner, 100 μ M of each lead compound was tested overnight against *S. aureus* MN8 which is clinical isolate (Li et al., 2011). FR02, FR05 and FR49 which had low efficacy in reducing TSST-1 production by KH1187A were used as negative controls alongside untreated and DMSO-treated cultures.

Western blot analysis revealed that MN8 treated with FR41 and FR44 reduced TSST-1 production but enhanced protein A production, whereas FR37 had no effect on TSST-1 (Figure 3.4a). Secreted protein profiles of the MN8 cultures treated with FR37, FR41 and FR44 were noticeably different to the negative controls as pointed by arrows (Figure 3.4b). This suggested that the active compounds reduced the secreted virulence factors without interfering with bacterial growth. Coomassie blue staining of SDS-polyacrylamide gels confirmed that amount of proteins in each sample was similar.

Since, FR41 and FR44 both reduced exotoxin production in a clinical *S. aureus* strain, they were selected for further investigation.

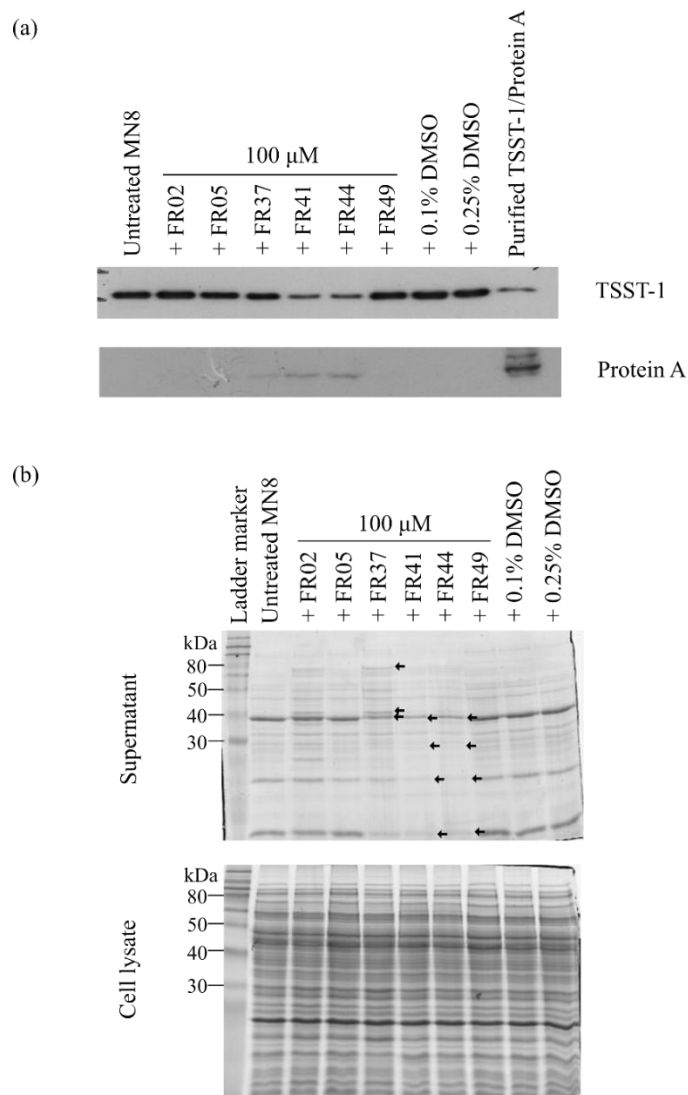


Figure 3.4 Effect of FR37, FR41 and FR44 on *S. aureus* MN8. To determine the effect of these active compounds on exotoxin production in different *S. aureus* belonging to different agr groups, *S. aureus* strain MN8, *agr*-III, was treated with 100 μ M each of FR37, FR41 and FR44 for 18 h. FR02, FR05 and FR33 which were not active against strain KH1187A were selected as negative controls together with untreated and DMSO-treated cultures. (a) Western blot analysis of TSST-1 and protein A. FR37, FR41 and FR44 all increased protein A whereas only FR41 and FR44 reduced TSST-1. (b) The secreted and intracellular protein profiles from the cell lysates stained with Coomassie blue. FR37, FR41 and FR44 influenced the secreted protein profile of MN8 but not the intracellular and membrane protein profiles. The difference is pointed by arrow. The experiment was repeated independently twice. The data presented is representative.

3.2.5 FR41 and FR44 are active against CA-MRSA strain USA300 JE2

To determine whether FR41 and FR44 had activity against CA-MRSA strains a preliminary experiment was carried out with USA300 strain JE2 treated with a range of concentrations from 1 - 100 μ M for 6 h and 18 h, respectively. FR49 was selected as negative control together with untreated and DMSO-treated groups. At each time point, the supernatant and cells were harvested for Coomassie blue staining of SDS-polyacrylamide gels, Western blot analysis and haemolysin assay.

Preliminary Western blot analysis showed that both FR41 and FR44 at 50 and 100 μ M down-regulated α -haemolysin production and up-regulated protein A production at 6 h and 18 h after treatment (Figure 3.5a). To confirm the Western blot data, haemolysin assays were carried out to measure the erythrocyte lytic activity of the culture supernatants. The left panel of Figure 3.5a shows that haemolytic activity was reduced to 7 % and 22 %, respectively following treatment with 50 μ M or 100 μ M FR41. For F44, only the 100 μ M concentration reduced the haemolytic activity (to 30 %). However, no reduction of haemolytic activity was observed for the 18 h treated samples (Figure 3.5b, right panel). The secreted protein profiles at 6 h and 18 h also showed changes when the cultures were treated with FR41 or FR44 at 50 and 100 μ M (Figure 3.5c). The intracellular protein profile was unaffected at 6 or 18 h (data not shown).

Both FR41 and FR44 not only affected virulence expression of MSSA strains as shown in earlier data but also MRSA.

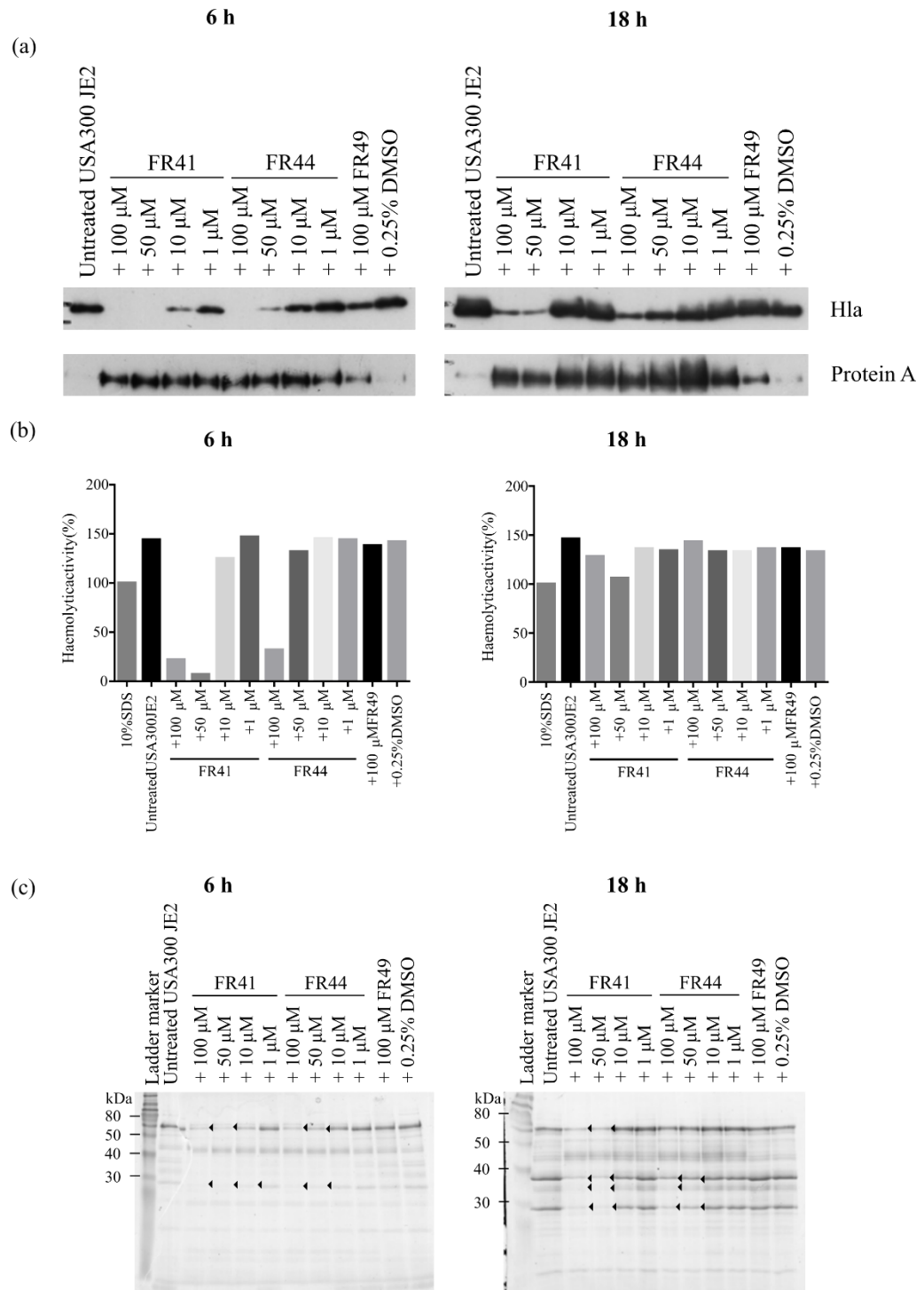


Figure 3.5 Effects of FR41 and FR44 on MRSA USA300 JE2. Bacteria were cultured in the presence and absence of either FR41 or FR44 at the indicated concentrations. Untreated, DMSO-treated and FR49-treated groups were negative controls. Samples were collected at 6 h (left panel) and 18 h (right panel) after treatment. The effect of these compounds on exotoxin production and protein profile of MRSA strain was determined. (a) Western blot analysis determined the α -haemolysin (Hla) and protein A production. FR41 and FR44 down-regulated α -haemolysin and up-regulated protein A at both time points. (b) Haemolysin assays using rabbit blood showed that after 6 h of treatment, the haemolytic activity of cultures treated with 100 μ M and 50 μ M FR41 were reduced to 22 % and 7 % respectively. For FR44, 100 μ M reduced haemolysis to around 30 %. One hundred microliters of 10 % SDS was used as a positive control treatment and the activity was adjusted to 100 %. (c) Coomassie blue staining of SDS-polyacrylamide gels shows the secreted protein profiles of bacteria. The difference between treated groups and controls is pointed out by arrow head. The experiment was done once.

3.2.6 Determination of the minimum effective concentration of FR41 and FR44.

To determine the lowest effective concentration of the two lead compounds, KH1187A and MN8 were treated with FR41 or FR44 at concentrations ranging from 1 to 100 μ M for 18 h. FR49 was used as a negative control together with untreated and DMSO-treated groups. Western blotting was used to determine the minimum concentration required to affect *S. aureus* exotoxin production.

The results clearly show that 1 μ M FR41 effectively abolished TSST-1 while increasing protein A in KH1187A, whereas FR44 required at least 10 μ M (Figure 3.6a, left panel). FR41 and FR44 failed to completely inhibit TSST-1 production by MN8. The data indicate that for both FR41 and FR44, 10 μ M is required to reduce TSST-1 production by MN8 while as little as 1 μ M of either compound up-regulated protein A (Figure 3.6a, right panel). The secreted protein profiles from both KH1187A and MN8 were only slightly modified depending to the concentration of FR41 and FR44 used (Figure 3.6b).

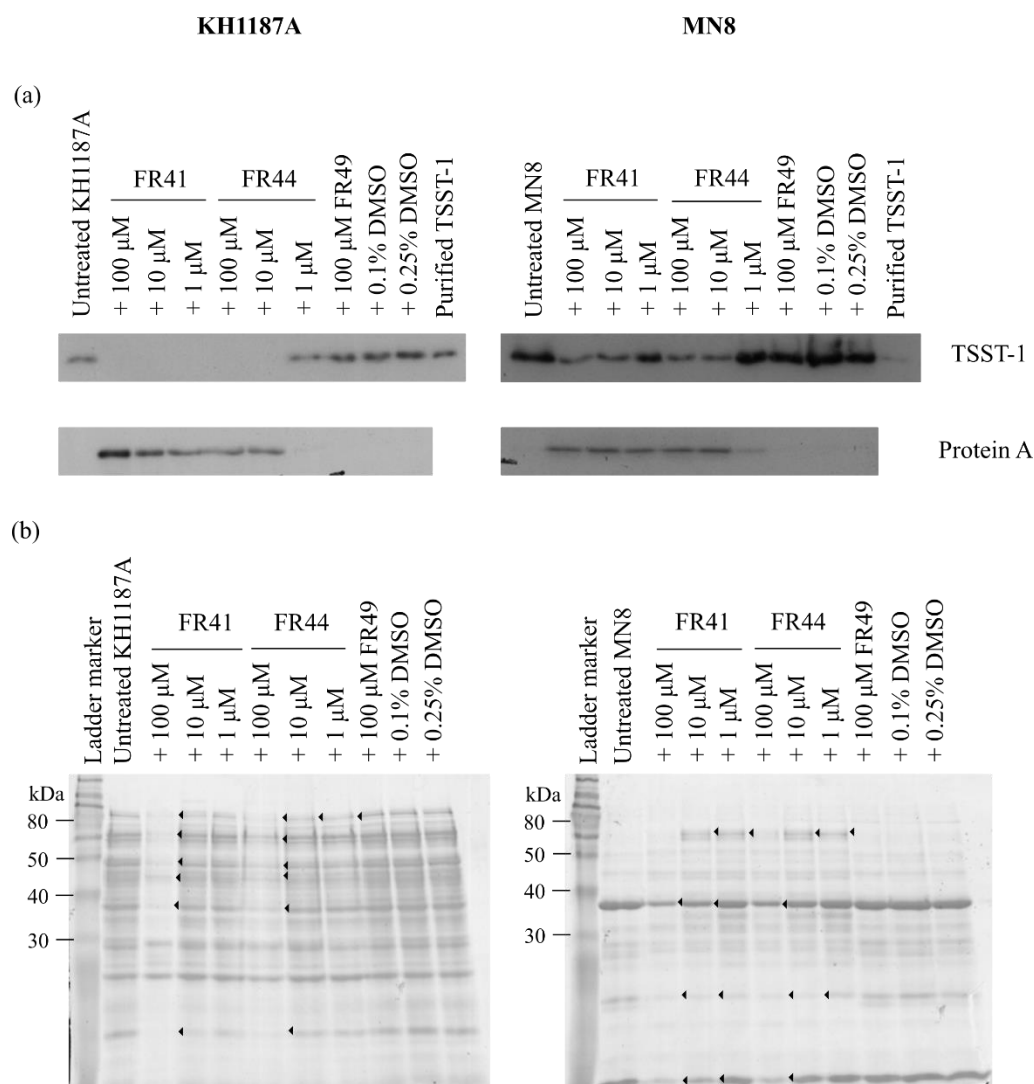


Figure 3.6 Minimum effective concentrations of FR41 and FR44. *S. aureus* KH1187A and MN8 cultures were treated with either FR41 or FR44 at 1, 10 and 100 μ M for 18 h in order to observe the minimum concentration of the compounds that actively against the virulence factor production of *S. aureus*. Untreated, DMSO-treated and FR49-treated cultures were used as controls. The left panel shows the results from KH1187A cultures and the right panel shows MN8. (a) The production of TSST-1, α -haemolysin (Hla) and protein A were analysed by Western blotting. (b) Coomassie blue staining showed secreted protein profiles of KH1187A and MN8. The difference between the treated and control cultures is depicted by arrow head. The experiment was carried out two separate occasions. The data presented are representative.

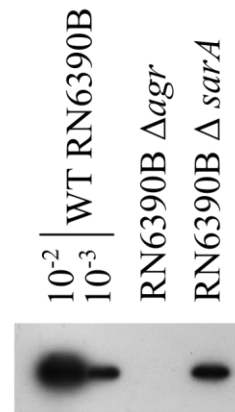
3.2.7 Identification of the FR41 and FR44 target

3.2.7.1 Construction of Δagr and $\Delta sarA$ mutants in *S. aureus* RN6390B

TSST-1 and α -haemolysin are positively regulated by *agr* and SarA, whereas protein A is negatively regulated (Dunman et al., 2001). The response of *S. aureus* to FR41 and FR44 suggests that they may target either *agr* or SarA. To address this possibility, isogenic *agr* and *sarA* mutants were generated by phage transduction as described in the Materials and Methods (chapter 2 section 2.3).

The mutants generated were initially confirmed by antibiotic resistance screening and PCR (data not shown). Western blotting showed that the *agr* mutant produced no detectable α -haemolysin while the *sarA* mutant showed a much reduced signal compared with the wild-type (Figure 3.7a). Phenotypic analysis using rabbit blood agar revealed that a clear zone of haemolysis could only be observed for the wild-type and *sarA* mutant colonies (Figure 3.7b).

(a)



(b)



Figure 3.7 Haemolysin production and haemolytic activity of RN6390B Δagr and $\Delta sarA$ mutants. To validate the constructed mutant strains, the exotoxin production of mutants was determined as it was under the control of knock-out genes. (a) α -haemolysin Western blot of parental WT RN6390B that was diluted 100- and 1000-fold, *agr* and *sarA* mutants cultured in TSB broth at 37 °C for 18 h shaking at 200 rpm. The α -haemolysin could be detected only from wild-type and *sarA* mutant. (b) Ten percent rabbit blood agar showing the haemolytic phenotype of wild-type, *agr* and *sarA* strains. A clear zone can be observed around the wild-type and *sar* mutant.

3.2.7.2 FR41 and FR44 do not target SarA

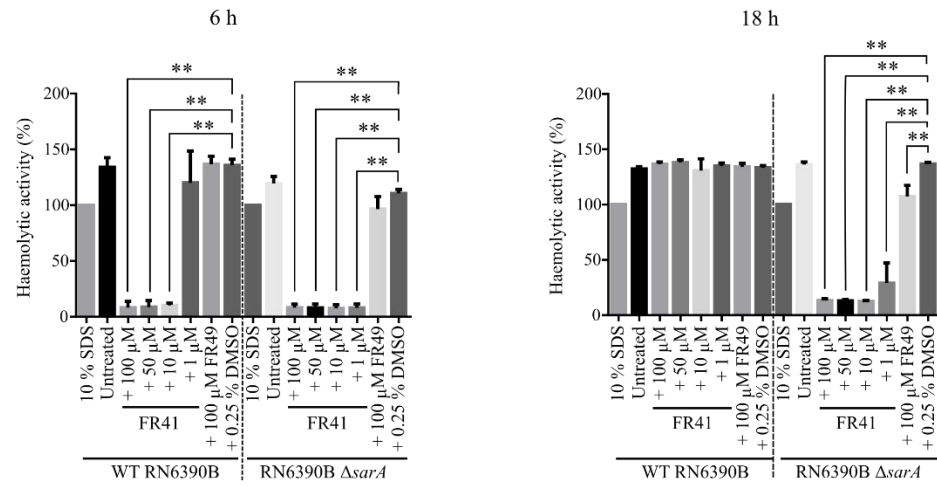
If the lead compounds target *agr* or SarA, the level of residual protein expression should remain unaltered after the addition of compound to the respective mutants. Therefore, WT, Δagr and $\Delta sarA$ strains were treated with either FR41 or FR44 from 1 to 100 μ M for 6 h or 18 h and the haemolytic activity and protein A production investigated. FR49-treated cultures were used as controls together with untreated and DMSO-treated cultures.

After 6 h of treatment, the haemolysin activity of wild-type RNA6390B significantly reduced to around 10 % in the 10, 50 and 100 μ M FR41-treated groups compared with the untreated controls ($p < 0.01$) as shown in Figure 3.8a, left panel. Moreover, FR41 at lower concentrations was more efficient at reducing the haemolytic activity of *sarA* mutant compared with the wild-type. After 6 h of treatment, the haemolysin activity of the FR41-treated group was significantly reduced to less than 10 % even at concentrations as little as to 1 μ M ($p < 0.01$). Although, after 18 h of treatment FR41 did not reduce the haemolytic activity of wild-type, it still significantly reduced the activity of *sarA* mutant to 13 % when treated with 10 - 100 μ M FR41 and to 30 % when treated with 1 μ M FR41 ($p < 0.01$) as shown in Figure 3.8a, right panel. Western blot result for wild-type, Δagr and $\Delta sarA$ strains treated with FR41 for 18 h shows that FR41 up-regulated protein A in the wild-type and *sarA* mutant (Figure 3.8b). In addition, at 1 μ M FR41 increased protein A production by the *sarA* mutant, but not in the wild-type. Coomassie blue staining of SDS-polyacrylamide gels in Figure 3.8c shows that FR41 induced changes in the secreted protein profile of both wild-type and *sarA* mutant while it had no effect on the *agr* mutant. FR41

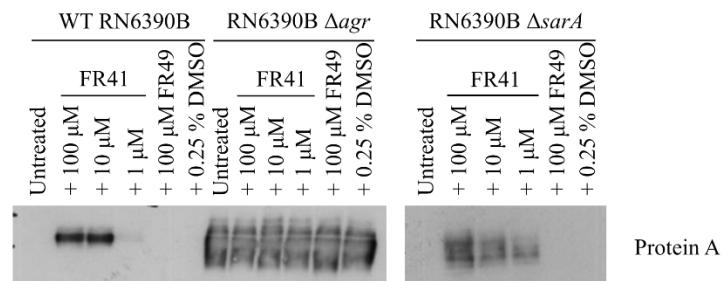
did not influence the intracellular or membrane-bound protein profiles of the wild-type, *agr* or *sarA* mutants (data not shown).

FR41 reduced haemolytic activity, increased protein A and induced changes in the of secreted protein profile of the *sarA* mutant. Since, FR41 was reduced haemolytic activity in the *sarA* mutant, this indicates that SarA is unlikely to be the primary target of FR41.

(a)



(b)



(c)

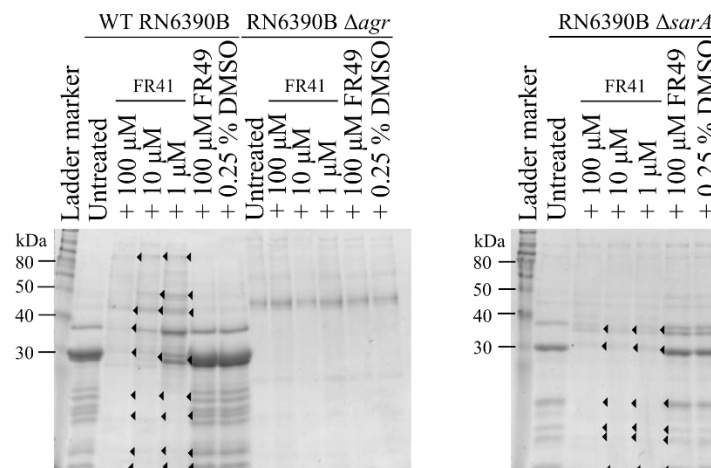


Figure 3.8 SarA is not the primary target of FR41. Wild-type RN6390B, *agr* and *sarA* mutants were treated with FR41 at concentration of 1, 10, 50 and 100 μ M. The FR49-treated, DMSO-treated and untreated groups were used as negative controls. (a) Haemolytic activity to defibrinated rabbit blood in spent culture supernatants was determined at OD₅₄₂ using a spectrophotometer. The lytic activity of 100 μ l of 10 % SDS was used as a positive control and normalized as 100 % activity. The left hand panel of the figure shows the data obtained after 6 h growth whereas the right hand panel shows 18 h of treatment. The data represent mean $\pm$ standard deviations of three independent experiments. **, $p < 0.01$. (b) Western blot shows protein A production in the cultures treated with FR41 for 18 h. (c) Secreted protein profile from overnight cultures is revealed by Coomassie blue staining of SDS-polyacrylamide gels. The difference between treated and control cultures is indicated by arrow head. The Western blot and Coomassie blue staining experiments were carried out in independently biological triplicate. The data presented are representative.

Next, we investigated the potential target of FR44. A similar experiment as that described for FR41 was carried out. Figure 3.9a shows the haemolytic activity present in supernatants treated with FR44 for 6 h. FR44 reduced the activity of wild-type samples from 130 % of negative controls to 40 % and 16 % of 50 and 100 μ M FR44-treated groups, respectively. The *sarA* mutant showed further reductions in haemolytic activity when treated with FR44. At 10 μ M FR44, the haemolysin activity was reduced to 10 % from around 100 % of the negative control groups. After 18 h of treatment, no further reduction in haemolytic activity was noted for the parent strain, whereas the *sarA* mutant treated with FR44 still showed a substantial decrease in haemolytic activity (Figure 3.9a). The impact of FR44 on protein A is shown in Figure 3.9b. After 18 h of treatment FR44 enhanced protein A production by both wild-type and *sarA* mutant but not the *agr* mutant. Figure 3.9c shows that changes in the secreted protein profile of both wild-type and *sarA* mutant but not the *agr* mutant treated with FR44 for 18 h are apparent.

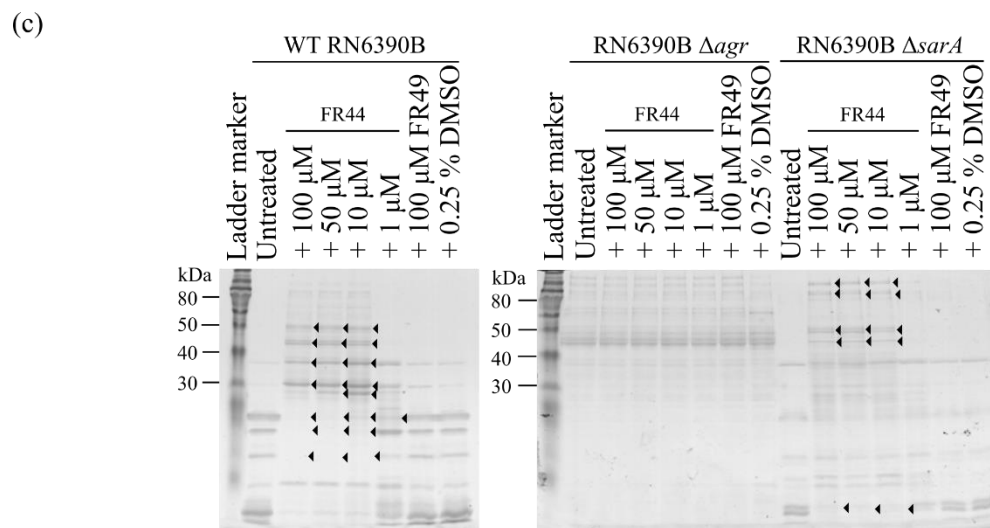
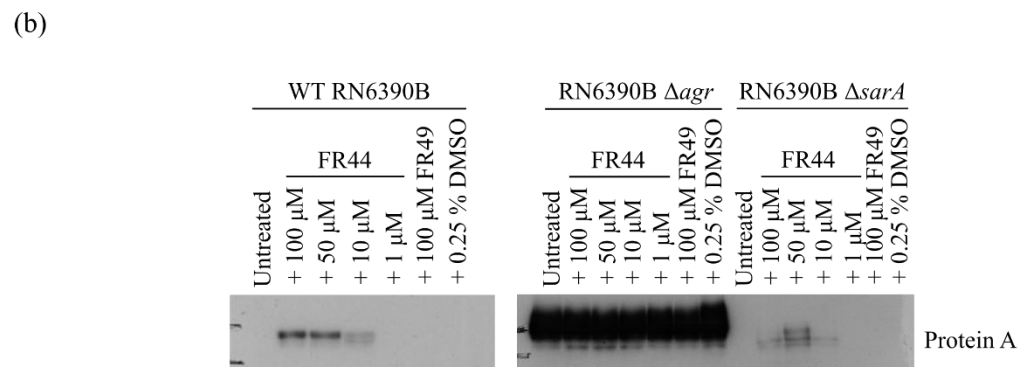
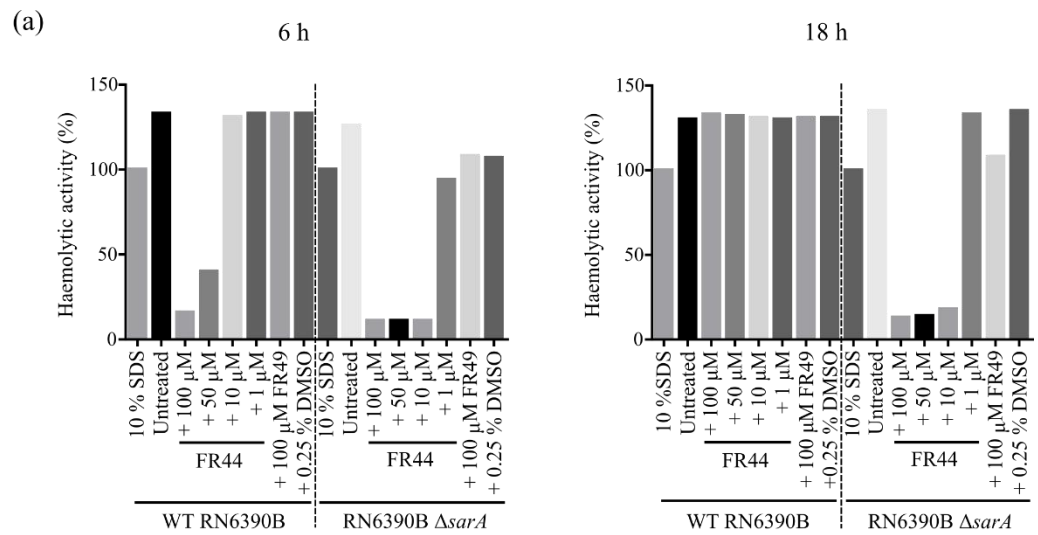


Figure 3.9 Investigation of the target of FR44. Wild-type RN6390B, *agr* and *sarA* mutants were treated with FR44 at concentrations of 1, 10, 50 or 100 μ M to identify target of FR44. The FR49-treated, DMSO-treated and untreated cultures were used as negative controls. (a) Haemolysin assay showed haemolytic activity in supernatants. The lytic activity of 10 % SDS was used as a positive control and adjusted to 100 % of the activity. The left panel of the figure shows the results from 6 h whereas the right panel shows 18 h of treatment. (b) Western blot showing protein A production by the cultures treated with FR44 for 18 h. (c) Secreted protein profiles from overnight cultures as revealed by Coomassie blue staining of SDS-polyacrylamide gels.

These results showed that both FR41 and FR44 retain their activity against the *sarA* mutant but did not affect the *agr* mutant. This suggests that these compounds do not reduce *S. aureus* virulence factor production by inhibiting SarA but are more likely to target the *agr* system.

3.2.8 Re-synthesis FR41 and FR37 resulted in batches that lacked inhibitory activity

To replenish stocks, new batches of FR37 and FR41 were synthesised. FR37 batch 2 was termed FR37_2. The stocks of FR41 batch 2 were prepared at 2 different concentrations, 100 μ M and 40 μ M termed FR41_2_s1 and FR41_2_s2, respectively. Their efficacy was compared with the original batches of FR41 and FR37 (now termed FR41_1 and FR37_1, respectively). In addition, a third batch of FR41 (FR41_3) after the synthetic process was finished either a production from crystallization (FR41_3_cry) which has high purity or a precipitate (FR41_3_ppt) of a solution after crystallisation were collected. *S. aureus* KH1187A was treated overnight as previously described with each new compound. Untreated, FR49-treated and DMSO-treated cultures were used as negative controls. FR44 was used as a positive control together with FR37_1.

Western blot analysis (Figure 3.10a) showed that the new batches of FR41 were not as active as the first batch. One hundred micromolar FR41_3_cry and FR41_3_ppt both only slightly reduced TSST-1 and in contrast to FR41_1 had no effect on α -haemolysin. FR37_2 showed no TSST-1 inhibitory activity (Figure 3.10b). Coomassie blue staining of SDS-polyacrylamide gels confirmed that equivalent amounts of total protein had been loaded onto the SDS-polyacrylamide gels for each sample prior to Western blotting (data not shown).

The level of TSST-1 and α -haemolysin were decreased in FR49-treated group was likely caused by losing protein pellet during preparation step because no active activity was found in further experiments.

Dr. Fabio Rui carried out ^1H NMR and HPLC analysis to confirm the nature and assess the purity of each compound (Figure 1 in Appendix). The purity of FR41_2 was 96.8 %, whereas the first batch was 79.1 %. No obvious differences between FR37_1 and FR37_2 were apparent. This data suggest that losing the activity of new batches of FR41 is not caused by low purity and no obvious impurity was found.

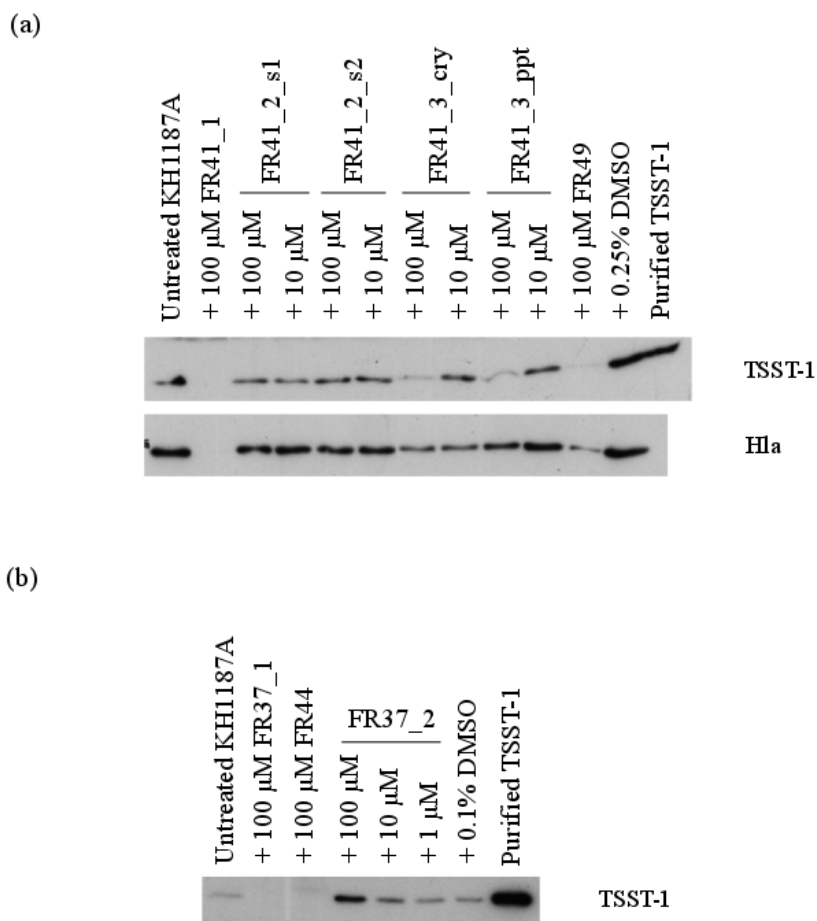


Figure 3.10 Comparison of different batches of FR41 and FR37. Two new batches of FR41 and new one batch of FR37 were produced. The activity of new batches at the indicated concentrations towards KH1187A was compared with 100 μ M batch 1 (FR41_1). Two different stock concentrations of FR41 batch 2 (FR41_2) of 100 μ M (s1) and 40 μ M (s2) were assayed. FR41 batch 3 (FR41_3) was collected after re-crystallisation (cry) or precipitation (ppt). Untreated, 100 μ M FR49 and 0.25 % DMSO-treated groups were used as negative controls. (a) Western blotting was used to determine the effect of FR41 on TSST-1 and α -haemolysin (Hla) production. (b) Comparison of the activity of the first batch of FR37 (FR37_1) with the second batch (FR37_2). The experiment was carried out in duplicate. The presented data is a representative.

3.2.9 Re-synthesis FR44 resulted in a batch that lacked inhibitory activity

3.2.9.1 Investigation of FR44 batch 2

Given that the new batches of both FR41 and FR37 were inactive, FR44 was resynthesized. The activity of this new batch (FR44_2) was compared with the first batch (FR44_1) by testing their inhibitory activity towards against KH1187A grown overnight.

The Western blot results presented in Figure 3.11a show that FR44_2 did not down-regulate TSST-1 or α -haemolysin or up-regulate protein A production compared with FR44_1. The Coomassie blue staining of a SDS-polyacrylamide showed that the total secreted protein used in the Western blot contained the same amount (Figure 3.11b).

¹H- nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC) analysis were carried out by Dr. Fabio Rui revealed some differences between FR44_1 and FR44_2 (Figure 2 in Appendix). ¹H NMR showed an extra-peak from FR44_1 at approximately 2 ppm surrounded by dashed-circle (Figure 2a in Appendix) and HPLC analysis revealed another peak in FR44_1 with a retention time of 14.934 min as surrounded by dashed-circle in (Figure 2b in Appendix). These data suggest that FR44_1 contained a possible contaminant or remaining by-product that might be the active compound.

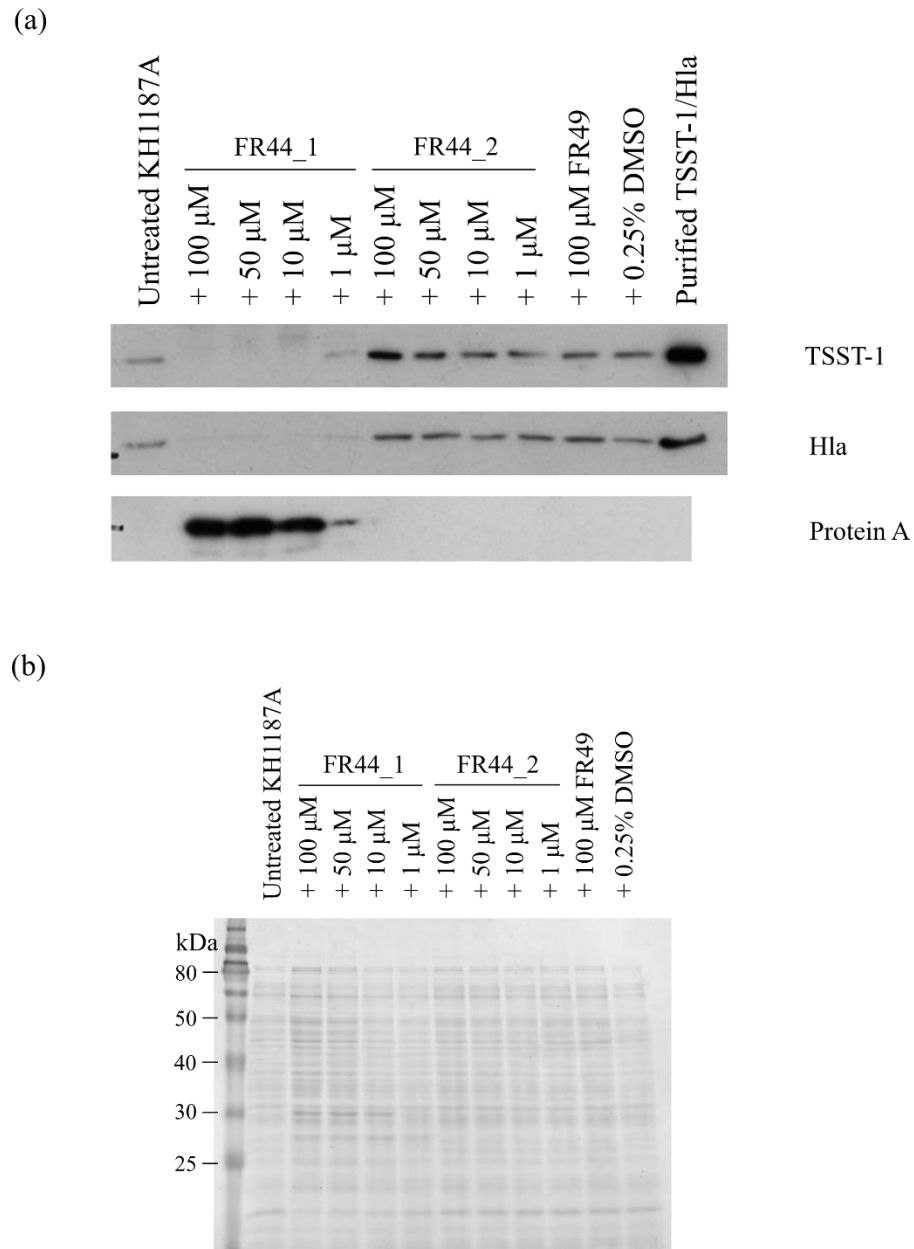


Figure 3.11 Comparison of the activity of FR44 batches 1 and 2. KH1187A was treated with either FR44 batch 1 or batch 2 (FR44_1 and FR44_2, respectively) at concentrations ranging from 1 to 100 μ M overnight. Untreated, 100 μ M FR49-treated and 0.25 % DMSO-treated cultures were used as negative controls. (a) Western blot analysis was carried out to determine the effect of FR41 on production of TSST-1, α -haemolysin (Hla) and protein A. (b) Coomassie blue staining of SDS-polyacrylamide determined amount of total secreted protein in each sample.

3.2.9.2 Purification and analysis of contaminating by-product from FR44

Dr. Fabio purified the compound conferring the extra-peak (termed FR44_1b) found on HPLC analysis (Figure 2 in Appendix). In order to determine whether FR44_1b was the active compound, KH1187A was treated with FR44_1b overnight. Untreated, FR49-treated and DMSO-treated groups were used as negative controls. The activity of FR44_1b was compared with FR44_1 by evaluating TSST-1, α -haemolysin and protein A production by Western blot analysis

FR44_1b did not reduce the production of TSST-1 or α -haemolysin and did not increase protein A production compared with FR44_1 (Figure 3.12a). The Coomassie blue staining of a SDS-polyacrylamide gel showed that the amount of total protein in each sample was equal (Figure 3.12b).

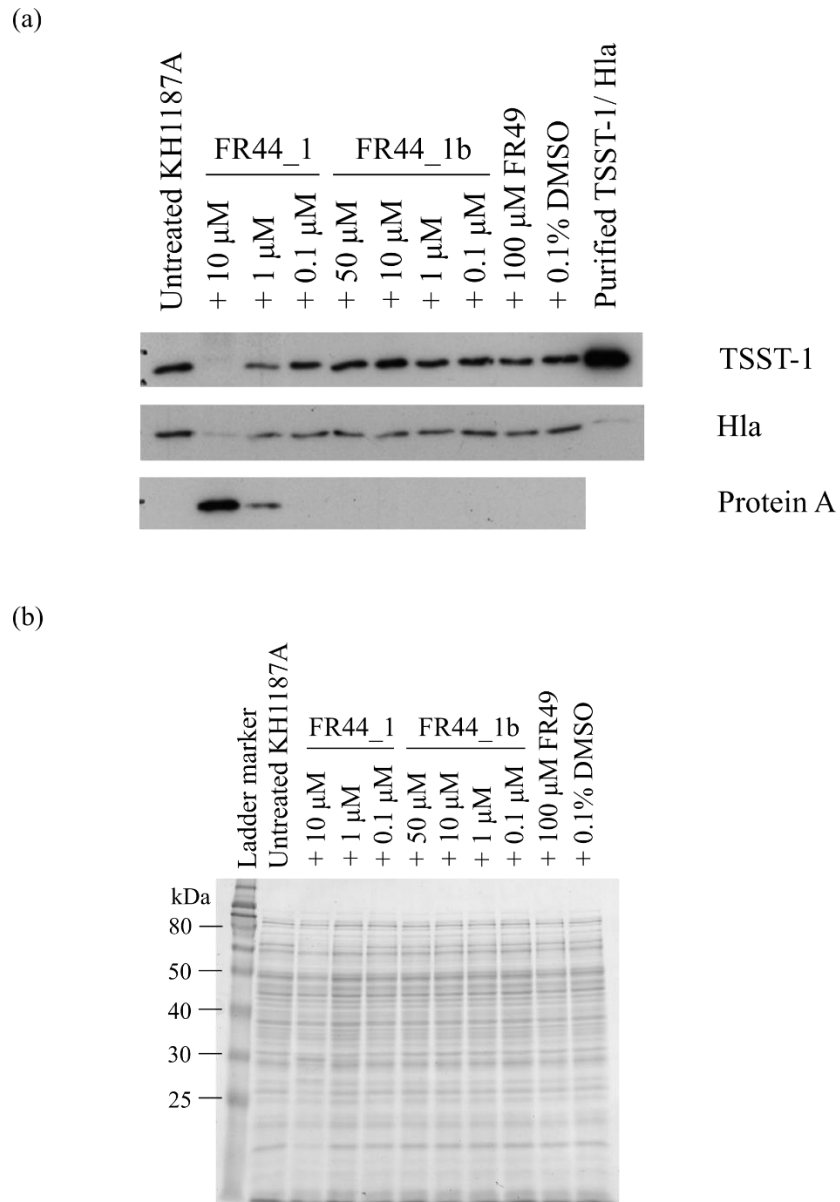


Figure 3.12 Comparison of the activities of FR44 batches. The effect of FR44_1b on TSST-1, α -haemolysin (Hla) and protein A production by KH1187A after overnight growth was determined using Western blot (a). The total amount of secreted protein in each sample was determined using Coomassie blue staining of SDS-polyacrylamide (b). FR44_1 was used as a positive control, whereas untreated, 100 μ M FR49 and 0.1 % DMSO-treated groups were used as negative controls.

3.2.9.3 Evaluation of the activity of FR44 side reaction products

Since, FR44_1b lacked inhibitory activity towards TSST-1, α -haemolysin and induction of protein A, it was possible that the additional compound(s) present in FR44_1 were responsible for the biological activity observed either alone or in combination. To investigate this possibility the highly purified FR44_1b was mixed with FR44 batch 2 at two different ratios, 50:50 and 75:25 of FR44_2 and FR44_1b, respectively. The ratios were selected to mimic the ratio between the extra-peak and the major peak of FR44_1 from HPLC analysis (Figure 2 in Appendix). Therefore, the mixture of FR44_2 and FR44_1b should have the same concentration of each component as batch 1. This mixture was added to KH1187A cultures and incubated overnight. Western blot analysis showed that the mixture of FR44_1b and FR44_2 at either ratio did not reduce TSST-1 and or induce protein A production (Figure 3.13a). The Coomassie staining of a SDS-polyacrylamide showed that the total amount of each protein sample was equal (Figure 3.13b).

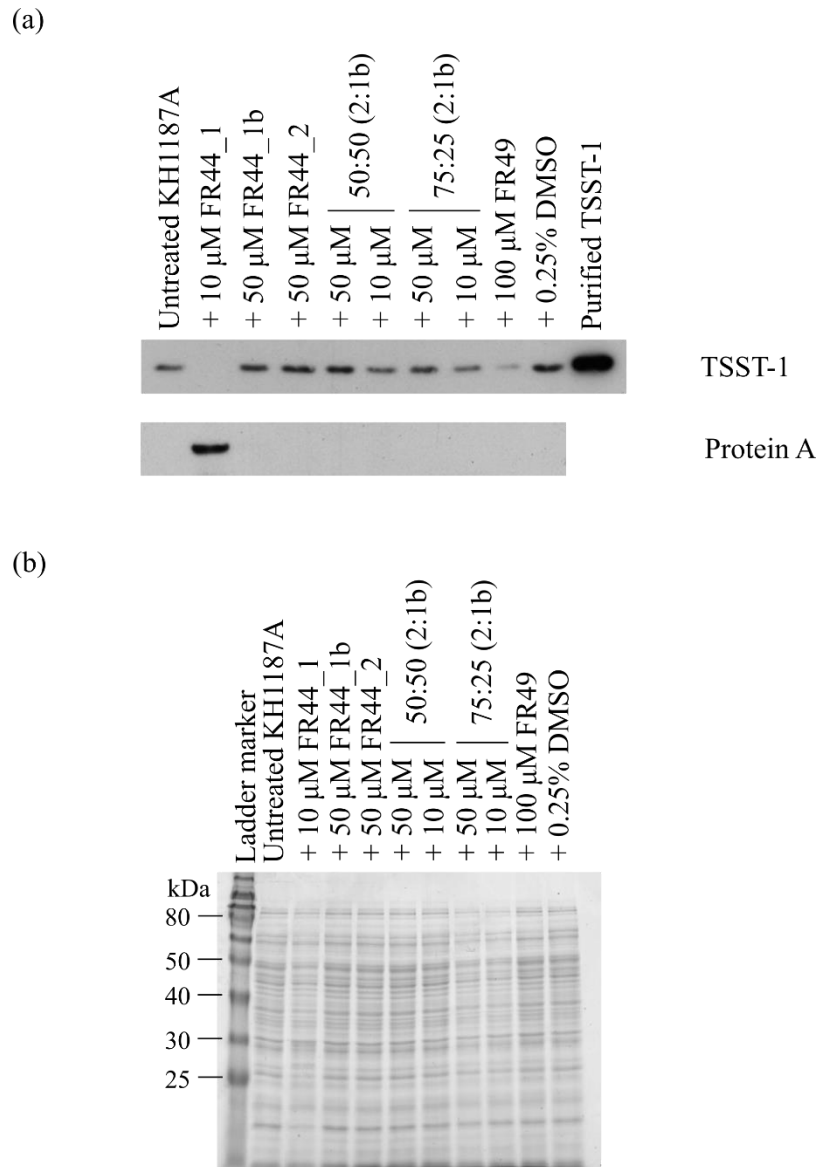


Figure 3.13 Comparison of the activities of mixture of FR44 batch 2 and 1b. The effect of FR44_2 and _1b at different ratios on TSST-1, α -haemolysin (Hla) and protein A production by KH1187A after overnight growth was determined using Western blot (a). The total amount of secreted protein was determined by Coomassie blue staining of a SDS-polyacrylamide gel. FR44_1 was used as a positive control, whereas untreated, 100 μ M FR49 and 0.1 % DMSO-treated groups were used as negative controls.

3.3 Discussion and Conclusions

S. aureus is an opportunistic bacterial pathogen. A recent increase in the prevalence of drug resistant clinical isolates, including vancomycin resistant strains, requires the development of new drug therapies.

Virulence determinants of *S. aureus* play a key role, permitting *S. aureus* to invade and evade the host immune response. Virulence factor expression is tightly regulated by a number of transcriptional regulators, such as SarA and *agr* (Foster, 2005, Dunman et al., 2001). Several studies have shown that the virulence of a *sarA* or an *agr* mutant is attenuated in multiple animal infection models (Cheung et al., 1994, Blevins et al., 2003). The genes regulated by SarA and *agr* are not crucial to bacterial growth and viability. Therefore, therapies that target SarA or *agr* reduce bacterial virulence but do not place a strong selective pressure that generates drug resistant mutants (Gordon et al., 2013).

In this study a small-compound library of quinones and rhodanines was synthesized by Dr. Fabio Rui. Table 3.1 summarises the data obtained for these compounds. Compounds FR37, FR41 and FR44 reduce *S. aureus* exotoxins and increase protein A production without killing or inhibiting growth of the bacteria. FR37 was the only active quinone in this study, suggesting that the thiazole ring, a feature that only FR37 contains is crucial for the activity. FR49 was the only rhodanine in this study that was inactive. The major difference between FR49 and the other active rhodanines, FR41 and FR44, is the carboxylic acid group on the side chain of FR49 that may reduce the affinity of interaction to a targeted protein or reduce the permeability of the compound. Moreover, all active compounds in this study display higher activity than MDSA, an MgrA

inhibitor (Sun et al., 2011), suggesting that the lead compounds have promising anti-*virulence* activity.

FR41 and FR44 reduced TSST-1 and α -haemolysin production, while FR37 reduced TSST-1 but did not reduce α -haemolysin production. These data indicate that the rhodanines and quinones evaluated have different modes of action. FR41 and FR44 may target the *agr* system or SarA (Andrey et al., 2010, Dunman et al., 2001) while FR37 may target a different regulatory system. FR41 and FR44 differ from FR37 in that they display broader activity against different strains, suggesting that the target of FR41 and FR44 is more available or conserved between *S. aureus* strains. FR41 and FR44 reduced exotoxin production by MRSA strain USA300 JE2 despite this clone having a highly up-regulated *agr* system (Li et al., 2009).

Data suggest that FR41 and FR44 are likely to target the *agr* system as they both reduced α -haemolysin production in $\Delta sarA$ mutant indicating that the target of these compounds is not SarA.

The anti-*virulence* activity of a newly synthesized batch of FR41, FR44 and FR37 was absent. NMR and HPLC analysis of FR44 from batch 1 uncovered an extra peak in the HPLC chromatogram which was not present in FR37 and FR41. The extra peak was purified and tested against KH1187A on its own and by mixing it with FR44 batch 2 in order to make the compound like FR44 batch 1; however, the combination was still inactive.

In this study, the quinone FR37 and the rhodanines FR41 and FR44 show activity against *S. aureus* virulence factor production in different strains in a concentration-dependent manner in comparison with the inactive compounds from the same chemical classes. This indicates that these analogues are

inhibitors, not pan-assay interference compounds as defined by (Baell and Walters, 2014). At the moment, these 3 compounds have a synthesis problem. It is possible that the active compound is an as yet unidentified contaminating species and is effective against *S. aureus* at a nanomolar level, well below the detection limit of ^1H - NMR and HPLC. This problem is waiting for a chemist in our group to address further.

Chapter 4: *Pseudomonas aeruginosa* metabolite IQS and analogues reduce production of *Staphylococcus aureus* virulence determinants

4.1 Introduction

S. aureus and *Pseudomonas aeruginosa* are both important opportunistic pathogens that are often found at the same infection site, for example the airway of a cystic fibrosis or severe bronchiectasis patient. It is commonly found that *S. aureus* infects a patient suffering with cystic fibrosis before *P. aeruginosa*, however, *P. aeruginosa* eventually outcompetes *S. aureus* for their shared niche (Machan et al., 1992). *P. aeruginosa* can inhibit *S. aureus* growth because it produces several anti-staphylococcal substances. For example, 4-hydroxy-2-heptylquinoline-*N*-oxide suppresses growth of *S. aureus* and other Gram-positive bacteria species (Machan et al., 1992, Hoffman et al., 2006). 4-hydroxy-2-heptylquinoline-*N*-oxide transforms *S. aureus* into a small-colony variant with decreased respiratory and altered metabolism (Hoffman et al., 2006). *N*-(3-Oxododecanoyl)-*L*-homoserine lactone which is utilised by *P. aeruginosa* as a QS signalling molecule reduces exotoxin production and induces protein A expression via inhibition of *S. aureus* *sarA* and *agr* regulatory systems (Qazi et al., 2006). It is also known that *P. aeruginosa* can lyse *S. aureus* and utilise key nutrients such as iron to maintain growth in a nutrient limited environment (Mashburn et al., 2005).

IQS is produced by *P. aeruginosa*. It is generated from dihydroaeruginic acid that is a by-product from the biosynthesis of the siderophore pyochelin (Ye et al., 2014, Serino et al., 1997). The structure of IQS is shown in Table 4.1 coded IQS5. Dihydroaeruginic acid which is a precursor

of IQS has antifungal and antibiotic properties towards phytopathogens (Carmi et al., 1994). IQS is thought to play a key role in regulating signal molecule production in both the *pqs* and *rhl* quorum sensing systems in low phosphate growth conditions and so enables virulence factor expression (Lee et al., 2013). Here we investigated the effect of IQS on *S. aureus* virulence factor production.

In this results chapter, we present data showing that IQS reduces *S. aureus* exotoxin and increases protein A production, however, the efficacy was low and the spectrum in inhibiting different *S. aureus* strains was narrow. Therefore, analogues were synthesized by Alex Truman and Dr. Siri Ram Chabra (School of Life Sciences, University of Nottingham) and their activity was screened. Some analogues showed improvement in anti-*S. aureus* activity and also broader spectrum with respect to inhibiting virulence factor production by different strains.

4.2 Results

4.2.1 Determination of IQS activity on *S. aureus* virulence factor production

To address this question, *S. aureus* strain KH1187A was cultured in TSB at 37 °C in the presence and absence of IQS for 18 h. AIP-84 (tAIP-III D2A) which is an AgrC competitive inhibitor (Tal-Gan et al., 2013) was used as a positive control for the inhibition of the *agr* system.

Western blot analysis showed that the presence of 100 µM IQS down-regulated *S. aureus* TSST-1 and α -haemolysin and up-regulated protein A production (Figure 4.1a). The minimum concentration of IQS required to effectively inhibit TSST-1 and α -haemolysin production was 10 and 50 µM, respectively. IQS increased protein A production at 50 µM. IQS did not change the secreted or intracellular protein profile of KH1187A visualisation of which confirms that the amount of protein in each sample was similar (Figure 4.1b). The growth curve in Figure 4.1c shows that 100 µM IQS, the highest concentration used, did not affect the growth of KH1187.

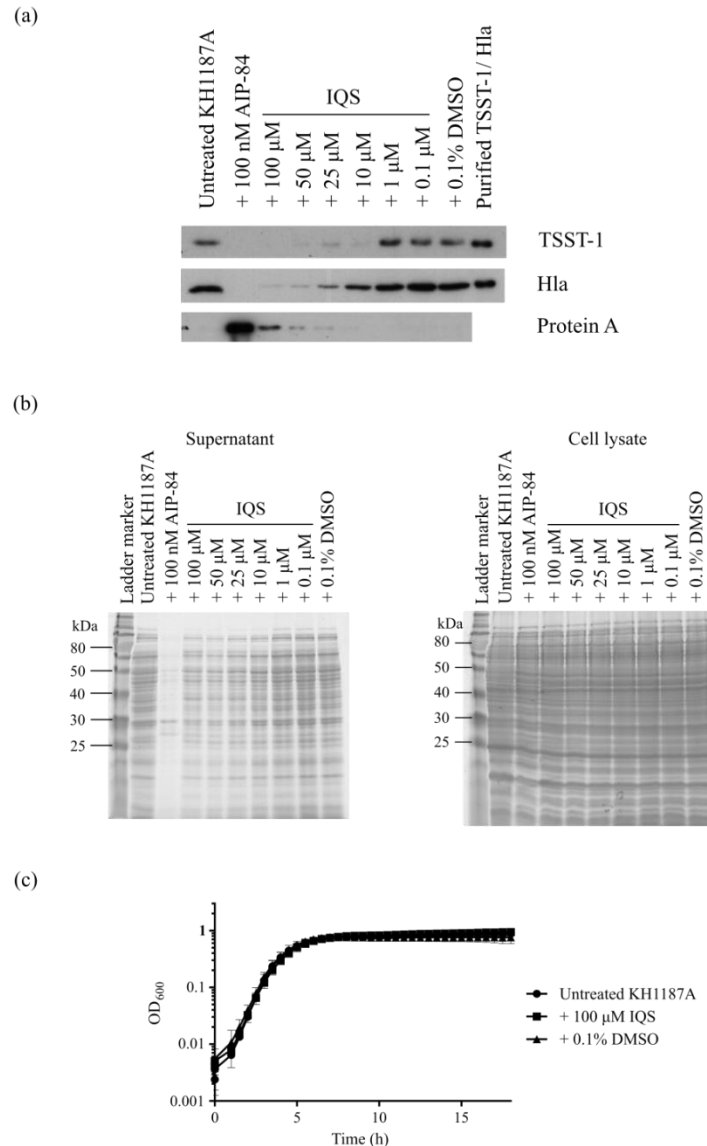


Figure 4.1 Virulence factor production by *S. aureus* KH1187A in the presence of IQS. To determine the effect of IQS on virulence factor production of *S. aureus*, strain KH1187A was cultured in the presence and absence of IQS for 18 h. AIP84, an AgrC inhibitor, was used as a positive control. (a) Western blot showing TSST-1, α -haemolysin (Hla) and protein A production in KH1187A cultures. (b) Coomassie blue staining of SDS-polyacrylamide gels shows the secreted, intracellular and membrane-bound protein profiles. (c) Growth curve of KH1187A treated with IQS cultured in TSB was observed at OD₆₀₀ using a microplate reader (F200, Tecan). All experiments were carried out three separate occasions and the data presented are representative.

4.2.2 IQS reduces toxin production is dependent on the *S. aureus* strains used

S. aureus has high genetic variation between each strain/isolate as its genome contains mobile genetic elements (Malachowa and DeLeo, 2010). To determine if the IQS reduces toxin production in different *S. aureus* strains, *agr* group I strains SH1000 (MSSA), KH1187A (MSSA, TSST-1 producer), and USA300 LAC (MRSA, Panton-Valentine producer) and the *agr* group 3 strain MN8 (MSSA, TSST-1 producer) were treated with IQS at different concentrations for 18 h. AIP-84 was used as a positive control.

Western blot analysis showed that 10 μ M IQS reduced α -haemolysin production by SH1000 (Figure 4.2a). However, IQS did not reduce TSST-1 or α -haemolysin produced by MN8 and USA300 LAC (Figure 4.2b and c). In addition, protein profiles and growth of SH1000, MN8 and USA300 LAC were also not affected by IQS treatment (data not shown); therefore inhibition of toxin production by IQS is strain specific

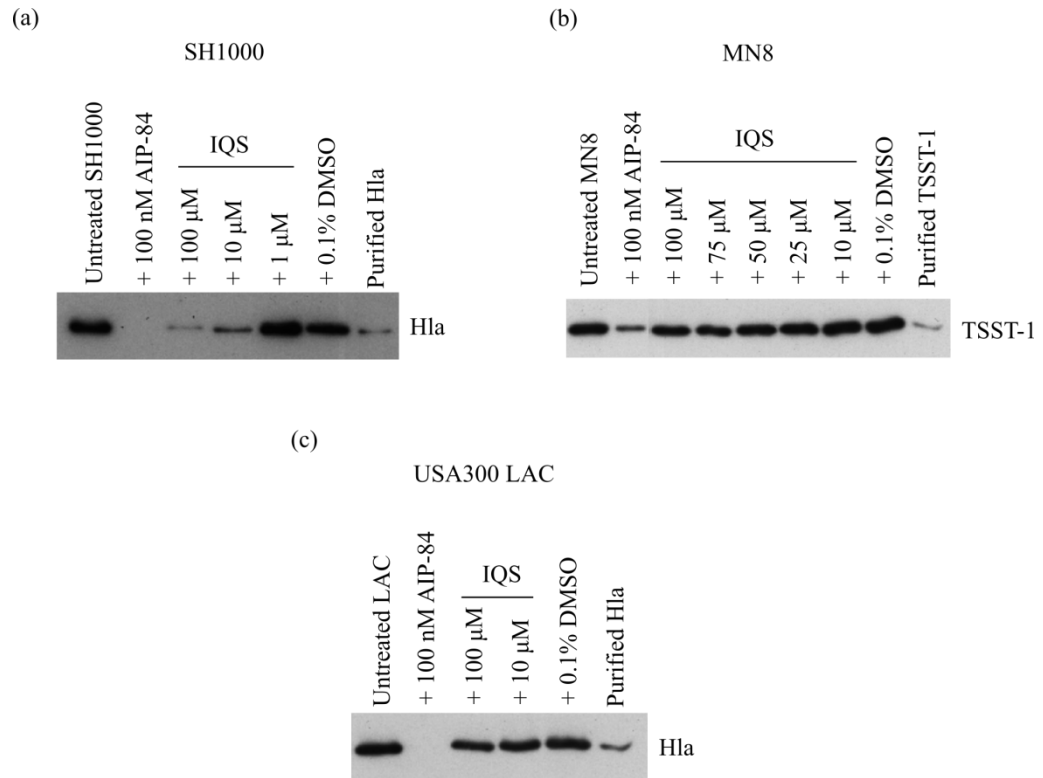


Figure 4.2 Effect of IQS on exotoxin production in SH1000, MN8 and USA300 LAC. To determine the effect of IQS on exotoxin production of other different strains of *S. aureus*, strain SH1000, MN8 and USA300 LAC were cultured in the presence and absence of IQS for 18 h. AIP-84 was used as a positive control. Western blots show the production of α -haemolysin (Hla) by SH1000 and USA300 LAC (a and c) and TSST-1 produced by MN8 (b). The experiment was repeated on at least two separate occasions, with data shown representative of the results obtained.

4.2.3 Screening a small IQS library for activity against *S. aureus* exotoxin production

In an attempt to overcome the strain specificity of IQS activity (see 4.2.2) 14 IQS analogues were synthesized by Dr. Ram Chhabra and Alex Truman (School of Pharmacy, University of Nottingham), Table 4.1. The activity of the compound library was tested by growing KH1187A for 18 h in the presence of all compounds and comparing the reduction in exotoxin production with the native IQS (IQS5) and the *agr* competitive antagonist AIP-84.

Figure 4.3a shows that analogues, IQS1, 4, 6, 7, 8 and 10 had similar activity to the natural IQS compound (IQS5). Interestingly, IQS2, 3, 9 and 11 reduced toxin production to a greater extent when compared to native IQS. Of these four analogues, IQS11 did not up-regulate protein A production (Figure 4.3a). IQS12, IQS13 and IQS14 increased both TSST-1 and α -haemolysin production relative to the untreated control. IQS15 had no activity. Coomassie blue staining showed that 100 μ M IQS10, IQS11 and IQS13 altered the secreted protein profiles (Figure 4.3b). None of the compounds resulted in a visible change of the intracellular and cell membrane-bound protein profiles (data not shown) and did not affect *S. aureus* growth (Figure 4.3c).

Here, we found that IQS2, IQS3, IQS9 and IQS11 had higher efficacy in reducing TSST-1 and α -haemolysin produced in KH1187A than the natural product, IQS5. IQS2 and IQS3 have methylester and ethylester groups replacing the aldehyde group at 4' of thiazole ring of native IQS. Losing hydroxyl group in benzene ring of IQS9 improved the activity in against KH1187A compared with natural IQS, IQS5. IQS11 that was only analogue that had side chain at 5' in thiazole ring effectively reduced exotoxin production without up-regulating

protein A and it changed the secreted protein profile. Additionally, none of these active analogues repressed the bacterial growth.

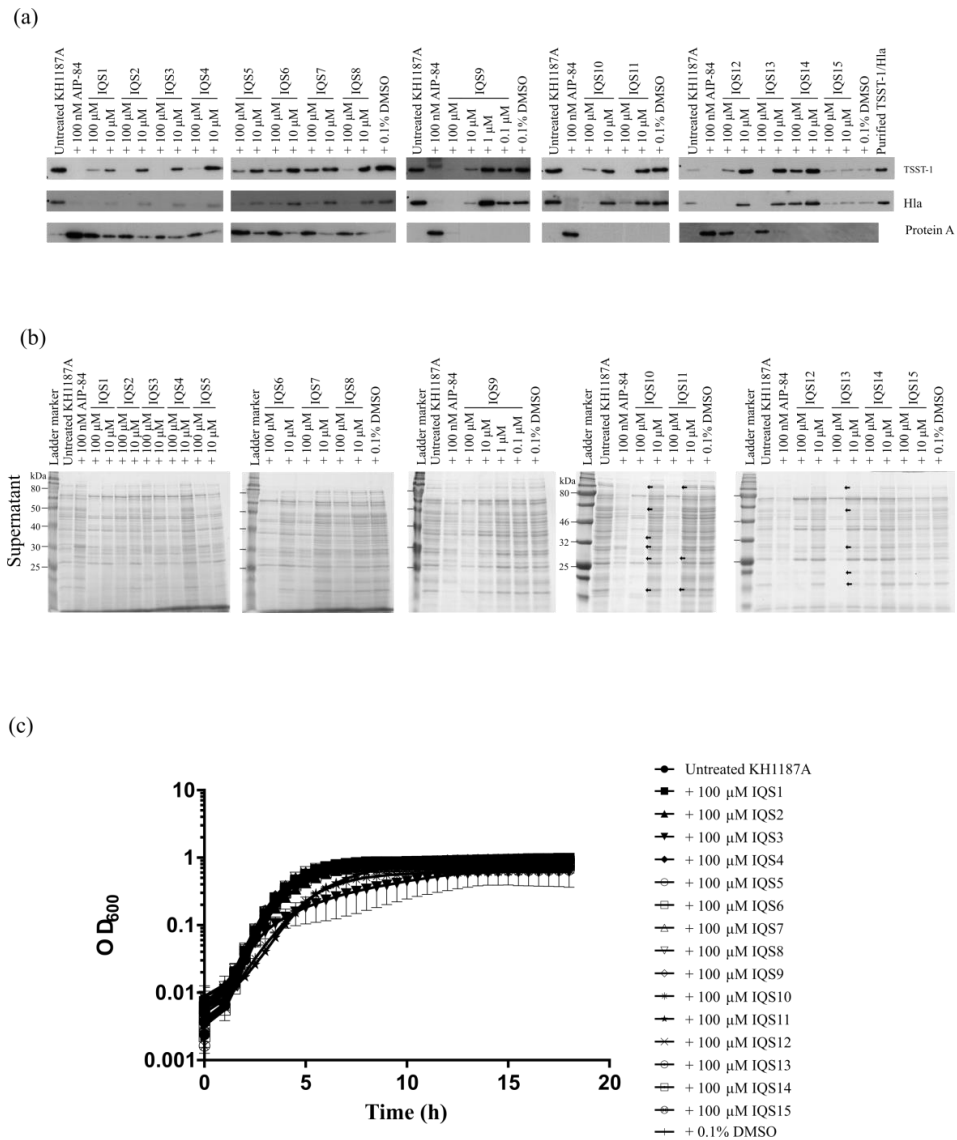


Figure 4.3 Exotoxin production and growth of *S. aureus* in the presence of IQS analogues. To observe and compare the activity of IQS analogues to native IQS (IQS5), KH1187A was cultured in TSB and treated with each analogue at the indicated concentrations for 18 h. AIP-84 was used as a positive control. (a) TSST-1, α -haemolysin (Hla) and protein A production was determined using Western blot. (b) Coomassie blue staining of SDS-polyacrylamide gels showing the secreted protein profiles. The difference between the treated sample and control is indicated by an arrow. (c) Growth of culture treated with each IQS analogue was also determined at OD₆₀₀ using a microplate reader (F200, Tecan). The data shown are the mean $\pm$ standard deviation. This experiment was repeated at least twice.

Table 4.1 Chemical structures of IQS and synthetic analogues with its activity against KH1187A

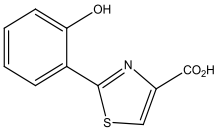
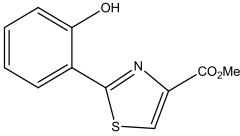
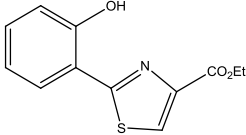
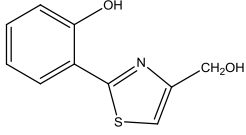
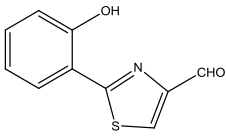
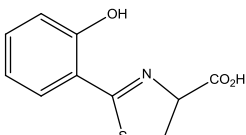
Activity	Code	Compound	Structure	Ref
✓	IQS1	IQS-CO ₂ H (aeruginoic acid)	 2-(2-hydroxyphenyl)thiazole-4-carboxylic acid	(Carmi et al., 1994)
✓✓	IQS2	IQS-CO ₂ Me	 methyl 2-(2-hydroxyphenyl)thiazole-4-carboxylate	
✓✓	IQS3	IQS-CO ₂ Et	 ethyl 2-(2-hydroxyphenyl)thiazole-4-carboxylate	
✓	IQS4	IQS-CH ₂ OH (aeruginol)	 2-(4-(hydroxymethyl)thiazol-2-yl)phenol	(Yang et al., 1993)
✓	IQS5	IQS (aeruginaldehyde)	 2-(4-(hydroxy-1,2-methyl)thiazol-2-yl)phenol	(Lee et al., 2013, Ye et al., 2014)
✓	IQS6	IQS-2H-CO ₂ H (dihydroaeruginoic acid)	 2-(2-hydroxyphenyl)thiazole-4-carboxylic acid	(Carmi et al., 1994)

Table 4.1 (continue)

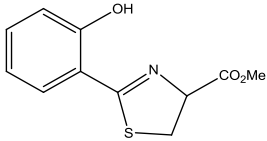
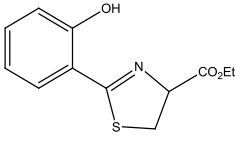
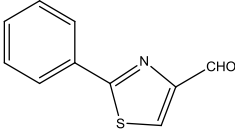
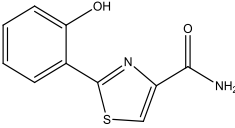
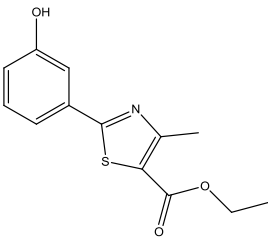
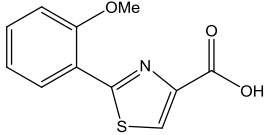
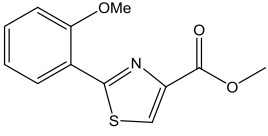
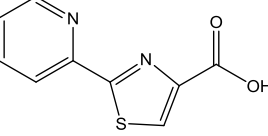
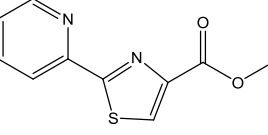
Activity	Code	Compound	Structure	Ref
✓	IQS7	IQS-2H- CO ₂ Me	 methyl 2-(2-hydroxyphenyl)-4,5- dihydrothiazole-4-carboxylate	
✓	IQS8	IQS-2H- CO ₂ Et	 ethyl 2-(2-hydroxyphenyl)-4,5- dihydrothiazole-4-carboxylate	
✓✓	IQS9	deOH-IQS	 2-phenylthiazole-4-carbaldehyde	
✓	IQS10	IQS-CONH ₂	 2-(2-hydroxyphenyl)thiazole-4-carboxamide	

Table 4.1 (continue)

Activity	Code	Compound	Structure	Ref
✓✓	IQS11	mIQS-4Me- SCO ₂ Et	 ethyl 2-(2-hydroxyphenyl)-4-methylthiazole-5-carboxylate	
✕✕	IQS12	2OMeIQS- CO ₂ H	 2-(2-hydroxyphenyl)thiazole-4-carboxylic acid	
✕✕	IQS13	2OMeIQS-CO ₂ Me	 methyl 2-(2-methoxyphenyl)thiazole-4-carboxylate	
✕✕	IQS14	2-PyIQS-COOH	 2-(pyridin-2-yl)thiazole-4-carboxylic acid	
✕	IQS15	2-PyIQS- COOMe	 methyl 2-(pyridin-2-yl)thiazole-4-carboxylate	

Note. ✓, has positive activity level against KH1187A similar to natural IQS (IQS5); ✓✓, activity level against KH1187A is higher than natural IQS; ✕ has no activity in against KH1187A; ✕✕, has inverse activity in against KH1187A

4.2.4 Toxin reduction is not caused by iron-chelating activity of the IQS analogues

IQS is a by-product from the hydrolysis of dihydroaeruginoate during the production of pyochelin, an iron-chelating siderophore produced by *Pseudomonas spp.* (Serino et al., 1997, Ye et al., 2014). To evaluate potential iron-chelating activity of the compounds, a CAS assay (Schwyn and Neilands, 1987) was carried out with the IQS analogues at 100 μ M. Desferrioxamine was used as a positive control.

The CAS assay showed the lack of or low iron-chelating activity of natural IQS and most of the analogues, i.e. IQS2, IQS3, IQS4, IQS9, IQS11, IQS12, IQS13, IQS14 and IQS15 relative to desferrioxamine (less than 10 %). The iron-chelating activity of IQS7, IQS8 and IQS10 were approximately 30 %. IQS7 and IQS8 had a very similar structure to IQS2 and IQS3, only difference is at 5' in thiazole ring as IQS7 and IQS8 which had CH₂ instead of CH. This suggests an important of the position 5' in thiazole in iron-chelating property. IQS1 and IQS6 chelated iron nearly equal to the positive control adjusted to 100 %. They both contain a carboxylic group at the 4' of the thiazole. In addition, IQS10 which has similar structure to IQS9 but contains a carboxylic group at 4' in the thiazole had iron-chelating activity up to 30 %. This suggests that position 4' in thiazole is crucial in iron-chelating.

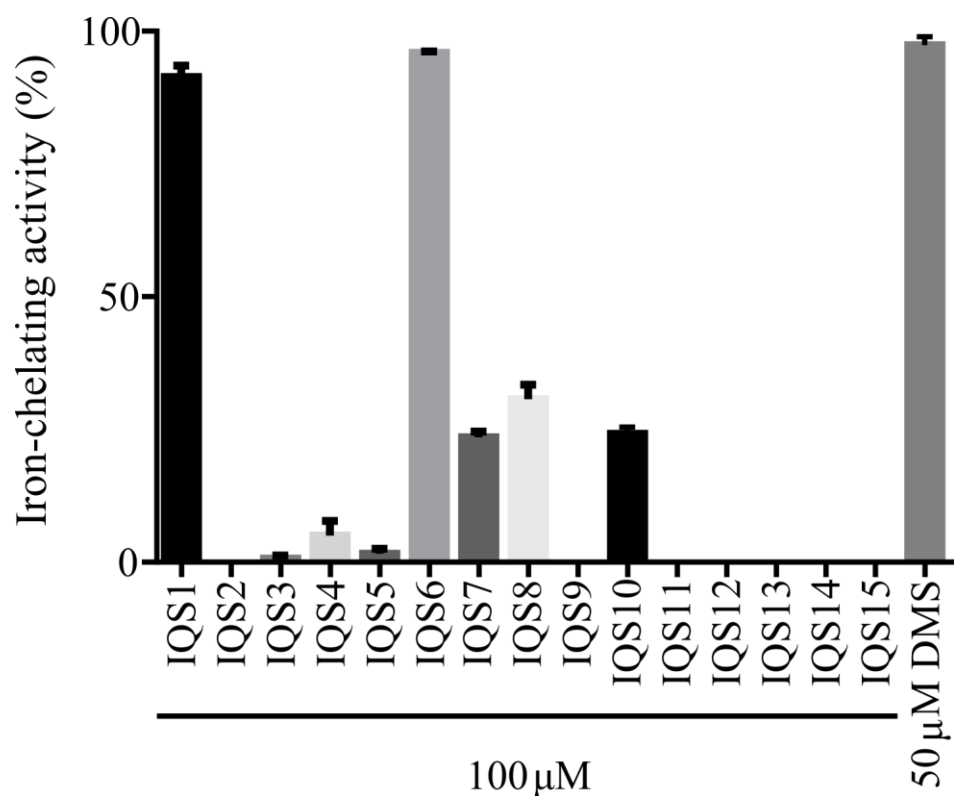


Figure 4.4 Iron-chelating activities of IQS analogues. The iron-chelating activity of IQS and analogues at 100 μ M was evaluated using a CAS assay (Schwyn and Neilands, 1987). Fifty micromolar desferrioxamine mesylate salt (DMS) was used as a positive control. The bar chart represents mean $\pm$ standard deviation. The experiment was repeated in triplicate and on two separate occasions.

4.2.5 Determination of the efficacy of IQS3 and IQS11 as inhibitors of exotoxin production by different *S. aureus* strains

To determine structure activity relationships and the mode of action of the active analogues, IQS3 and IQS11 were selected as representatives of the lead compounds. IQS3 has a similar structure to IQS2 and IQS9 (Table 4.1) but is more hydrophobic which may aid entry into the cell. IQS11 was selected as it does not induce protein A production of *S. aureus* compared with the other analogues and it is only an active IQS analogue containing a side chain at position 5 of the thiazole ring (compare the structure of IQS11 and other analogues in Table 4.1).

4.2.5.1 Determination of the efficacy of IQS3 and IQS11 against CA-MRSA strain

Previous data has shown that IQS (IQS5) was ineffective against certain *S. aureus* strains. In order to investigate the activity of IQS3 and IQS11 against a CA-MRSA strain, USA300 LAC was chosen for further studies and cultured in the presence and absence of IQS3 and IQS11, respectively for 18 h. AIP-84 was used as positive control.

Western blot analysis showed α -haemolysin production of USA300 LAC treated with IQS11 slightly reduced, whereas IQS3 was not active against USA300 LAC, neither analogue influenced protein A production (Figure 4.5a). Coomassie blue staining showed that IQS11 slightly decreased the secreted proteins of USA300 LAC (Figure 4.5b). Both analogues did not alter the intracellular and membrane-bound protein profile (data not shown) and did not affect the growth of USA300 LAC (Figure 4.5c).

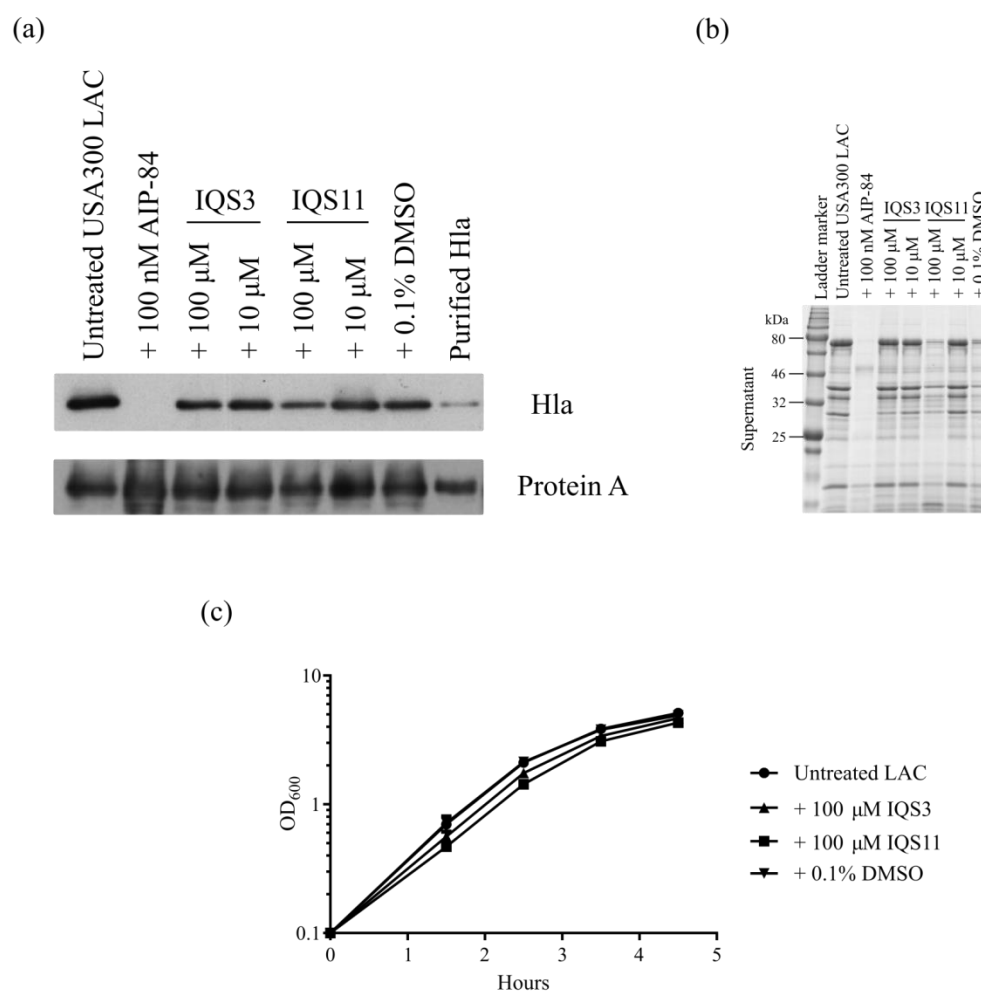


Figure 4.5 Effect of IQS3 and IQS11 on CA-MRSA USA300 LAC. To observe the effect of active IQS analogue 3 and 11 on MRSA strain, USA300 LAC was treated in the presence and absence of IQS3 and IQS11 for 18 h. AIP-84 was used as a positive control. (a) α -haemolysin (Hla) and protein A production was determined using Western blot. (b) Secreted protein profile was investigated from supernatant by Coomassie blue staining of SDS-polyacrylamide gels. (c) Growth curve of USA300 LAC in the presence of IQS analogues 3 and 11 was determined at OD₆₀₀ using a spectrophotometer. The experiment was carried out on two separate occasions, data shown is representative.

4.2.5.2 IQS3 and IQS11 are active against different *agr* groups

To determine the effect of IQS3 and IQS11 on *S. aureus* strains belonging to different *agr* groups, TS2 and TS5 (*agr*-II), MN8 (*agr*-III) and TS3 (*agr*-IV) were cultured in the presence of IQS3 and IQS11 for 18 h. AIP-84 was used as a positive control.

Western blot analysis shows that IQS3 and IQS11 reduced α -haemolysin production by *S. aureus* TS2 (Figure 4.6a), whereas these analogues slightly reduced α -haemolysin production by TS5 (Figure 4.6b). Interestingly, 100 μ M IQS11 reduced production of protein A in both *agr* group II strains (Figure 4.6a and b). The secreted protein profiles of TS2 and TS5 were slightly changed when treated with IQS11 (Figure 4.6c and d). IQS3 and IQS11 did not affect intracellular or membrane-bound protein profiles or the growth of TS2 and TS5 (data not shown). IQS3 and IQS11 did not reduce production of TSST-1 in MN8 which is *agr*-III (Figure 4.7a). IQS3 slightly up-regulated protein A in MN8 but IQS11 did not (Figure 4.7a). Coomassie blue staining of SDS-polyacrylamide gels showed that IQS11 had a minor effect on the secreted protein profile of MN8 (Figure 4.7b). IQS3 reduced α -haemolysin production by TS3 which is *agr*-IV, whereas IQS11 had no effect on TS3's α -haemolysin or protein A production (Figure 4.7c). IQS11 did not affect the secreted protein profile of TS3 (Figure 4.7d).

IQS3 and IQS11 have shown extensive reduction of exotoxin production in KH1187A *agr*-I, here it is shown that IQS3 reduced α -haemolysin production by *agr*-II and IV strains. Meanwhile, IQS11 showed striking results in reducing protein A production by *agr*-II strains but it did not reduce virulence factor production of the *agr*-III and -IV strains.

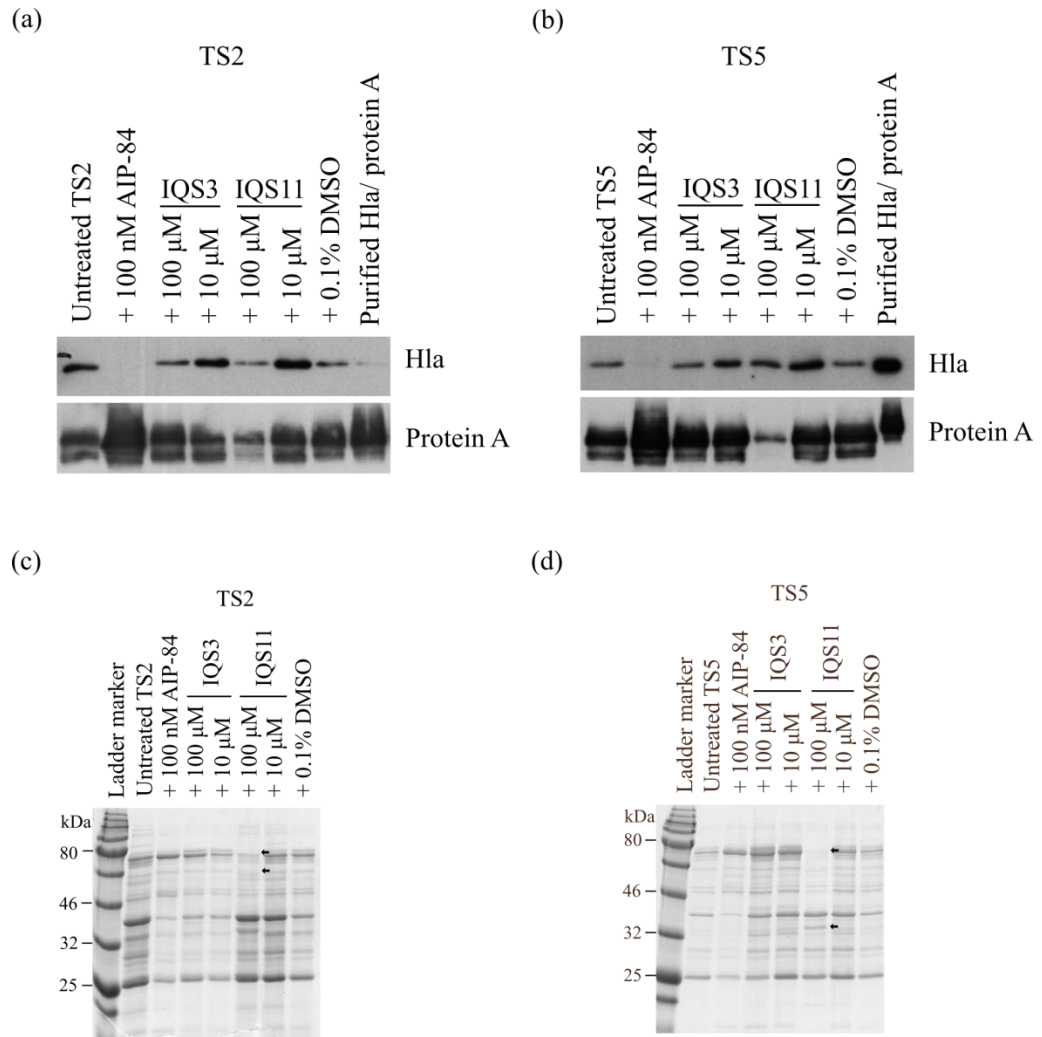


Figure 4.6 Effect of IQS3 and IQS11 on *S. aureus* *agr*-II strains. To determine the effect of IQS3 and IQS11 on the production of *S. aureus* *agr*-II, TS2 and TS5 which are clinical isolates belonging to *agr*-II were cultured in the presence or absence of IQS3 and IQS11 for 18 h. (a and b) Western blot was performed to determine α -haemolysin (Hla) and protein A production by TS2 and TS5. (c and d) Coomassie blue staining of SDS-polyacrylamide gels show the secreted protein profiles of TS2 and TS5 cultures. The differences between treated samples and controls are indicated by arrows. The experiment was carried out twice and representative data are shown.

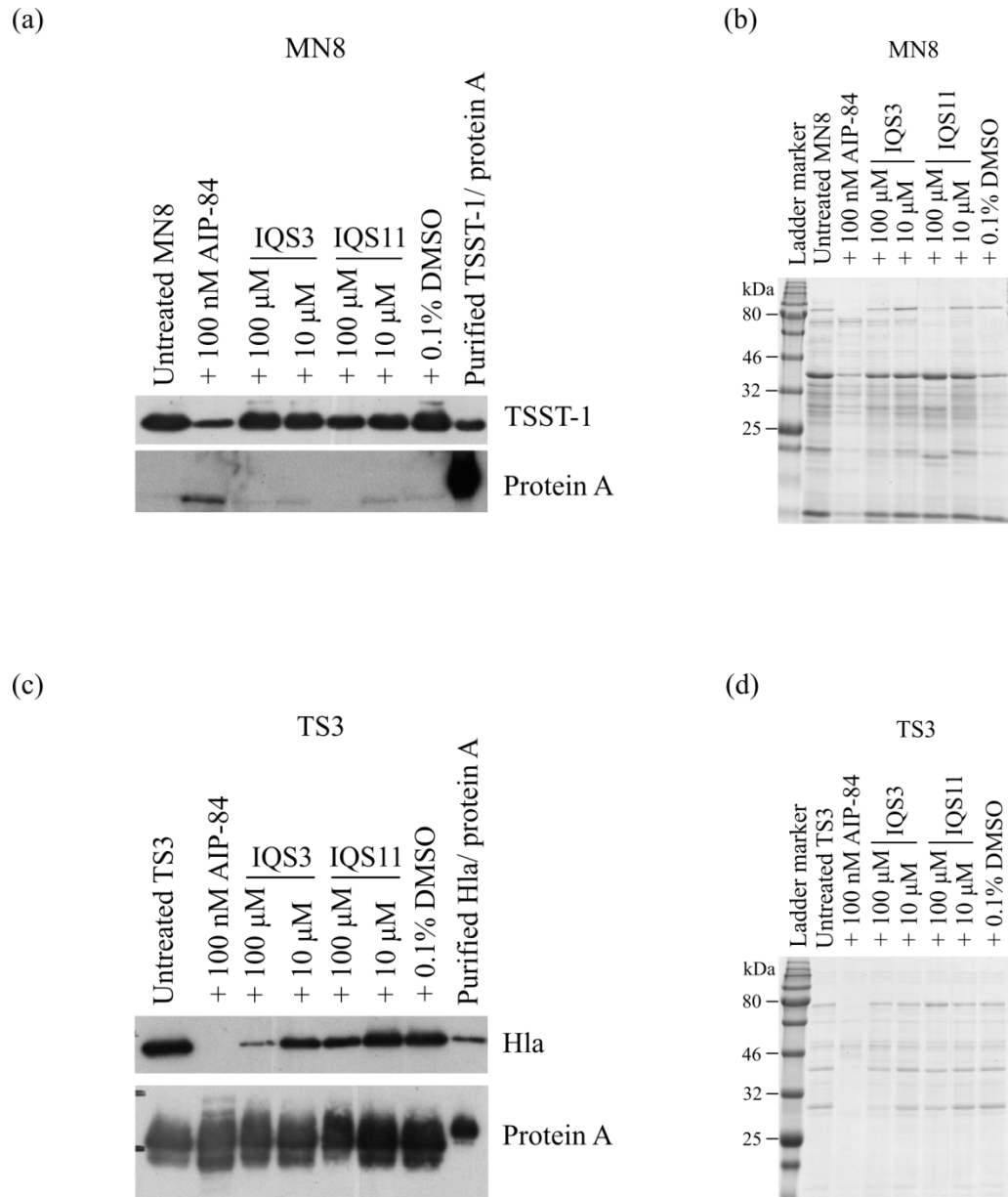


Figure 4.7 Effect of IQS3 and IQS11 on *S. aureus* *agr*-III and -IV. *S. aureus* strain MN8 which is *agr*-III and strain TS3, a clinical isolate *agr*-IV, were cultured in the presence and absence of IQS3 and IQS11 for 18 h to investigate the effect of the analogues on exotoxin production of these *agr* groups. (a) TSST-1 production in MN8 and (c) α -haemolysin (Hla) and protein A production in TS3 was determined using Western blot. (b and d) Coomassie blue staining of an SDS-polyacrylamide gel shows the secreted protein profiles of MN8 and TS3, respectively. The experiment was carried out twice and representative data are shown.

4.2.6 Determining the mode of action of the lead IQS analogues

As IQS3 inhibited exotoxin and increased protein A production in some *S. aureus* strains, this suggested that the target of IQS3 could be the *agr* system or SarA. To determine whether *agr* or SarA is a target, Δagr and $\Delta sarA$ reporter strains were generated.

4.2.6.1 IQS3 and IQS11 do not inhibit the light generation by bacterial *lux* reporters

In order to investigate the target of IQS3 and IQS11, an *agrP3:lux* reporter strain was generated. To ensure that IQS3 and IQS11 specifically reduced *agrP3* promoter activity and not the luminescent signal, *S. aureus* RN4220 harbouring *Pxyl/tet:gfp:luxCDABE* (Bethany Mills's unpublished reporter strain) was treated with IQS3 and IQS11 in the presence of 200 ng/ml anhydrotetracycline, an activator of *xyl/tet* promoter

Figure 4.8 shows that 100 μ M of either IQS3 or IQS11 did not affect luminescence of the culture compared with DMSO-treated group. The AgrC inhibitor AIP-84 also did not affect luminescence.

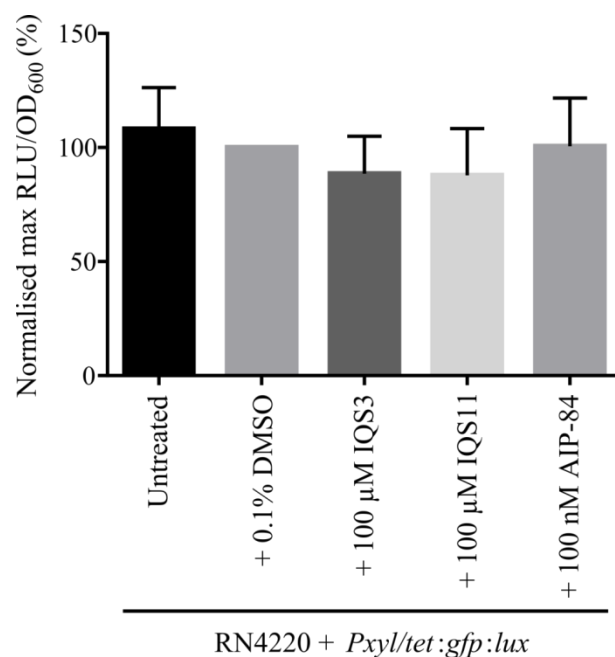


Figure 4.8 IQS analogues do not inhibit light production by an inducible *luxCDABE* reporter strain. In order to determine the specificity of the lead analogues, IQS3 and IQS11, RN4220 harbouring *Pxyl/tet:gfp:luxCDABE* was treated with either 100 μM IQS3 or IQS11. 200 ng/ml of anhydrotetracyclin was added to the culture to activate the *xyl/tet* promoter. The luminescent signal of DMSO-treated culture was used as control and adjusted to 100 %. The data represent mean of three independent experiments ± standard deviation.

4.2.6.2 Construction of $\Delta sarA$ SH1000 *agrP3:lucDABE*

As the presence of IQS 3 or 11 mimics the reduced toxin phenotype of both *agr* and *sarA* mutants (see Figures 4.9a and b), a $\Delta sarA$ mutant was generated to assess whether the IQS analogues targeted these regulatory systems. It is known that both SarA and AgrA bind directly to and enhance expression from the RNAlII promoter, *P3*. Therefore, to assess SarA and AgrA regulatory function indirectly a *sarA* mutant was generated in a *S. aureus* strain with a chromosomal *agrP3-lux* reporter. The hypothesis being that IQS activity would be reduced if the IQS target protein, in this case SarA, had been deleted. The $\Delta sarA$ was transduced into SH1000 *attB2::agrP3-lux* as described in the methods.

Successful construction of a *sarA* mutant was confirmed by erythromycin resistance and PCR (data not shown). The expression of α -haemolysin was, subsequently, determined by Western blot. Figure 4.9a shows that the *sarA* mutant produced less α -haemolysin than the wild-type, whereas, α -haemolysin could not be detected in the *agr* mutant cultures. The result was confirmed by streaking each strain onto sheep blood agar. It was found that the wild-type and *sarA* mutant created a clear zone, indicating haemolysis, but an *agr* mutant did not (Figure 4.9b). Finally, we investigated the *agrP3* promoter activity. Figure 4.9c, in the presence of a saturating concentration of activator, i.e. 1 μ M of AIP-1, *agrP3* expression by the *sarA* mutant was approximately half that of wild-type (966 ± 387 vs $2,168 \pm 981$, respectively). In addition, the maximum RLU/OD₆₀₀ did not rise above the basal level.

As the $\Delta sarA$ reporter strain produced a detectable amount of α -haemolysin and *agrP3* promoter activity, any further reduction in the presence

of the IQS analogues would suggest that SarA was not the target of IQS inhibition.

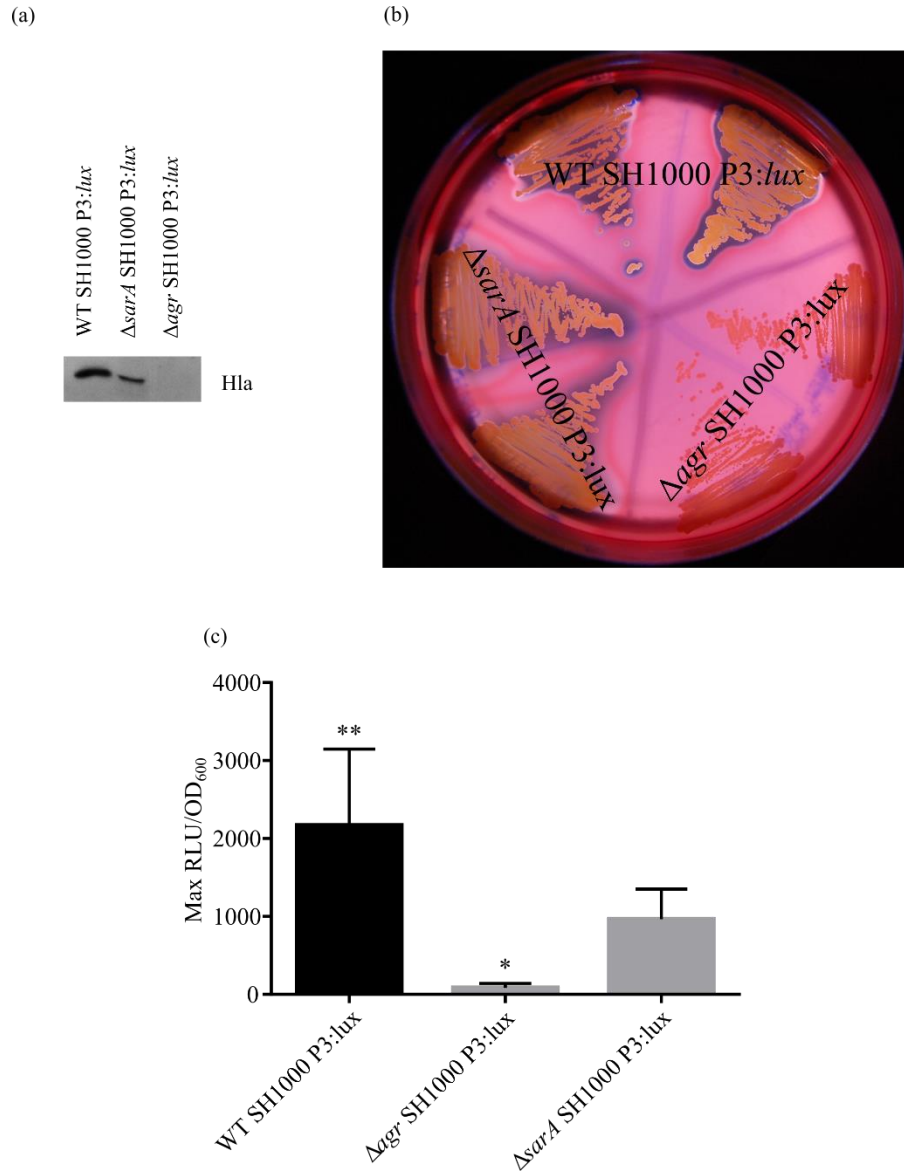


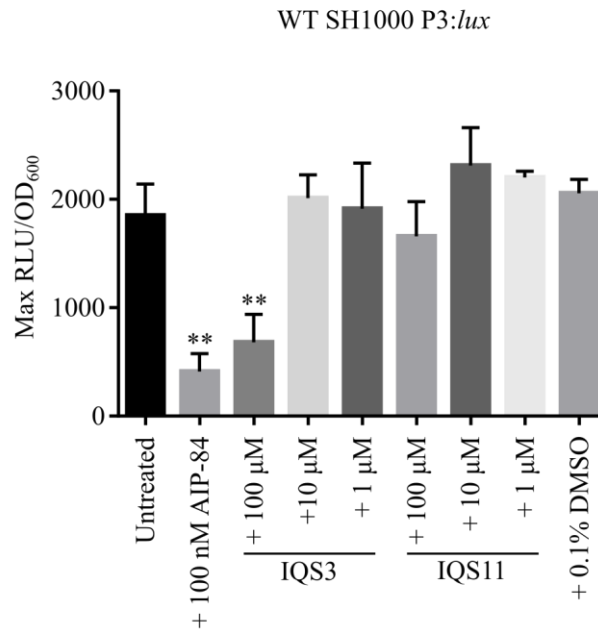
Figure 4.9 Validation of the $\Delta sarA$ *agrP3:luxCDABE* reporter strain. To validate the constructed $\Delta sarA$ *agrP3:luxCDABE*, it was investigated using different approaches on different targets. (a) Western blot analysis comparing α -haemolysin (Hla) production of overnight wild-type, *sarA* and *agr* mutants. (b) Sheep blood agar was used to visualise haemolysin activity of the cultures. (c) *P3* promoter activity which is also under the control of SarA was evaluated using an *agrP3-lux* fusion reporter gene in the presence of 1 μ M AIP-1. The data represents mean $\pm$ standard deviation of three independent experiments. The means of each tested group were compared with the DMSO-treated group. *; $p < 0.05$. **; $p < 0.01$.

4.2.6.3 Neither IQS3 nor IQS11 reduced *agrP3* expression?

To determine the effect of IQS3 and IQS11 on the *agrP3* promoter activity of the wild-type reporter strain, SH1000 *agrP3:luxCDABE* was treated with IQS11 and IQS3. Figure 4.10a shows that 100 μ M IQS3 significantly reduced *agrP3* promoter expression of the wild-type approximately 3-fold from $1,850 \pm 290$ to 681 ± 257 ($p < 0.01$). This suggests that the target of IQS3 regulates the *agrP3* promoter. Figure 4.10a also shows that IQS11 did not affect *agrP3* promoter activity, indicating that IQS11 has a different target to IQS3.

To determine whether SarA was the target of IQS3 or IQS11, the Δ *sarA* SH1000 *agrP3:lux* reporter strain was grown with IQS3 or IQS11. Figure 4.10b indicates that 100 μ M IQS3 significantly reduced expression of the *agrP3* promoter by ~3 fold (from 794 ± 32 to 269 ± 68) when compared to the DMSO-treated group ($p < 0.01$). This suggests that IQS3 does not target SarA. IQS11 did not inhibit *agrP3* promoter activity, confirming that IQS11 targets another protein/system which regulates virulence factor production via an *agrP3* promoter-independent pathway.

(a)



(b)

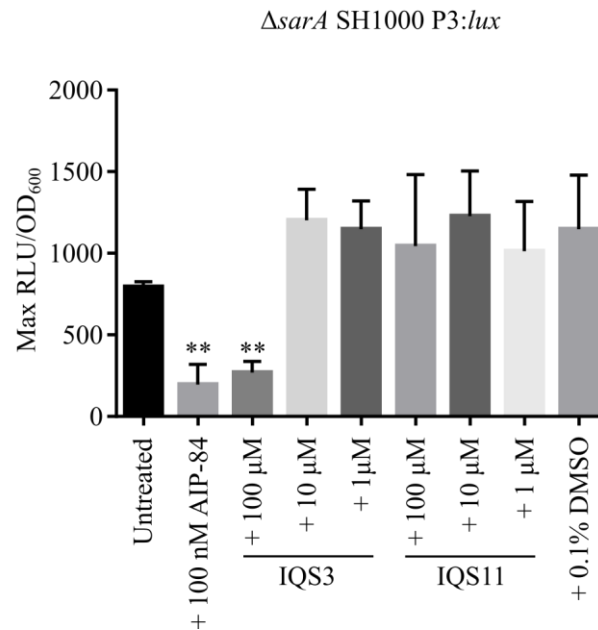


Figure 4.10 Effect of IQS3 and IQS11 on wild-type and SH1000 Δ *sarA* *agrP3:luxCDABE* expression. (a) wild-type and (b) SH1000 Δ *sarA* *agrP3:luxCDABE* were treated either with IQS3 or IQS11 at indicated concentrations and the activity of *agrP3* that is affected was determined through luminescence fusion. The data represents the mean $\pm$ standard deviation of three independent experiments. **; $p < 0.01$.

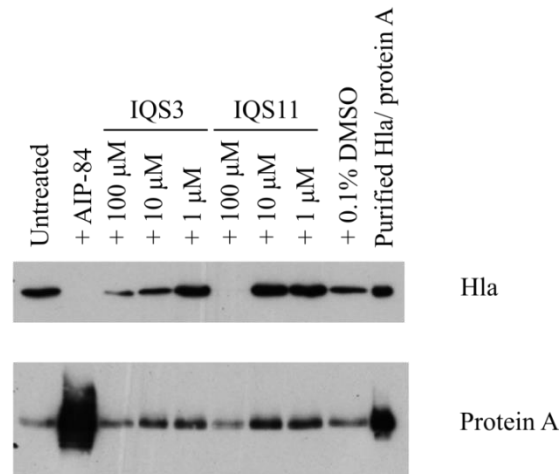
4.2.6.4 IQS3 and IQS11 both reduce α -haemolysin production in wild-type *S. aureus* SH1000 *agrP3:luxCDABE*

Although IQS11 did not inhibit *agrP3* promoter activity in SH1000 *agrP3:luxCDABE*, it was questioned whether IQS11 inhibited exotoxin production in this *S. aureus* strain. SH1000 *P3:lux* was treated with IQS3 and IQS11 for 18 h. AIP-84 was used as a positive control.

Western blot analysis showed that 100 μ M IQS3 and IQS11 both reduced the production of α -haemolysin, and did not increase protein A production as much as the AIP-84-treated group (Figure 4.11a). Coomassie blue staining of an SDS-polyacrylamide gel showed that 100 μ M IQS11 had a slightly greater effect on the secreted protein profile than IQS3 (Figure 4.11b).

The results showed that IQS3 and IQS11 both reduced α -haemolysin expression by SH1000. Overall it suggests that IQS3 and IQS11 have different cellular targets, with IQS3 likely to target regulation of RNAIII via inhibition of *agr*.

(a)



(b)

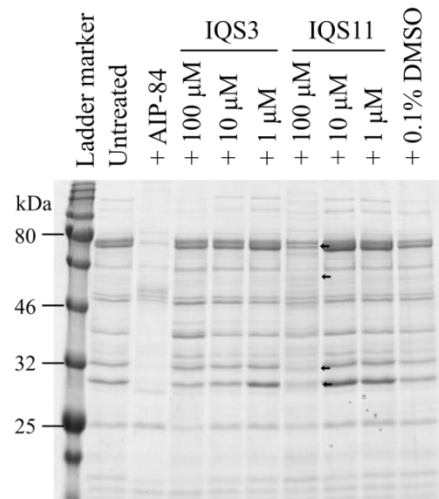


Figure 4.11 IQS3 and IQS11 inhibit α -haemolysin production by *S. aureus* SH1000 *agrP3:lucCDABE*. To determine the effect of IQS3 and IQS11 on α -haemolysin production of the constructed reporter strain, SH1000 *agrP3:lucCDABE*, the reporter strain was treated with IQS3 and IQS11 for 18 h. (a) α -haemolysin and protein A production was examined by Western blotting. (b) Secreted protein profile was visualized by Coomassie blue staining of an SDS-polyacrylamide gel. The arrows highlight the differences between the treated group and negative controls. The experiment was repeated on two independent occasions with representative data shown.

4.2.7 IQS3 targets either AgrC or AgrA

Data suggest that IQS3 reduced *S. aureus* virulence determinants by targeting the *S. aureus agr* system which is composed of four proteins, AgrA/B/C and D. The next aim was to determine which Agr protein was the likely target for IQS3. To directly investigate the effect of IQS3 on each *agr* group and to help identify the molecular target of IQS3, ROJ48 strains (RN4220 Δagr *agrP3:luxCDABE*) harbouring a pSKerm-P2:*agrCA* plasmid carrying one of the four *agr* group *agrC* and *agrA* genes (Jensen, 2008) was used. Each strain was cultured in the presence of a saturating concentration of the cognate AIP (10-fold higher than the EC₅₀ value) together with increasing concentrations of either IQS3 or IQS11. If the compound reduced *agrP3:lux* activity, it would confirm that it targeted either AgrC or AgrA.

Figure 4.12 indicated that IQS3 inhibited all *agr* groups in a concentration dependent manner. Inhibition by IQS3 of *agr*-I, -II, -III and -IV gave estimated IC₅₀ values of 84 μ M, 75 μ M, 114 μ M and 45 μ M, respectively. Surprisingly, IQS11 inhibited the activity of the *agrP3 agr*-II construct with an IC₅₀ of ~34 μ M.

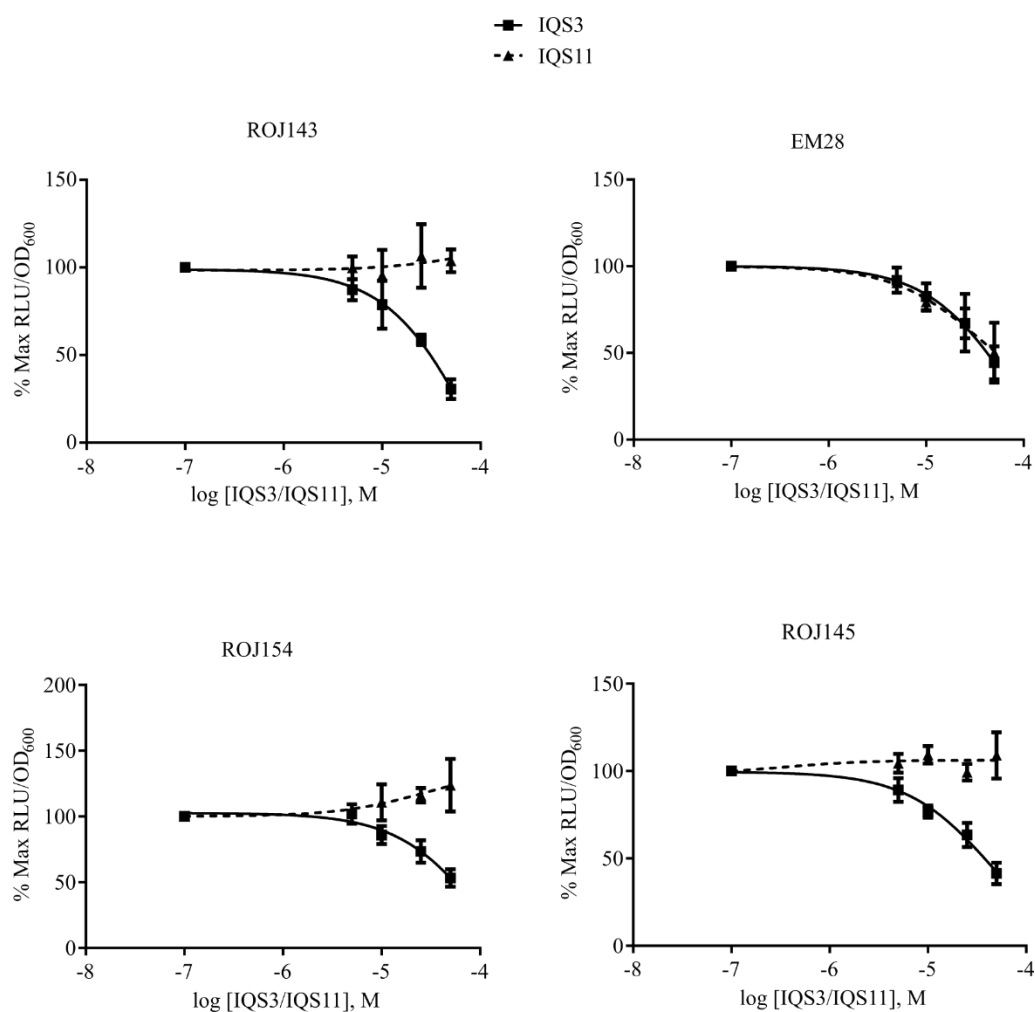


Figure 4.12 Effect of IQS3 and IQS11 on *S. aureus* agr groups I to IV. To determine the effect of IQS3 and IQS11 on reporter strains of all *agr* groups and identify the target of IQS3, reporter strains (*agrP3::lux* carrying AgrC1A1, AgrC2A2, Agr C3A3 or AgrC4A4) strains, ROJ143 (*agr*-I), EM28 (*agr*-II), ROJ154 (*agr*-III) and ROJ145 (*agr*-IV) were treated with IQS3 or IQS11 ranging from 5 - 100 μ M in the presence of 100 nM AIP-1, 100 nM AIP-2, 1 μ M AIP-3 or 100 nM AIP-4, respectively. The normalised data represent the mean $\pm$ standard deviation of three independent experiments. Squares represent cultures treated with IQS3. Triangles with IQS11.

4.2.8 IQS3 and IQS11 are non-competitive inhibitors of *agr*

The data presented in Figure 4.12 indicates that IQS3 is a cross group antagonist of the *S. aureus agr* system and that IQS11 is an antagonist of *agr*-II only. To determine whether the IQS analogues were competitive or non-competitive antagonists each *agrCA/agrP3::lux* reporter was incubated with a fixed concentration of antagonist (5, 10, 25, 50 μ M) and a variable concentration of activator (AIP). If IQS3 was a competitive antagonist, the dose-response curve would shift rightwards without decreasing the maximum response (Murray et al., 2014).

All *agr* group reporters treated with IQS3 showed a similar response curve, the maximum response was reduced as the concentration of IQS3 increased and there was no rightward shift of the curves (Figure 4.13). These results suggest that IQS3 is a non-competitive antagonist, inhibiting sensing or signal transduction of the *agr* QS system.

IQS11 which inhibited the activity of the *agr*-II reporter was also a non-competitive antagonist (Figure 4.14).

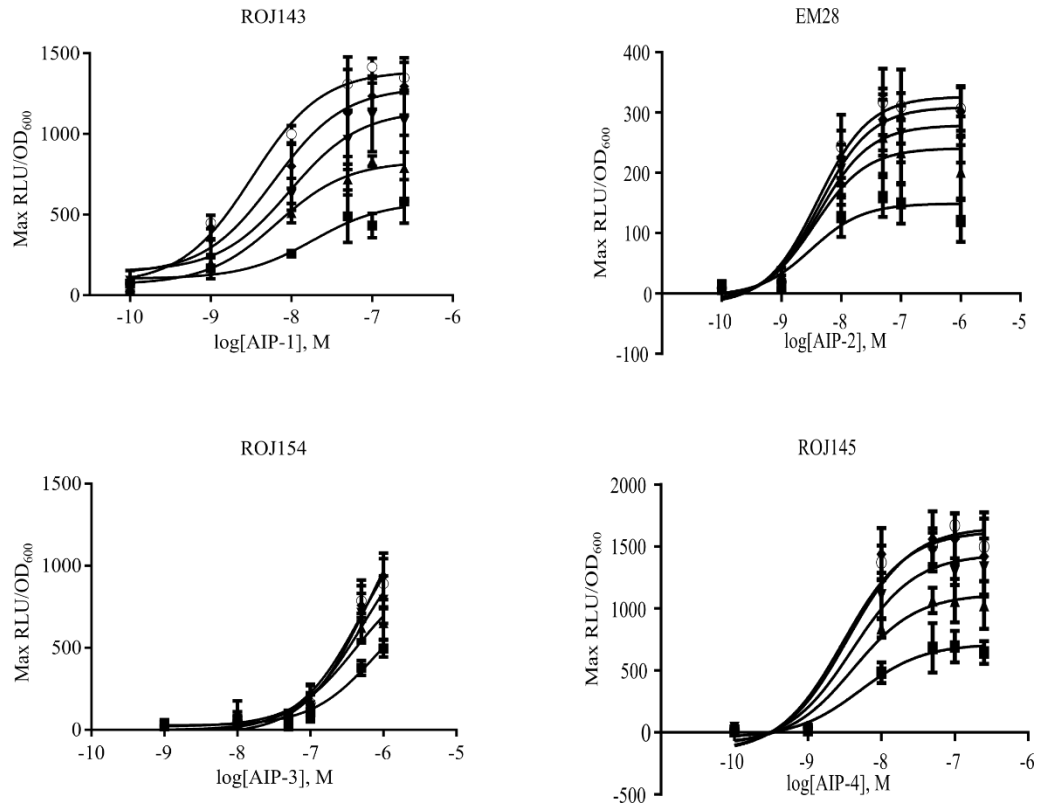


Figure 4.13 IQS3 is non-competitive inhibitor of AgrCA/agrP3::lux for all four *agr* groups. ROJ143 (*agr*-I), EM28 (*agr*-II), ROJ154 (*agr*-III) and ROJ145 (*agr*-IV) were incubated with various concentrations of AIPs and IQS3 in order to identify type of inhibition of IQS3. The *agrP3:lucCDABE* activity was evaluated. The Max RLU/OD₆₀₀ was plotted as a dose-response curve. The data represent mean ± standard deviation of three independent experiments. ○, + 0.1 % DMSO; ■, + 50 μM IQS3; ▲, + 25 μM IQS3; ▼, + 10 μM IQS3; ◆, + 5 μM IQS3.

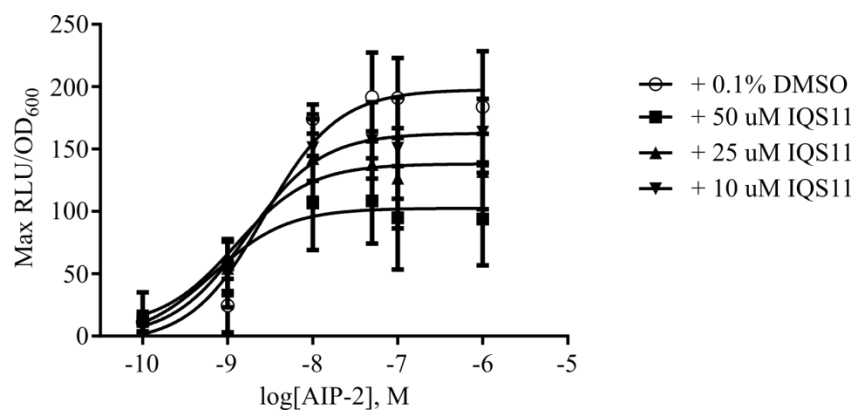


Figure 4.14 IQS11 is non-competitive inhibitor of AgrC group-II. Strain EM28 (*agr-II*) was incubated with fixed concentrations of IQS11 and increasing concentrations of AIP-2 to identify type of inhibition of IQS11. The *agrP3:luxCDABE* activity was evaluated. The Max RLU/OD₆₀₀ was plotted as a dose-response curve. The data represent mean $\pm$ standard deviation. The experiment was carried out in triplicate. $\circ$, + 0.1 % DMSO; $\blacksquare$, + 50 μ M IQS3; $\blacktriangle$, + 25 μ M IQS3; $\blacktriangledown$, + 10 μ M IQS3; $\blacklozenge$, + 5 μ M IQS3.

4.2.9 IQS3 is antagonist of constitutive AgrC reporter strain suggesting AgrA is the target

Geisinger and coworkers have previously described a set of AgrC point mutations that lead to constitutive activation of AgrC (Geisinger et al., 2009). Point mutations were broadly split into two groups. Firstly, mutations within the receptor binding domain that confer constitutive activity by promoting the ‘on’ state/confirmation of AgrC that could be attenuated by an inverse agonist, such as AIP-2. Secondly, mutations within the kinase domain that are constitutively active but do not respond to an inverse agonist (Geisinger et al., 2009). In order to further understand the inhibition caused by the lead IQS analogues, it was decided to determine whether they could inhibit constitutive AgrC receptor activity. AgrC-1 point mutations T197K and L205R as described by Geisinger were generated in our *agr-I* reporter (Dr Victoria Steele, unpublished data) as well as a novel constitutive reporter AgrC-1 S116K (Fabio Rui et al, manuscript in preparation).

4.2.9.1 Determination of the response of an AgrC group-1 S116K mutant to an agonist, an antagonist and a neutral antagonist.

To determine whether the S116K mutation of AgrC which lies within the receptor binding domain behaves like other constitutive receptor domain mutants reported previously by Geisinger (Geisinger et al., 2009), the activity of the reporter was measured in the presence of increasing concentrations of a known agonist (AIP-1), a neutral antagonist (AIP-3) and an inverse agonist (AIP-2).

Figure 4.15 shows that as with the previously published constitutive mutants AIP-1 activated *agrP3:lux* expression, AIP-2 inhibited *agrP3:lux* and AIP-3 was neutral. These data suggest that AgrC S116K behaves like and is comparable to the other AgrC constitutive receptor mutants previously described.

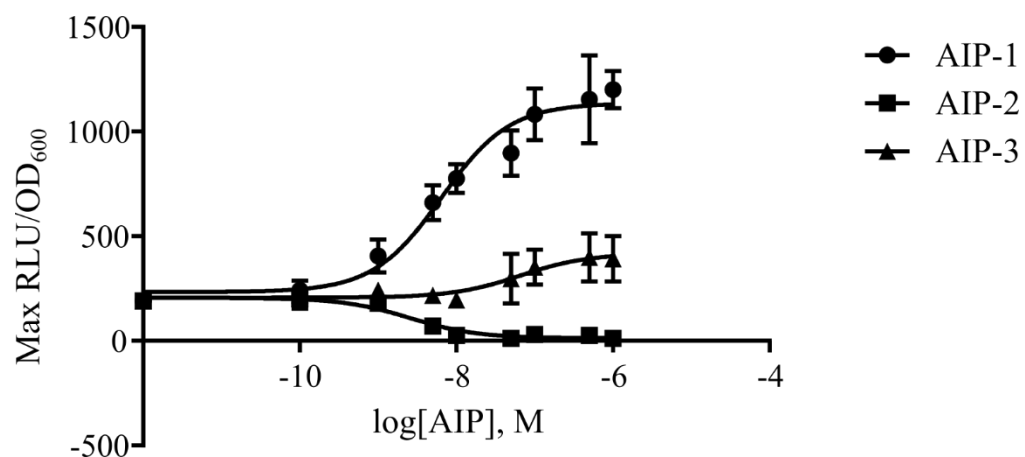


Figure 4.15 Effect of AIP-1, AIP-2 and AIP-3 on a constitutively AgrC (S116K) *agrP3::lux* reporter strain. To determine the response of constitutive AgrC-I (S116K) to AIP, it was incubated with AIP-1 (●), AIP-2 (■) or AIP-3 (▲) at concentrations ranging from 0.1 - 100 nM. The data represents mean $\pm$ standard deviation generated from two independent experiments.

4.2.9.2 IQS3 inhibits constitutive AgrC-I reporters S116K, T197K and L205R

To determine whether AgrC is the target of IQS3 or IQS11, all constitutive AgrC-I (S116K, T197K and L205R) mutant reporters were treated with IQS3. AIP-84, a competitive antagonist of wild type AgrC that displays no inverse agonism, was used as negative control.

Figure 4.16 shows that as expected, competitive inhibitor AIP-84 does not reduce *agrP3::lux* expression by any of the AgrC constitutive mutants. However, 50 and 100 μ M IQS3 significantly decreased *agrP3::lux* expression in each of the reporter strains ($p < 0.05$). IQS11 that was shown above not to inhibit *agrAC* group-1, -3 and -4 *agrP3::lux* reporters did not affect any of the AgrC mutants.

These results suggest that IQS3 does not perturb signal recognition as inhibition is maintained in a constitutive reporter system. Therefore, IQS3 likely targets either the kinase activity of AgrC, phospho-transfer from AgrC to AgrA or AgrA/DNA binding.

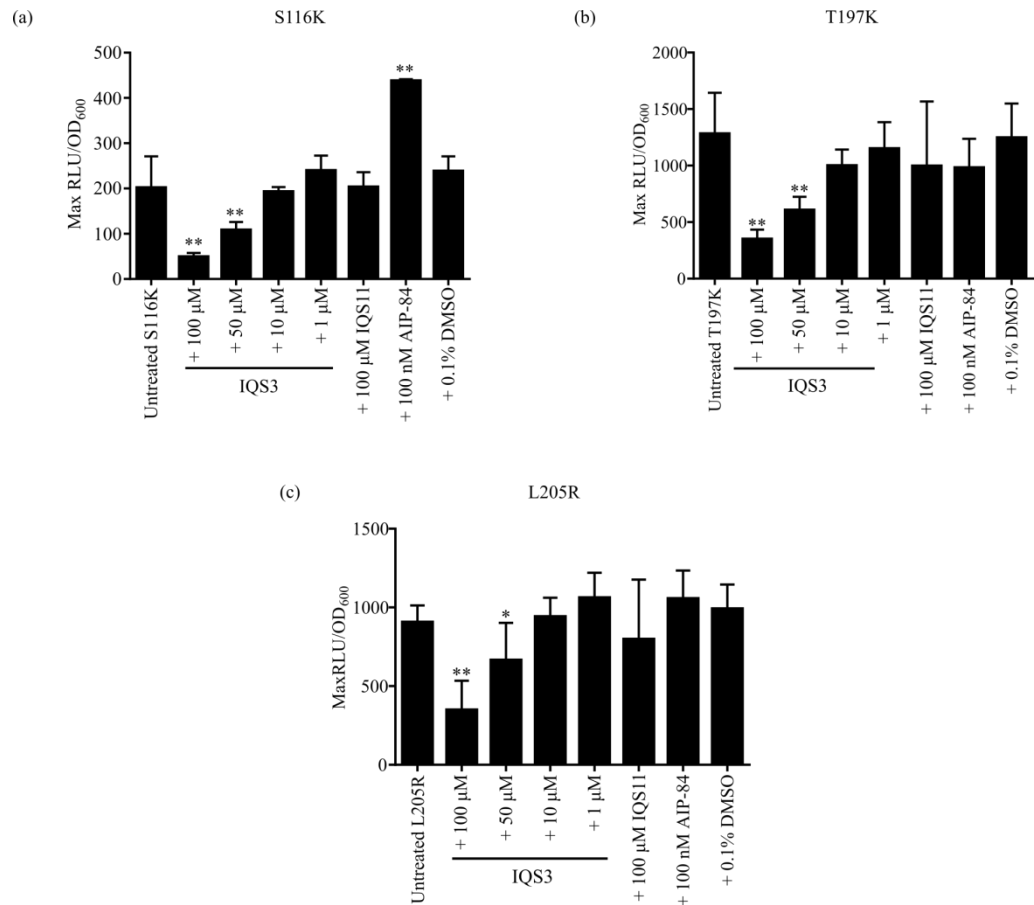


Figure 4.16 IQS3 inhibits constitutive AgrC activity in *agrP3:lux* reporter strains. To identify whether AgrC is the target of IQS3 and IQS11, S116K (a), T197K (b), L205R (c) are constitutive AgrC reporter strains that were treated with IQS3 or IQS11. AIP-84, DMSO-treated and untreated were used as negative controls. The data represent mean $\pm$ standard deviation of three independent experiments. The mean of each group tested was compared relative to the DMSO-treated group. *, $p < 0.05$; **, $p < 0.01$.

4.2.10 AgrA is the target of IQS3

IQS3 reduces expression from the *S. aureus agrP3* promoter in all four AgrCA reporter strains. As the amino acid sequence and therefore structure of AgrC is different in each of these reporters a likely hypothesis is that IQS3 likely targets AgrA which has a much more conserved amino acid sequence across all four *agr* groups.

4.2.10.1 Construction and optimisation of inducible AgrA reporter strain

In order to determine whether AgrA is the target of IQS3, an inducible AgrA reporter was constructed that would by-pass the requirement for AgrC. The reporter *P_{xyl}/tet:agrA* was transformed into *S. aureus* SH1000 Δ *agr attB2::P3:lux*, therefore any light produced was driven by induced AgrA expression.

To determine the optimal concentration of inducer for *agrA* expression and therefore maximal light output, the reporter strain was grown in increasing concentrations of anhydrotetracycline. Figure 4.17a shows that at concentrations at or above 500 ng/ml the inducer significantly reduced bacterial growth. Figure 4.17b shows that optimal light output was achieved at a concentration of 400 ng/ml anhydrotetracycline. However, 500 ng/ml anhydrotetracycline caused stress to the bacteria and reduced the growth. Therefore, the expression was induced by 250 ng/ml anhydrotetracycline throughout the experiment. In addition, area under the curve of bioluminescence signal divided by optical density was presented instead of maximal luminescence signal divided by optical density. Because the maximal signal was low even it can be distinguished from the background (data not shown). This is an unsurprising occurrence as in this system there was no AgrC which phosphorylates and activates AgrA resulting

in less affinity between AgrA and the *agrP3* promoter (Koenig et al., 2004, Rajasree et al., 2016).

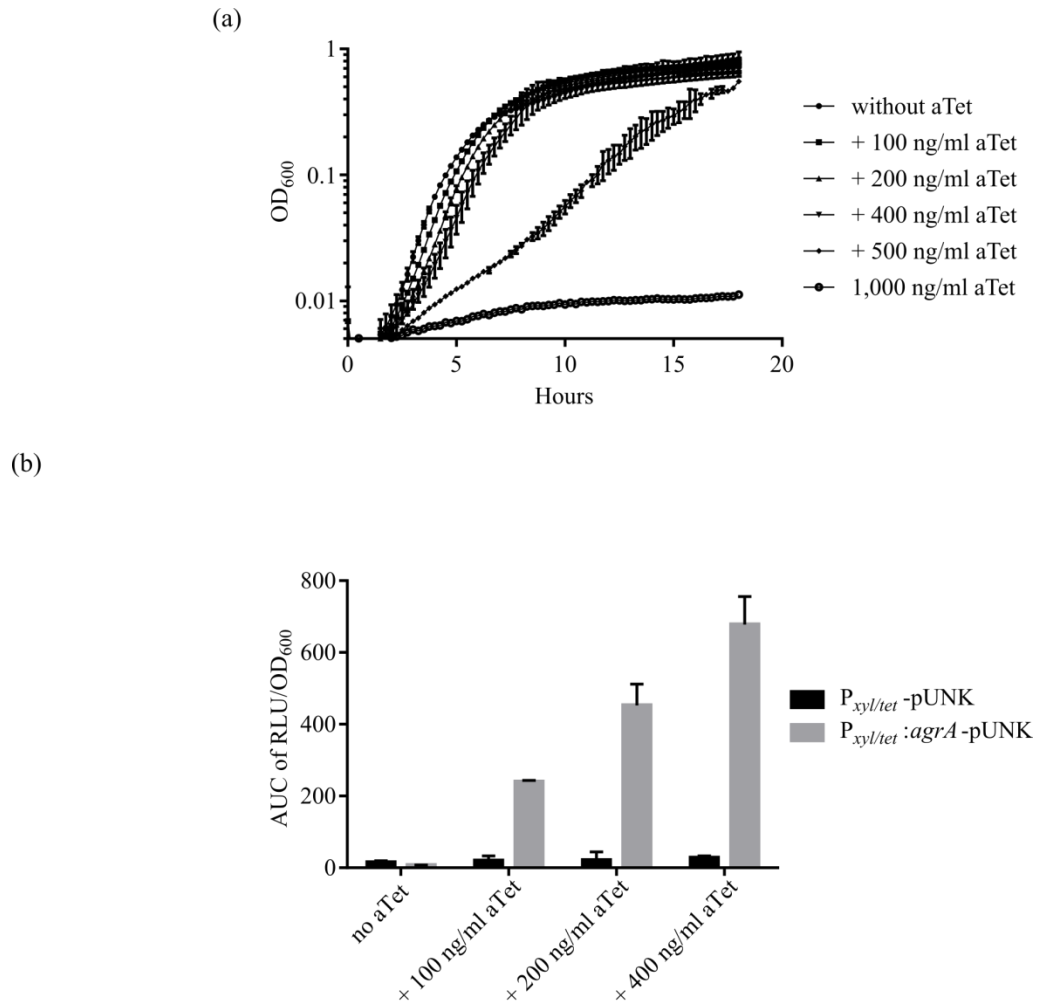


Figure 4.17 Optimisation of *agrA* induction. *S. aureus* SH1000 Δagr *attB::agrP3:lux* was transformed either with $P_{xyl/tet}:agrA$ or an empty vector control. Both were inoculated into BHI broth in the presence of anhydrotetracycline (aTet) at the concentrations indicated. The growth of the culture (a) and the activity of *agrP3:lux* (b) were quantified in a microplate reader (F200, Tecan). The data presented are area under curve (AUC) divided by OD₆₀₀. The experiment was carried out on at least two independent occasions.

4.2.10.2 Savirin inhibits the bacterial bioluminescence

Savirin (*S. aureus* virulence inhibitor; 3-(4-propan-2-ylphenyl) sulfonyl-1H-triazolo [1,5-a] quinazolin-5-one) is a small molecule that has been shown to reduce *S. aureus* virulence factor production without affecting bacterial growth in a study that screened compounds from the NIH Molecular Library Small Molecule Repository, USA. Savirin inhibited *agr* activity by binding directly to AgrA and inhibiting its ability to bind DNA (Sully et al., 2014). In order to determine whether this was also the case for the IQS analogues, the constitutive *agrA* reporter constructed above was used together with savirin as a positive control. To confirm that any changes in light output by our constitutive *agrA* reporter was due to a specific reduction in AgrA activity and not to non-specific inhibition of bacterial luminescence generated via LuxCDABE, the reporter was grown in increasing concentrations of savirin, Figure 4.18 shows that concentrations at or above 5 μ M savirin significantly reduced the luminescence signal ($p < 0.05$). Therefore, the concentrations of savirin that could be used with the inducible *agrA* reporter strain would be up to 1 μ M.

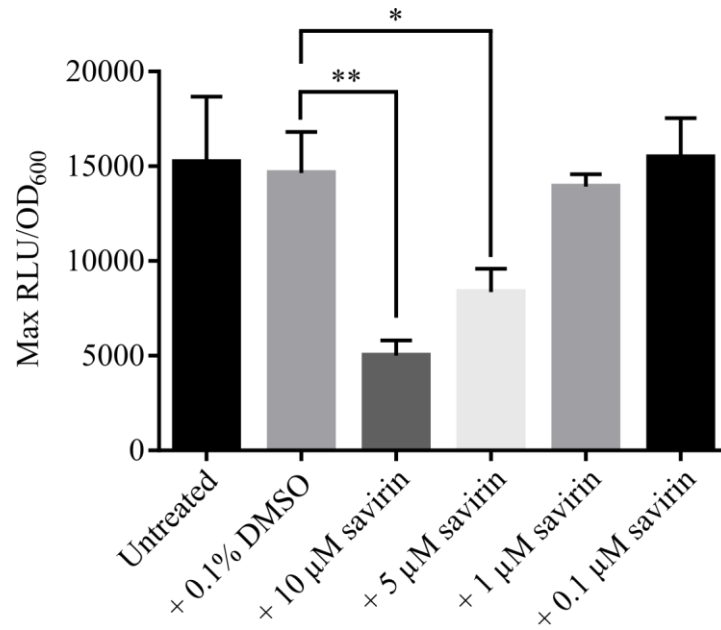


Figure 4.18 Inhibition of an inducible *luxCDABE* cassette by savirin. *S. aureus* RN4220 with *Pxyl/tet:gfp:lux* was treated with savirin in order to determine non-specificity inhibition of savirin on luminescence. The luminescence output of the culture was quantified using a microplate reader (Infinite F200, Tecan). The data represents the mean $\pm$ standard deviation of 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$.

4.2.10.3 IQS3 inhibits the inducible AgrA reporter

To determine whether IQS3 targets AgrA, the inducible AgrA reporter strain was treated with IQS3, savirin, or AIP-84 a competitive inhibitor of AgrC that should not be effective in this assay.

Figure 4.19 shows that 1 μ M savarin, a known AgrA inhibitor significantly reduces *P3:lux* activity ($p < 0.01$), whereas AIP-84 which targets AgrC competitively does not have any effect. IQS3 at 1 - 100 μ M significantly reduces the activity of *P3:lux* ($P < 0.05$). The results suggest that IQS3 targets AgrA. Surprisingly, IQS11 significantly increased *P3:lux* activity ($p < 0.01$).

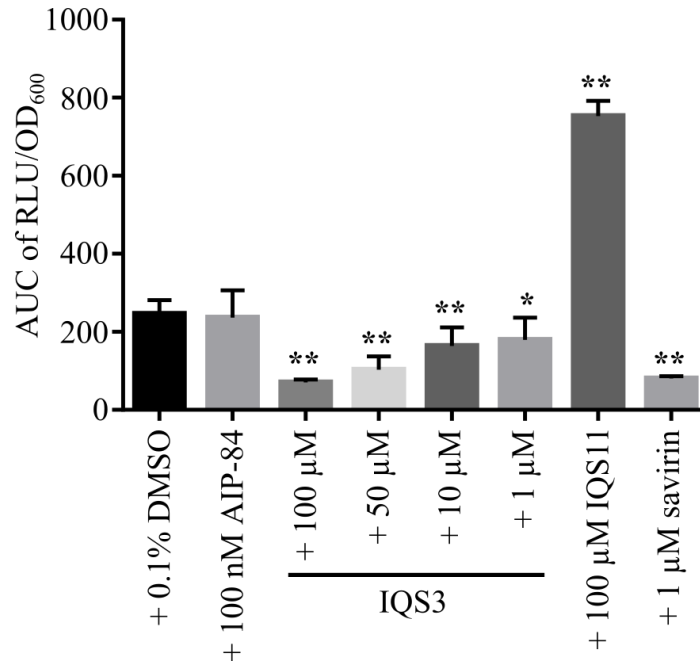


Figure 4.19 IQS3 reduces *agrP3:lux* expression from an inducible AgrA reporter. To determine the effect of IQS3 and IQS11 on the constructed inducible AgrA reporter strain, SH1000 $\Delta agr attB2::P3:lux$ harbouring *Pxyl/tet:agrA* was inoculated with either IQS3, AIP-84 and DMSO-treated groups were used as negative controls. The Savirin-treated group was a positive control. The *agrP3:lux* activity was measured by microplate reader (F200, Tecan). The bar chart represents the mean $\pm$ standard deviation of three independent experiments. **, $p < 0.01$; *, $p < 0.05$.

4.3 Discussion and Conclusions

Vancomycin is the drug of last resort for treating MRSA infections. The number of vancomycin-resistance *S. aureus* (VRSA) isolates is increasing. Moreover, VRSA which used to be found only in hospital-settings was isolated from a community-acquired *S. aureus* infections for the first time from a case in 2012 in the USA (Limbago et al., 2014). Finding new therapies for the treatment of *S. aureus* infections is an urgent issue. Here we determine the effect of IQS and IQS analogues on the production of *S. aureus* virulence determinants alongside compounds of the quinone and rhodanine classes as shown in Chapter 3.

It has been reported that *P. aeruginosa* can outcompete *S. aureus* using several types of metabolites (Machan et al., 1992, Hoffman et al., 2006, Qazi et al., 2006). IQS also known as ‘aeruginaldehyde’ is a by-product from the biosynthesis of pyochelin, one of two main siderophores produced by *P. aeruginosa* (Ye et al., 2014). IQS is thought to act as a signal molecule in the *pqs* and *rhl* quorum sensing systems of *P. aeruginosa* regulating expression of virulence factors (Lee et al., 2013). Dihydroaeruginoic acid which is a direct by-product of pyochelin is an antibiotic against phytopathogens (Carmi et al., 1994). In this study the effect of IQS which is a derivative of dihydroaeruginoic acid on *S. aureus* was determined.

IQS was first tested against *S. aureus* KH1187A which is an *agr*-I strain. It reduced exotoxin production and increased protein A. However, when IQS was tested against the CA-MRSA *agr*-I USA300 LAC and *agr*-III MN8, it did not display any activity against these strains. The data indicate that IQS activity is strain-dependent.

Given the activity of the ‘natural’ IQS, a series of IQS analogues were synthesised by Alex Truman and Dr. Siri Ram Chhabra (School of Life Sciences, University of Nottingham) and their activities were evaluated. Production of exotoxins and protein A by *S. aureus* strain KH1187A was assessed. IQS analogues that had more efficacy in reducing exotoxin production in KH1187A than natural IQS were IQS2, IQS3, IQS9 and IQS11 as shown in Table 4.1.

In this study we found the improvement of the activity of analogues by changing the structure as follows (i) the side chain at position 4 of the thiazole was rendered more non-polar and more hydrophobic as the aldehyde of natural IQS was changed to either a methyl ester (IQS2) or ethyl ester (IQS3), (ii) removal of the side chain at the position 2 of the benzene ring (IQS9). Interestingly, adding a side chain at position 5 of the thiazole ring not only lead to an increase in activity, but also a change in the mechanism of action, e.g. compare IQS11 with IQS5 (Table 4.1).

Analogues that had the highest iron-chelating activity were IQS6 (dihydroaeruginoic acid) which is a direct by-product of pyochelin biosynthesis and a precursor of natural IQS (Ye et al., 2014) and IQS1 (aeruginoic acid) which is an oxidised form of dihydroaeruginoic acid (Ye et al., 2014). Both analogues contains a hydroxyl group at the position 2 of the benzene ring and a carboxylic acid at the position 4 of thiazole ring suggesting that the two oxygens are important in stabilising the IQS-Fe (III) complex (Hider and Kong, 2010). As IQS1 and IQS6 displayed similar activity to that of natural IQS (IQS5) and that the most active analogues (IQS2, 3, 9 and 11) did not chelate free iron suggesting that the reduction of exotoxin production is independent of iron chelation.

IQS3 which has a similar structure to IQS2 and IQS9 was selected for further study together with IQS11 as it has a distinct structure from the other lead analogues used in this study. IQS3 and IQS11 were tested for activity against representative strains from all *agr* groups, as well as the CA-MRSA, USA300 LAC (*agr*-I). IQS11 displayed broader activity against strains from other *agr* groups and was active against USA300 LAC. Interestingly, IQS11 reduced exotoxin production without increasing protein A production of all tested strains suggesting a different mode of action.

In order to determine the IQS cellular target a Δ *sarA agrP3:lux* reporter strain was generated. The expression of *agrP3:lux* by the Δ *sarA* strain was around half of the parental wild-type. This result correlated with prior work indicating that SarA up-regulates the *agr* system (Cheung et al., 1997, Chien and Cheung, 1998, Reyes et al., 2011).

As the *agrP3:lux* was reduced in the presence of IQS3 in the Δ *sarA* background, this suggests that SarA is not the target of IQS3. Interestingly, IQS11 did not decrease *agrP3:lux* expression but still reduced α -haemolysin production in both the parental wild-type reporter and *sarA* mutant reporter suggesting that the target of IQS11 does not involve the *agrP3* pathway.

IQS3 reduced expression from the *agrP3* promoter in all *agr* groups in reporters, indicating that the target of IQS3 is either AgrC or AgrA. However, IQS3 did not reduce exotoxin production in every strain studied. This may be because the *agr* I-IV reporter constructs are harboured within *S. aureus* RN4220 an *agr*-I strain, this observation suggests that there is at least one other factor that can determine the efficacy of the IQS analogues. Furthermore, evidence showing other factors impact on the efficacy of IQS3 was found in the

experiment using the CA-MRSA USA300 LAC, an *agr*-I strain against which IQS3 did not effectively reduce α -haemolysin production. This could be because USA300 LAC has a robust up-regulated *agr* system (Li et al., 2009). These data suggest that possible factors influencing the activity of IQS3 include the structure of the cell wall barrier and the level of *agr* system activity.

Finally, AgrA was identified as the target of IQS3 as IQS3 could reduce *agrP3* expression of not only the AgrCA reporter strain, but also a constitutive AgrC and an inducible AgrA reporter. The target of IQS11 is less clear and mechanism of action seems more complicated than that of IQS3 as it reduced *agrP3* expression only in EM28 (*agr*-II AgrCA reporter strain), but not in other reporter strains. These data suggest that IQS11 may have more than one target.

Chapter 5 IQS3 binds to and inhibits AgrA

5.1 Introduction

A number of previously discovered compounds that have anti-virulence activity against *S. aureus*, such as savirin and the anti-inflammatory agent diflunisal target AgrA (Sully et al., 2014, Khodaverdian et al., 2013) whereas MDSA targets MgrA. None of these compounds are available in the market or in clinical trials as yet. Therefore, finding a new anti-*S. aureus* virulence agent is still an urgent need.

IQS analogues 3 and 11 are the lead anti-*virulence* compounds identified in this study. Data presented in chapter 4 suggest AgrA is the likely target of IQS3, whereas the target of IQS11 is still unknown.

Here the IQS3 - AgrA interaction was characterized by differential scanning fluorimetry and electrophoretic mobility shift assays and shown to disrupt the DNA-binding activity of AgrA. RNA-Seq data also support that AgrA is a target of IQS3. However, the target of IQS11 is still unclear as it also disrupts DNA-binding of AgrA but the transcriptomic response was different from the IQS3-treated group and *agr* mutant. Lastly, the cytotoxicity of IQS3 and IQS11 to a human Jurkat T cell line was assessed using the 7-aminoactinomycin D staining and resazurin reduction assays.

5.2 Results

5.2.1 Expression of recombinant AgrA

The whole cell data suggested that AgrA is the cellular target for IQS3. To verify this hypothesis, an *in vitro* demonstration of an AgrA interaction with IQS3 was required. To perform the *in vitro* experiments, *agrA* was cloned into an *E. coli* expression vector. Recombinant AgrA was subsequently expressed and purified as described in details in the Materials and Methods.

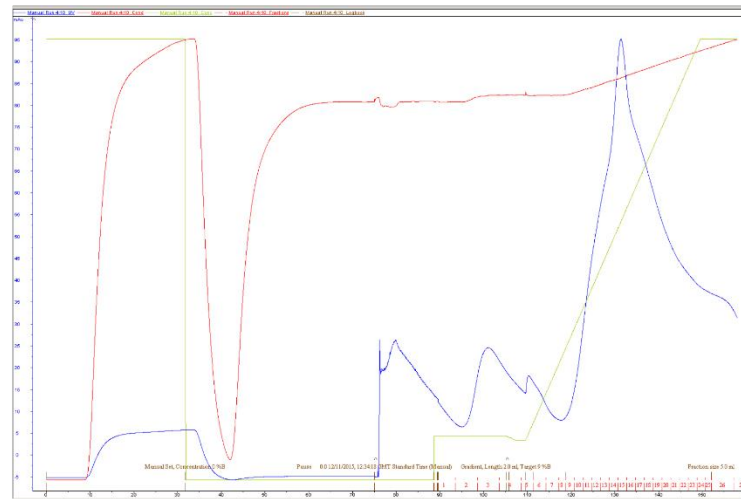
Initial expression of *agrA*-pCOLD-I by *E. coli* BL21 was undertaken but recombinant 6His-tagged AgrA was found to be insoluble (Figure 5.1a).

To address the insolubility of recombinant AgrA, *E. coli* BL21 harbouring *agrA*-pCOLD-I was expressed under osmotic stress conditions (1 M sorbitol) in the presence of various concentrations of betaine following Koenig's protocol (Koenig et al., 2004). Figure 5.1b shows that after *E. coli* was cultured in high osmotic conditions, recombinant AgrA can be found in the soluble fractions. Interestingly the addition of the compatible solute betaine did not improve solubility. Western blotting was carried out to confirm that the induced band from the Coomassie gel as highlighted in top panel of Figure 5.1b was recombinant AgrA, bottom panel of Figure 5.1b. Therefore, to express high levels of soluble AgrA, *E. coli* BL21 harbouring *agrA*-pCOLD-I was cultured in terrific broth supplemented with 1 M sorbitol throughout the rest of this study.

5.2.2 Purification of recombinant AgrA

Induced *E. coli* BL21 harbouring *agrA*-pCOLD-I biomass was lysed by sonication and the soluble fraction containing recombinant AgrA containing a hexa-histidine tag at the N'-terminus was separated from the insoluble cell debris by centrifugation. Purification of recombinant AgrA then followed by application of soluble material to a nickel charged IMAC column, the retained AgrA protein was eluted with increasing imidazole concentrations; for more specific details see the Material and Methods chapter section 2.8. The UV recording and the SDS-PAGE of proteins from fractions taken throughout the purification are shown in Figure 5.2a. This typical purification shows that a proportion of recombinant AgrA expressed remains soluble, binds to the column and elutes with an increasing imidazole concentrations. Fractions 14 – 19 were pooled as the Coomassie stained gel indicates the presence of AgrA with no major contaminating proteins present (Figure 5.2b). Desalting of the recombinant AgrA was carried out using a PD-10 column (GE Healthcare) to remove salt and imidazole (which may interfere with the assay downstream) into storage buffer preceded storage of aliquots of the recombinant protein at -80°C until used.

(a)



(b)

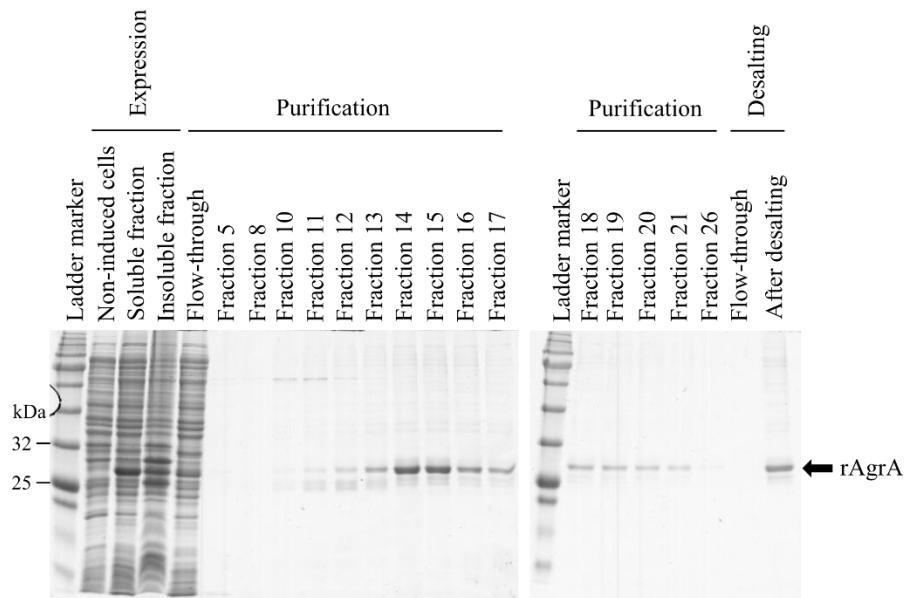


Figure 5.2 Purification and desalting of recombinant AgrA. The recombinant AgrA was purified prior to further analysis. (a) Chromatogram during AgrA purification using Ni-NTA column detected by UV recording. The blue line is protein eluted from the column, green is the percentage of elution buffer mixed with wash buffer and red is concentration of salt. (b) Coomassie blue staining of an SDS-polyacrylamide gel shows protein samples collected during expression, purification and desalting. The arrow highlights the position of recombinant AgrA (rAgrA).

5.2.3 Interaction between recombinant AgrA and IQS3 as measured by nanoDSF

Differential scanning fluorimetry (DSF) is commonly used for studying protein stability and protein unfolding and is often applied to investigate the interaction between a protein and low-molecular weight ligand (Lea and Simeonov, 2012, Sorrell et al., 2010). Here we used a recent automated instrument, a Prometheus NT.48 (Figure 3a and b in Appendix) which offers nanoDSF technology; protein unfolding can be determined directly by measuring fluorescence from intrinsic tryptophan and tyrosine residues. Native proteins usually have tryptophan and tyrosine residues buried in the hydrophobic moiety of protein that emit higher energy fluorescence at 330 nm. When a protein unfolds, by thermal or chemical denaturation, these residues become exposed to more hydrophilic environments where they emit lower energy fluorescence at 350 nm. The Prometheus NT.48 measures these changes in fluorescence (Appendix Figure 3c) and plots the changes in the ratio of 350/330 nm with increasing temperature to derive a thermal melting T_m value for the protein, where is the peak of its first derivative from fitting the experimental data using a polynomial function (see Appendix Figure 3d). This T_m value describes the stability of the protein, such that in the presence of a (de)stabilizing ligand, an altered T_m is measured and a ligand-protein interaction can be identified.

Preliminary nanoDSF showed that in the presence of 1 mM IQS3, the melting temperature of recombinant AgrA increased from 40 °C to 48 °C compared with AgrA and the DMSO only solvent control (Table 5.1 and Figure 5.3). These data indicate that IQS3 binds to the recombinant AgrA and increases the stability of the protein. However, at 0.1 mM IQS3 the melting temperature

of recombinant AgrA was no different from AgrA alone, suggesting that the binding affinity of IQS3 for AgrA is low. At this time, IQS15, an inactive IQS analogue, was tested in parallel with IQS3 and failed to increase the T_m of AgrA when present at 1 mM (data not shown). These preliminary experiments were performed only once, when NanoTemper Technologies demonstrated the Prometheus NT.48 in the laboratory of Dr Ian Kerr, at Nottingham University. However, the results showed a direct interaction between AgrA and IQS3.

Table 5.1: Melting temperatures of recombinant AgrA in the presence and absence of IQS3.

Sample	Melting temperature (°C)
rAgrA	40.4
rAgrA + 10 % DMSO	39.9
rAgrA + 1 mM IQS3	48.4
rAgrA + 0.1 mM IQS3	40.8

Note: rAgrA is instant of recombinant AgrA

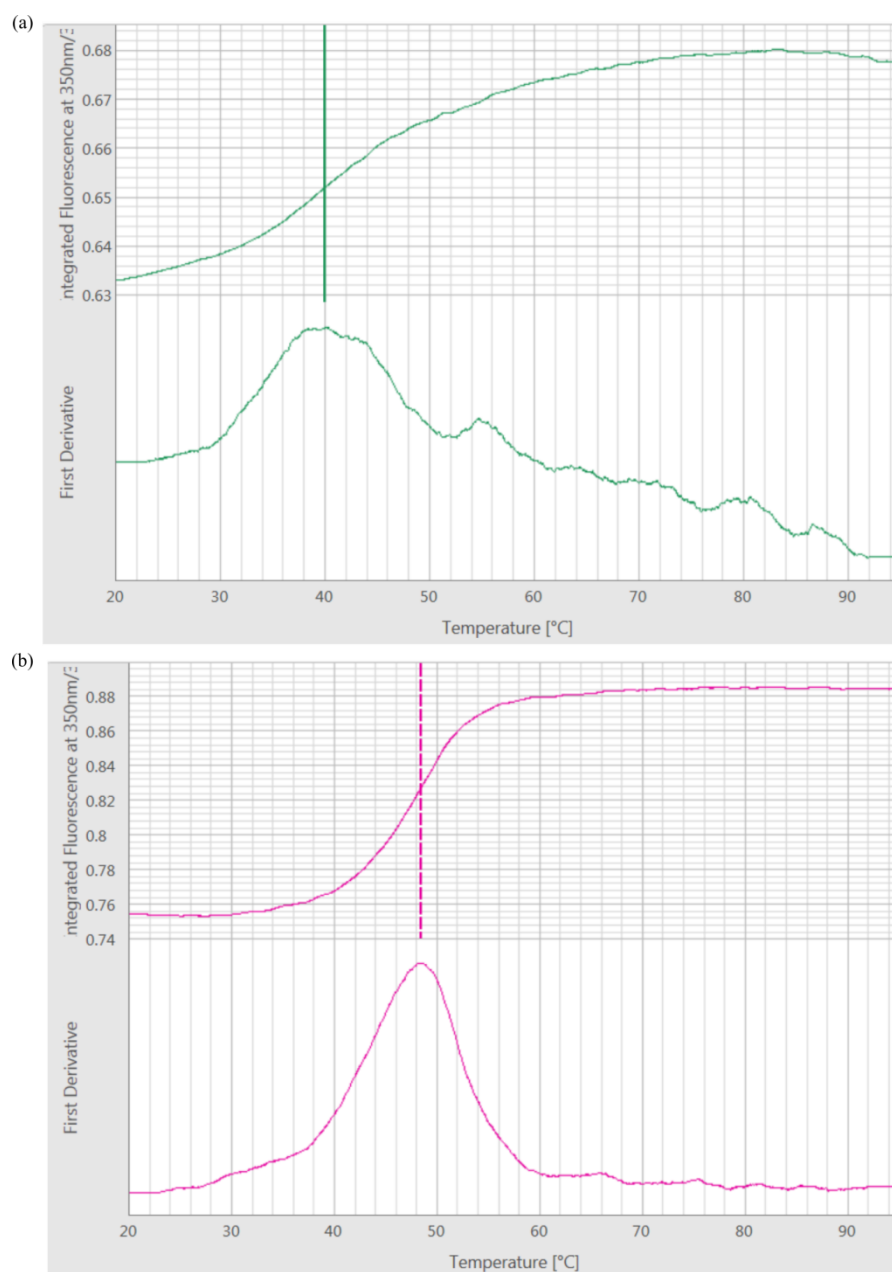


Figure 5.3 Melting temperatures of recombinant AgrA in the presence and absence of IQS3 by nanoDSF analysis. The interaction between recombinant AgrA and IQS3 was determined using nanoDSF technique. 600 nM of recombinant AgrA in the presence of 10 % DMSO (a) and in the presence of 1 mM IQS3 (b). Top panel shows the ratio of 350/330 nm against the temperature. The bottom panel shows unfolding and aggregation of protein as a function of temperature. The peak displayed in the bottom panel represents the melting temperature at which 50 % of the protein has unfolded.

5.2.4 Electrophoretic mobility shift assay (EMSA)

AgrA is a response regulator within the *agr* system. AgrA is activated by phosphorylation via AgrC, the sensor kinase, leading to direct binding of AgrA to the promoter of the target so regulating expression (Koenig et al., 2004, Novick, 2003, Queck et al., 2008). The binding of AgrA to the *agrP2* and *agrP3* promoters is well established. The *P2* and *P3* promoters both have 2 AgrA recognition sites (Koenig et al., 2004). In order to assess the effect of IQS3 on the DNA-binding activity of AgrA with respect to the *P2* and *P3* promoters, electrophoretic mobility shift assays (EMSA) were carried out.

EMSA is a classical assay that is commonly used to detect protein – DNA interactions (Holden and Tacon, 2011). The DNA probe which is bound by protein migrates slower than the free DNA probe in native polyacrylamide gels. Therefore, this assay was selected to indirectly confirm the interaction between IQS3 and protein. In addition, this technique would establish whether the interaction of IQS3 with AgrA interferes with AgrA function by blocking its interaction with DNA.

5.2.4.1 Optimisation of AgrA binding to the *P2-P3* intergenic region and *P2* promoter

Before performing an EMSA experiment to determine if our lead IQS analogues prevent AgrA binding to the *P2-P3* intergenic region and *P2* promoter, the experimental conditions were optimised. The primers were designed and adapted from (Koenig et al., 2004). A *P2-P3* DNA probe at a concentration of 10 nM gave a good fluorescent signal without too much non-specific noise (Figure 5.4a and 5.5a). To optimise the protein concentration required to cause a shift, increasing concentrations of AgrA were incubated with

10 nM of fluorescent probe. Figure 5.4b and 5.5b indicate that 600 nM recombinant AgrA was the lowest concentration of protein required to shift all of the DNA probe. To determine the specificity of the labelled probe – protein interaction, bovine serum albumin (BSA) was used as a non-specific protein. Figure 5.4c and 5.5c indicate that BSA did not shift the labelled probe. Furthermore, the AgrA – DNA probe interaction could be outcompeted by incubation with unlabelled probe (Figure 5.4d and 5.5d) which did not occur when non-specific salmon sperm DNA was used (Figure 5.4e and 5.5e). These results show that only a specific inhibitor can compete with the binding of recombinant AgrA to the promoter regions used in this study.

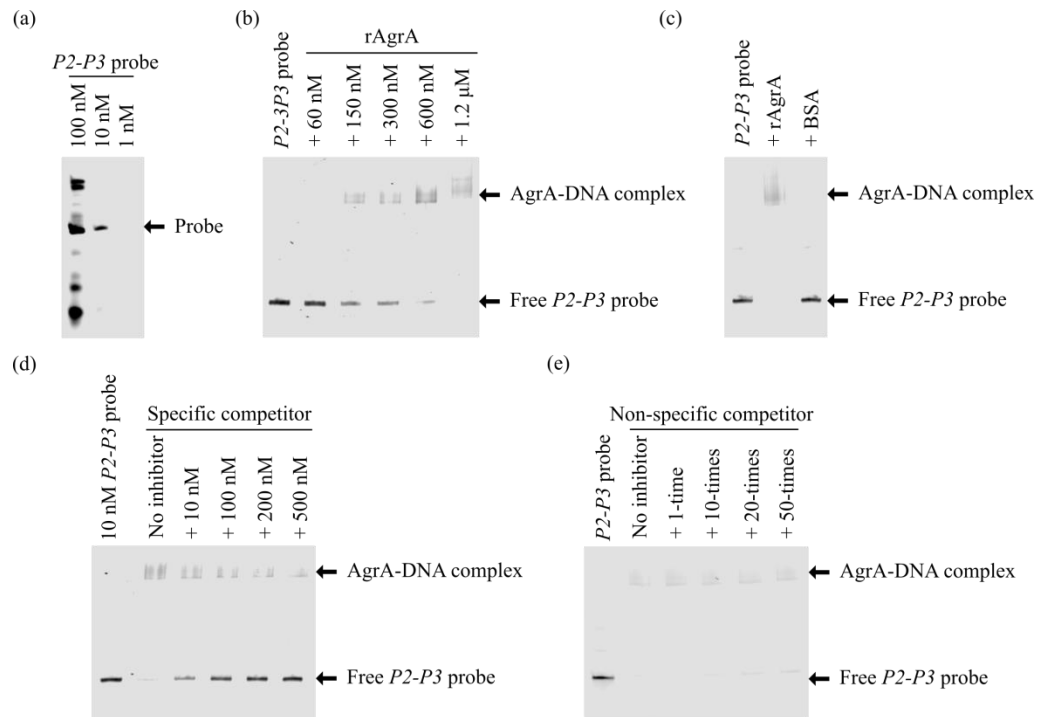


Figure 5.4 Optimisation of EMSA using a *P2-P3* intergenic probe. To optimise the experimental conditions, the concentrations of *P2-P3* intergenic probe (a) and the concentrations of recombinant AgrA (rAgrA) (b) were varied. The specificity of probe was investigated (c). The specificity of recombinant protein was investigated using a specific competitor which was unlabelled probe (d) and a nonspecific competitor as salmon sperm DNA (e).

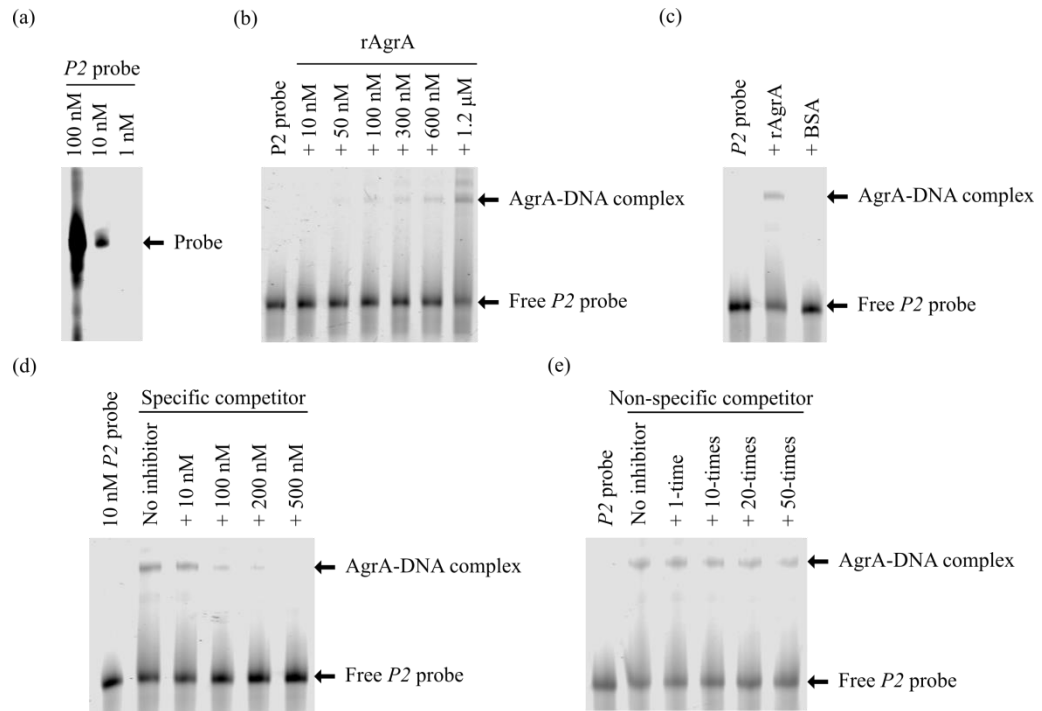


Figure 5.5 Optimisation of EMSA using a P2 promoter probe. To optimise the experimental conditions, the concentrations of promoter P2 probe (a) and the concentrations of recombinant AgrA (rAgrA) (b) were varied. The specificity of probe was investigated (c). The specificity of recombinant protein was investigated using specific competitor which was unlabelled probe (d) and nonspecific competitor as salmon sperm DNA (e).

5.2.4.2 Phosphorylation of AgrA did not enhance DNA probe binding

It has been demonstrated *in vitro* that the binding of AgrA to the *P2-P3* intergenic region could be enhanced by phosphorylation of AgrA using acetyl phosphate as shown by improved DNase I protection by phosphorylated AgrA compared with unphosphorylated AgrA (Koenig et al., 2004). Here, it was investigated whether phosphorylated AgrA would enhance binding to the target promoter regions. AgrA was phosphorylated using two different methods adapted from (Belcheva et al., 2012, Koenig et al., 2004).

DNA-binding activity of unphosphorylated and phosphorylated AgrA by lithium potassium acetyl phosphate, a high-energy phosphate donor, was assessed by EMSA. The *P2-P3* probe and *P2* probe both of which contain the AgrA binding site were selected to determine activity (Koenig et al., 2004). Figure 5.6 shows the appearance of AgrA-DNA complex at 600 nM for both unphosphorylated and phosphorylated recombinant AgrA following Belcheva's and Koenig's methods (Belcheva et al., 2012, Koenig et al., 2004). These results suggest that there is no significant increase in DNA-binding of phosphorylated recombinant AgrA.

Therefore, unphosphorylated recombinant AgrA was used in the EMSA experiments for the rest of this study.

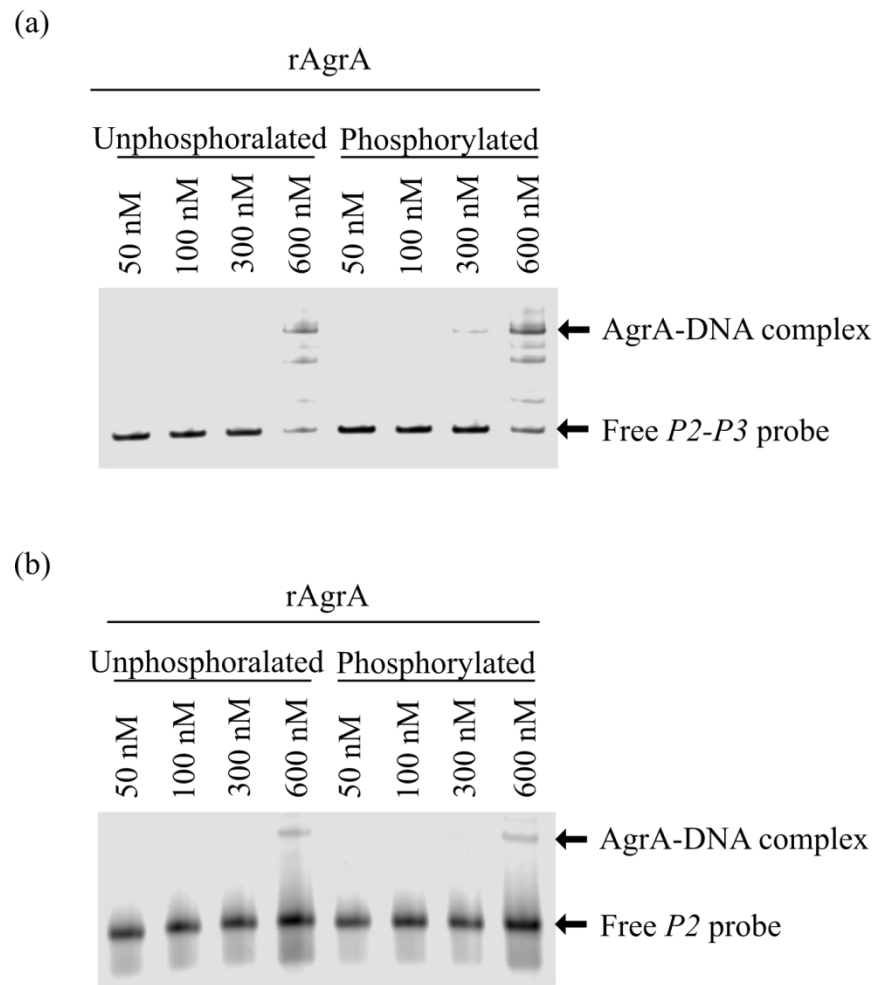


Figure 5.6 Comparison of the DNA-binding efficacy of unphosphorylated and phosphorylated recombinant AgrA. The DNA-binding activity of unphosphorylated and phosphorylated recombinant AgrA (rAgrA) was determined using EMSA with two different probes, the *P2-P3* intergenic region (a) and the *P2* promoter (b). AgrA binding efficacy is as described by (Koenig et al., 2004).

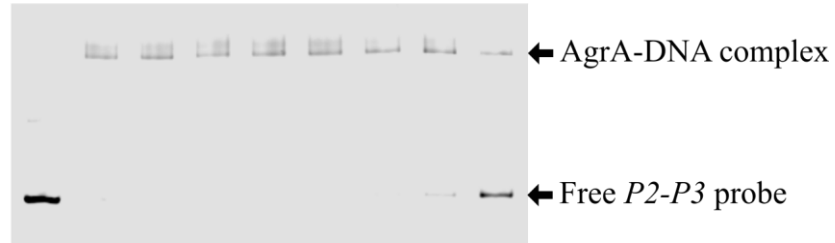
5.2.4.3 Savirin inhibits the binding of AgrA to the *P2-P3* and the *P2* probe

Savirin inhibits the ability of AgrA to bind to target DNA (Sully et al., 2014). Therefore, it was used as a positive control. The experimental conditions used in this study differ from Sully and co-workers's. Here full length recombinant AgrA was used, not just the DNA-binding domain. The target DNA also differs, here the *P2-P3* intergenic region and *P2* promoter probes were used instead of a 16-mer oligonucleotide which is part of *P2* promoter (Sully et al., 2014).

Figure 5.7a and b show that 500 μ M savirin clearly inhibits the binding of full length recombinant AgrA to *P2-P3* and *P2* probes, respectively. Therefore, savirin was used at 500 μ M as a positive control throughout the rest of this study.

(a)

Savirin (μ M)	-	-	-	-	10	50	100	250	500
Vehicle	-	-	5%	1%	-	-	-	-	-
rAgrA	-	+	+	+	+	+	+	+	+
P2-P3 probe	+	+	+	+	+	+	+	+	+



(b)

Savirin (μ M)	-	-	-	-	10	50	100	250	500
Vehicle	-	-	5%	1%	-	-	-	-	-
rAgrA	-	+	+	+	+	+	+	+	+
P2 probe	+	+	+	+	+	+	+	+	+

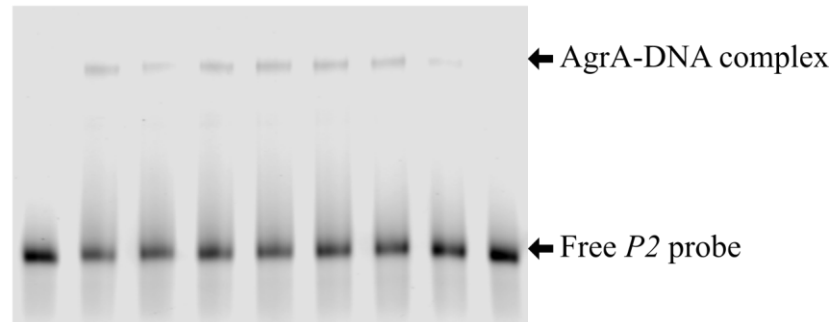


Figure 5.7 Savirin inhibits AgrA DNA-binding. Savirin at the indicated concentrations was added into the reaction buffer with 600 nM recombinant AgrA (rAgrA) and 10 nM DNA probe. Savirin blocks AgrA binding to the *P2-P3* intergenic region (a) and the *P2* promoter probe (b) as determined using EMSAs. Vehicle was DMSO. The experiment was carried out on two different occasions using different stocks of purified recombinant AgrA, the data shown are representative.

5.2.4.4 IQS3 and IQS11 block the binding of recombinant AgrA to DNA

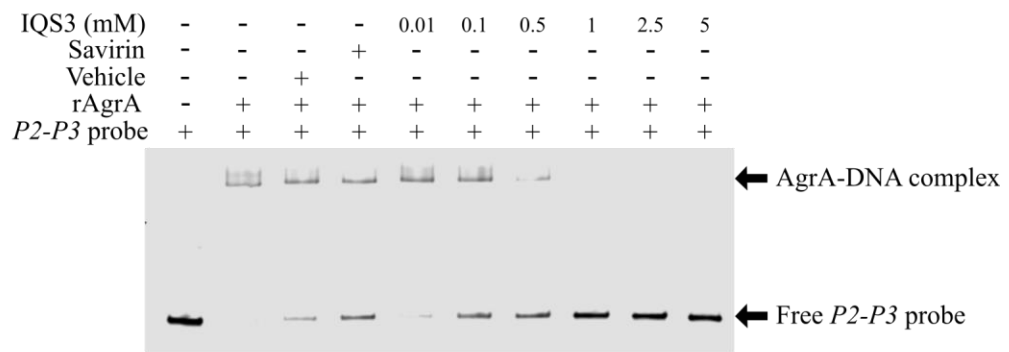
It has been shown previously (chapter 4, section 4.2.10.3) that IQS3 reduces expression of the *P3* promoter in an inducible *agrA* reporter strain, suggesting that IQS3 elicits its activity by targeting the regulatory function of AgrA. Furthermore, preliminary nanoDSF data indicate that IQS3 binds to recombinant AgrA. In order to determine if the mechanism of IQS3 inhibition was to block DNA binding an EMSA was employed. The data obtained shows that IQS3 inhibits the binding of recombinant AgrA to both the *P2-P3* intergenic region and the *P2* promoter at concentrations above 100 μ M (Figure 5.8a and b, respectively).

IQS11 which inhibits virulence factor production via an *agr* system-independent pathway as shown in Chapter 4 was also included in our analysis. Surprisingly, IQS11 inhibited the DNA-binding of recombinant AgrA with a similar efficacy to that of IQS3 (Figure 5.9a and b).

IQS15 is structurally related to IQS3 (Table 4.1) but did not inhibit *S. aureus* toxin production, as determined by Western blot. It was included in the EMSA analysis to determine the effect of an inactive IQS analogue on DNA binding activity of recombinant AgrA. EMSA analysis showed that IQS15 slightly decreases the signal of the protein-DNA complex, however, the complex does not disappear completely, even in the presence of 5 mM IQS15 (Figure 5.10a and b). This suggests that IQS15 is less effective at interfering with the DNA-binding of recombinant AgrA compared with IQS3 and IQS11. Since, IQS11 interferes with AgrA-DNA binding but does not inhibit the *agr* system in whole cell assays (see chapter 4 section 4.2.7), these results suggest that AgrA

is not the primary target of IQS11 but an alternative, as yet unidentified protein that may share a similar IQS11 binding site with AgrA.

(a)



(b)

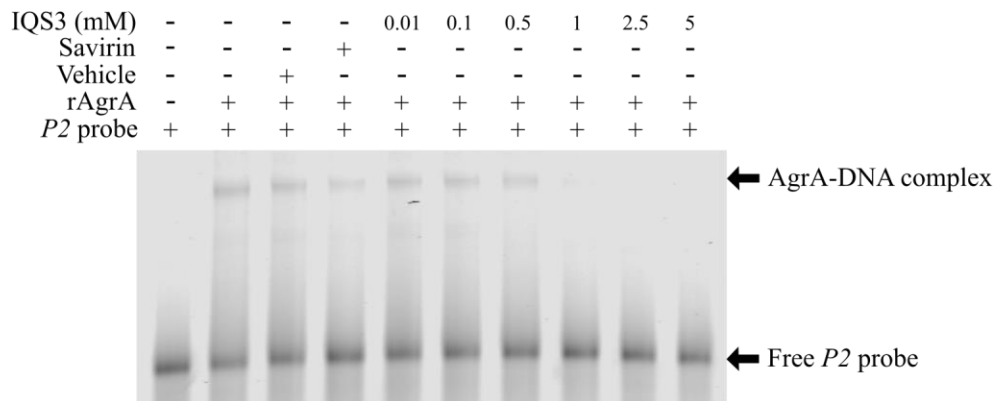


Figure 5.8 Effect of IQS3 on DNA binding of AgrA. Disruption of DNA binding activity of AgrA was observed when IQS3 was mixed with EMSA reaction buffer which contains 600 nM recombinant AgrA (rAgrA) and 10 nM of the *P2-P3* intergenic region DNA probe (a) and *P2* promoter probe (b). Vehicle was DMSO. The experiment was carried out on two different occasions and with two different stocks of purified recombinant AgrA. The data shown are representative.

(a)

IQS11 (mM)	-	-	-	-	0.01	0.1	1	2.5	5	10
Savirin	-	-	-	+	-	-	-	-	-	-
Vehicle	-	-	+	-	-	-	-	-	-	-
rAgrA	-	+	+	+	+	+	+	+	+	+
<i>P2-P3</i> probe	+	+	+	+	+	+	+	+	+	+



(b)

IQS11 (mM)	-	-	-	-	0.01	0.1	1	2.5	5	10
Savirin	-	-	-	+	-	-	-	-	-	-
Vehicle	-	-	+	-	-	-	-	-	-	-
rAgrA	-	+	+	+	+	+	+	+	+	+
<i>P2</i> probe	+	+	+	+	+	+	+	+	+	+

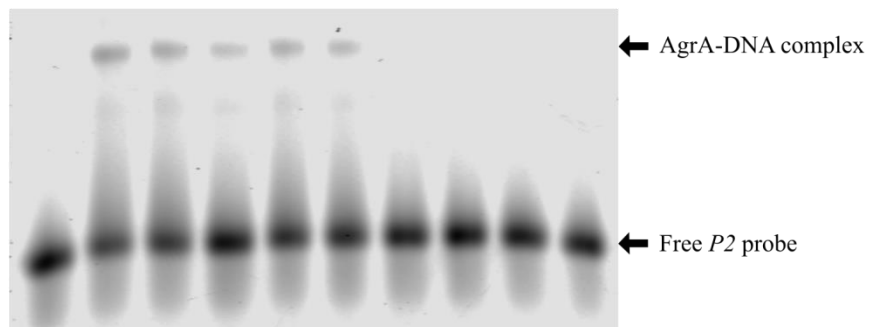
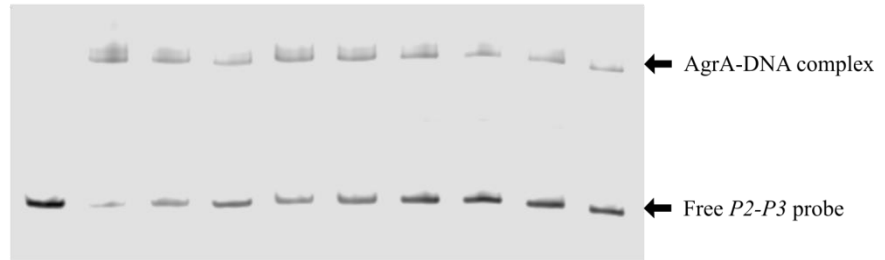


Figure 5.9 Effect of IQS11 on DNA binding of AgrA. Disruption of DNA binding activity of AgrA was observed when IQS11 was mixed in EMSA reaction buffer which contains 600 nM recombinant AgrA (rAgrA) and 10 nM of the *P2-P3* intergenic DNA probe (a) and *P2* promoter probe (b). Vehicle was DMSO. The experiment was carried out on two separate occasions and used two different stocks of purified recombinant AgrA. The data shown are representative.

(a)

IQS15 (mM)	-	-	-	-	0.01	0.1	1	2.5	5	10
Savirin	-	-	-	+	-	-	-	-	-	-
Vehicle	-	-	+	-	-	-	-	-	-	-
rAgrA	-	+	+	+	+	+	+	+	+	+
<i>P2-P3</i> probe	+	+	+	+	+	+	+	+	+	+



(b)

IQS15 (mM)	-	-	-	-	0.01	0.1	1	2.5	5	10
Savirin	-	-	-	+	-	-	-	-	-	-
Vehicle	-	-	+	-	-	-	-	-	-	-
rAgrA	-	+	+	+	+	+	+	+	+	+
<i>P2</i> probe	+	+	+	+	+	+	+	+	+	+

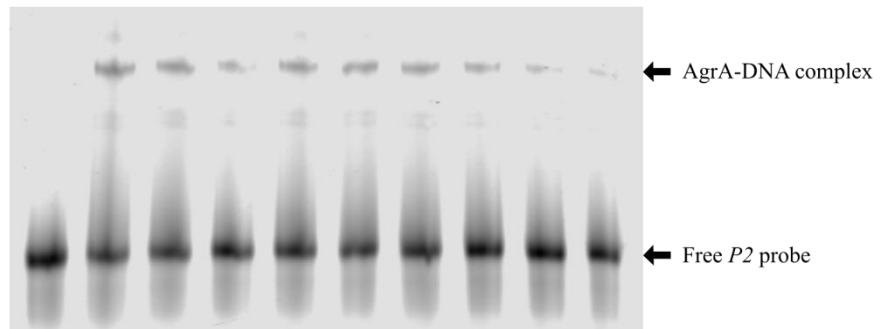


Figure 5.10 Effect of IQS15 on DNA binding of AgrA. IQS15 was mixed with EMSA reaction buffer which contains 600 nM recombinant AgrA (rAgrA) and 10 nM of the *P2-P3* intergenic region DNA probe (a) and *P2* promoter probe (b). IQS15 does not significantly disrupt DNA binding of AgrA. Vehicle was DMSO. The experiment was carried out on two separate occasions and used two different stocks of purified recombinant AgrA. The data shown are representative.

5.2.5 Determination of the transcriptional response of *S. aureus* CA-MRSA USA300 strain LAC to IQS3 and IQS11 using RNA-Seq

To investigate the global response of *S. aureus* to IQS3 and IQS11 treatment, RNA-Seq was selected. This technique is based on next generation sequencing. Since RNA-Seq is not a hybridization-based technique, it overcomes many limitations of microarray assays, such as detection of transcription without knowing the target annotation, higher sensitivity and no or higher threshold of dynamic range (Finotello and Di Camillo, 2015, Zhao et al., 2014, Croucher and Thomson, 2010).

USA300 LAC was selected because this strain is resistant to several antibiotics (Diep et al., 2006), is highly virulent due to robust expression of *agr* (Li et al., 2009) and is a well-studied *S. aureus* strain with a myriad of genetic tools available such as the genome sequence (Kennedy et al., 2008) and the ordered transposon library available from BEI Resources (USA).

5.2.5.1 RNA sample collection for RNA-Seq

As AgrA is the target of IQS3, it is necessary to collect RNA samples at a time point when the *agr* system would normally be active. In order to assess this in USA 300, AIP production was evaluated throughout growth. The cell-free culture supernatant of USA300 LAC was collected at different time points ranging from early exponential phase to stationary phase (Figure 5.11a). Supernatants were then added to the AIP reporter strain ROJ143. Figure 5.11b shows that there is high concentration of AIP-1 in a USA300 LAC culture after 3 h of growth, indicating that the *agr* system is active at this time point. To ensure that the *agr* system is on, RNA samples were collected at 4.5 h after the treatment.

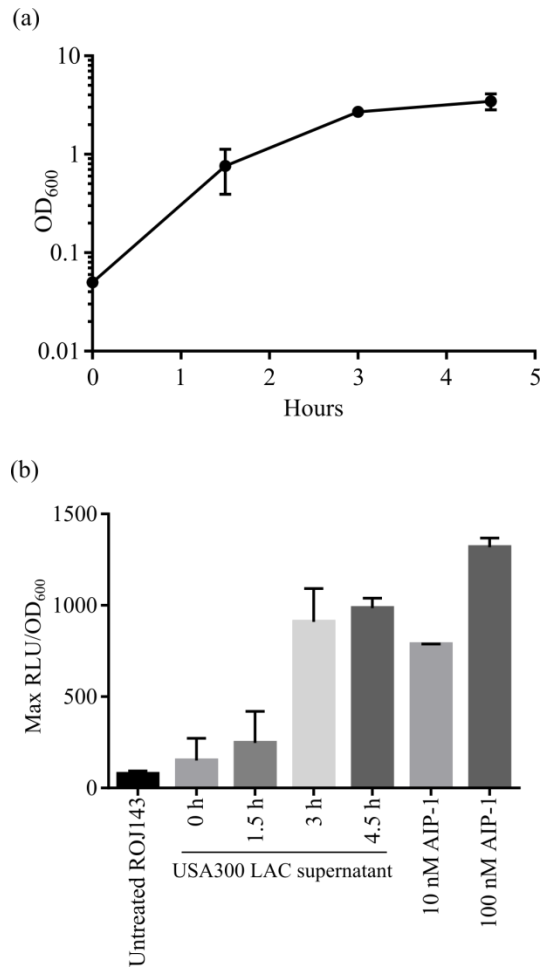


Figure 5.11 The *agr* system of USA300 LAC is active 3 h after inoculation.

USA300 LAC was inoculated into TSB and the activity of *agr* system was observed. (a) The growth curve was plotted. (b) Culture supernatants were collected and incubated with an *agr* group 1 reporter strain. Bioluminescence of the reporter indicates the production of AIP-1 by USA300 LAC. The data presented are the mean of two independent biological assays $\pm$ the standard deviation.

5.2.5.2 Preparation of the data before differential gene expression analysis

RNA samples were isolated and prepared as described in the Materials and Methods chapter 2 section 2.17.1. Samples were sent to the Deep Sequencing Facility, Queen's Medical Centre, University of Nottingham for RNA-Seq analysis.

After a cDNA library had been constructed and, tRNAs and rRNAs filtered out, the numbers of short sequences (reads) were counted. Table 5.5 shows that all samples gave a similar number of reads ranging from 7 million to nearly 12 million. The data suggest that each repeat in each group had a similar number of reads indicative of a good quality data set.

Table 5.2. Number of reads was counted by Deep Sequencing facility,
University of Nottingham

Sample	Number of paired reads (millions)
WT treated with DMSO_1	8.3
WT treated with DMSO_2	8.1
WT treated with DMSO_3	11.8
WT treated with IQS3_1	8.8
WT treated with IQS3_2	7.1
WT treated with IQS3_3	9.3
WT treated with IQS11_1	7.5
WT treated with IQS11_2	11.1
WT treated with IQS11_3	9.5
<i>Δagr</i> treated with DMSO_1	9.3
<i>Δagr</i> treated with DMSO_2	7.0
<i>Δagr</i> treated with DMSO_3	7.7

Next, variability between each repeat in each group was analysed using principal component analysis as a statistical tool to visualise the variability of a dataset by plotting in two or three dimensions (here a two dimensional plot is presented) (Paliy and Shankar, 2016). The first principle component (PC) axis which is the x-axis represents the largest variability in the data set and the second PC axis or y-axis represents the second largest variability (Paliy and Shankar, 2016). Samples that distribute separately from other samples have more variation.

Each experimental group was paired and compared with the WT USA300 LAC treated with DMSO. Figure 5.12a, b and c show that the second repeat of the negative control had more variation compared to the other repeats. IQS11-treated cells repeat 2 was also an outlier (Figure 5.12a). This suggests that repeat 2 of wild-type treated with DMSO and repeat 2 of IQS11-treated culture are outliers and possibly an error occurred. Therefore, they were removed before performing DESeq2 differential expression analysis.

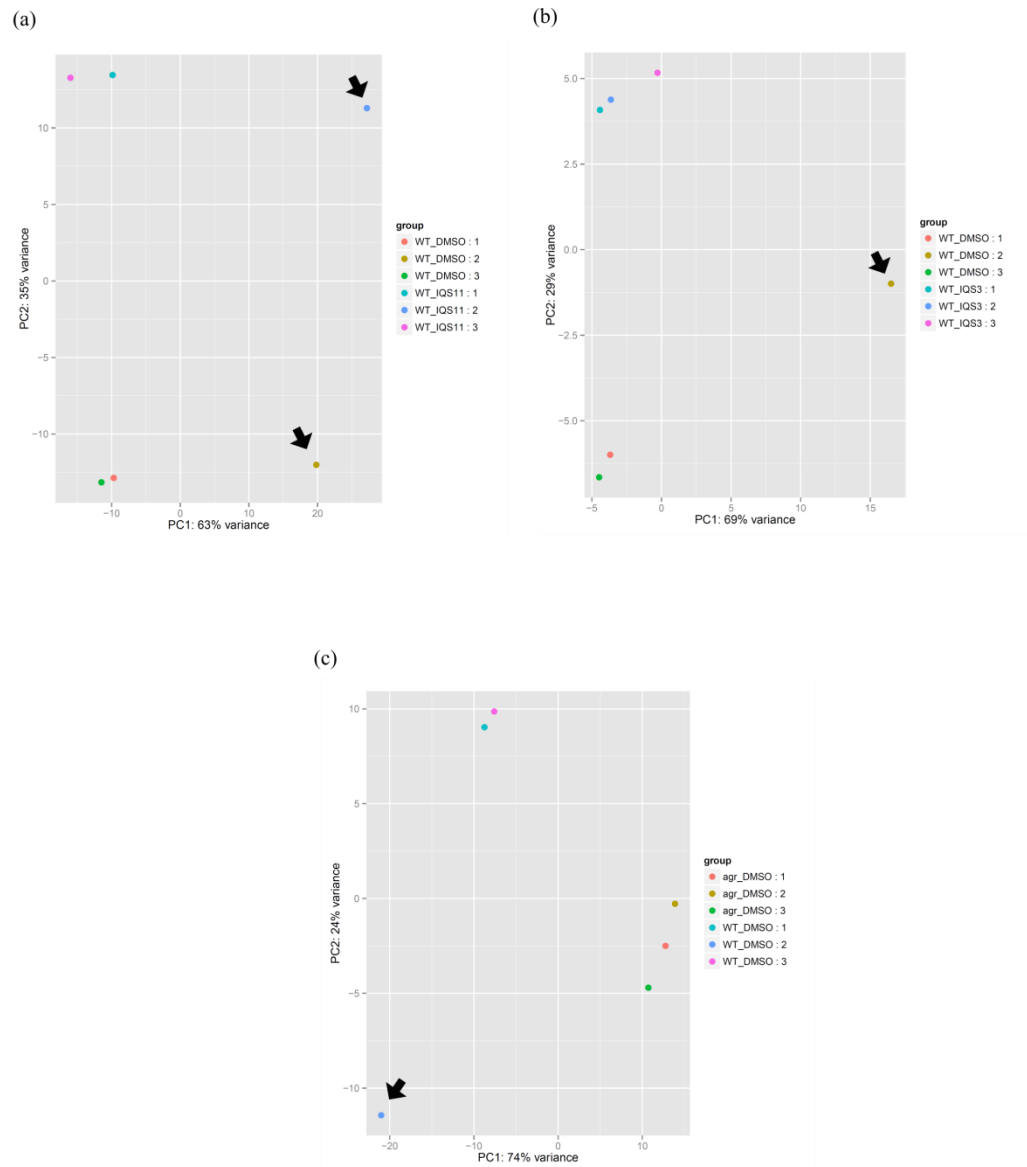


Figure 5.12 Principal component analysis. The variability among repeats was analysed using principal component analysis. (a) IQS11-treated (b) IQS3-treated and (c) LAC Δagr treated with DMSO were paired with wild-type LAC treated with DMSO for principal component analysis. The arrows indicate a repeat that has a large degree of variation and was therefore removed from differential expression analysis. The analysis was carried out by Victoria Wright, Deep Sequencing Facility, University of Nottingham.

5.2.5.3 Transcriptional responses of USA300 LAC to IQS3 and IQS11

Differential expression of the whole transcriptome was analysed using DESeq2 analysis (Love et al., 2014) by Victoria Wright, a bioinformatician at the Deep Sequencing Facility, University of Nottingham.

DESeq2 compared expression between the IQS treated groups and the DMSO-treated wild-type LAC control group. Genes that were differentially expressed compared to the wild type at the time point chosen (4.5 h) by at least 2-fold with a p-value less than 0.05 were considered significantly different. Figure 5.13a shows that the expression of 298 genes was increased and 172 genes were decreased in the IQS11-treated group, whereas only 97 genes were up-regulated and 36 genes were down-regulated in IQS3-treated group relative to the wild type. 56 genes were up-regulated and 160 down-regulated in the *agr* mutant reference group.

To determine the similarity of the response between each group, overlapping genes were determined and represented in Venn diagrams (Figure 5.13b). Only 14 out of 298 (0.3 %) and 28 out of 170 (16 %) up-regulated and down-regulated genes, respectively overlapped between IQS11-treated and LAC Δagr . In addition, when comparing IQS11-treated group with the IQS3-treated group, 25 out of 298 (8 %) up-regulated genes and 10 out of 170 (6 %) down-regulated genes overlapped between these two groups. The data indicate and agree with previous data that the mechanism of action of IQS11 is different from IQS3 and that it is unlikely that the *agr* system is target of IQS11 in this strain. Interestingly, 12 out of 97 (12 %) up-regulated genes and 8 out of 36 (22 %) down-regulated genes in IQS3-treated cultures overlapped with the LAC Δagr strain.

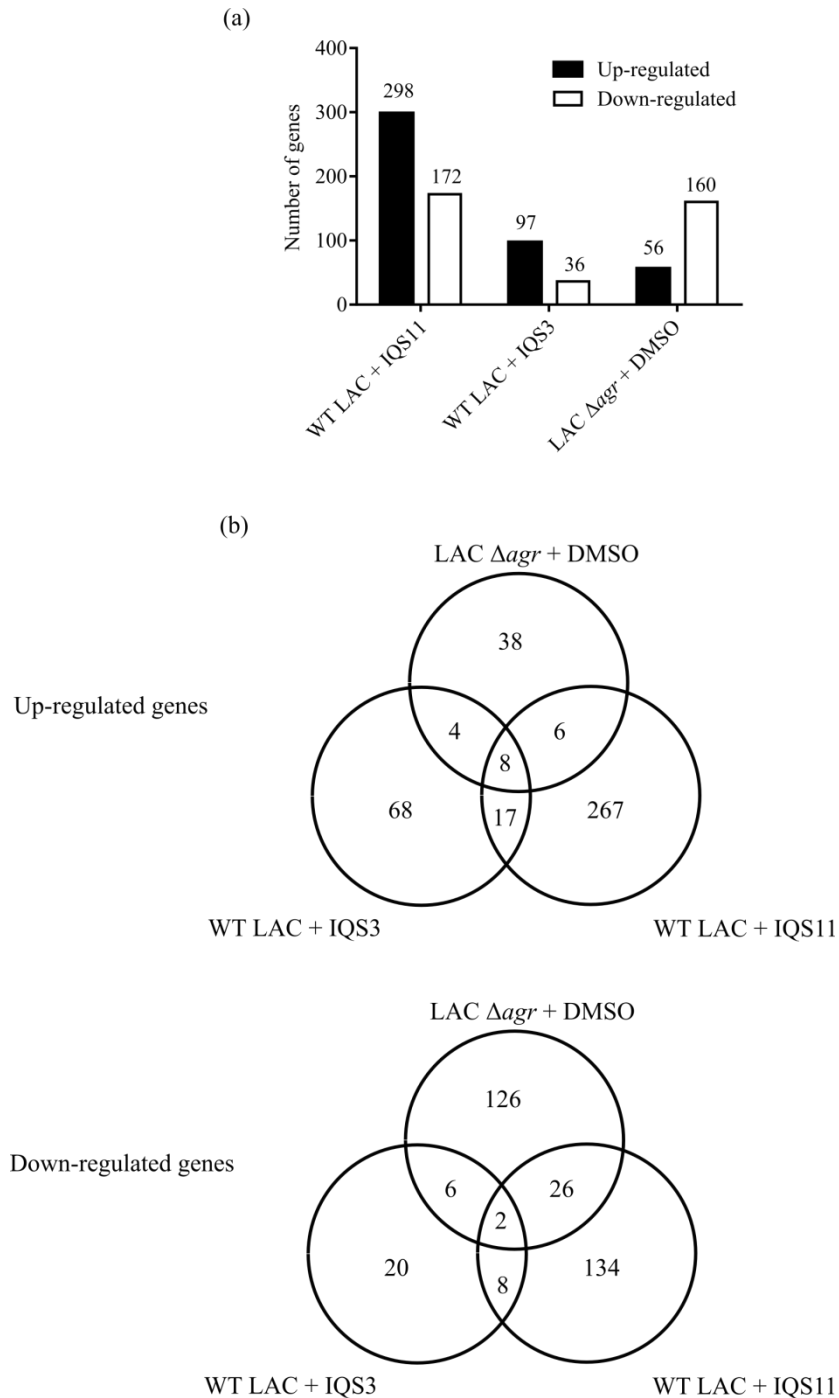


Figure 5.13 Differentially expressed genes after treatment with IQS analogues. (a) The height of the bar represents the number of differentially expressed genes in USA300 LAC compared with IQS3, IQS11 treatment or and agr mutation (LAC Δ agr). The number on the bar represents the number of differentially expressed genes. The up-regulated genes are indicated in black and the down-regulated genes are in white. (b) The Venn diagrams show the number of overlapping differentially expressed genes between each group.

5.2.5.4 Comparison of functional categories shows that the transcriptional response to IQS11-treatment differs from IQS3-treatment and *agr* mutation

Genes that were differentially expressed by more than 2-fold relative to the control groups were categorised based on the function of their encoded-protein. Figure 5.14 shows the number of genes in a functional category responding to treatment with IQS3, IQS11 and the *agr* mutation.

Even though, for all three groups, the expression of metabolic genes changed the most relative to the wild type (Tables 1, 9 and 14 in the Appendix), several genes up-regulated by the IQS11 treatment group only are involved in nitrate respiration (*nar*, *nir* and *arc* genes) and growth under anaerobic conditions (Fuchs et al., 2007, Makhlin et al., 2007). In addition, 32 phages and phage-like genes were up-regulated in the IQS11-treated group as shown in Figure 5.14 and Table 2 in the Appendix. This indicates that IQS11-treated cultures appear to be stressed as indicated by the anaerobic gene upregulation and increased expression of phage loci. Furthermore, genes involved in capsular biosynthesis and many virulence factor genes were up-regulated in the IQS11-treated cultures but were down-regulated in the Δ *agr* culture (Table 3, 4, 15 and 17 in Appendix). These data suggest and support the mode of action of IQS11 as involving an *agr*-independent pathway.

Among the virulence factor genes in the IQS3-treated group, 9 out of 12 showed a similar response as the *agr* mutant. This supports that the target of IQS3 as being AgrA. Although, genes in other categories did not show similar responses, this could be because IQS3 did not effectively inhibit AgrA to the same extent as deleting *agr* or induce the up-regulation of transcription factors,

such as genes encoding SarA, SarR and MarR as note in the *agr* mutant transcriptome (Table 17 in the Appendix).

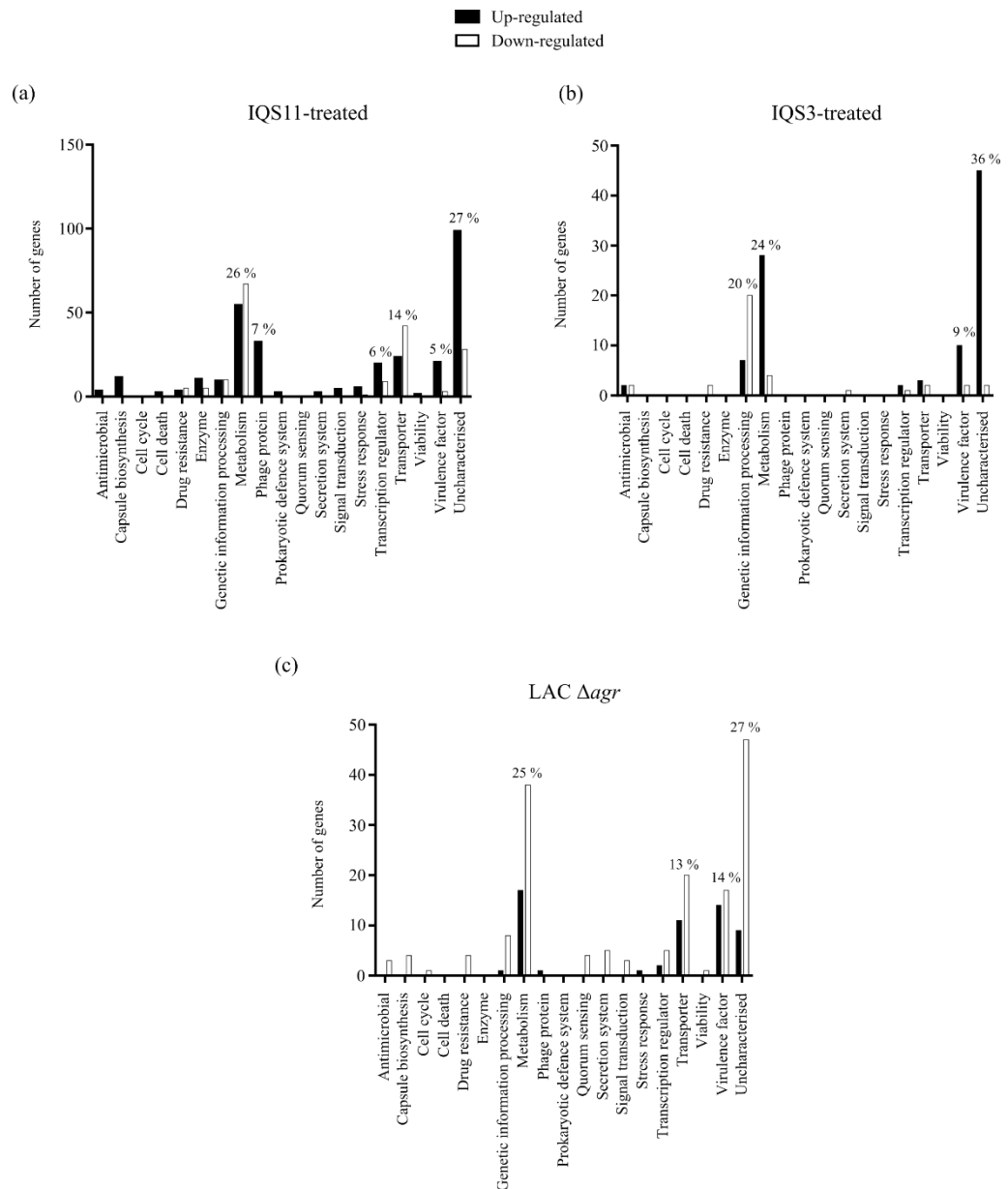


Figure 5.14 Number of genes differential expressed grouped by encoded function. The height of bar represents the number of differentially expressed genes. The percentage figures above each bar indicates the number of differentially expressed genes (up- and down-regulated) relative to the total number of differentially expressed genes within a treatment group (a) IQS11-treated group, (b) IQS3-treated group and (c) Δagr . Up-regulated expression is indicated in black, down-regulated expression white.

5.2.6 Validation of RNA-Seq data

To validate the RNA-Seq data, the expression of two uncharacterised genes which were highly up-regulated in IQS11-treated group and six virulence factor genes was quantified using qRT-PCR. Figure 5.15 shows the pattern of gene expression observed by qRT-PCR (as shown on the left-hand side of each graph) is comparable with the RNA-Seq data shown on the right-hand side. The qRT-PCR analysis data show high up-regulation of SAUSA300_2310 and SAUSA300_2311 IQS11-treated group as determined by RNA-Seq. Also, *clfA*, *hlgC* and *lukS-PV*, which RNA-Seq analysis shows that these genes up-regulated only in IQS11-treated group, show the similar results assessed by qRT-PCR analysis. Protein A-encoding gene (*spa*) assessed was found high up-regulation only in Δagr mutant. The pattern expression of other genes determined by RNA-Seq has similar pattern as shown in qRT-PCR.

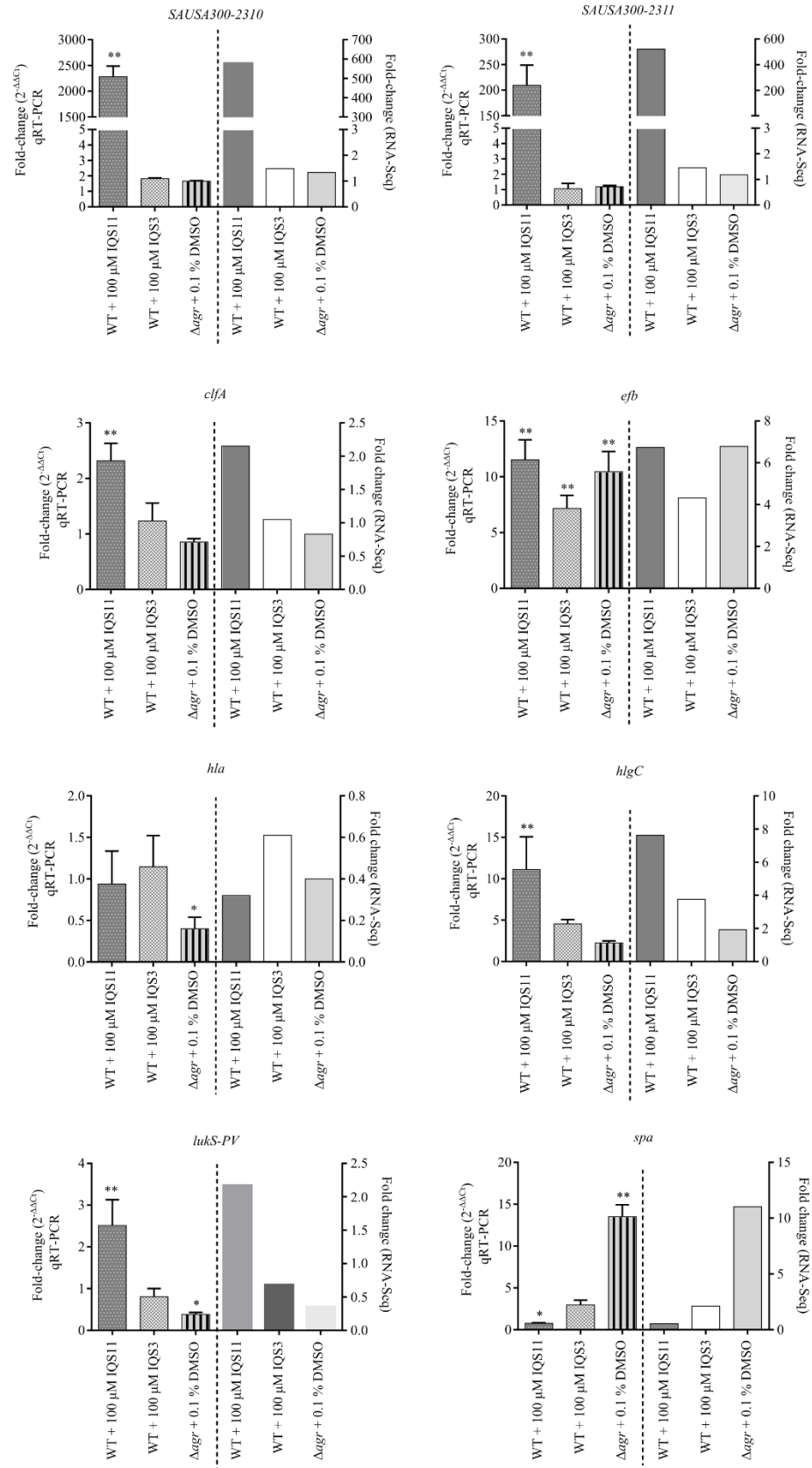


Figure 5.15 Validation of RNA-Seq data using qRT-PCR. The expression of genes SAUSA300_2310 and _2311 and 6 other virulence factor genes were evaluated using qRT-PCR. The expression levels were calculated using the comparative Ct method and represent mean $\pm$ standard deviation of three independent RNA samples and is shown at the left-hand side of graph, whereas the right-hand side of each graph represents RNA-Seq data. The mean of each group tested was compared relative to the DMSO-treated wild-type LAC group. *, $p < 0.05$; **, $p < 0.01$.

5.2.7 Promoter prediction of an uncharacterized operon containing a predicted HTH-type transcriptional regulator (SAUSA300_2310) and a hypothetical protein (SAUSA300_2311)

SAUSA300_2310 and SAUSA300_2311 genes which were up-regulated more than 500 fold when *S. aureus* was treated with 100 μ M IQS11 have no known function. SAUSA300_2310 is predicted to contain a helix-turn-helix motif (Ramos et al., 2005, Liu et al., 2006, Luong et al., 2003). Here SAUSA300_2310 was questioned whether it is a transcription regulator and causes changes of expression of nearly 500 genes. This would suggest that SAUSA300_2310 could be a new discovered *S. aureus* global regulator.

The NCBI genome database indicates that the sequence of these two genes overlap by a single nucleotide, suggesting that they share a common promoter (Figure 5.16a). As the expression of these two genes appears to be regulated by the presence of IQS 11 the promoter region that regulates transcription was investigated.

BPROM is a piece of software inform the Fgenesb_annotator which uses a predictive algorithm to identify bacterial promoters by comparing the upstream DNA sequence to a set of known *E. coli* promoters stored in databases' such as COG, KEGG and NR (Solovyev and Salamov, 2011). Using this methodology, a promoter is identified using five relatively conserved sequence motifs. The software has been widely used with a number of validated predictions published (Mishra et al., 2015, Hu et al., 2015).

Figure 5.16 shows the predicted promoter region of the uncharacterised transcription factor SAUSA300_2310 and the hypothetical gene SAUSA300_2311 that was over 500-fold up-regulated in IQS11-treated

cultures. BPROM predicted that there are 3 potential promoters and 4 transcription factor binding sites.

5.2.8 Cytotoxicity of IQS3 and IQS11

Lastly, the cytotoxicity of IQS3 and IQS11 was evaluated using two different assays, the resazurin reduction assay and 7-aminoactinomycin D (7-AAD) staining. Resazurin is non-toxic and permeable through cell membrane and also is water-soluble and stable in culture medium. It is blue and non-fluorescent and can be reduced by nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide and cytochromes to the pink-coloured fluorescent compound resorufin in a cell that is metabolically active (Rampersad, 2012). Therefore, the resazurin reduction assay can be used to evaluate cellular health (Rampersad, 2012) and to detect signs of early cell death (Rampersad, 2012). 7-AAD binds to genomic DNA and fluoresces but it is cell membrane impermeable. Therefore, it detects apoptotic and early cell necrosis when the cell loses membrane integrity (Lecoeur et al., 2002).

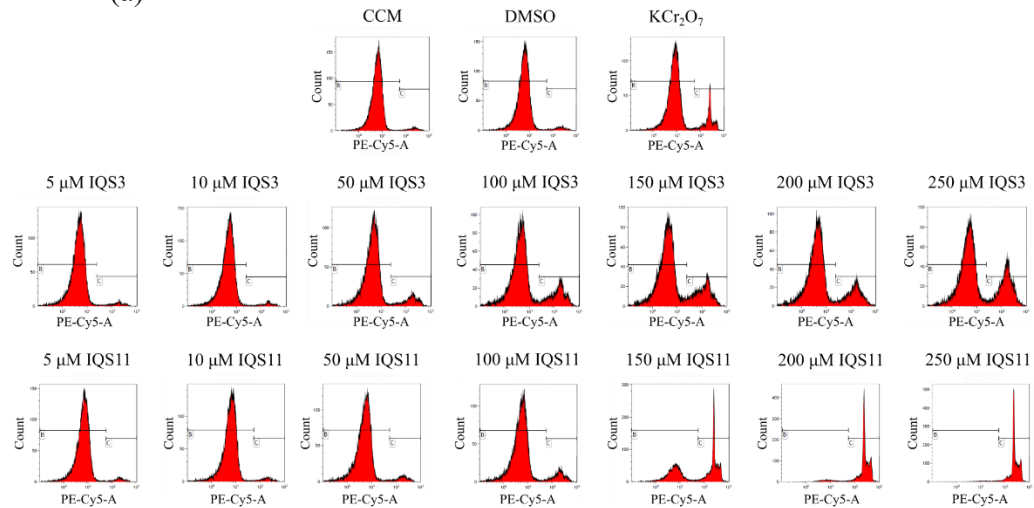
As the main objective of this study is to find new potential therapeutic agents that can inhibit *S. aureus* virulence, it is important to evaluate efficacy in the context of cytotoxicity. The cytotoxicity of IQS3 and IQS11 on eukaryotic cells was therefore assayed using a Jurkat human T cell line.

5.2.8.1 7-AAD staining assay

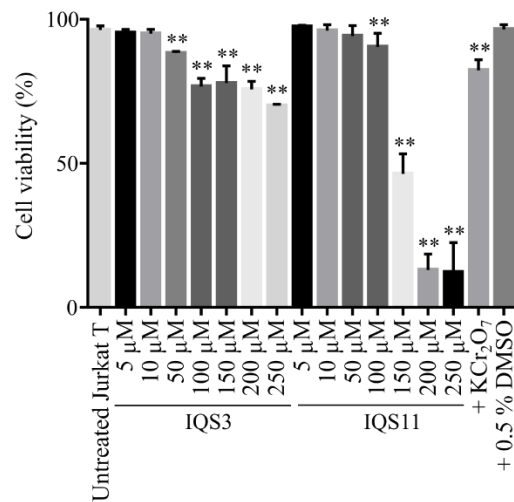
To determine whether IQS3 and IQS11 induce cell death, 7-AAD staining of Jurkat T cells combined with flow cytometry was employed to determine the number of viable cells after 24 h of treatment with increasing concentrations of IQS3 or IQS11. The toxic compound potassium dichromate at 200 μ M was used as a positive control.

7-AAD staining showed that at 24 h after treatment with IQS3 or IQS11, cell death increased in a concentration-dependent manner (Figure 5.17a and b). The proportion of viable cells decreased from 95 % to 70 % when treated with 5 to 250 μ M IQS3, respectively. The viability of cells treated with IQS11 reduced from 97 % to 12 % when they were treated with 5 to 250 μ M (Figure 5.17b) generating an LC_{50} for IQS11 of 145.83 ± 14.24 μ M (Figure 5.17c). Whereas the predicted LC_{50} for IQS3 was 333.53 ± 4.77 μ M which was higher than the concentration used (data not shown).

(a)



(b)



(c)

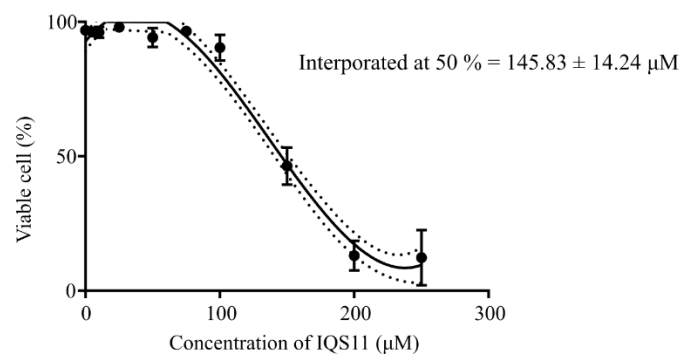


Figure 5.17 Toxicity of IQS3 and IQS11 as determined by 7-AAD staining.

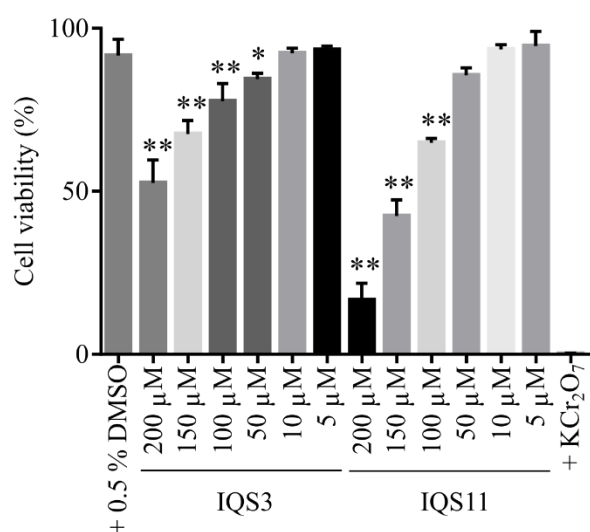
Jurkat T cell viability was evaluated by 7-AAD after treatment for 24 h. CCM, cells treated with cell culture media; KCr_2O_7 , cells treated with 200 μM potassium dichromate. The fluorescent cells were detected using flow cytometry. Twenty thousand gated events were counted. (a) The events were gated to distinguish live and dead cells. (b) IQS3 and IQS11 decreased cell viability of Jurkat T cells. (c) Mean viabilities of cells treated with IQS11 were plotted against the concentration of test compound to generate an LC_{50} . Data shown are the mean $\pm$ standard deviation relative to the DMSO-treated group. **, $p < 0.01$. The experiments were carried out in biological triplicate with two technical replicates each.

5.2.8.2 Resazurin reduction assay

In order to determine cell death based on metabolic activity, Jurkat T cells were treated with IQS3 or IQS11 for 24 h before being evaluated in the resazurin reduction assay. Two hundred μM Potassium dichromate was used as a positive control.

The resazurin reduction assay showed that IQS3 and IQS11 reduced cell viability of Jurkat T cells in a concentration-dependent manner (Figure 5.18). The LC_{50} of IQS11 was lower i.e. IQS11 is more cytotoxic than that of IQS3 ($132.87 \pm 4.12 \mu\text{M}$ vs $213.76 \pm 3.73 \mu\text{M}$).

(a)



(b)

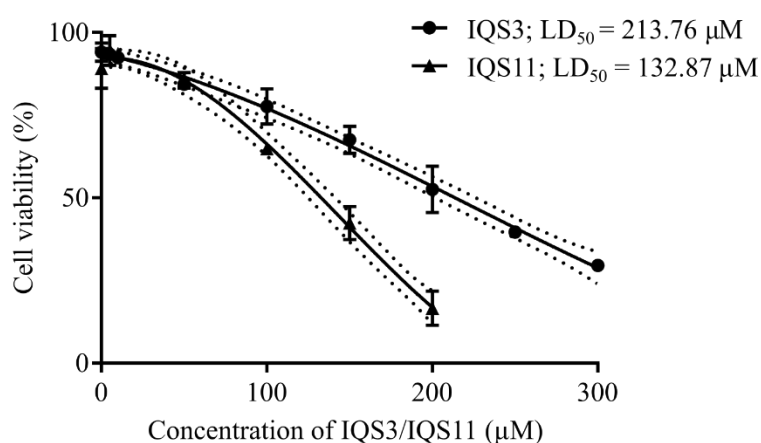


Figure 5.18 Toxicity of IQS3 and IQS11. Jurkat T cells were treated with IQS3 or IQS11 for 24 h. Cell viability was determined by the resazurin reduction assay. (a) Cell viability of each experimental group. (b) Plotted graph to determine the correlation between concentrations of IQS3 and IQS11 to cell viability. The group treated with 0.5 % DMSO was used as a negative control and the cell viability is adjusted to 100 %. 200 μM potassium dichromate (KCr₂O₇)-treated group is the positive control for cell death. Data shown are the mean ± standard deviation relative to the DMSO-treated group. **, $p < 0.01$. The experiments were carried out in biological triplicates with two technical replicates each.

5.3 Discussion and Conclusions

In the previous chapter AgrA was identified as the target of IQS3, whereas the target(s) for IQS11 has not yet to be discovered. In this chapter *in vitro* assays were carried out in order to confirm the whole cell data and RNA-Seq was employed to determine the response of *S. aureus* treated with either IQS3 or IQS11.

RNA-Seq analysis showed that IQS3 changed gene expression, especially virulence factor genes in USA300 LAC similar to the *agr* mutant supporting the *in vivo* assays that AgrA is the target of IQS3. Employing *in vitro* assays, IQS3 directly interaction with AgrA was assessed by nanoDSF analysis. The data indicate and support AgrA as the target of IQS3. As the recombinant AgrA used in the EMSA analysis was not phosphorylated, this suggests that IQS3 inhibits the AgrA protein–DNA interaction rather than phosphorylation or dimerisation of AgrA (Srivastava et al., 2014). Since, the full-length AgrA was used in this study, the topology would be more similar to the wild-type protein than utilising only the AgrA DNA-binding domain resulting in more reliable results.

In addition, IQS15 which is structurally similar to IQS3 but inactive in reducing *S. aureus* exotoxin production was included as a negative control in the *in vitro* assays. IQS15 did not show evidence of an interaction with AgrA as assessed by nanoDSF. EMSA analysis showed that IQS15 was much less effective at blocking DNA-binding compared with IQS3.

EMSA using the AgrA DNA-binding domain alone failed because it aggregated under the experimental conditions used. We propose that IQS3 could directly inhibit DNA-binding by direct steric hindrance, close to the DNA-

binding motif, such as at positions H169, N201 and R233 (Sidote et al., 2008) or by destabilisation of protein folding, for example by interaction near residues, H174, D176, K223 and E226 (Sidote et al., 2008) or by binding IQS3 to AgrA at an allosteric site that may be outside the DNA-binding domain (Laskowski et al., 2009).

Gene expression by IQS11-treated *S. aureus* was different from the IQS3-treated group and the *agr* mutant. This observation supports the whole cell data analysis that IQS11 does not target the *agr* system. IQS11-treated cultures had up-regulated transcription of a number of phage-like genes and several genes involved in anaerobic growth i.e. *arc*, *nar* and *nir* (Fuchs et al., 2007) see Appendix, Tables 1 and 2 . Furthermore, genes involved in glycolysis, such as *gapA1*, *eno* and *pgm* (Fuchs et al., 2007) were also up-regulated (Table 1, Appendix). Up-regulation of such metabolic genes suggests that the cells were ‘stressed’ in the presence of IQS11. Surprisingly, many virulence factor genes were also up-regulated in the presence of IQS11 as shown in Appendix Table 3. This potentially suggests that IQS11 could increase the virulence/pathogenicity of the bacterium. Furthermore, cytotoxicity assays showed that IQS11 is toxic to eukaryotic cells with an LD₅₀ of 150 µM in both assays utilised. In total, these data suggest that IQS11 may not be suitable candidate to be taken forward as an anti-virulence agent.

Interestingly, RNA-Seq analysis showed that two genes were up-regulated 500-fold in the IQS11-treated group. According to NCBI database, one of them is gene encoding an uncharacterised HTH-type transcription regulator, SAUSA300_2310 and the other an uncharacterised gene SAUSA300_2311 located directly upstream (see Figure 5.16a). One possible explanation for such

a dramatic increase in transcription of these two genes would be that IQS11 may bind to a repressor of this operon leading to the increased transcription.

To conclude, here it has been shown that the IQS analogue 3 binds to and inhibits the function of AgrA, leading to a reduction of exotoxin and increase of protein A by *S. aureus*. The effective concentration of IQS3 in cells is between 25 - 100 μM and is strain specific. Cytotoxicity of IQS3 was at least more than 2-fold higher than the active concentration (LD_{50} was 214 μM and 333 μM from resazurin reduction assay and 7-AAD staining analysis, respectively). Overall, IQS3 is an excellent 'starter' molecule that hopefully can be modified to improve potency whilst reducing toxicity.

Chapter 6 General Discussion

S. aureus is an important pathogen that can cause a variety of infections, ranging from mild skin infections (impetigo) to much more severe life threatening conditions such as endocarditis or sepsis. With the misuse of available antibiotics and the inherent plasticity of the microbial genome a number of methicillin resistant *S. aureus* isolates have evolved (Jevons, 1961). Traditional antibacterial therapy has focused on reducing the bacterial load with administration of an antibiotic that kills the bacteria (bactericidal) or reduces bacterial growth (bacteriostatic). However recent advances in understanding how bacteria regulate the production of virulence factors, essential for invasion and life within a host has provided a number of new potential drug targets, which instead of inhibiting bacterial growth, block the ability of the bacteria to cause disease (Foster, 2005, McCallum et al., 2010, Gordon et al., 2013)

Employing this anti-virulence strategy could reduce the selective pressure required to develop drug resistance, with the added benefit that any impact on the host's normal microbial flora would be reduced (Alksne and Projan, 2000).

The *S. aureus* quorum sensing system *agr* and the transcriptional regulator SarA regulate the expression of several virulence factors produced by *S. aureus*, each of which play a key role in causing an infection (Dunman et al., 2001, Alksne and Projan, 2000, Foster, 2005, Roberts et al., 2006). Virulence of both an *agr* and *sarA* mutant is attenuated in different animal models (Cheung 1994, Blevins 2003). Therefore, drug therapies that target *agr* or SarA could aid immune clearance of the pathogen without strongly selecting for the development of resistance (Gordon 2013).

In this study compounds were screened against different strains of *S. aureus* with care taken to avoid false positive data that is often found in studies of new virulence inhibitors (Defoirdt et al., 2013, Baell and Walters, 2014).

6.1 Quinones and rhodanines

Table 3.1 displays the structures of a series of quinone and rhodanine analogues that were synthesised by Dr. Fabio Rui (School of Pharmacy, University of Nottingham). They were designed to target the 9'-cysteine residue of SarA which has been shown to be a phosphorylation site and essential to the function of the protein (Liu et al., 2006, Sun et al., 2012). From screening this small library, three compounds, FR37, FR41 and FR44 were identified that reduced *S. aureus* exotoxin production and increased protein A. FR37 was the only one of eight quinone compounds that significantly reduced TSST-1 production by *S. aureus* KH1187A. FR37 is the only quinone tested that contains a thiazole ring whereas others all contain a thiophene ring (Figure 6.1). This striking result suggests that the nitrogen at the position 3 of the thiazole ring is crucial to activity, therefore in order to further develop the quinones as anti-virulence agents, new derivatives should incorporate a thiazole ring.

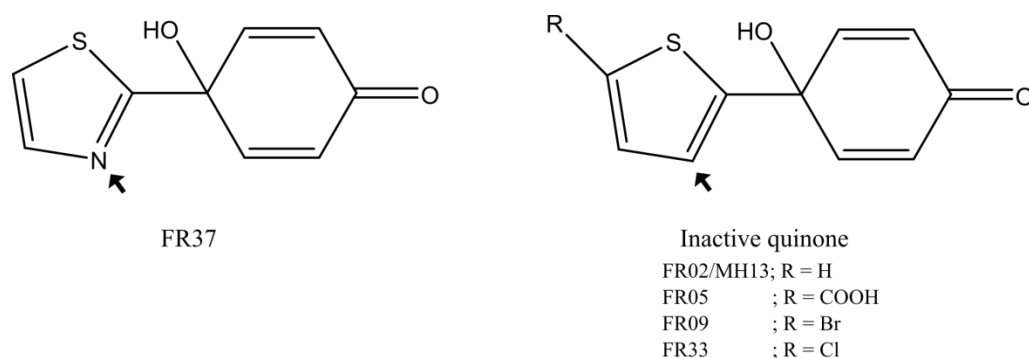


Figure 6.1 Chemical structures of quinones used in this study. FR37 which is an active quinone is compared with inactive quinones. FR37 contains a thiazole ring, whereas others have thiophenes as indicated by arrows.

FR41 and FR44 are rhodanines (Figure 6.2 and Table 3.1) both of which showed activity in reducing *S. aureus* TSST-1 and α -haemolysin production whilst increasing protein A. FR41 was the most potent rhodanine tested. FR41 contains a biphenyl moiety improve the activity compound compared with the methoxybenzene found in the FR44 structure (Figure 6.2). FR49 was inactive in this study (Table 3.1 and Figure 6.2). FR49 although structurally similar to the other active rhodanines tested is charged due to a carboxyl substituent that would increase hydrophilicity potentially interfering with cellular uptake.

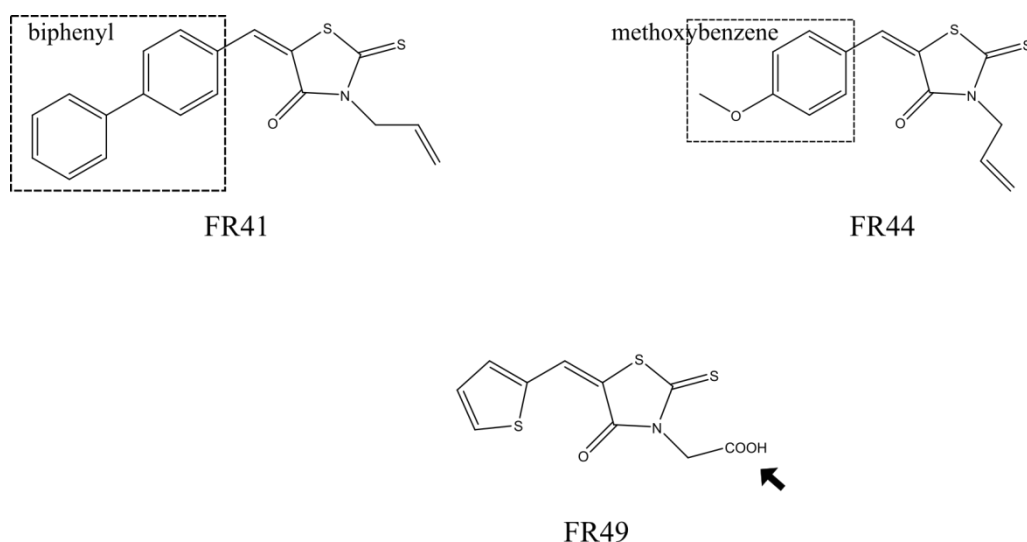


Figure 6.2 Comparison of the structures of rhodanines FR41, FR44 and FR49. The dashed lines highlight the difference between FR41 and FR44. The arrow indicates the differences between FR49, FR41 and FR44.

As FR37 (100 μ M) did not reduce α -haemolysin production but FR41 and FR44 did, it would suggest that active rhodanines and quinones have different modes of action.

This study revealed that FR37, FR41 and FR44 were potent inhibitors that reduced production of TSST-1 and α -haemolysin, more so than MDSA which was used as control and used at the same concentration. MDSA, an MgrA inhibitor, is able to bind to MgrA and alter the conformation of MgrA resulting in disruption of DNA binding and a reduction in α -haemolysin production (Sun et al., 2011). FR37, FR41 and FR44 did not kill or reduce bacterial growth and so may exhibit a reduced tendency to select for resistance (Gordon et al., 2013, Alksne and Projan, 2000).

Virulence determinants produced and the *agr* systems encoded by *S. aureus* vary between strains (McCarthy and Lindsay, 2010, Traber et al., 2008, Lyon et al., 2002). Therefore, an investigation of any strain-dependent effects of

FR37, FR41 and FR44 was important. *S. aureus* MN8 was selected as it belongs to a different *agr* group from KH1187A. MN8 belongs to *agr*-III, whereas KH1187A belongs to *agr*-I. FR41 and FR44 were found to be active against MN8. FR41 and FR44 were active not only against the MSSA strain KH1187A and MN8, but also the CA-MRSA USA300 strain JE2, which has a robust *agr* system (Seidl et al., 2014, Li et al., 2009). The data implies that FR41 and FR44, in contrast to FR37, possess strain-independent inhibitory activity.

It would be interesting to expand this study to an HA-MRSA strain, such as those containing SCCmec type II that have a different cell wall structure that leads to a reduced quorum sensing and therefore less toxin production compared to a CA-MRSA or clinical MSSA strains (Otto, 2012, Rudkin et al., 2012). It would also be interesting to test FR41 and FR44 on *S. aureus* *agr*-II as its AIP is distinct from the other groups (Lyon et al., 2002, Goerke et al., 2005).

Rhodanines FR41 and FR44 were selected for more detailed investigations of their mode of action and target. Phage transduction was used to generate isogenic *agr* and *sarA* mutants in *S. aureus* strain RN6390B. Alpha-haemolysin production in the *sarA* mutant was reduced compared with the wild-type whereas α -haemolysin in the *agr* mutant could not be detected using Western blotting or streaking onto blood agar plates. Therefore, the *sarA* mutant was used in this investigation to determine if FR41 targeted SarA or *agr*. Both FR41 and FR44 were shown to reduce haemolytic activity whilst inducing protein A production in the *sarA* mutant. This suggests that they do not target SarA and are therefore likely to target the *agr* system.

Unfortunately, the newly synthesised batches of FR41, FR44 and FR37 were not as active as the first batches. ^1H -NMR and HPLC analysis were

employed by Dr. Fabio Rui to identify the differences between the batches. There was no apparent difference between batches of FR37 and FR41. FR44 had an extra-peak as shown by ^1H - NMR and HPLC analysis suggesting that a by-product from the synthesis process may still be present. Dr. Fabio Rui purified the contaminating species and it was tested for activity against KH1187A by itself alone and by mixing it with FR44 batch 2 in order to make the compound like FR44 batch1; however, no activity was observed.

Quinones and rhodanines are classified as pan-assay interference compounds (PAINS) as hundreds of compounds in both classes have been published but none have progressed to the clinic (Baell and Walters, 2014). However, the results of our screening test, in this study showed inactive quinones and rhodanines. This suggests that the technique and activity was specific. In addition, the compounds were tested directly against *S. aureus* with respect to toxin production and growth, not via the use of reporter genes, such as bioluminescence or green fluorescence which act as a proxy for toxin production and which may signify a false positive if they are perturbed by the presence of the inhibitor (Defoirdt et al., 2013). Here, the active compounds reduced *S. aureus* exotoxin production without interfering with the intracellular protein profile or growth, strongly suggesting that they are actively targeting a regulatory mechanism such as *agr*. It is possible that the contaminating compound identified by HPLC was the active component, however, it was present at a nanomolar concentration, below which ^1H - NMR and HPLC analysis can detect. Therefore, these three compounds were kept in the hope that a more sensitive analysis technique can be developed in the future.

6.2 IQS

IQS is a by-product from the *P. aeruginosa* pyochelin biosynthesis pathway (Ye et al., 2014). In the presence of IQS the production of exotoxins, TSST-1 and α -haemolysin, was reduced, whilst protein A expression increased. Interestingly, the presence of IQS reduced toxin production by only MSSA *agr*-I strains such as KH1187A and SH1000. IQS did not reduce toxin production by the CA-MRSA USA300 LAC (Li et al., 2009) and had no effect on an *agr*-III strain MN8 (Li et al., 2011). The reason for this narrow spectrum of activity is unknown but may be to do with cellular access.

In order to improve the efficacy and increase the spectrum of IQS activity against various strains of *S. aureus*, fourteen IQS analogues were synthesised by Dr. Siri Ram Chhabra and Alex Truman (School of Life Sciences, University of Nottingham). Four out of fourteen analogues (IQS2, IQS3, IQS9 and IQS11) reduced TSST-1 and α -haemolysin more at a lower concentration than natural IQS. Comparing the structures of lead analogues with the activity as shown in Figure 6.3 it can be concluded that the crucial position of the chemical structure for activity are (i) the side chain at the position 4 of the thiazole ring and the (ii) side chain at the position 2 of the benzene ring. The sidechain at the position 4 of the thiazole ring, which is less polar like the methyl ester and ethyl ester group of IQS2 and IQS3 has increased activity compared to analogues with the same side chain as IQS e.g. IQS1, IQS4 and IQS10. Removal of the hydroxyl group at position 2 of the benzene ring also increased activity when compared with IQS9 or natural IQS or analogues that have a small side chain, such as IQS1 with IQS12.

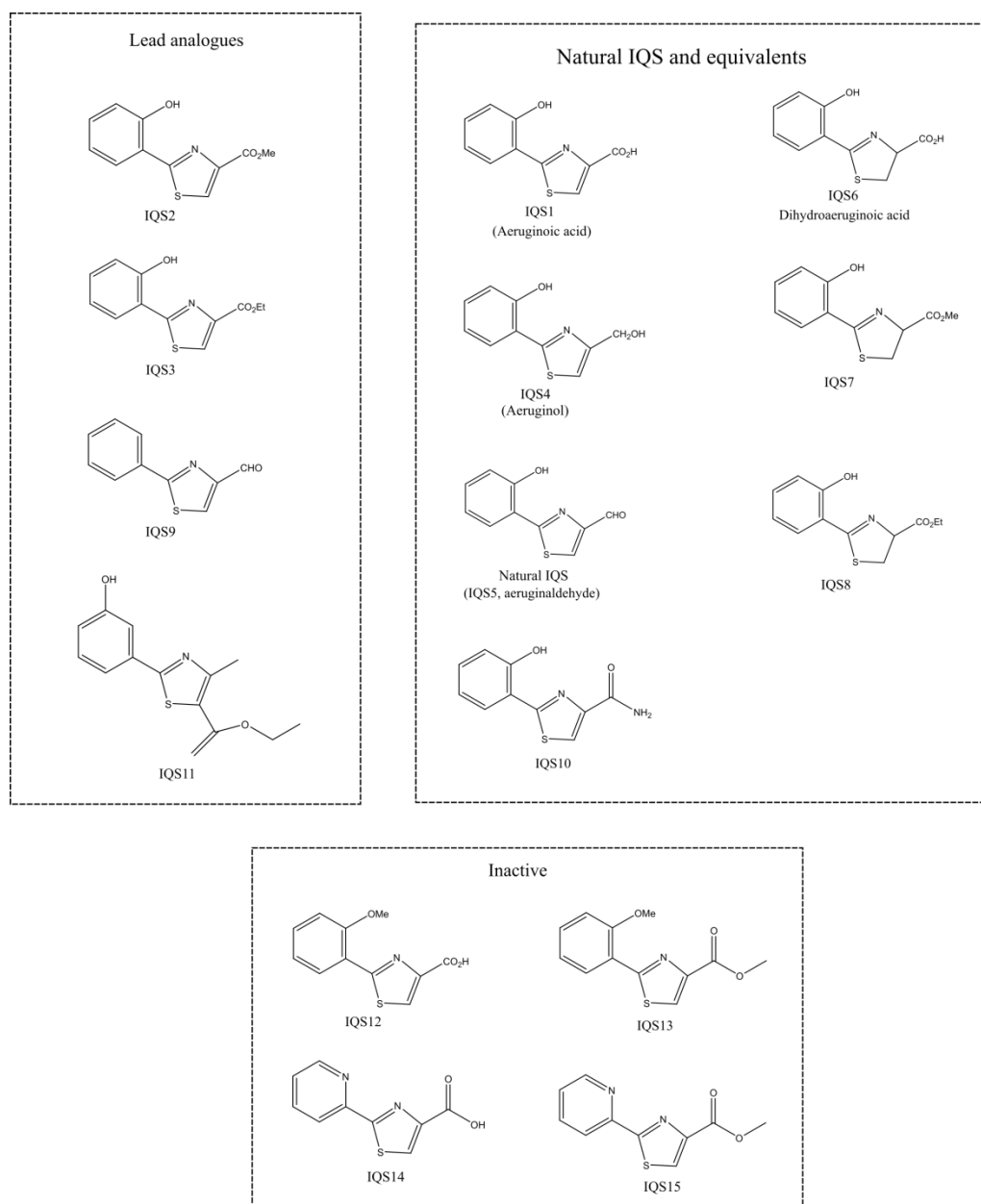


Figure 6.3 IQS analogues grouped by activity against *S. aureus* exotoxin production. IQS analogues used in this study are classified according to their anti-*S. aureus* virulence activity showing a relationship between the chemical structure and their activities.

Iron chelators usually bind to iron (III) with high affinity using negatively charged oxygen as an electron donor although nitrogen and sulphur can also act as donors (Hider and Kong, 2010). As IQS is generated from the biosynthesis of the siderophore pyochelin by *Pseudomonas spp.* (Carmi et al., 1994, Ye et al., 2014), therefore the iron-chelating activity of all IQS analogues synthesised in this study was assessed using a CAS assay (Schwyn and Neilands, 1987). Here we show that the reduced exotoxin production by IQS is independent of iron chelation, thus they would not directly interfere with bacterial iron metabolism. In addition, all active analogues did not inhibit growth or kill the bacteria, therefore, are less likely to trigger the development of a resistance (Gordon et al., 2013).

IQS3 and IQS11 were selected as lead analogues for further studies due to similarity in chemical structure of IQS3 to IQS2 and IQS9 and the distinct structure of IQS11 which was the only analogue containing a sidechain at position 5 of the thiazole ring. In addition, unlike the other analogues used in this study, IQS11 did not increase the production of protein A. As IQS3 reduced exotoxin and increased protein A production, it was proposed that it targeted either the *agr* system or the transcriptional regulator SarA (Dunman et al., 2001, Andrey et al., 2010). Employing a chromosomal *agrP3:lux* reporter strain, it was shown that IQS3 reduced *agrP3* expression, supporting the hypothesis that IQS3 reduced exotoxin production and increased protein A via inhibition of either the *agr* system or SarA. In order to rule out SarA as a target for IQS3, a $\Delta sarA$ *agrP3:lux* strain was generated. It was found to be sensitive to IQS3 suggesting that SarA is not the target for IQS3 mediated-inhibition of *agrP3:lux*. Furthermore, change of virulence factor gene expression in IQS3-treated wild

type bacteria was similar to the *agr* mutant assessed by RNA-Seq supporting the proposal that the mechanism of action of IQS3 is through AgrA inhibition.

The effect of IQS3 on the *agr* system was further assessed using *agr*-I – *agr*-IV reporter strains (ROJ143, EM28, ROJ154 and ROJ145, respectively). The results showed that IQS3 inhibited *agrP3* expression of all four *agr* groups in a non-competitive manner. These data suggest that IQS3 inhibited the function of either the membrane kinase AgrC or the response regulator AgrA. The data also raised an interesting observation as IQS3 inhibited every *agr* group utilised but it did not inhibit exotoxin production in all clinical isolates. This might be because the AgrCA expression construct is harboured within the same *agr* null strain. Whereas, physiological differences between clinical isolates especially within the cell envelope are widely reported. For example, an HA-MRSA isolate BH1CC that expresses high levels of PBP2a results in structural changes in its cell wall resulting in a defective *agr* system (Rudkin et al., 2012). Therefore, it is possible that different isolates contain different cell wall and cell membrane structures leading to different susceptibility to IQS3.

AgrA was confirmed as the target of IQS3 using an *in vivo* assay employing an inducible AgrA construct and by *in vitro* assays, such as nanoDSF and EMSA. The nanoDSF data indicate a direct interaction between IQS3 and AgrA. Even though, the binding affinity is low, it was shown to be a specific interaction as an inactive analogue (IQS15) failed to increase the melting temperature of AgrA. Interestingly, recombinant AgrA used in this study was cloned from *S. aureus* strain MN8, a strain against which, IQS3 lacked activity. These data together with the *in vivo* assays suggest that there is at least one additional factor that effects the activity of IQS3 which could be (i) the structure

of cell membrane and cell wall or (ii) activity of *agr* system which may be different as it was found that USA300 lineage has high activity of the *agr* system that may dampen the effect of IQS3 (Li et al., 2009).

The EMSA assays utilised in this study showed that the disruption of AgrA binding to a target promoter, either the *agrP2-P3* intergenic region or the entire *agrP2* promoter was inhibited in the presence of IQS3. As the EMSA reaction used in this study did not use phosphorylated AgrA, it indicates that IQS3 probably disrupts DNA-binding and, not the phosphor transfer or dimerization of AgrA (Novick, 2003, Srivastava et al., 2014, Koenig et al., 2004). The protein domain to which IQS3 binds could not be narrowed down as attempts to purify the DNA binding domain alone failed. *In silico* protein docking analyses could be used to identify the binding site of IQS3, particularly at the amino acid residues that interact directly with DNA and those that stabilise the folding of protein (Sidote et al., 2008) which will be discussed later in section 6.3.

The molecular target of IQS11 is still unidentified. However, the *agr* system and SarA can be ruled out as incubation with IQS11 did not reduce *agrP3* expression. RNA-Seq analysis was employed using CA-MRSA USA300 LAC as a model in order to identify the target protein of IQS11 and to determine the response of *S. aureus* to treatment with IQS3 and IQS11. Expression of nearly 500 genes changed in response to IQS11 treatment, whereas 133 and 216 genes changed respectively in the response to IQS3 treatment and the *agr* mutant. The expression of only 20 and 42 genes among genes responding to IQS11 also changed in the same pattern with IQS3-treated group and the *agr* mutant,

respectively. These data support that suggestion that IQS11 has a different mechanism of action from IQS3 and the *agr* system is not the target.

Several operons that had a significant increase in expression due to the presence of IQS11 such as *nar*, *nir* and *arc* are involved in anaerobic growth (Fuchs et al., 2007, Makhlin et al., 2007). Aerobic conditions were used for RNA preparation, suggesting the presence of IQS11 resulted in a growth condition that mimics an anaerobic environment. Interestingly genes differentially regulated following treatment should not be anaerobic condition related as genes that are involved in glycolysis in aerobic conditions were also up-regulated, such as glyceraldehyde-3-phosphate dehydrogenase, type I (*gapA*), phosphoglyceromutase (*pgm*) and enolase (*eno*) (Fuchs et al., 2007) as shown in Table 1 in Appendix. Treatment with IQS11 also up-regulated a number of phage and phage-like genes. Upregulation of phage has been proposed to be associated with stressful growth conditions like in anaerobic environments. Prophage can switch to a lytic phase in *P. aeruginosa* to find a new less ‘stressed’ host (Platt et al., 2008). The presence of IQS11 also up-regulated many virulence factor genes, such as *hlgABC*, *efb* and *sspAB*. It is therefore likely that IQS 11 although at the concentrations tested did not impact on growth it elicited general stress on the cell that results in a reduction in specific virulence factor determinants.

6.3 Future plan

In this study we found that IQS analogues 2, 3, 9 and 11 reduced *S. aureus* exotoxin production more so than natural IQS (IQS5). However, the spectrum of activities of IQS3 and IQS11 were not broad as they did not effectively reduce exotoxin of many different isolates. Generating a model of the

interaction between IQS3 and AgrA is important as it would help to develop the analogue so that it could bind to AgrA with higher affinity, this can be achieved by *in silico* protein docking using software, such as Autodock Vina and Pymol (Seeliger and de Groot, 2010) or online tool SwissDock (Grosdidier et al., 2011). The amino acid residues of AgrA that directly bind to DNA are H169, N201 and R233 (Sidote et al., 2008), residues that play a key role in folding are H174, D176, K223 and E226 (Sidote et al., 2008) these should be considered a starting point to analyse an IQS-AgrA interaction. However, due to Sidote's crystallography study that used only the AgrA DNA-binding domain (Sidote et al., 2008), the residues at the N-terminus of the protein which help stabilise the protein structure have not been analysed and essential residues may have been neglected.

Expression of the phenol soluble modulins, small amphipathic toxins that lyse host immune cells, are positively regulated by the binding of AgrA to their promoter element leading to biofilm detachment (Periasamy et al., 2012). It has been shown that an *agr* mutant induces biofilm formation and increases the size of a biofilm causing an increased tolerance to antibiotics (Urish et al., 2016, Costerton et al., 1999, Bendouah et al., 2006, Periasamy et al., 2012). Since, IQS3 targets AgrA, it may enhance biofilm formation of a treated culture. Therefore, studies that evaluate biofilm formation after treatment with IQS analogues would be advisable.

HA-MRSA that carries SCC*mec* type II is less virulent because it is impaired in its response to AIP (Rudkin et al., 2012). As AgrA is a target of IQS3, it raises a question of the effect of IQS3 on these isolates. The experiment should be undertaken. Moreover, HA-MRSA USA500 clonal which contains

SCCmec type IV and a progenitor of CA-MRSA USA300 is as virulent as USA300 (Li et al., 2009, Li et al., 2010). It suggests that the effect of IQS3 on different HA-MRSA clonal groups should be determined.

Other pathogenic gram-positive bacteria have *agr* or *agr-like* systems and play a role in virulence i.e., *Enterococcus faecalis* (Del Papa and Perego, 2011), *Clostridium difficile* (Martin et al., 2013) and *Listeria monocytogenes* (Autret et al., 2003). It would be interesting to determine the effect of IQS3 on the production of virulence factors in these bacteria.

Only the AgrA DNA-binding domain has been analysed using crystallography (Sidote et al., 2008). In this study AgrA was successfully expressed and purified to an extent that structural analysis should be possible.

This study that found IQS11 up-regulated a previously uncharacterised transcriptional regulator and its adjacent gene more than 500-fold and altered expression of large number of gene. This raised a question whether these uncharacterised genes are involved in the stress response. In order to determine the function of these genes, gene deletion technique would be employed. The predicted promoter and transcription factor binding sites using SoftBerry BPRON online tool (Solovyev and Salamov, 2011), details in the section 5.2.4 would be determined using further analysis and it will help to understand this operon.

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Appendix

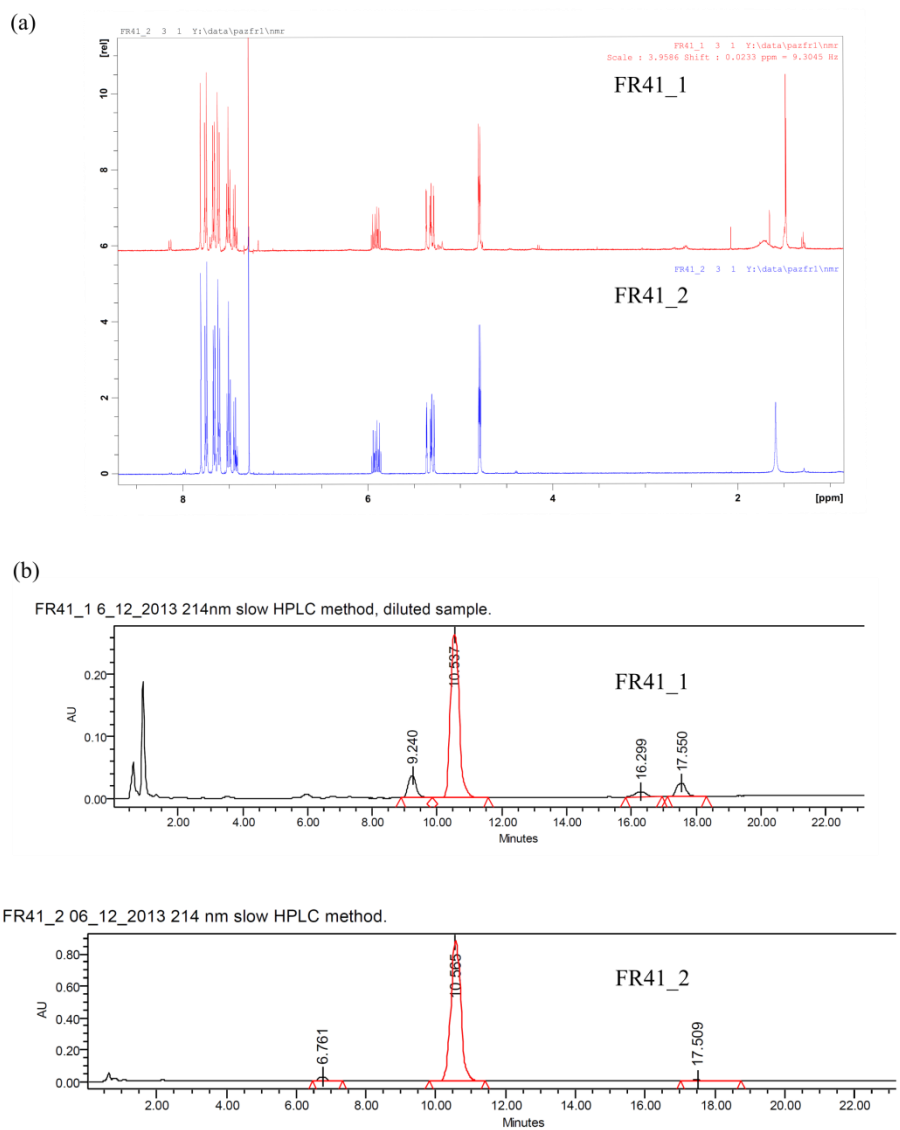
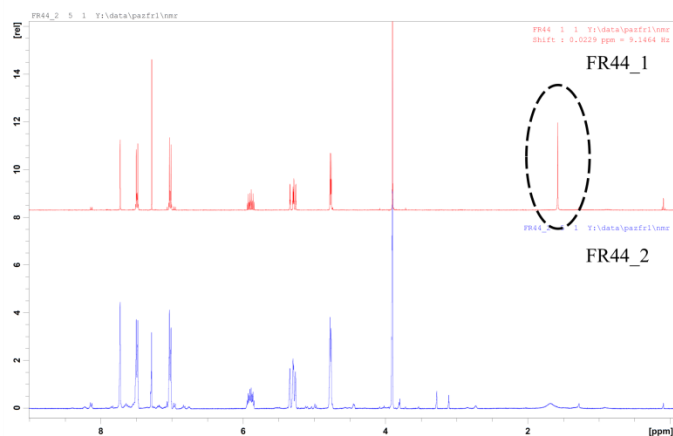


Figure 1 Comparison between FR41 batch 1 and 2 using ^1H NMR and HPLC analysis. (a) ^1H NMR and (b) HPLC carried out by Dr. Fabio Rui (School of Pharmacy, University of Nottingham; unpublished) to compare FR41 batch 1 and 2 (FR41_1, FR41_2, respectively).

(a)



(b)

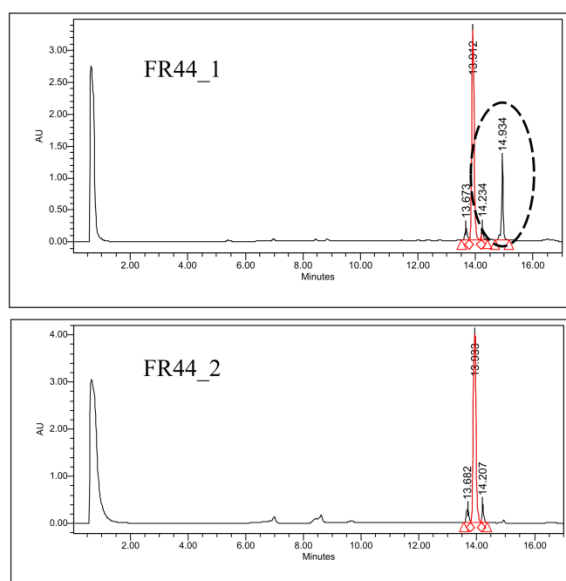


Figure 2 Comparison between FR44 batch 1 and 2 using ^1H NMR and HPLC analysis. (a) ^1H NMR and (b) HPLC carried out by Dr. Fabio Rui (School of Pharmacy, University of Nottingham; unpublished data) to compare FR44 batch 1 and 2 (FR44_1, FR44_2, respectively). Dotted lines circle around extra-peak that found in FR44 batch 1.

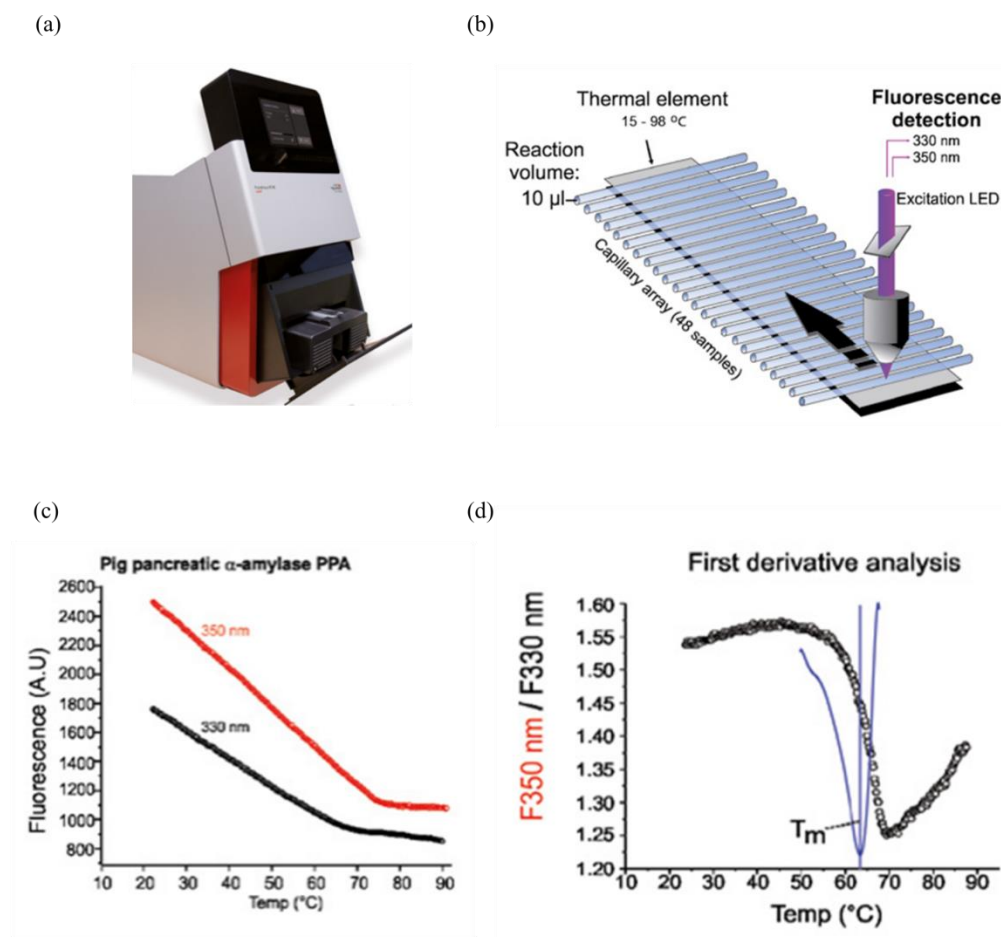


Figure 3 Nano Differential Scanning Fluorimetry using Prometheus NT.48 platform. (a) Prometheus NT.48 (NanoTemper). (b) Illustration of the platform. (c) Two wavelengths, 330 and 350 nm, are recorded. (d) The ratio of 350nm and 330 nm was plotted against the temperature. A polynomial function is used to derive the first derivative as shown as a trough which indicates melting temperature (T_m) (Prometheus NT.48 Production Information).

Table 1. Differentially expressed metabolic genes in IQS11-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_2342	<i>narH</i>	respiratory nitrate reductase, beta subunit	64.80	0
SAUSA300_2341	<i>narJ</i>	respiratory nitrate reductase, subunit delta	63.70	1.81E-264
SAUSA300_2343	<i>narZ</i>	respiratory nitrate reductase, alpha subunit	50.29	0
SAUSA300_0684	<i>fruB</i>	fructose 1-phosphate kinase	44.77	2.89E-261
SAUSA300_0685	<i>fruA</i>	fructose specific permease	35.02	0
SAUSA300_2345	<i>nirD</i>	nitrite reductase [NAD(P)H], small subunit	22.22	1.24E-136
SAUSA300_2344	-	uroporphyrin-III C-methyl transferase	20.16	0
SAUSA300_2346	<i>nirB</i>	nitrite reductase [NAD(P)H], large subunit	15.69	5.57E-287
SAUSA300_2340	<i>narI</i>	respiratory nitrate reductase, gamma subunit	12.45	2.44E-270
SAUSA300_0966	<i>purE</i>	phosphoribosylaminoimidazole carboxylase, catalytic subunit	10.47	1.72E-134
SAUSA300_0756	<i>gapA</i>	glyceraldehyde-3-phosphate dehydrogenase, type I (GAPDH)	9.13	1.14E-279
SAUSA300_0386	<i>xpt</i>	xanthine phosphoribosyltransferase	8.80	2.45E-41
SAUSA300_0757	<i>pgk</i>	phosphoglycerate kinase	8.66	3.20E-244
SAUSA300_0758	<i>tpiA</i>	triosephosphate isomerase	8.66	6.06E-253
SAUSA300_0759	<i>pgm</i>	phosphoglyceromutase	8.49	6.69E-232
SAUSA300_0967	<i>purK</i>	phosphoribosylaminoimidazole carboxylase ATPase subunit	7.50	6.08E-162
SAUSA300_0538	-	NAD-dependent epimerase/dehydratase family protein	6.13	1.83E-191
SAUSA300_0692	-	thiosulfate dehydrogenase	5.99	9.13E-95
SAUSA300_0968	<i>purC</i>	phosphoribosylaminoimidazole-succinocarboxamide synthase	5.49	1.14E-77
SAUSA300_2516	-	short chain dehydrogenase/reductase family oxidoreductase	4.90	1.92E-30
SAUSA300_1949	<i>dut</i>	dUTP diphosphatase	4.77	3.99E-08
SAUSA300_0970	<i>purQ</i>	phosphoribosylformylglycinamide synthase I	4.24	1.97E-64
SAUSA300_0969	<i>purS</i>	phosphoribosylformylglycinamide synthase	4.22	3.26E-28
SAUSA300_1976	-	succinyl-diaminopimelate desuccinylase	3.77	4.64E-60
SAUSA300_0320	-	triacylglycerol lipase	3.36	3.55E-127
SAUSA300_0711	-	putative lipid kinase	3.20	3.74E-74
SAUSA300_RS03005		VraX	3.16	3.17E-39
SAUSA300_2076	-	aldehyde dehydrogenase family protein	3.09	2.34E-94
SAUSA300_2281	<i>hutG</i>	formimidoylglutamate	3.01	8.00E-83
SAUSA300_0971	<i>purL</i>	phosphoribosylformylglycinamide synthase II	3.01	1.08E-59
SAUSA300_0555	-	putative hexulose-6-phosphate synthase	2.47	1.77E-44
SAUSA300_0972	<i>purF</i>	amidophosphoribosyltransferase	2.43	2.85E-44
SAUSA300_0556	-	SIS domain-containing protein	2.33	2.60E-36
SAUSA300_2166	<i>alsS</i>	acetolactate synthase	2.30	1.26E-24
SAUSA300_2457	-	phospholipase/carboxylesterase family protein	2.24	2.59E-05
SAUSA300_0181	-	non-ribosomal peptide synthetase	2.21	1.49E-36
SAUSA300_0179	-	formate dehydrogenase	2.14	7.25E-07

Table 1 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_1125	<i>acpP</i>	acyl carrier protein	2.12	1.37E-32
SAUSA300_0955	<i>atl</i>	autolysin	2.06	2.49E-31
SAUSA300_2458	-	glyoxalase	2.05	1.69E-05
SAUSA300_0865	<i>pgi</i>	glucose-6-phosphate isomerase	2.03	7.30E-39
SAUSA300_0973	<i>purM</i>	phosphoribosylaminoimidazole synthetase	2.01	7.82E-23
SAUSA300_1098	<i>pyrE</i>	orotate phosphoribosyltransferase	0.50	5.78E-06
SAUSA300_0424	-	putative cobalamin synthesis protein	0.49	6.52E-03
SAUSA300_2014	<i>ilvA</i>	threonine dehydratase	0.49	6.75E-05
SAUSA300_0594	<i>adhP</i>	alcohol dehydrogenase	0.48	6.78E-30
SAUSA300_2277	<i>hutI</i>	imidazolonepropionase	0.46	2.00E-03
SAUSA300_0225	-	putative acyl-CoA acetyltransferase FadA	0.46	2.02E-04
SAUSA300_2108	<i>mtlD</i>	mannitol-1-phosphate 5-dehydrogenase	0.46	1.06E-36
SAUSA300_2013	<i>leuD</i>	isopropylmalate isomerase small subunit	0.46	5.15E-03
SAUSA300_2081	<i>pyrG</i>	CTP synthetase	0.45	1.53E-26
SAUSA300_1896	<i>pheA</i>	prephenate dehydratase	0.45	2.89E-22
SAUSA300_1331	<i>ald</i>	alanine dehydrogenase	0.44	1.15E-09
SAUSA300_0697	<i>queC</i>	7-cyano-7-deazaguanine synthase	0.44	1.20E-03
SAUSA300_0170	-	aldehyde dehydrogenase	0.43	4.41E-56
SAUSA300_0007	<i>nnrD</i>	ADP-dependent (S)-NAD(P)H-hydrate dehydratase	0.43	2.18E-29
SAUSA300_2319	-	pyridine nucleotide-disulfide oxidoreductase	0.42	2.38E-15
SAUSA300_0315	<i>nanA</i>	N-acetylneuraminate lyase	0.42	2.76E-07
SAUSA300_1097	<i>pyrF</i>	orotidine 5'-phosphate decarboxylase	0.42	4.03E-08
SAUSA300_1262	<i>trpE</i>	anthranilate synthase component I	0.36	2.36E-02
SAUSA300_2551	<i>nrdD</i>	anaerobic ribonucleoside triphosphate reductase	0.36	1.10E-52
SAUSA300_2154	<i>lacB</i>	galactose-6-phosphate isomerase subunit LacB	0.36	5.27E-15
SAUSA300_2611	<i>hisD</i>	histidinol dehydrogenase	0.36	1.24E-02
SAUSA300_0193	<i>murQ</i>	N-acetylmuramic acid-6-phosphate etherase	0.35	2.13E-38
SAUSA300_0222	-	glycerophosphoryl diester phosphodiesterase	0.34	5.57E-13
SAUSA300_0260	<i>bglA</i>	6-phospho-beta-glucosidase	0.34	1.39E-09
SAUSA300_1227	<i>thrC</i>	threonine synthase	0.34	3.22E-40
SAUSA300_0151	<i>adhE</i>	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	0.33	1.18E-47
SAUSA300_1330	-	serine/threonine dehydratase	0.32	9.06E-24
SAUSA300_2012	<i>leuC</i>	isopropylmalate isomerase large subunit	0.32	7.83E-12
SAUSA300_0132	-	glycosyl transferase, group 1 family protein	0.30	1.83E-03
SAUSA300_1094	<i>pyrC</i>	dihydroorotase	0.30	3.41E-55
SAUSA300_2469	<i>sdaAA</i>	L-serine dehydratase, iron-sulfur-dependent, alpha subunit	0.29	2.60E-48
SAUSA300_1095	<i>carA</i>	carbamoyl phosphate synthase small subunit	0.29	5.01E-48
SAUSA300_0220	<i>pflB</i>	formate acetyltransferase	0.29	1.11E-77
SAUSA300_2471	-	perfringolysin O	0.29	4.51E-48

Table 1 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_1096	<i>carB</i>	carbamoyl phosphate synthase large subunit	0.28	2.70E-67
SAUSA300_2008	<i>ilvN</i>	acetolactate synthase 1 regulatory subunit	0.28	9.21E-06
SAUSA300_2539	-	4-aminobutyrate aminotransferase	0.28	1.43E-40
SAUSA300_2570	<i>arcA</i>	arginine deiminase	0.28	7.43E-06
SAUSA300_2569	<i>arcB</i>	ornithine carbamoyltransferase	0.26	2.60E-07
SAUSA300_2470	<i>sdaAB</i>	L-serine dehydratase, iron-sulfur-dependent, beta subunit	0.26	5.33E-34
SAUSA300_0221	<i>pflA</i>	pyruvate formate-lyase activating enzyme	0.24	2.15E-117
SAUSA300_2153	<i>lacC</i>	tagatose-6-phosphate kinase	0.24	3.82E-42
SAUSA300_2152	<i>lacD</i>	tagatose 1,6-diphosphate aldolase	0.24	1.62E-43
SAUSA300_1226	-	homoserine dehydrogenase	0.24	4.13E-72
SAUSA300_1062	<i>argF</i>	ornithine carbamoyltransferase	0.23	5.88E-17
SAUSA300_2577	<i>manA</i>	mannose-6-phosphate isomerase	0.22	2.62E-17
SAUSA300_2006	<i>ilvD</i>	dihydroxy-acid dehydratase	0.22	5.05E-55
SAUSA300_2149	<i>lacG</i>	6-phospho-beta-galactosidase	0.21	6.00E-72
SAUSA300_2011	<i>leuB</i>	3-isopropylmalate dehydrogenase	0.21	3.55E-19
SAUSA300_2010	<i>leuA</i>	2-isopropylmalate synthase	0.20	6.59E-38
SAUSA300_0987	-	cytochrome D ubiquinol oxidase, subunit II	0.23	4.79E-22
SAUSA300_0986	-	cytochrome D ubiquinol oxidase, subunit I	0.18	2.11E-46
SAUSA300_2007	<i>ilvB</i>	acetolactate synthase large subunit	0.17	4.59E-56
SAUSA300_2009	<i>ilvC</i>	ketol-acid reductoisomerase	0.16	1.13E-40
SAUSA300_1063	<i>arcC</i>	carbamate kinase	0.14	9.00E-54
SAUSA300_1225	-	aspartate kinase	0.10	1.61E-49

Table 2. Differentially expressed phage and phage-like genes in IQS11-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_1962	-	phiPVL ORF39-like protein	9.11	1.626E-13
SAUSA300_1959	-	phiPVL ORF044-like protein	8.43	6.94E-16
SAUSA300_1966	-	phi77 ORF014-like protein, phage anti-repressor protein	8.39	3.715E-64
SAUSA300_1957	-	phiPVL ORF046-like protein	8.06	8.41E-26
SAUSA300_1961	-	phiPVL ORF41-like protein	7.98	4.058E-59
SAUSA300_1938	-	phi77 ORF006-like protein putative capsid protein	7.88	1.18E-18
SAUSA300_1947	-	phi77 ORF031-like protein	7.50	1.396E-09
SAUSA300_1963	-	conserved hypothetical phage protein	6.90	5.18E-12
SAUSA300_1945	-	phi77 ORF071-like protein	5.99	8.69E-05
SAUSA300_1956	-	conserved hypothetical phage protein	5.80	5.06E-04
SAUSA300_1934	-	phi77 ORF020-like protein, phage major tail protein	5.64	8.44E-09
SAUSA300_1936	-	conserved hypothetical phage protein	4.73	4.11E-04
SAUSA300_1935	-	phi77 ORF029-like protein	4.62	3.47E-04
SAUSA300_1941	-	phi77 ORF003-like protein, phage terminase, large subunit	4.41	1.22E-08
SAUSA300_1948	-	phi77 ORF069-like protein	4.39	4.02E-03
SAUSA300_1965	-	conserved hypothetical phage protein	4.03	9.87E-76
SAUSA300_1944	-	phi77 ORF026-like protein phage transcriptional activator	3.99	1.66E-12
SAUSA300_1943	-	phi77 ORF040-like protein	3.65	4.83E-03
-	-	phage head-tail adapter protein	3.61	1.54E-02
SAUSA300_1937	-	phi77 ORF045-like protein	3.32	2.09E-02
SAUSA300_1954	-	phiPVL ORF050-like protein	3.11	2.58E-03
SAUSA300_1939	-	phi77 ORF015-like protein protease	3.09	7.64E-06
SAUSA300_1930	-	phi77 ORF001-like protein, phage tail tape measure protein	3.04	2.52E-10
SAUSA300_1953	-	phiPVL ORF051-like protein	3.03	4.02E-02
SAUSA300_1952	-	phiPV083 ORF027-like protein	3.02	9.94E-03
SAUSA300_1924	-	holin	2.84	5.95E-02
SAUSA300_1409	-	Conserved hypothetical phage protein	2.49	3.62E-02
SAUSA300_1392	-	phiSLT ORF191-like protein	2.39	3.55E-02
SAUSA300_1929	-	phi77 ORF004-like protein phage tail component	2.38	1.89E-02
SAUSA300_1940	-	phage portal protein	2.26	4.93E-03
SAUSA300_1932	-	Conserved hypothetical phage protein	2.25	2.52E-03
SAUSA300_1925	-	phiPVL ORF17-like protein	2.18	7.48E-02
SAUSA300_1397	-	phiSLT ORF213-like protein, major tail protein	2.15	2.81E-02
SAUSA300_1396	-	phiSLT ORF151-like protein, major tail protein	2.09	1.03E-02

Table 3. Differentially expressed virulence factor genes in IQS11-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_1918	-	truncated beta-haemolysin	8.10	8.16E-22
SAUSA300_2366	<i>hlgC</i>	gamma-haemolysin component C	7.63	2.42E-52
SAUSA300_1052	-	fibrinogen-binding protein	7.28	4.09E-122
SAUSA300_1055	<i>efb</i>	fibrinogen-binding protein	6.74	8.29E-83
SAUSA300_1056	-	staphylococcal complement inhibitor	5.89	1.34E-44
SAUSA300_0951	<i>sspA</i>	V8 protease	5.18	9.60E-88
SAUSA300_0950	<i>sspB</i>	staphopain B, cysteine protease	4.48	7.65E-74
SAUSA300_2365	<i>hlgA</i>	gamma-haemolysin component A	4.22	1.14E-35
SAUSA300_2364	<i>sbi</i>	IgG-binding protein SBI	3.70	1.02E-38
SAUSA300_2367	<i>hlgB</i>	gamma-haemolysin component B	3.66	4.24E-20
SAUSA300_0647	-	ABC transporter ATP-binding protein	3.27	2.39E-46
SAUSA300_0278	<i>esxA</i>	Virulence factor EsxA	3.02	7.93E-87
SAUSA300_2499	<i>crtM</i>	squalene desaturase	2.63	1.50E-50
SAUSA300_1975	-	aerolysin/leukocidin family protein	2.53	1.62E-09
SAUSA300_0395	-	superantigen-like protein	2.52	3.27E-21
SAUSA300_1920	<i>chs</i>	chemotaxis-inhibiting protein CHIPS	2.47	7.65E-03
SAUSA300_2498	<i>crtN</i>	squalene synthase	2.31	1.16E-46
SAUSA300_1988	-	delta-haemolysin	2.20	5.65E-42
SAUSA300_1382	<i>lukS-PV</i>	Panton-Valentine leukocidin, LukS-PV	2.17	1.21E-17
SAUSA300_0772	<i>clfA</i>	clumping factor A	2.15	8.51E-52
SAUSA300_1059		superantigen-like protein, putative exotoxin 1	0.35	4.44E-04
SAUSA300_1061	-	superantigen-like protein	0.34	1.97E-04
SAUSA300_1058	<i>hla</i>	alpha-haemolysin	0.32	3.20E-51

Table 4. Differentially expressed capsular biosynthesis genes in IQS11-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_0159	<i>cap5H</i>	capsular polysaccharide biosynthesis protein Cap5H	3.85	3.58E-05
SAUSA300_0158	<i>cap5G</i>	capsular polysaccharide biosynthesis protein Cap5G	3.70	4.34E-09
SAUSA300_0157	<i>cap5F</i>	capsular polysaccharide biosynthesis protein Cap5F	3.64	2.92E-09
SAUSA300_0160	<i>cap5I</i>	capsular polysaccharide biosynthesis protein Cap5I	3.16	2.80E-09
SAUSA300_0161	<i>cap5J</i>	capsular polysaccharide biosynthesis protein Cap5J	2.82	2.03E-06
SAUSA300_0163	<i>cap5L</i>	capsular polysaccharide biosynthesis protein Cap5L	2.76	9.47E-07
SAUSA300_0156	<i>cap5E</i>	capsular polysaccharide biosynthesis protein Cap5E	2.56	5.41E-05
SAUSA300_0165	<i>cap5N</i>	capsular polysaccharide biosynthesis protein Cap5N	2.42	4.29E-05
SAUSA300_0154	<i>cap5C</i>	capsular polysaccharide biosynthesis protein Cap5C	2.25	2.78E-03
SAUSA300_0166	<i>cap5O</i>	capsular polysaccharide biosynthesis protein Cap5O	2.19	5.31E-06
SAUSA300_0162	<i>cap5K</i>	capsular polysaccharide biosynthesis protein Cap5K	2.16	5.46E-03

Table 5 Differentially expressed transporter genes in IQS11-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_2333	<i>narK/T</i>	nitrite extrusion protein	40.37	0
SAUSA300_0387	<i>pbuX</i>	xanthine permease	8.35	2.59E-101
SAUSA300_1244	<i>mscL</i>	large-conductance mechanosensitive channel	7.61	1.01E-203
SAUSA300_1874	<i>ftnA</i>	Bacterial non-heme ferritin	3.27	5.60E-92
SAUSA300_2099	-	cation efflux family protein	3.22	7.355E-25
SAUSA300_0309	-	ABC transporter ATP-binding protein	3.17	1.04E-62
SAUSA300_2399	-	ABC transporter ATP-binding protein	3.16	7.798E-38
SAUSA300_0308	-	ABC transporter permease	3.04	2.61E-75
SAUSA300_2398	-	putative ABC transport system permease protein	2.90	3.43E-60
SAUSA300_0566	-	amino acid permease	2.85	3.07E-80
SAUSA300_1002	<i>potD</i>	spermidine/putrescine ABC transporter spermidine/putrescine-binding protein	2.82	7.27E-51
SAUSA300_0983	<i>ptsH</i>	phosphocarrier protein HPr	2.81	3.69E-87
SAUSA300_2145	-	glycine betaine transporter	2.67	3.17E-71
SAUSA300_0984	<i>ptsI</i>	phosphoenolpyruvate-protein phosphotransferase	2.49	2.56E-73
SAUSA300_2390	<i>opuCd</i>	glycine betaine/carnitine/choline transport system permease	2.38	3.08E-17
SAUSA300_0851	<i>mnhE</i>	putative monovalent cation/H ⁺ antiporter subunit E	2.11	1.44E-07
SAUSA300_2391	<i>opuCc</i>	glycine betaine/carnitine/choline ABC transporte	2.09	5.60E-11
SAUSA300_1273	<i>opp-2F</i>	oligopeptide permease, ATP-binding protein	2.06	7.59E-08
SAUSA300_1628	<i>lysP</i>	lysine-specific permease	2.04	4.45E-32
SAUSA300_0115	<i>sirC</i>	iron compound ABC transporter permease SirC	2.04	1.13E-17
SAUSA300_0707	-	osmoprotectant ABC transporter permease	2.04	1.05E-29
SAUSA300_0706	-	putative osmoprotectant ABC transporter ATP-binding protein	2.04	1.33E-27
SAUSA300_0818	<i>sufC</i>	FeS assembly ATPase SufC	2.04	6.92E-36
SAUSA300_0337	<i>glpT</i>	glycerol-3-phosphate transporter	0.50	7.49E-09
SAUSA300_2107	<i>mtlA</i>	PTS system, mannitol specific IIA component	0.50	2.48E-21
SAUSA300_0201	-	peptide ABC transporter permease	0.50	5.36E-03
SAUSA300_2139	-	putative transporter	0.49	1.24E-02
SAUSA300_2410	-	oligopeptide ABC transporter permease	0.49	9.05E-04
SAUSA300_1726	-	CrcB family protein	0.48	1.99E-02
SAUSA300_0634	<i>fhuB</i>	ferrichrome transport permease FhuB	0.48	3.92E-06
SAUSA300_0633	<i>fhuA</i>	ferrichrome transport ATP-binding protein FhuA	0.48	9.58E-16
SAUSA300_2324	-	PTS system, sucrose-specific IIBC component	0.47	5.06E-32
SAUSA300_0202	-	peptide ABC transporter permease	0.47	1.56E-02
SAUSA300_2235	-	iron compound ABC transporter iron compound-binding protein	0.47	9.95E-14
SAUSA300_1847	-	Putative multidrug export ATP-binding/permease protein	0.46	5.33E-23

Table 5 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_2442	<i>gntP</i>	gluconate permease	0.46	3.70E-26
SAUSA300_1913	-	ABC transporter ATP-binding protein	0.46	4.41E-06
SAUSA300_2329	<i>gltT</i>	proton/sodium-glutamate symport protein	0.45	2.08E-37
SAUSA300_1001	<i>potC</i>	spermidine/putrescine ABC transporter permease	0.44	1.02E-03
SAUSA300_1808	-	amino acid ABC transporter permease/substrate-binding protein	0.42	3.91E-05
SAUSA300_0259	-	PTS system, IIA component	0.42	1.62E-05
SAUSA300_1030	<i>isdC</i>	Iron-regulated surface determinant protein C	0.41	2.26E-03
SAUSA300_0306	<i>brnQ</i>	branched-chain amino acid transport system II carrier protein	0.41	2.02E-46
SAUSA300_2411	<i>opp-1A</i>	oligopeptide permease, peptide-binding protein	0.41	1.12E-21
SAUSA300_1000	<i>potB</i>	spermidine/putrescine ABC transporter permease	0.40	1.04E-04
SAUSA300_0999	<i>potA</i>	spermidine/putrescine ABC transporter ATP-binding protein	0.40	2.00E-13
SAUSA300_2103	-	ABC transporter ATP-binding protein	0.39	1.24E-19
SAUSA300_2488	<i>feoA</i>	ferrous iron transport protein A	0.39	1.75E-02
SAUSA300_2105	<i>mtlF</i>	PTS system, mannitol specific IIBC component	0.38	1.31E-40
SAUSA300_2487	<i>feoB</i>	ferrous iron transport protein B	0.38	5.47E-09
SAUSA300_0203	-	RGD-containing lipoprotein, peptide/nickel transport system substrate-binding protein	0.37	1.26E-04
SAUSA300_2538	-	amino acid permease family protein	0.37	3.48E-53
SAUSA300_1092	<i>pyrP</i>	uracil permease	0.35	8.25E-48
SAUSA300_0216	<i>uhpT</i>	sugar phosphate antiporter	0.33	2.69E-12
SAUSA300_0314	-	sodium:solute symporter family protein	0.33	2.83E-14
SAUSA300_0914	-	sodium:alanine symporter family protein	0.31	2.24E-23
SAUSA300_1329	-	amino acid permease	0.29	2.23E-31
SAUSA300_2568	<i>arcD</i>	arginine/ornithine antiporter	0.28	1.42E-09
SAUSA300_1996	<i>amt</i>	ammonium transporter	0.27	2.10E-03
SAUSA300_2151	<i>lacF</i>	PTS system, lactose-specific IIA component	0.25	1.81E-12
SAUSA300_0194	-	sucrose-specific PTS transporter protein	0.25	1.10E-74
SAUSA300_0332	-	PTS system, IIA component	0.20	6.14E-40
SAUSA300_2150	<i>lacE</i>	PTS system, lactose-specific IIBC component	0.20	3.68E-69
SAUSA300_0331	-	PTS system, ascorbate-specific IIB component	0.16	2.15E-35
SAUSA300_0330	<i>ulaA</i>	PTS system ascorbate-specific transporter subunit IIC	0.10	1.58E-156

Table 6 Differentially expressed transcriptional regulator genes in IQS11-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_2310	-	Uncharacterized HTH-type transcriptional regulator	577.64	0
SAUSA300_0683	-	DeoR family transcriptional regulator	60.55	4.11E-203
SAUSA300_2347	<i>nirR</i>	nitrite reductase transcriptional regulator NirR	13.03	8.749E-89
SAUSA300_0755	<i>gapR</i>	glycolytic operon regulator	13.00	0.00E+00
WP_000854072.1	-	transcriptional regulator (predicted)	8.05	1.012E-09
SAUSA300_1457	<i>malR</i>	maltose operon transcriptional repressor	4.53	3.92E-84
SAUSA300_2515	<i>tetR</i>	TetR family transcriptional regulator	4.52	1.42E-19
SAUSA300_0605	<i>sarA</i>	staphylococcal accessory regulator A	4.06	3.82E-174
SAUSA300_2490		TetR family regulatory protein	3.96	1.49E-24
SAUSA300_2098	<i>arsR</i>	ArsR family transcriptional regulator, transcriptional repressor	3.88	1.14E-15
SAUSA300_0475	<i>spoVG</i>	regulatory protein SpoVG (stage V sporulation protein G)	3.78	7.32E-152
SAUSA300_0928	<i>comK</i>	competence transcription factor ComK2	3.29	4.05E-34
SAUSA300_0804	-	putative transcriptional regulator	3.00	3.49E-03
SAUSA300_0265	<i>rbsR</i>	putative ribose operon repressor	2.73	8.82E-24
SAUSA300_2245	<i>sarR</i>	staphylococcal accessory regulator R	2.60	9.75E-55
SAUSA300_0473	<i>purR</i>	Pur operon repressor	2.13	6.25E-30
SAUSA300_2640	-	putative transcriptional regulator	2.10	3.48E-08
SAUSA300_1295	<i>cspA</i>	CSD family cold shock protein	2.07	1.66E-47
SAUSA300_1469	<i>argR</i>	arginine repressor	2.06	1.72E-15
SAUSA300_2639	<i>cspB</i>	cold shock protein, CspB	0.50	8.46E-03
SAUSA300_1200	<i>glnR</i>	glutamine synthetase repressor	0.49	9.19E-24
SAUSA300_2566	<i>arcR</i>	Crp/FNR family transcriptional regulator, ArcR	0.46	1.22E-07
SAUSA300_1009	<i>typA</i>	GTP-binding protein	0.45	1.76E-43
SAUSA300_2326	-	transcription regulatory protein (predicted)	0.37	3.59E-29
SAUSA300_2106	-	putative transcriptional regulator	0.32	1.12E-47
SAUSA300_0195	<i>rpiRB</i>	RpiR transcriptional regulator	0.18	1.36E-82

Table 7 Differentially expressed genes in IQS11-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
Antimicrobial				
SAUSA300_1762	<i>epiF</i>	lantibiotic epidermin immunity protein F	2.91	7.89E-46
SAUSA300_1763	<i>epiP</i>	lantibiotic epidermin leader peptide processing serine protease EpiP	2.771091	1.20E-36
SAUSA300_1761	<i>epiE</i>	lantibiotic epidermin immunity protein F	2.17	1.57E-31
Cell death				
SAUSA300_2479	<i>cidA</i>	holin-like protein CidA	4.70	3.76E-06
SAUSA300_2478	<i>cidB</i>	Holin-like protein CidB	2.57	8.08E-55
Drug resistance				
SAUSA300_2489	-	putative drug exporter of the RND superfamily	257.00	0.00E+00
SAUSA300_1653	-	metal-dependent hydrolase	3.00	4.04E-60
SAUSA300_0630	-	ABC transporter ATP-binding protein	2.60	1.02E-67
SAUSA300_0887	<i>oppB</i>	oligopeptide ABC transporter permease	0.42	2.63E-19
SAUSA300_0889	<i>oppD</i>	oligopeptide ABC transporter ATP-binding protein	0.40	2.87E-14
SAUSA300_0888	<i>oppC</i>	oligopeptide ABC transporter permease	0.36	1.26E-22
SAUSA300_0268	-	putative drug transporter	0.35	5.19E-08
SAUSA300_1328	<i>norB</i>	Quinolone resistance protein NorB	0.33	1.13E-12
Enzyme				
SAUSA300_1862	<i>ptpA</i>	Low molecular weight protein-tyrosine-phosphatase PtpA	5.37	2.45E-201
SAUSA300_2275	-	short chain dehydrogenase/reductase family oxidoreductase	4.59	2.16E-154
SAUSA300_0474	-	putative endoribonuclease L-PSP	4.26	6.51E-177
SAUSA300_0776	<i>nuc</i>	thermonuclease	3.17	1.24E-06
SAUSA300_0070	-	putative lysophospholipase	2.94	2.23E-42
SAUSA300_1923	-	N-acetylmuramoyl-L-alanine amidase	2.90	2.10E-02
SAUSA300_0105	-	M20/M25/M40 family peptidase	2.85	1.58E-12
SAUSA300_0688	-	aldo/keto reductase family oxidoreductase	2.55	2.13E-58
SAUSA300_0689	-	glycosyl transferase, group 2 family protein	2.28	1.01E-44
SAUSA300_2251	-	dehydrogenase family protein	2.01	3.04E-21
SAUSA300_2317	-	putative zinc-binding dehydrogenase	0.49	1.86E-20
SAUSA300_1571	-	O-methyltransferase family protein	0.47	4.99E-07
SAUSA300_0641	-	putative lipase/esterase	0.43	8.57E-07
SAUSA300_1222	<i>nuc</i>	thermonuclease	0.42	8.58E-06
SAUSA300_0991	<i>def</i>	peptide deformylase	0.40	5.37E-24

Table 7 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
Genetic information processing				
SAUSA300_1958	-	single-strand binding protein	7.42	6.01E-12
SAUSA300_1117	<i>rpmB</i>	50S ribosomal protein L28	7.33	5.69E-275
SAUSA300_1955	-	putative endodeoxyribonuclease RusA	6.33	3.02E-06
SAUSA300_1960	-	putative phage-related DNA recombination protein, recT	6.12	9.04E-30
SAUSA300_1525	<i>glyS</i>	glycyl-tRNA synthetase	4.22	3.25E-140
SAUSA300_1362	<i>hup</i>	DNA-binding protein HU	3.87	4.68E-127
SAUSA300_2052	-	single-stranded DNA- binding protein family	2.99	1.89E-09
SAUSA300_0809	-	putative DNA primase	2.72	5.97E-14
SAUSA300_0794	-	TOPRIM domain-containing protein	2.50	3.41E-19
SAUSA300_1242	<i>sbcD</i>	exonuclease SbcD	0.48	1.99E-07
SAUSA300_0037	<i>ccrB</i>	cassette chromosome recombinase B	0.48	3.11E-06
SAUSA300_1143	<i>topA</i>	DNA topoisomerase I	0.46	7.24E-34
SAUSA300_1595	<i>tgt</i>	queuine tRNA-ribosyltransferase	0.45	6.25E-32
SAUSA300_1131	<i>rpsP</i>	30S ribosomal protein S16	0.44	7.07E-26
SAUSA300_2037	-	ATP-dependent RNA helicase	0.43	1.06E-58
SAUSA300_1259	-	ImpB/MucB/SamB family protein	0.42	1.34E-07
SAUSA300_1133	<i>trmD</i>	tRNA (guanine-N(1)-)-methyltransferase	0.40	1.03E-08
SAUSA300_1132	<i>rimM</i>	16S rRNA-processing protein RimM	0.39	5.55E-07
SAUSA300_1629	<i>thrS</i>	threonyl-tRNA synthetase	0.38	1.75E-35
Prokaryotic defence system				
SAUSA300_2353	-	antitoxin YefM	3.41	2.13E-19
SAUSA300_2352		addiction module antitoxin, Txe/YoeB family	2.88	2.35E-26
Secretion system				
SAUSA300_1480	-	putative traG membrane protein, mannosyl-glycoprotein endo-beta-N-acetylglucosamidase	3.74	2.46E-17
SAUSA300_0283	<i>essC</i>	essC protein	2.58	1.10E-30
Signal transduction				
SAUSA300_2034	<i>kdpA</i>	potassium-transporting ATPase subunit A	4.92	1.31E-10
SAUSA300_2338	<i>nreB</i>	sensor histidine kinase NreB	5.81	2.14E-153
SAUSA300_2337	<i>nreC</i>	Oxygen regulatory protein NreC	4.89	8.58E-110
SAUSA300_2033	<i>kdpB</i>	potassium-transporting ATPase subunit B	2.75	3.62E-09
Stress response				
SAUSA300_0536	<i>hchA</i>	chaperone protein HchA	3.24	5.61E-111
SAUSA300_0067	-	universal stress protein	3.00	9.29E-95
SAUSA300_2142	<i>asp23</i>	alkaline shock protein 23	2.99	1.25E-94
SAUSA300_1983	<i>groES</i>	co-chaperonin GroES	2.35	1.37E-11
SAUSA300_0786	-	OsmC/Ohr family protein	2.11	3.77E-25
SAUSA300_0490	<i>hslO</i>	Hsp33-like chaperonin	0.43	3.06E-29
Viability				
SAUSA300_0949	<i>sspC</i>	staphostatin B, cysteine protease inhibitor	4.26	7.06E-36

Table 8 Differentially expressed uncharacterised genes in IQS11-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_2311	-	hypothetical protein	521.81	0.00E+00
SAUSA300_0409	-	hypothetical protein	11.02	4.16E-273
SAUSA300_0693	-	hypothetical protein	10.86	2.85E-190
SAUSA300_1964	-	hypothetical protein	10.78	3.96E-22
SAUSA300_RS10795	-	hypothetical protein	7.09	4.42E-19
SAUSA300_2321	-	hypothetical protein	6.35	1.38E-67
SAUSA300_2339	-	hypothetical protein	5.88	5.03E-149
SAUSA300_1581	-	hypothetical protein	5.31	2.18E-217
SAUSA300_RS03680	-	hypothetical protein	5.22	1.62E-03
SAUSA300_RS10770	-	hypothetical protein	5.20	9.04E-05
SAUSA300_RS10655	-	hypothetical protein	5.11	2.33E-04
SAUSA300_1582	-	hypothetical protein	4.88	1.33E-169
SAUSA300_2592	-	hypothetical protein	4.84	2.45E-134
SAUSA300_2418	-	hypothetical protein	4.72	2.51E-157
SAUSA300_1863	-	hypothetical protein	4.61	2.61E-131
SAUSA300_RS10610	-	hypothetical protein	4.57	1.00E-03
SAUSA300_RS05270	-	hypothetical protein	4.56	7.47E-87
SAUSA300_0374	-	hypothetical protein	4.45	3.49E-185
SAUSA300_0805	-	hypothetical protein	4.45	1.99E-07
SAUSA300_0816	-	hypothetical protein	4.45	4.67E-166
SAUSA300_RS12420	-	hypothetical protein	4.25	2.93E-81
SAUSA300_2543	-	hypothetical protein	4.25	2.39E-68
SAUSA300_0982	-	hypothetical protein	4.17	1.32E-157
SAUSA300_0372	-	hypothetical protein	4.10	7.74E-110
SAUSA300_1856	-	hypothetical protein	3.78	1.17E-102
SAUSA300_0781	-	hypothetical protein	3.62	1.84E-104
SAUSA300_2144	-	hypothetical protein	3.53	1.74E-116
SAUSA300_2517	-	amidohydrolase family protein	3.47	2.11E-54
SAUSA300_2518	-	uncharacterised hydrolase protein	3.47	3.23E-58
SAUSA300_RS05060	-	hypothetical protein	3.45	2.28E-54
SAUSA300_2493	-	hypothetical protein	3.44	1.38E-04
SAUSA300_RS10690	-	membrane protein	3.41	2.43E-02
SAUSA300_1788	-	hypothetical protein	3.39	5.61E-111
SAUSA300_2143	-	hypothetical protein	3.31	5.78E-101
SAUSA300_0749	-	hypothetical protein	3.30	8.51E-88
SAUSA300_1685	-	hypothetical protein	3.27	4.79E-119
SAUSA300_1684	-	hypothetical protein	3.23	1.79E-101
SAUSA300_1759	-	hypothetical protein	3.18	1.37E-05
SAUSA300_0668	-	hypothetical protein	3.17	3.11E-103
SAUSA300_2447	-	hypothetical protein	3.12	4.45E-89
SAUSA300_2097	-	hypothetical protein	3.10	4.37E-52
SAUSA300_0111	-	hypothetical protein	3.09	7.13E-32

Table 8 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_0808	-	hypothetical protein	3.08	7.81E-03
SAUSA300_2629	-	hypothetical protein	3.05	2.22E-27
SAUSA300_2138	-	hypothetical protein	3.03	4.64E-53
SAUSA300_0172	-	hypothetical protein	3.03	1.13E-40
SAUSA300_0753	-	hypothetical protein	3.02	3.37E-58
SAUSA300_1804	-	hypothetical protein	3.00	1.43E-96
SAUSA300_0284	-	hypothetical protein	2.98	2.69E-05
SAUSA300_1803	-	hypothetical protein	2.90	1.60E-71
SAUSA300_1440	-	hypothetical protein	2.90	1.00E-10
SAUSA300_0778	-	hypothetical protein	2.88	6.17E-16
SAUSA300_RS00820	-	hypothetical protein	2.86	9.36E-10
SAUSA300_0285	-	hypothetical protein	2.84	5.11E-05
SAUSA300_2632	-	hypothetical protein	2.81	3.63E-63
SAUSA300_2525	-	hypothetical protein	2.80	1.33E-36
SAUSA300_RS11950	-	membrane protein	2.80	6.29E-09
SAUSA300_0779	-	hypothetical protein	2.78	2.64E-15
SAUSA300_RS14095	-	membrane protein	2.77	1.70E-25
SAUSA300_2327	-	hypothetical protein	2.76	1.27E-69
SAUSA300_0806	-	hypothetical protein	2.74	9.08E-03
SAUSA300_2041	-	hypothetical protein	2.73	2.17E-72
SAUSA300_0750	-	hypothetical protein	2.66	8.76E-67
SAUSA300_0807	-	hypothetical protein	2.66	6.76E-03
SAUSA300_1606	-	hypothetical protein	2.65	4.38E-12
SAUSA300_0287	-	hypothetical protein	2.65	9.16E-05
SAUSA300_0604	-	alpha/beta fold family hydrolase	2.63	6.72E-36
SAUSA300_1698	-	hypothetical protein	2.62	1.70E-71
SAUSA300_2315	-	hypothetical protein	2.62	7.76E-72
SAUSA300_2093	-	hypothetical protein	2.60	5.00E-16
SAUSA300_1898	-	hypothetical protein	2.51	5.31E-18
SAUSA300_2626	-	hypothetical protein	2.50	1.88E-43
SAUSA300_2514	-	hypothetical protein	2.50	4.39E-05
SAUSA300_2513	-	hypothetical protein	2.50	6.85E-11
SAUSA300_2137	-	hypothetical protein	2.45	3.01E-22
SAUSA300_2056	-	hypothetical protein	2.41	3.35E-25
SAUSA300_1864	-	putative membrane protein	2.39	1.41E-56
SAUSA300_0754	-	hypothetical protein	2.38	8.23E-46
SAUSA300_2415	-	hypothetical protein	2.37	1.47E-24
SAUSA300_0385	-	hypothetical protein	2.35	2.18E-57
SAUSA300_0664	-	hypothetical protein	2.34	1.93E-41
SAUSA300_0810	-	hypothetical protein	2.30	8.47E-04
SAUSA300_2376	-	hypothetical protein	2.28	3.73E-15

Table 8 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_0329	-	hypothetical protein	2.28	1.14E-24
SAUSA300_0286	-	hypothetical protein	2.25	1.15E-05
SAUSA300_1552	-	hypothetical protein	2.24	1.17E-22
SAUSA300_RS13415	-	hypothetical protein	2.22	4.74E-23
SAUSA300_1904	-	hypothetical protein	2.20	1.94E-34
SAUSA300_1211	-	hypothetical protein	2.11	2.56E-07
SAUSA300_1230	-	hypothetical protein	2.11	3.92E-14
SAUSA300_RS05995	-	hypothetical protein	2.10	5.93E-41
SAUSA300_RS10500	-	MAP domain-containing protein	2.10	8.49E-14
SAUSA300_2593	-	hypothetical protein	2.09	2.20E-09
SAUSA300_2380	-	hypothetical protein	2.08	9.58E-06
SAUSA300_0795	-	hypothetical protein	2.06	2.56E-09
SAUSA300_RS07120	-	hypothetical protein	2.03	1.15E-03
SAUSA300_1716	-	hypothetical protein	2.03	9.39E-12
SAUSA300_1003	-	hypothetical protein	2.02	4.08E-37
SAUSA300_RS14210	-	hypothetical protein	0.50	2.91E-02
SAUSA300_1199	-	hypothetical protein	0.50	2.94E-11
SAUSA300_1744	-	hypothetical protein	0.48	3.01E-05
SAUSA300_RS01390	-	hypothetical protein	0.48	4.06E-02
SAUSA300_0192	-	hypothetical protein	0.47	6.15E-23
SAUSA300_2330	-	hypothetical protein	0.46	3.86E-09
SAUSA300_2133	-	membrane protein	0.44	7.03E-07
SAUSA300_0413	-	hypothetical protein	0.42	8.45E-03
SAUSA300_2425	-	hypothetical protein	0.42	2.92E-02
SAUSA300_1060	-	hypothetical protein	0.41	5.83E-03
SAUSA300_0723	-	hypothetical protein	0.41	3.38E-04
SAUSA300_0940	-	hypothetical protein	0.39	8.50E-05
SAUSA300_0133	-	hypothetical protein	0.38	3.41E-02
SAUSA300_0169	-	hypothetical protein	0.37	8.72E-07
SAUSA300_RS02410	-	hypothetical protein	0.35	1.93E-06
SAUSA300_RS01165	-	hypothetical protein	0.35	1.75E-02
SAUSA300_0934	-	membrane protein	0.35	1.19E-04
SAUSA300_1797	-	hypothetical protein	0.34	3.35E-25
SAUSA300_2619	-	hypothetical protein	0.30	7.17E-10
SAUSA300_2618	-	hypothetical protein	0.29	2.06E-06
SAUSA300_1334	-	hypothetical protein	0.28	2.25E-29
SAUSA300_RS13785	-	hypothetical protein	0.24	2.06E-27
SAUSA300_0953	-	hypothetical protein	0.24	4.35E-19
SAUSA300_RS07275	-	hypothetical protein	0.23	8.50E-03
SAUSA300_RS13775	-	hypothetical protein	0.22	5.88E-23
SAUSA300_RS06715	-	hypothetical protein	0.21	3.97E-09
SAUSA300_RS13780	-	hypothetical protein	0.20	8.75E-12
SAUSA300_2507	-	hypothetical protein	0.16	2.95E-12

Table 9. Differentially expressed metabolic genes in IQS3-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_1714	<i>ribE</i>	riboflavin synthase subunit alpha	5.30	7.33E-163
SAUSA300_1713	<i>ribBA</i>	riboflavin biosynthesis protein	4.92	2.71E-197
SAUSA300_1715	<i>ribD</i>	riboflavin biosynthesis protein	4.29	2.5E-170
SAUSA300_1712	<i>ribH</i>	6,7-dimethyl-8-ribityllumazine synthase	4.11	7.82E-102
SAUSA300_0756	<i>gapA</i>	glyceraldehyde-3-phosphate dehydrogenase, type I (GAPDH)	3.64	6.288E-36
SAUSA300_0757	<i>pgk</i>	phosphoglycerate kinase	3.47	2.05E-25
SAUSA300_0758	<i>tpiA</i>	triosephosphate isomerase	3.38	6.917E-23
SAUSA300_0759	<i>gpmI</i>	phosphoglyceromutase	3.37	2.02E-23
SAUSA300_2240	<i>ureC</i>	urease subunit alpha	3.17	2.46E-92
SAUSA300_0538	-	NAD-dependent epimerase/dehydratase family protein	3.17	1.14E-197
SAUSA300_2242	<i>ureF</i>	urease accessory protein UreF	2.89	7.16E-33
SAUSA300_2516	-	short chain dehydrogenase/reductase family oxidoreductase	2.89	5.63E-20
SAUSA300_2244	<i>ureD</i>	urease accessory protein UreD	2.78	9.58E-49
SAUSA300_2241	<i>ureE</i>	urease accessory protein UreE	2.66	2.10E-23
SAUSA300_2238	<i>ureA</i>	urease subunit gamma	2.58	1.05E-12
SAUSA300_0684	<i>fruB</i>	fructose 1-phosphate kinase	2.48	5.16E-09
SAUSA300_2239	<i>ureB</i>	urease subunit beta	2.46	2.20E-16
SAUSA300_2243	<i>ureG</i>	urease accessory protein UreG	2.35	4.01E-30
SAUSA300_0966	<i>purE</i>	phosphoribosylaminoimidazole carboxylase, catalytic subunit	2.34	2.66E-18
SAUSA300_0227	<i>fadD</i>	acyl-CoA dehydrogenase FadD	2.20	1.76E-34
SAUSA300_0967	<i>purK</i>	phosphoribosylaminoimidazole carboxylase ATPase subunit	2.18	4.19E-27
SAUSA300_2612	<i>hisG</i>	ATP phosphoribosyltransferase catalytic subunit	2.14	2.36E-03
SAUSA300_0225	-	putative acyl-CoA acetyltransferase FadA	2.12	4.51E-12
SAUSA300_0228	<i>fadE</i>	acyl-CoA synthetase FadE	2.11	4.25E-36
SAUSA300_0226	-	3-hydroxyacyl-CoA dehydrogenase	2.09	3.32E-20
SAUSA300_0055	-	alcohol dehydrogenase, zinc-containing	2.05	8.54E-56
SAUSA300_2610	<i>hisC</i>	histidinol-phosphate aminotransferase hisC	2.02	1.48E-03
SAUSA300_0968	<i>purC</i>	phosphoribosylaminoimidazole-succinocarboxamide synthase	2.02	1.50E-16
SAUSA300_0260	<i>bglA</i>	6-phospho-beta-glucosidase	0.49	1.65E-08
SAUSA300_0987	-	cytochrome D ubiquinol oxidase, subunit II	0.45	4.01E-17
SAUSA300_0986	-	cytochrome D ubiquinol oxidase, subunit I	0.37	1.45E-33
SAUSA300_1225	-	aspartate kinase	0.31	9.36E-23

Table 10. Differentially expressed virulence factor genes in IQS3-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_1055	<i>efb</i>	fibrinogen-binding protein	4.32	7.945E-67
SAUSA300_1052	-	fibrinogen-binding protein	4.14	4.30E-73
SAUSA300_1056	-	staphylococcal complement inhibitor	4.13	4.294E-45
SAUSA300_2366	<i>hlgC</i>	gamma-haemolysin component C	3.75	9.30E-29
SAUSA300_2364	<i>sbi</i>	IgG-binding protein SBI	3.16	3.853E-44
SAUSA300_2367	<i>hlgB</i>	gamma-haemolysin component B	2.53	4.75E-16
SAUSA300_1920	<i>chs</i>	chemotaxis-inhibiting protein CHIPS	2.48	1.314E-05
SAUSA300_1918	-	truncated beta-haemolysin	2.36	4.52E-05
SAUSA300_2365	<i>hlgA</i>	gamma-haemolysin component A	2.20	3.68E-16
SAUSA300_0113	<i>spa</i>	immunoglobulin G binding protein A	2.12	3.07E-30
SAUSA300_1754	<i>splE</i>	serine protease SplE	0.45	6.94E-05
SAUSA300_1753	<i>splF</i>	serine protease SplF	0.39	8.78E-08

Table 11. Differentially expressed genetic information processing genes in IQS3-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_RS08965	-	transposase	2.91	9.37E-06
SAUSA300_2242	<i>ureF</i>	urease accessory protein UreF	2.89	7.16E-33
SAUSA300_0034	-	IS1272, transposase	2.66	2.13E-05
SAUSA300_0038	<i>ccrA</i>	cassette chromosome recombinase A	2.63	6.63E-06
SAUSA300_RS09450	-	transposase	2.31	1.49E-03
SAUSA300_RS11800	-	transposase	2.16	3.66E-03
SAUSA300_2263	-	putative transposase	2.10	2.10E-05
SAUSA300_1133	<i>trmD</i>	tRNA (guanine-N(1)-)-methyltransferase	0.50	1.24E-11
SAUSA300_2192	<i>rplE</i>	50S ribosomal protein L5	0.50	5.95E-37
SAUSA300_2202	<i>rplW</i>	50S ribosomal protein L23	0.48	2.58E-31
SAUSA300_2194	<i>rplN</i>	50S ribosomal protein L14	0.48	4.77E-31
SAUSA300_2195	<i>rpsQ</i>	30S ribosomal protein S17	0.48	1.26E-27
SAUSA300_2199	<i>rplV</i>	50S ribosomal protein L22	0.48	1.61E-44
SAUSA300_2193	<i>rplX</i>	50S ribosomal protein L24	0.48	2.09E-43
SAUSA300_0524	<i>rplJ</i>	50S ribosomal protein L10	0.47	2.65E-64
SAUSA300_2196	<i>rpmC</i>	50S ribosomal protein L29	0.47	9.50E-21
SAUSA300_2197	<i>rplP</i>	50S ribosomal protein L16	0.46	4.04E-45
SAUSA300_2201	<i>rplB</i>	50S ribosomal protein L2	0.45	3.88E-43
SAUSA300_2198	<i>rpsC</i>	30S ribosomal protein S3	0.45	6.20E-55
SAUSA300_2205	<i>rpsJ</i>	30S ribosomal protein S10	0.45	4.57E-74
SAUSA300_2203	<i>rplD</i>	50S ribosomal protein L4	0.44	5.81E-54
SAUSA300_2200	<i>rpsS</i>	30S ribosomal protein S19	0.44	4.20E-45
SAUSA300_2204	<i>rplC</i>	50S ribosomal protein L3	0.43	2.69E-66
SAUSA300_2037	-	ATP-dependent RNA helicase	0.42	1.97E-109
SAUSA300_1666	<i>rpsD</i>	30S ribosomal protein S4	0.40	2.57E-168
SAUSA300_1629	<i>thrS</i>	threonyl-tRNA synthetase	0.34	2.37E-58
SAUSA300_2052	-	single-stranded DNA- binding protein family	0.33	5.21E-31

Table 12. Differentially expressed genes in IQS3-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
Antimicrobial				
SAUSA300_RS09675	-	lantibiotic epidermin	2.48	4.84E-11
SAUSA300_1767	<i>epiA</i>	lantibiotic epidermin biosynthesis protein EpiA	2.15	7.30E-16
SAUSA300_1067	-	antibacterial protein	0.32	2.12E-105
SAUSA300_1068	-	antibacterial protein	0.29	8.18E-111
Drug resistance				
SAUSA300_2299	-	multidrug resistance protein A, drug resistance transporter	0.50	4.22E-16
SAUSA300_2298	-	multidrug resistance protein B, drug resistance transporter	0.46	8.43E-26
Secretion system				
SAUSA300_0928	<i>comK</i>	competence transcription factor	0.46	2.45E-14
Transcription regulator				
SAUSA300_0755	<i>gapR</i>	glycolytic operon regulator, GapR	3.19	8.25E-174
SAUSA300_2515	<i>tetR</i>	TetR family transcriptional regulator	2.81	6.98E-17
SAUSA300_2639	<i>cspB</i>	cold shock protein, CspB	0.47	5.17E-63
Transporter				
SAUSA300_2465	-	ABC transporter ATP-binding protein	2.37	1.56E-05
SAUSA300_2393	<i>opuCa</i>	glycine betaine/carnitine/choline ABC transporter ATP-binding protein	2.11	2.15E-20
SAUSA300_2391	<i>opuCc</i>	glycine betaine/carnitine/choline ABC transporter	2.03	4.38E-20
SAUSA300_1009	<i>typA</i>	GTP-binding protein	0.48	3.92E-70
SAUSA300_2549	<i>bccT</i>	BCCT family choline/carnitine/betaine transporter	0.46	6.96E-54

Table 13. Differentially expressed uncharacterised genes in IQS3-treated LAC

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_RS02980	-	membrane protein	4.75	4.98E-11
SAUSA300_0693	-	hypothetical protein	3.83	9.33E-53
SAUSA300_RS13590	-	hypothetical protein	3.42	4.30E-07
SAUSA300_RS03440	-	hypothetical protein	3.28	2.38E-07
SAUSA300_RS00405	-	hypothetical protein	3.12	6.99E-07
SAUSA300_RS08945	-	hypothetical protein	2.99	4.26E-06
SAUSA300_RS06795	-	hypothetical protein	2.82	3.19E-05
SAUSA300_RS06140	-	hypothetical protein	2.68	1.44E-04
SAUSA300_RS12190	-	hypothetical protein	2.66	5.31E-05
SAUSA300_RS03385	-	hypothetical protein	2.62	2.09E-04
SAUSA300_RS13065	-	hypothetical protein	2.57	2.79E-04
SAUSA300_RS14250	-	hypothetical protein	2.56	4.71E-05
SAUSA300_RS10515	-	hypothetical protein	2.47	5.14E-05
SAUSA300_RS14480	-	hypothetical protein	2.45	3.43E-04
SAUSA300_RS07065	-	hypothetical protein	2.45	5.59E-04
SAUSA300_RS02735	-	hypothetical protein	2.45	5.85E-04
SAUSA300_RS04035	-	hypothetical protein	2.40	6.49E-04
SAUSA300_RS10110	-	hypothetical protein	2.37	2.71E-04
SAUSA300_0409	-	hypothetical protein	2.37	1.13E-40
SAUSA300_RS14080	-	hypothetical protein	2.33	1.28E-03
SAUSA300_0929	-	hypothetical protein	2.31	4.01E-30
SAUSA300_RS14220	-	hypothetical protein	2.31	9.75E-04
SAUSA300_RS03680	-	hypothetical protein	2.30	1.36E-03
SAUSA300_2493	-	hypothetical protein	2.29	2.33E-10
SAUSA300_RS09120	-	hypothetical protein	2.25	2.07E-03
SAUSA300_RS02470	-	hypothetical protein	2.24	2.20E-03
SAUSA300_RS12625	-	hypothetical protein	2.23	1.77E-03
SAUSA300_RS10405	-	hypothetical protein	2.22	2.52E-03
SAUSA300_0232	-	hypothetical protein	2.20	5.83E-16
SAUSA300_RS05900	-	hypothetical protein	2.20	1.37E-03
SAUSA300_RS09095	-	hypothetical protein	2.20	2.06E-03
SAUSA300_2512	-	glyoxalase family protein	2.19	1.83E-09
SAUSA300_RS11510	-	hypothetical protein	2.19	3.03E-03
SAUSA300_RS11885	-	hypothetical protein	2.19	3.07E-03
SAUSA300_RS01000	-	hypothetical protein	2.16	3.27E-03
SAUSA300_RS01880	-	hypothetical protein	2.15	3.15E-03
SAUSA300_1663	-	hypothetical protein	2.14	4.12E-03
SAUSA300_RS07040	-	hypothetical protein	2.13	4.30E-03

Table 13 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_RS13420	-	hypothetical protein	2.13	4.36E-03
SAUSA300_RS07155	-	hypothetical protein	2.12	3.68E-03
SAUSA300_RS12395	-	hypothetical protein	2.12	4.55E-03
SAUSA300_1106	-	putative lipoprotein	2.11	4.66E-31
SAUSA300_RS05700	-	hypothetical protein	2.10	2.04E-03
SAUSA300_2513	-	hypothetical protein	2.07	2.19E-13
SAUSA300_RS02040	-	hypothetical protein	2.06	1.54E-03
SAUSA300_1334	-	hypothetical protein	0.49	3.86E-18
SAUSA300_2414	-	hypothetical protein	0.48	8.50E-04

Table 14. Differentially expressed metabolic genes in LAC Δagr

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_0108	-	myosin-cross-reactive antigen, oleate hydratase	4.09	2.449E-65
SAUSA300_1712	<i>ribH</i>	6,7-dimethyl-8-ribityllumazine synthase	2.79	5.18E-21
SAUSA300_0557	-	HAD family hydrolase, subfamily IA, variant 1	2.75	3.514E-17
SAUSA300_0604	-	alpha/beta fold family hydrolase	2.49	1.39E-66
SAUSA300_2462	<i>frp</i>	NAD(P)H-flavin oxidoreductase	2.44	1.662E-55
SAUSA300_0692	-	thiosulfate dehydrogenase	2.28	4.24E-15
SAUSA300_1713	<i>ribBA</i>	riboflavin biosynthesis protein	2.17	3.641E-12
SAUSA300_2613	<i>hisZ</i>	ATP phosphoribosyltransferase regulatory subunit	2.16	9.68E-03
SAUSA300_2356	<i>fmhA</i>	fmhA protein	2.15	5.87E-12
SAUSA300_1498	<i>gcvT</i>	aminomethyltransferase T	2.15	3.50E-11
SAUSA300_0862	<i>glpQ</i>	glycerophosphoryl diester phosphodiesterase	2.13	9.16E-08
SAUSA300_0315	<i>nanA</i>	N-acetylneuraminate lyase	2.12	6.58E-13
SAUSA300_0138	<i>deoD</i>	purine nucleoside phosphorylase	2.11	7.18E-04
SAUSA300_0538	-	NAD-dependent epimerase/dehydratase family protein	2.10	3.45E-03
SAUSA300_0318	-	N-acetylmannosamine-6-phosphate 2-epimerase	2.04	7.07E-13
SAUSA300_0504	-	pyridoxal biosynthesis lyase PdxS	2.01	6.05E-12
SAUSA300_1714	<i>ribE</i>	riboflavin synthase subunit alpha	2.01	1.71E-10
SAUSA300_2242	<i>ureF</i>	urease accessory protein UreF	0.50	6.36E-07
SAUSA300_2012	<i>leuC</i>	isopropylmalate isomerase large subunit	0.50	3.18E-10
SAUSA300_1896	<i>pheA</i>	prephenate dehydratase	0.49	5.76E-25
SAUSA300_2166	<i>alsS</i>	acetolactate synthase	0.49	8.68E-16
SAUSA300_2161	<i>hysA</i>	hyaluronate lyase	0.48	1.78E-08
SAUSA300_1895	-	nitric oxide synthase oxygenase	0.48	1.94E-24
SAUSA300_2341	<i>narJ</i>	respiratory nitrate reductase, subunit delta	0.47	4.17E-03
SAUSA300_0262	<i>rbsK</i>	ribokinase	0.46	5.80E-28
SAUSA300_0446	<i>gltD</i>	glutamate synthase subunit beta	0.46	1.93E-25
SAUSA300_2545	<i>betA</i>	choline dehydrogenase	0.44	1.94E-15
SAUSA300_2008	<i>ilvN</i>	acetolactate synthase 1 regulatory subunit	0.44	5.94E-06
SAUSA300_2007	<i>ilvB</i>	acetolactate synthase large subunit	0.43	1.33E-28
SAUSA300_2165	<i>budA</i>	alpha-acetolactate decarboxylase	0.43	3.58E-18
SAUSA300_2011	<i>leuB</i>	3-isopropylmalate dehydrogenase	0.43	1.43E-15
SAUSA300_0179	-	formate dehydrogenase	0.43	1.90E-07
SAUSA300_2009	<i>ilvC</i>	ketol-acid reductoisomerase	0.43	1.38E-24
SAUSA300_2010	<i>leuA</i>	2-isopropylmalate synthase	0.42	9.75E-24
SAUSA300_2006	<i>ilvD</i>	dihydroxy-acid dehydratase	0.42	2.39E-35
SAUSA300_0783	-	phosphoglycerate mutase family protein	0.42	1.41E-22
SAUSA300_0424	-	putative cobalamin synthesis protein	0.38	3.09E-08

Table 14 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_0182	-	4'-phosphopantetheinyl transferase superfamily protein	0.37	3.56E-27
SAUSA300_2469	<i>sdaAA</i>	L-serine dehydratase, iron-sulfur-dependent, alpha subunit	0.33	1.91E-59
SAUSA300_2238	<i>ureA</i>	urease subunit gamma	0.32	7.11E-07
SAUSA300_2240	<i>ureC</i>	urease subunit alpha	0.32	1.95E-37
SAUSA300_0756	<i>gapA</i>	glyceraldehyde-3-phosphate dehydrogenase, type I (GAPDH)	0.32	1.46E-46
SAUSA300_0757	<i>pgk</i>	phosphoglycerate kinase	0.30	1.81E-31
SAUSA300_2239	<i>ureB</i>	urease subunit beta	0.29	8.71E-13
SAUSA300_0758	<i>tpiA</i>	triosephosphate isomerase	0.28	3.44E-54
SAUSA300_0759	<i>gpmI</i>	phosphoglyceromutase	0.26	1.43E-52
SAUSA300_2470	<i>sdaAB</i>	L-serine dehydratase, iron-sulfur-dependent, beta subunit	0.23	7.11E-66
SAUSA300_0449	<i>treC</i>	alpha,alpha-phosphotrehalase	0.22	5.58E-75
SAUSA300_2603	<i>lip</i>	triacylglycerol lipase	0.21	1.03E-258
SAUSA300_0193	<i>murQ</i>	N-acetylmuramic acid-6-phosphate etherase	0.20	7.54E-132
SAUSA300_0235	-	L-lactate dehydrogenase	0.18	4.03E-228
SAUSA300_2443	<i>gntK</i>	gluconate kinase	0.15	6.24E-97
SAUSA300_0099	<i>plc</i>	1-phosphatidylinositol phosphodiesterase	0.10	1.89E-74
SAUSA300_0151	<i>adhE</i>	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	0.09	6.76E-113
SAUSA300_0320	-	triacylglycerol lipase	0.05	6.27E-68

Table 15 Differentially expressed virulence factor genes in LAC Δagr

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_2364	<i>sbi</i>	IgG-binding protein SBI	11.50	1.26E-97
SAUSA300_0113	<i>spa</i>	immunoglobulin G binding protein A	11.03	0.00E+00
SAUSA300_1055	<i>efb</i>	fibrinogen-binding protein	6.78	2.28E-111
SAUSA300_1056	-	staphylococcal complement inhibitor	6.30	2.79E-52
SAUSA300_1052	-	fibrinogen-binding protein	6.02	4.46E-102
SAUSA300_0773	-	putative staphylocoagulase	5.54	1.21E-24
SAUSA300_2365	<i>hlqA</i>	gamma-hemolysin component A	4.59	3.799E-34
SAUSA300_1920	<i>chs</i>	chemotaxis-inhibiting protein CHIPS	2.74	3.54E-06
SAUSA300_0223	-	staphylococcal complement inhibitor	2.70	2.61E-26
SAUSA300_0224	<i>coa</i>	staphylocoagulase	2.59	1.60E-06
SAUSA300_2440	<i>fnbB</i>	fibronectin binding protein B	2.49	3.35E-15
SAUSA300_0546	<i>sdrC</i>	SdrC protein, serine-aspartate repeat-containing protein C/D/E	2.12	1.37E-32
SAUSA300_0547	<i>sdrD</i>	SdrD protein, serine-aspartate repeat-containing protein C/D/E	2.10	2.22E-48
SAUSA300_2573	<i>isaB</i>	immunodominant antigen B	2.03	3.48E-22
SAUSA300_1922	<i>sak</i>	staphylokinase	0.47	4.03E-07
SAUSA300_1058	<i>hla</i>	alpha-haemolysin	0.40	3.34E-63
SAUSA300_0278	<i>esxA</i>	Virulence factor EsxA	0.39	1.05E-67
SAUSA300_1382	<i>lukS-PV</i>	Panton-Valentine leukocidin, LukS-PV	0.35	6.22E-29
SAUSA300_1758	<i>splA</i>	serine protease SplA	0.32	1.29E-07
SAUSA300_1757	<i>splB</i>	serine protease SplB	0.30	5.90E-08
SAUSA300_0950	<i>sspB</i>	staphopain B	0.27	7.60E-42
SAUSA300_1756	<i>splC</i>	serine protease SplC	0.22	1.97E-13
SAUSA300_0951	<i>sspA</i>	V8 protease	0.21	5.18E-54
SAUSA300_1755	<i>splD</i>	serine protease SplD	0.21	3.23E-12
SAUSA300_1890	-	staphopain A	0.18	3.67E-39
SAUSA300_2471	-	perfringolysin O	0.17	5.19E-123
SAUSA300_1753	<i>splF</i>	serine protease SplF	0.16	1.79E-19
SAUSA300_0277	-	putative staphyloxanthin biosynthesis protein	0.14	1.02E-77
SAUSA300_1754	<i>splE</i>	serine protease SplE	0.12	1.60E-17

Table 16 Differentially expressed transporter genes in LAC Δagr

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_245	-	ABC transporter ATP-binding protein	15.53	3.37E-107
SAUSA300_2454	-	membrane spanning protein	10.77	2.34E-68
SAUSA300_2307	-	ABC transporter permease	3.06	1.094E-11
SAUSA300_2306	-	ABC transporter ATP-binding protein	2.82	2.68E-08
SAUSA300_0236	-	PTS system, IIBC components	2.62	2.415E-17
SAUSA300_1283	<i>pstS</i>	phosphate ABC transporter phosphate-binding protein PstS	2.48	4.66E-05
SAUSA300_2291	<i>gltS</i>	sodium/glutamate symporter	2.48	8.292E-16
SAUSA300_0257	-	antiholin-like protein LrgB	2.32	8.60E-27
SAUSA300_2270	<i>glvC</i>	PTS system, arbutin-like IIBC component	2.28	2.87E-67
SAUSA300_0256	-	murein hydrolase regulator LrgA	2.09	2.22E-02
SAUSA300_2449	-	putative transporter	2.04	3.59E-16
SAUSA300_0432	-	sodium dependent transporter	0.50	3.14E-10
SAUSA300_0263	<i>rbsD</i>	D-ribose pyranase	0.50	5.19E-13
SAUSA300_2349	-	formate/nitrite transporter family protein	0.49	3.93E-21
SAUSA300_0264	-	ribose transporter RbsU	0.49	3.24E-25
SAUSA300_0144	<i>phnC</i>	phosphonate ABC transporter ATP-binding protein	0.49	7.22E-03
SAUSA300_1274	-	peptide ABC transporter ATP-binding protein	0.49	2.94E-04
SAUSA300_2395	-	amino acid permease	0.48	1.00E-17
SAUSA300_2237	-	putative urea transporter	0.44	2.73E-10
SAUSA300_0448	<i>treP</i>	PTS system, trehalose-specific IIBC component	0.41	2.34E-22
SAUSA300_2442	<i>gntP</i>	gluconate permease	0.40	5.75E-28
SAUSA300_0145	-	phosphonate ABC transporter phosphonate-binding protein	0.37	2.20E-09
SAUSA300_1275	-	peptide ABC transporter permease	0.36	2.51E-09
SAUSA300_0142	<i>phnE</i>	phosphonate ABC transporter permease	0.35	3.76E-05
SAUSA300_0231	-	ABC transporter substrate-binding protein	0.32	3.04E-49
SAUSA300_1005	-	manganese transport protein MntH	0.31	4.47E-69
SAUSA300_1276	<i>opp-2B</i>	oligopeptide permease, channel-forming protein	0.29	2.29E-16
SAUSA300_2487	<i>feoB</i>	ferrous iron transport protein B	0.28	1.96E-25
SAUSA300_2488	<i>feoA</i>	ferrous iron transport protein A	0.28	3.88E-06
SAUSA300_0382	-	sodium:dicarboxylate symporter family protein	0.25	2.13E-43
SAUSA300_0194	-	sucrose-specific PTS transporter protein	0.21	1.27E-146

Table 17 Differentially expressed genes in LAC Δagr

Gene tag	Gene	Encoded protein	Fold change	Padj
Antimicrobial				
SAUSA300_1766	<i>epiB</i>	lantibiotic epidermin biosynthesis protein EpiB	0.45	1.13E-05
SAUSA300_1068	-	antibacterial protein	3.84E-03	4.49E-240
SAUSA300_1067	-	antibacterial protein	2.73E-03	4.14E-252
Capsular biosynthesis				
SAUSA300_0156	<i>cap5E</i>	capsular polysaccharide biosynthesis protein Cap5E	0.38	2.03E-04
SAUSA300_0154	<i>cap5C</i>	capsular polysaccharide biosynthesis protein Cap5C	0.35	9.915E-05
SAUSA300_0153	<i>cap5B</i>	capsular polysaccharide biosynthesis protein Cap5B	0.33	2.41E-05
SAUSA300_0152	<i>cap5A</i>	capsular polysaccharide biosynthesis protein Cap5A	0.31	6.00E-05
Cell cycle				
SAUSA300_1140	<i>lytN</i>	cell wall hydrolase	0.37	2.67E-15
Drug resistance				
SAUSA300_0630	-	ABC transporter ATP-binding protein	0.46	2.55E-56
SAUSA300_0033	-	methicillin-resistance MecR1 regulatory protein	0.40	1.74E-68
SAUSA300_0180	-	integral membrane protein LmrP	0.30	7.28E-61
SAUSA300_0032	<i>mecA</i>	penicillin-binding protein 2'	0.26	5.08E-115
Genetic information processing				
SAUSA300_0026	-	rRNA large subunit methyltransferase	2.04	3.63E-18
SAUSA300_1503	-	putative competence protein ComGB	0.48	9.64E-03
SAUSA300_1629	<i>thrS</i>	threonyl-tRNA synthetase	0.44	2.82E-14
SAUSA300_RS01575	<i>queA</i>	QueA protein, S-adenosylmethionine:tRNA ribosyltransferase-isomerase	0.43	3.51E-05
SAUSA300_RS09410	<i>comK</i>	competence transcription factor ComK1	0.40	8.15E-07
SAUSA300_1504	-	putative competence protein ComGA	0.31	2.15E-08
SAUSA300_0928	<i>comK</i>	competence transcription factor ComK2	0.07	1.59E-71
SAUSA300_2052	-	single-stranded DNA- binding protein family	0.04	1.18E-164
Phage protein				
SAUSA300_1962	-	phiPVL ORF39-like protein	2.08	1.53E-02
Quorum sensing				
SAUSA300_1990	<i>agrD</i>	accessory gene regulator protein D	3.22E-03	6.24E-146
SAUSA300_1989	<i>agrB</i>	accessory gene regulator protein B	3.14E-04	0.00E+00
SAUSA300_1991	<i>agrC</i>	accessory gene regulator protein C	2.03E-04	0.00E+00
SAUSA300_1992	<i>agrA</i>	accessory gene regulator protein A	1.88E-04	0.00E+00

Table 17 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
Secretion system				
SAUSA300_2587	-	accessory secretory protein Asp1	0.46	2.00E-13
SAUSA300_2184	<i>secY</i>	preprotein translocase subunit SecY	0.34	1.18E-16
SAUSA300_0283	<i>essC</i>	EssC protein	0.25	4.20E-51
SAUSA300_0282	<i>essB</i>	EssB protein	0.18	1.55E-18
SAUSA300_0279	<i>esaA</i>	EsaA protein	0.15	2.11E-136
Signal transduction				
SAUSA300_2036	<i>kdpE</i>	DNA-binding response regulator, KdpE	0.41	3.33E-06
SAUSA300_2034	<i>kdpA</i>	potassium-transporting ATPase subunit A	0.39	6.08E-04
SAUSA300_2035	<i>kdpD</i>	sensor histidine kinase, KdpD	0.16	8.58E-101
Stress response				
SAUSA300_0135	-	Fe/Mn family superoxide dismutase	2.07	8.59E-32
Transcription regulator				
SAUSA300_0954	-	MarR family transcriptional regulator	2.49	2.73E-16
SAUSA300_0114	<i>sarA</i>	staphylococcal accessory regulator SarA	2.01	2.28E-35
SAUSA300_2245	<i>sarR</i>	staphylococcal accessory regulator R	0.44	1.20E-30
SAUSA300_2098	<i>arsR</i>	ArsR family transcriptional regulator, transcriptional repressor	0.38	7.14E-06
SAUSA300_0450	<i>treR</i>	trehalose operon repressor	0.29	7.62E-56
SAUSA300_0195	<i>rpiRB</i>	RpiR transcriptional regulator	0.25	4.46E-76
SAUSA300_2444	<i>gntR</i>	gluconate operon transcriptional repressor	0.15	9.06E-67
Viability				
SAUSA300_0949	<i>sspC</i>	staphostatin B, cysteine protease inhibitor	0.28	1.57E-16

Table 18 Differentially expressed uncharacterised genes in LAC Δagr

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_0693	-	hypothetical protein	4.34	2.92E-56
SAUSA300_0136	-	cell wall surface anchor family protein	3.09	2.08E-107
SAUSA300_2614	-	hypothetical protein	2.76	6.93E-35
SAUSA300_0793	-	hypothetical protein	2.19	2.13E-25
SAUSA300_0775	-	hypothetical protein	2.14	5.42E-04
SAUSA300_0804	-	putative transcriptional regulator	2.11	5.57E-03
SAUSA300_1652	-	hypothetical protein	2.11	6.43E-31
SAUSA300_1054	-	hypothetical protein	2.10	7.92E-10
SAUSA300_RS04285	-	hypothetical protein	2.07	1.96E-12
SAUSA300_RS07275	-	hypothetical protein	0.49	2.90E-05
SAUSA300_0285	-	hypothetical protein	0.49	8.79E-03
SAUSA300_2482	-	hypothetical protein	0.48	5.85E-05
SAUSA300_0421	-	hypothetical protein	0.47	8.13E-11
SAUSA300_1722	-	hypothetical protein	0.47	1.28E-06
SAUSA300_1724	-	hypothetical protein	0.46	2.98E-07
SAUSA300_RS03135	-	hypothetical protein	0.45	2.34E-03
SAUSA300_0415	-	tandem lipoprotein	0.45	1.98E-03
SAUSA300_0287	-	hypothetical protein	0.45	2.41E-03
SAUSA300_RS04215	-	membrane protein	0.44	8.43E-12
SAUSA300_0205	-	tandem lipoprotein	0.44	8.29E-16
SAUSA300_1106	-	putative lipoprotein	0.44	1.60E-17
SAUSA300_2100	-	lytic regulatory protein	0.43	1.69E-59
SAUSA300_RS13775	-	hypothetical protein	0.43	2.20E-11
SAUSA300_0035	-	uncharacterised protein	0.42	4.07E-27
SAUSA300_0422	-	hypothetical protein	0.42	1.88E-16
SAUSA300_1248	-	hypothetical protein	0.40	2.00E-31
SAUSA300_0294	-	hypothetical protein	0.40	1.08E-04
SAUSA300_0883	-	putative surface protein	0.40	1.03E-41
SAUSA300_RS13780	-	hypothetical protein	0.40	3.10E-08
SAUSA300_0127	-	hypothetical protein	0.38	1.67E-10
SAUSA300_0178	-	hypothetical protein	0.38	7.77E-49
SAUSA300_RS13785	-	hypothetical protein	0.38	2.26E-27
SAUSA300_RS05035	-	membrane protein	0.38	1.08E-12
SAUSA300_0769	-	hypothetical protein	0.37	2.72E-38
SAUSA300_0304	-	hypothetical protein	0.37	5.29E-52
SAUSA300_0183	-	hypothetical protein	0.36	5.53E-33
SAUSA300_0940	-	hypothetical protein	0.35	4.89E-09
SAUSA300_0286	-	hypothetical protein	0.32	2.27E-08
SAUSA300_RS00820	-	hypothetical protein	0.30	1.60E-09
SAUSA300_0303	-	hypothetical protein	0.30	3.36E-41

Table 18 (continue)

Gene tag	Gene	Encoded protein	Fold change	Padj
SAUSA300_0293	-	hypothetical protein	0.30	1.42E-07
SAUSA300_2522	-	hypothetical protein	0.30	2.56E-14
SAUSA300_2523	-	hypothetical protein	0.29	3.73E-12
SAUSA300_RS01255	-	hypothetical protein	0.29	1.46E-05
SAUSA300_2051	-	hypothetical protein	0.28	2.16E-05
SAUSA300_0281	-	hypothetical protein	0.28	6.98E-08
SAUSA300_2524	-	hypothetical protein	0.27	2.54E-12
SAUSA300_1334	-	hypothetical protein	0.24	1.15E-59
SAUSA300_0192	-	hypothetical protein	0.23	3.92E-170
SAUSA300_1214	-	hypothetical protein	0.23	1.21E-26
SAUSA300_RS02275	-	hypothetical protein	0.20	6.98E-08
SAUSA300_0292	-	hypothetical protein	0.20	2.35E-39
SAUSA300_2164	-	hypothetical protein	0.20	2.60E-99
SAUSA300_0280	-	hypothetical protein	0.19	2.14E-15
SAUSA300_RS10955	-	hypothetical protein	0.10	2.62E-15
SAUSA300_2448	-	hypothetical protein	0.10	3.45E-299

