

The Role of Hfq in *S. aureus* Gene Regulation



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Abstract

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Staphylococcus aureus is an important opportunistic pathogen, capable of causing a wide range of diseases. This ability to colonise and infect a variety of different tissues is due to the number of virulence factors it can produce. These factors are tightly regulated so they are only expressed when required and allow rapid adaptation to changing environments. In other bacteria the RNA binding protein Hfq is important for growth, resistance to stresses and virulence. Hfq regulation occurs through RNA stability, processing and translation. However the role of Hfq in *S. aureus* is controversial as conflicting reports on the subject have been published. Preliminary work in our laboratory indicated a role for Hfq in the positive regulation by the DNA binding protein Fur but the mechanisms involved are unknown. Therefore the aim of this study is to identify targets for Hfq regulation and investigate how Fur is involved in this regulation.

This work demonstrates that *S. aureus* Hfq, along with Fur, has a key role in the resistance to oxidative stress and the regulation of several important virulence genes including the immune evasion factor, Eap. Hfq was found to regulate *eap* expression at the post transcriptional level. In addition, both Hfq and Fur were found to regulate *eap* expression transcriptionally, possibly through regulation of *sae*, an important virulence gene regulator. Fe-Fur was shown to directly bind the *sae* promoters suggesting direct positive regulation of *sae* by Fur. But the mechanisms involved in Hfq regulation remain unclear as Hfq did not have a major affect *eap* or *sae* mRNA stability. The regulation by Hfq and Fur shows some strain variation indicating that other factors are involved. Therefore Hfq and Fur play key roles in *S. aureus* virulence regulation. Further understanding of this complex regulatory network may reveal new targets for antimicrobial development to combat this important pathogen.

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List of Abbreviations

ADP	adenosine diphosphate
AIP	auto-inducing peptide
Amp	Ampicillin
APS	ammonium persulphate
asRNA	antisense RNA
ATP	adenosine triphosphate
BHI	brain-heart infusion (medium)
CA-MRSA	community acquired MRSA
CC	clonal complex
ChIP	chromatin immunoprecipitation
Cm	chloramphenicol
CRPMI	chelexed RPMI (medium)
CTAB	cetyl trimethyl ammonium bromide
dH ₂ O	distilled H ₂ O
dNTPs	deoxyribonucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
EMRSA	epidemic MRSA
HA-MRSA	healthcare associated MRSA
H-NS	histone-like nucleoid-associated protein
IAA	isoamyl alcohol
IgG	Immunoglobulin G
IPTG	isopropyl β -D-1-thiogalactopyranoside
Kan	kanamycin
LA	Luria Bertani agar
LB	Luria Bertani broth
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation – time-of-flight
MIC	minimum inhibitory concentration
MLST	multi locus sequence typing
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
OD	optical density
PAGE	poly-acrylamide gel electrophoresis
PAP	poly-A polymerase
PBPs	penicillin binding proteins

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNACL	Protein and Nucleic Acid Chemistry Laboratory
PSM	Phenol-soluble modulins
PVL	Panton-Valentine leukocidin toxin
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RBS	ribosomal binding site
RIP	RNA immunoprecipitation
RNAP	RNA polymerase
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute – 1640 (medium)
RT	reverse transcription
SCCmec	Staphylococcal chromosomal cassette containing <i>mecA</i>
SDS	sodium dodecyl sulphate
SMM	supplemented minimal medium (buffer)
SMMP50	supplemented minimal medium Penassay 50 (medium)
SSI	surgical site infections
ST	sequence type
TAE	Tris base, acetic acid and EDTA containing buffer
TCA cycle	tricarboxylic acid cycle (Krebs cycle)
TCR	two component regulator
TEMED	N,N,N',N' - tetramethylethylenediamine
Tet	tetracycline
TSB	trypticase soy broth (medium)
TSST-1	toxic shock syndrome toxin - 1
U	unit
UV	ultraviolet
VISA	vancomycin intermediate-resistant <i>Staphylococcus aureus</i>
VRSA	vancomycin resistant <i>Staphylococcus aureus</i>

Chapter 1 Introduction

The aim of this study was to determine the role of Hfq in *Staphylococcus aureus* gene regulation and its involvement with Fur regulation. This introduction will begin by briefly outlining the importance of studying this pathogenic bacterium before describing how virulence is regulated. The latter half of this introduction will address regulation by regulatory RNA molecules and Hfq in other bacteria and comparing this to *S. aureus*. The following two results chapters firstly describe the phenotypic differences observed in *S. aureus hfq*, *fur* and *hfq/fur* mutant strains and secondly the investigation of the mechanisms involved in Hfq and Fur regulation. Each results chapter contains a discussion evaluating the results and suggestions for future work. Finally chapter 5 discusses these results in a wider context of the *S. aureus* virulence regulon.

1.1 *Staphylococcus aureus*

Staphylococcus aureus are gram positive, cocci bacteria which grow in grape-like clusters into yellow pigmented colonies. This bacterium, like many other staphylococci species, is a commensal of the skin and mucosal surfaces of healthy humans and animals. Approximately 20-30% of the population is persistently colonised with *S. aureus* and 50% intermittently colonised (Plata et al., 2009). Although *S. aureus* is a commensal it is also an opportunistic pathogen, causing infection when allowed to breach the skin or mucosal surface. Once inside the body it can infect a wide variety of tissues and cause a range of infections. These can be simple skin infections like boils or impetigo, to more deeply penetrating infections like endocarditis and pneumonia (Wertheim et al., 2005; Gordon & Lowy, 2008). More serious systemic infections tend to occur in those who are immunocompromised and without treatment infections can become life threatening. β -Lactam antibiotics are generally used for the treatment of *S.*

aureus infections, however resistance to such antibiotics has become widespread making the treatment of infections more difficult.

1.2 Methicillin Resistant *S. aureus* (MRSA)

The use of antibiotics to treat *S. aureus* infections has led to the selection of antibiotic resistant strains. The first cases of methicillin resistant *S. aureus* (MRSA) were identified in the 1960's shortly after its introduction into clinical practise (Jevons et al., 1963). Nowadays in Europe, in the USA and Japan 40-60% of hospital acquired *S. aureus* are methicillin resistant (Lindsay & Holden, 2004). The β -lactam set of antibiotics work by inhibiting the final stage of cell wall synthesis resulting in cell death. They bind to transpeptidase (a.k.a. penicillin binding protein, PBP), the enzyme responsible for cross linking peptidoglycan chains in the cell wall (Llarrull et al., 2009). Methicillin resistance is conferred by the *mecA* gene, which encodes a novel penicillin binding protein (PBP2A). This protein has a reduced affinity for β -lactam antibiotics, meaning that these strains are usually resistant to all penicillin-based antibiotics (Gordon & Lowy, 2008). The *mecA* gene is carried on a mobile genetic element known as the staphylococcal chromosomal cassette (SCC*mec*), which can be horizontally transferred between Staphylococcal strains (Ito et al., 1999; Wielders et al., 2001). Several types of these elements have been identified which range in size between 20 and 58kb and carry multiple antibiotic resistances (Plata et al., 2009).

1.2.1 Healthcare associated MRSA (HA-MRSA)

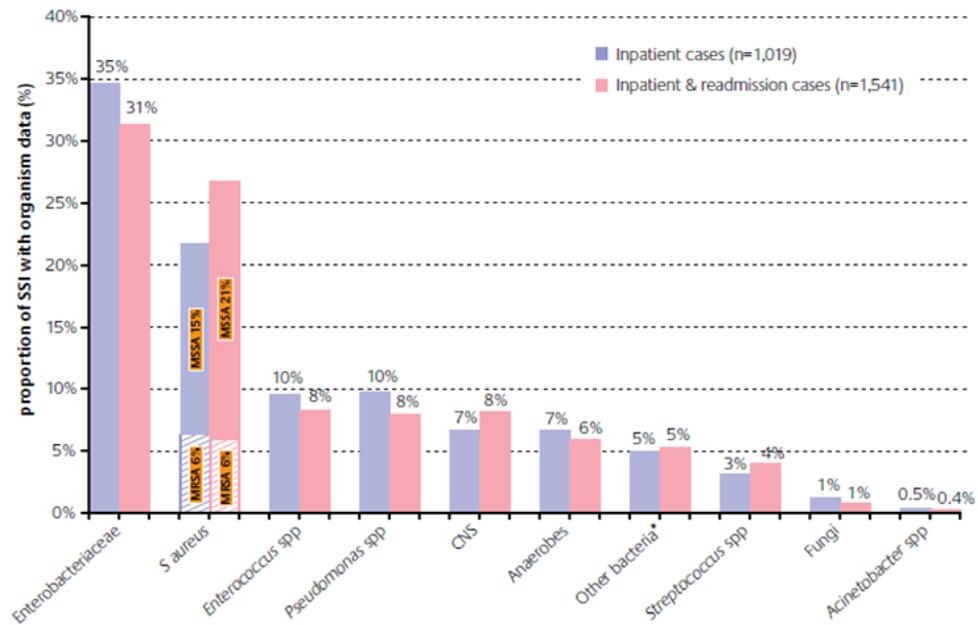
S. aureus is a common cause of hospital acquired, or nosocomial, infections due to its opportunistic nature. Surgical procedures and the use of devices such as catheters allow the bacteria to easily pass the hosts outer defences. In 2011, 27% of all surgery site

infections (SSI) were caused by *S. aureus* (Figure 1.1.A), with *S. aureus* being the leading cause of SSI in orthopaedic surgery (Figure 1.1.B). MRSA accounted for 23% of the total *S. aureus* SSI and 6% of total causative organisms in SSI (HPA - Surveillance of Surgical Site Infections in NHS hospitals in England). Although there is a drop in the number of MRSA cases the number of total *S. aureus* infections has not decreased, showing that *S. aureus* is still an important pathogen and causing a considerable burden to the health services. This burden is increased with MRSA infections due to the absence of an effective treatment which can mean prolonged and more intensive treatment. Therefore hospitals are trying to reduce the prevalence of *S. aureus* infections by reducing the opportunity for *S. aureus* to be transmitted to patients. This includes improved hospital and staff hygiene to prevent bacteria from surviving on surfaces that may come into contact with patients; and providing alcohol gel for visitors to use so that any bacteria on their skin does not infect patients.

1.2.2 Community acquired MRSA (CA-MRSA)

Serious *S. aureus* infections were initially only found in the hospital setting where the bacteria infected the very young or old and immuno-compromised patients. However community acquired MRSA (CA-MRSA) strains are becoming more prevalent and are more virulent than nosocomial MRSA. These CA-MRSA strains are capable of infecting healthy individuals commonly causing skin and soft tissue infections but can also cause more severe invasive infections such as necrotising pneumonia (King et al., 2006; Gillet et al., 2002; Vandenesch et al., 2003). This increased virulence was initially thought to be due to the production of Panton-Valentine leukocidine (PVL) which was produced by the majority of CA *S. aureus* strains. However studies conducted in animal models found conflicting results on the importance of this toxin

A



B

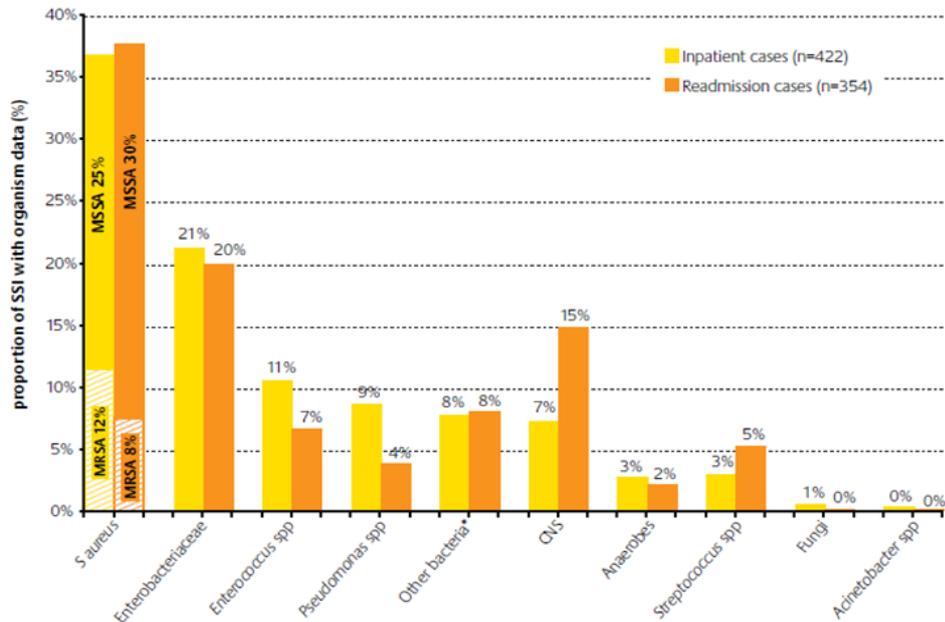


Figure 1.1 Distribution of organisms causing surgical site infections (SSI) in NHS hospitals between 2010/2011 for (A) all surgical categories and (B) orthopaedic category. *mostly comprising unspecified diphtheroids, bacilli and ‘other’ Gram-Positive organisms. CNS stands for coagulase negative staphylococci. Graphs taken from HPA -Surveillance of Surgical Site Infections in NHS hospitals in England (2010/2011).

(Otto, 2010; Moellering, 2012).

There are also CA-MRSA strains that do not contain this toxin and HA-MRSA strains that do, indicating that this is not a genetic marker for CA strains (Otto, 2010). Other factors such as alpha-toxin and phenol soluble modulins have also been shown to be important in CA-MRSA virulence (Otto, 2010). Although some contributing factors have been identified it is still not clear why these strains are more virulent.

1.2.3 Vancomycin resistant *S. aureus* (VRSA)

One of the antibiotics used to treat MRSA infections is vancomycin; however resistance to this antibiotic has also developed. In 1997 the first vancomycin intermediate-level resistant (VISA) isolate was reported in Japan, soon followed by reports from other countries (Hiramatsu et al., 1997b; Hiramatsu et al., 1997a; Smith et al., 1999; Srinivasan et al., 2002). Vancomycin resistant isolates (VRSA) were identified in the USA in 2002 (Anonymous, 2002; Chang et al., 2003; Tenover et al., 2004).

Vancomycin is a glycopeptide that functions by inhibiting cell wall synthesis through irreversibly binding the terminal D-alanyl-D-alanine residues of the cell wall precursor. This sequesters the molecules preventing them from being incorporated into the cell wall (Hiramatsu et al., 1997a; Sieradzki et al., 1999). Resistance in the VISA strains is thought to be due to changes in peptidoglycan synthesis by synthesising precursors with increased quantities of D-alanyl-D-alanine to bind and sequester the vancomycin (Sieradzki et al., 1999; Lowy, 2003). VRSA strains gained resistance through the acquisition of the *vanA* gene which produces peptidoglycan precursors with a terminal peptide of D-alanyl-D-lactate. This peptide has a reduced affinity for vancomycin allowing it to be incorporated into the cell wall.

1.2.4 Emerging resistance to linezolid and daptomycin

Linezolid and daptomycin are two newer drugs that are used to treat MRSA or VISA/VRSA infections. However, as with methicillin and vancomycin, resistance to these drugs is also emerging. Linezolid is a bacteriostatic antibiotic which binds the 50S subunit of the prokaryotic ribosome to prevent the formation of a function translation initiation complex (Swaney et al., 1998; Kloss et al., 1999). In 2001, Tsiodras et al. identified the first clinical *S. aureus* isolate with linezolid resistance. This resistance was found to be mediated through a G2576T mutation in all five copies of the 23S rRNA gene (Tsiodras et al., 2001; Pillai et al., 2002). Mutations present in the L3 and L4 ribosomal proteins, which are known to interact with the 50S ribosomal subunit, also facilitate linezolid resistance (Locke et al., 2009a; Locke et al., 2009b). In 2008 linezolid resistant isolates were obtained from 12 patients in a hospital in Spain; however the mechanism of resistance was not due to mutations in the 23S rRNA genes or L3/L4 ribosomal proteins. In these cases, resistance was mediated through the acquisition of the *cfr* gene encoding a methyltransferase which methylates 23S rRNA preventing linezolid binding (Morales et al., 2010).

Daptomycin is a cyclic lipopeptide that interacts with the bacterial cell membrane in a calcium dependent manner leading to membrane depolarisation with subsequent cell death (Silverman et al., 2003). In recent years there have been reports of daptomycin treatment failures from daptomycin resistant *S. aureus* infections (Hayden et al., 2005; Mangili et al., 2005; Marty et al., 2006; Skiest, 2006; Jacobson et al., 2009). A number of resistance mechanisms have been suggested which all affect cell membrane structure or function. Point mutations within MprF have been identified in strains with reduced daptomycin susceptibility (Friedman et al., 2006). The MprF protein is required for the

synthesis and translocation of lysyl-phosphatidylglycerol which is the only phospholipid with a net positive charge (Ernst et al., 2009). It is hypothesised that the point mutations led to accelerated translocation resulting in a reduction of the net negative charge of the membrane, which may electrostatically repel the calcium complexed daptomycin (Yang et al., 2009). This is similar to the mechanism due to mutations in the *dltABCD* operon which also contributes to the net positive charge of the membrane by D-alanylation of wall teichoic acids (Yang et al., 2009). Other genes carrying mutations associated with reduced daptomycin susceptibility include *walk* (*yycG*), involved with cell wall metabolism, and *rpoB/rpoC* encoding RNA polymerase subunits. However how these mutations increase daptomycin resistance is unknown (Friedman et al., 2006). The development of resistance to these antibiotics means that the methods of treatment for the MRSA are very limited. Further investigation into potential new antimicrobial targets and antibiotic development are crucial for the treatment of severe MRSA infections.

1.3 *S. aureus* is a highly adaptable organism

1.3.1 Genome and genetic diversity

S. aureus is capable of causing a range of diseases and can infect most sites of the human body. This ability to infect so many different sites is due to the adaptability of this bacterium. One factor which is important to the adaptability and versatility of *S. aureus* is genetic diversity.

The annotated sequences of around 31 *S. aureus* genomes are available online with an average length of ~2.8Mb. The core genome makes up approximately 75% of every *S. aureus* strain and consists of genes conserved across all strains. The majority of these

genes are associated with central metabolism and other house-keeping functions. There are also a number of genes that are not required for growth and survival but are associated with common species functions such as surface binding proteins, toxins and exoenzymes (Lindsay & Holden, 2004). Although the core genome is composed of genes found in all strains the sequence of these are not always identical. Small sequence variations in the genes can have effects on expression and protein function. This means that even the core genome affects diversity and causes phenotypic differences between strains (Lindsay & Holden, 2006).

The accessory genome accounts for the other 25% of the *S. aureus* genome. This component encodes genes for non-essential functions such as virulence, miscellaneous metabolism and drug and metal resistance (Lindsay & Holden, 2006). The accessory genome consists of mobile genetic elements which can be horizontally transferred between strains. These include bacteriophages, pathogenicity islands, genomic islands, plasmids and transposons. Since many of these elements contain virulence or drug resistance genes, it is easy to see how the evolution of more virulent and resistant strains occurs (Lindsay, 2010). The distribution of plasmids and bacteriophages appears to be *S. aureus* lineage specific, suggesting that there is some limitation to the acquisition of some mobile genetic elements (McCarthy & Lindsay, 2012; McCarthy et al., 2012). However some MRSA lineages are still acquiring new mobile elements and these can still be exchanged to other strains within that lineage.

1.3.2 *S. aureus* virulence determinants

S. aureus encode a number of different virulence determinants to allow colonisation, infection and evasion of the immune response. These factors include toxins, super

antigens, extracellular enzymes and nutrient-uptake systems. Virulence determinants can also add to the variability between strains as they are distributed and regulated differently between strains (Bronner et al., 2004). *S. aureus* virulence factors can be grouped into three categories; secreted factors, non-covalently attached surface factors and covalently attached surface factors.

Toxins and extracellular enzymes are secreted from the cell into the extracellular environment to attack host cells. A number of proteases and lipases are released to break down host cell membranes and host defence molecules (Archer, 1998). The α -toxin acts in both a cytotoxic and haemolytic manner forming pores in cell membranes causing cell damage and death (Bhakdi and Tranum-Jensen, 1991). Some of the toxins produced are the causative agents for a particular disease, for example the exfoliative toxin which breaks down the skin causing Scalded Skin Syndrome (Gemmell, 1995; Ladhani, 2001). The toxin responsible for Toxic Shock Syndrome is a superantigen, causing uncontrolled T-cell proliferation and cytokine release resulting in damage to the body (Musser et al., 1990; Fraser et al., 2000).

S. aureus produce a number of proteins covalently bound to the cell surface which aid in the initial adhesion and colonisation of infection sites. Covalently bound proteins are known as MSCRAMM (microbial surface components recognising adhesive matrix molecules) and include adhesins such as protein A and fibronectin-binding proteins (Patti et al., 1994; Wann et al., 2000). These surface proteins can also show multiple functions for example IsdA has been shown to bind to fibrinogen and fibronectin but is also part of a heme iron uptake system (Morrissey et al., 2002; Mazmanian et al., 2003; Clarke et al., 2004).

Examples of non-covalently bound virulence determinants are Eap and Emp. Both of these proteins bind a number of host proteins including fibronectin, fibrinogen, vitronectin, prothrombin and collagen which aids in colonisation (Bodén & Flock, 1992; Palma et al., 1999; Hussain et al., 2001; Chavakis et al., 2002). These proteins are also required for biofilm formation in low Fe conditions and in serum (Johnson et al., 2008; Thompson et al., 2010). Biofilms are multilayered clusters of cells that are coated with a protective layer of polysaccharide and can disseminate to spread infection. In addition to roles in colonisation, Eap has been shown to be important in immune evasion and modulation of host immune responses. *S. aureus* cells lacking Eap showed reduced binding to and internalisation into epithelial cells, a mechanism to avoid the host immune response (Palma et al., 1999; Hussain et al., 2002; Alvarez et al., 2011; Edwards et al., 2012). *In vivo* experiments using mouse models revealed Eap reduced T-cell proliferation and neutrophil recruitment (Lee et al., 2002; Chavakis et al., 2002; Wang et al., 2010). Interestingly, Eap displays both anti- and pro- inflammatory responses providing further evidence of immune modulation (Chavakis et al., 2002; Athanasopoulos et al., 2006; Scriba et al., 2008). The production of Eap is also responsible for impaired wound healing (Athanasopoulos et al., 2006). Therefore Eap is an incredibly important virulence factor involved in several stages of infection from colonisation to protection from the immune system.

1.3.3 Regulation of virulence determinants

Different virulence factors are required for the colonisation and infection of different body sites due to varying environmental factors the bacteria encounter. For example bacteria colonising skin will experience a different level of oxygen availability and pH compared to those in the gut. Therefore these factors need to be tightly regulated by

environmental signals such as pH, oxygen availability, osmotic stress and metal ions. A growing number of regulators have been identified that influence virulence gene expression including 16 two-component regulator (TCR) systems, DNA binding proteins and RNA regulators (Cheung et al., 2004; Bronner et al., 2004; Somerville & Proctor, 2009). Some of these regulators are known to co-regulate virulence factors directly or through regulation of each other. In this way a single virulence gene can be regulated by a number of regulatory factors and environmental stimuli. In this section three of the most well studied global regulators; Agr, SarA and Sae will be discussed in more detail.

Agr and Quorum sensing

One of the best characterised global regulators is the Accessory Gene Regulator (Agr) system. The Agr system is an example of a two-component regulator and is responsible for quorum sensing (Ji et al., 1995; Peng et al., 1988). The *agr* operon is transcribed from two divergent transcripts, RNAII and RNAIII. RNAII encodes four genes, *agrBDCA* whereas RNAIII acts as a regulatory RNA. However, RNAIII does also contain an ORF for haemolysin δ (Figure 1.2). These transcripts are controlled by two promoters, P2 and P3. The *agrC* and *agrA* genes encode a classical two component regulator producing the histidine kinase sensor and response regulator respectively. The auto inducing peptide (AIP), encoded by *agrD*, is modified and then exported out of the cell by AgrB. Accumulation of AIP leads to phosphorylation of AgrC which in turn phosphorylates AgrA to activate it (Lina et al., 1998). AgrA binds to the P2 and P3 promoters to increase transcription of both RNAII and RNAIII transcripts (Figure 1.2). The phosphorylated AgrA protein binds the P2 and P3 promoters to positively regulate them and therefore forms a positive feedback loop (Koenig et al., 2004; Reyes et al.,

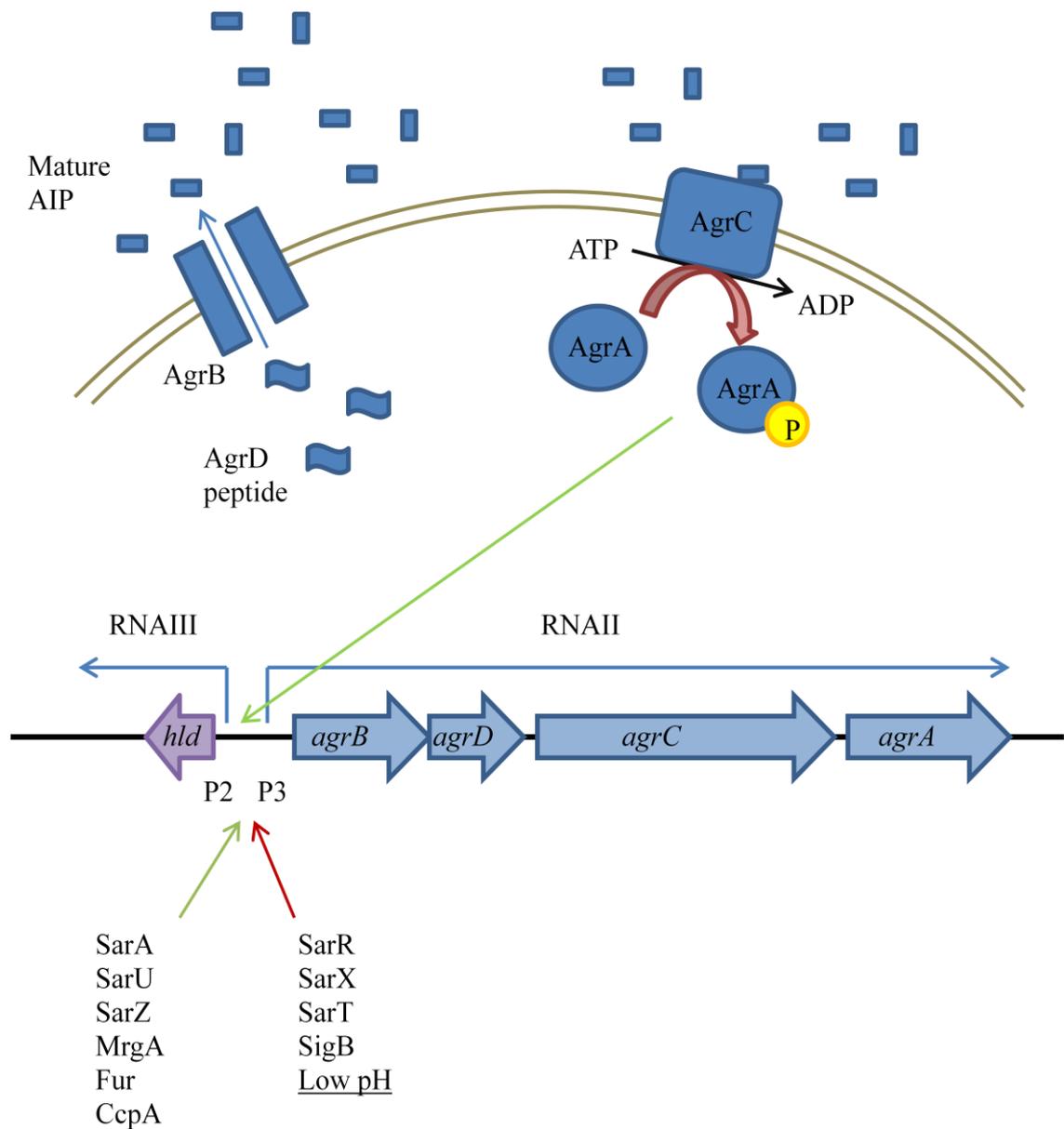


Figure 1.2 Schematic diagram of the *agr* operon and its regulation. AgrA and AgrC form the sensor kinase and response regulator of a classical TCR. The AgrD peptide is processed into AIP and transported by AgrB. The accumulation of AIP causes phosphorylation of AgrC which in turn phosphorylates, and therefore activates, AgrA. Activated AgrA binds to the P2 and P3 promoters to enhance transcription of RNAII and RNAIII. Positive and negative regulation of *agr* is indicated by green and red arrows respectively. The underlined text indicates environmental signals which affect *agr* expression.

regulator that can act positively or negatively on target genes. Although RNAIII is responsible for the majority of regulation by the *agr* system, AgrA can also directly bind target gene promoters (Queck et al., 2008).

The Agr system is an important global regulator modulating virulence gene expression in response to cell density. As cell density increases, the increase in RNAIII molecules results in repression of colonising factors such as Protein A and fibronectin binding proteins, but activates toxins such as *hla* and *eap* (Peng et al., 1988; Novick et al., 1993; Dunman et al., 2001). Through this regulator as cell density increases the focus of gene expression moves away from recruiting more cells and towards acquiring nutrients or spreading infection. In addition, Agr has been implicated in the regulation of carbohydrate metabolism, amino acid metabolism and staphyloxanthin biosynthesis indicating that cell density is used to regulate central metabolism as well as virulence (Queck et al., 2008).

The RNAIII molecule can regulate expression at the post transcriptional level as with other regulatory RNA molecules (discussed further in section 1.5) but can also act indirectly at the transcriptional level (Novick et al., 1993; Morfeldt et al., 1995; Huntzinger et al., 2005; Boisset et al., 2007; Liu et al., 2011). At the post transcriptional level, repression of *spa* and *rot* translation by RNAIII occurs through base pairing between the 5' of the mRNA and 3' of the RNAIII molecule. Binding of the two molecules results in repression of translation and degradation via the ribonuclease RNaseIII (Huntzinger et al., 2005; Boisset et al., 2007). Investigation into positive *hla* regulation by RNAIII found that intramolecular binding occludes the *hla* ribosomal binding site (RBS). Hybridisation between RNAIII and *hla* mRNA disrupts this

intramolecular pairing making the mRNA accessible for translation initiation (Morfeldt et al., 1995).

Although a central regulator, the *agr* operon is under complex regulatory control itself. A number of the SarA family of regulators positively or negatively regulate *agr* transcription. SarA, SarU, SarZ and MgrA all activate *agr* transcription whereas SarR, SarX and SarT repress transcription (Cheung & Projan, 1994; Heinrichs et al., 1996; Schmidt et al., 2001; Manna & Cheung, 2006a; Manna & Cheung, 2006b; Kaito et al., 2006; Tamber & Cheung, 2009; Ballal et al., 2009; Reyes et al., 2011). Another regulator, Sae, required for expression of exoproteins represses *agr* transcription (Johnson et al., 2011). Expression of *agr* is also controlled by regulators responding to environmental signals such as Fur, CcpA and SigB. The ferric uptake repressor (Fur) is required for Fe homeostasis but has been shown to positively regulate *agr* (Johnson et al., 2011). The carbon catabolite repressor, CcpA, responsible for regulating expression to adapt to a preferred carbon source has also been found to positively regulate *agr*. This supports the previously mentioned involvement of Agr in regulating metabolism (Seidl et al., 2006). Agr dependent expression of virulence factors is also influenced by environmental stresses as the alternate sigma factor SigB, involved in bacterial stress responses, represses *agr* expression (Lauderdale et al., 2009; Bischoff et al., 2001). This demonstrates that even global regulators are under tight control by a number of factors.

SarA family

The SarA family of regulators was originally identified by transposon mutagenesis, which identified a locus that affects exoprotein expression that was distinct from the *agr* operon (Cheung et al., 1992). This locus was termed the Staphylococcal Accessory

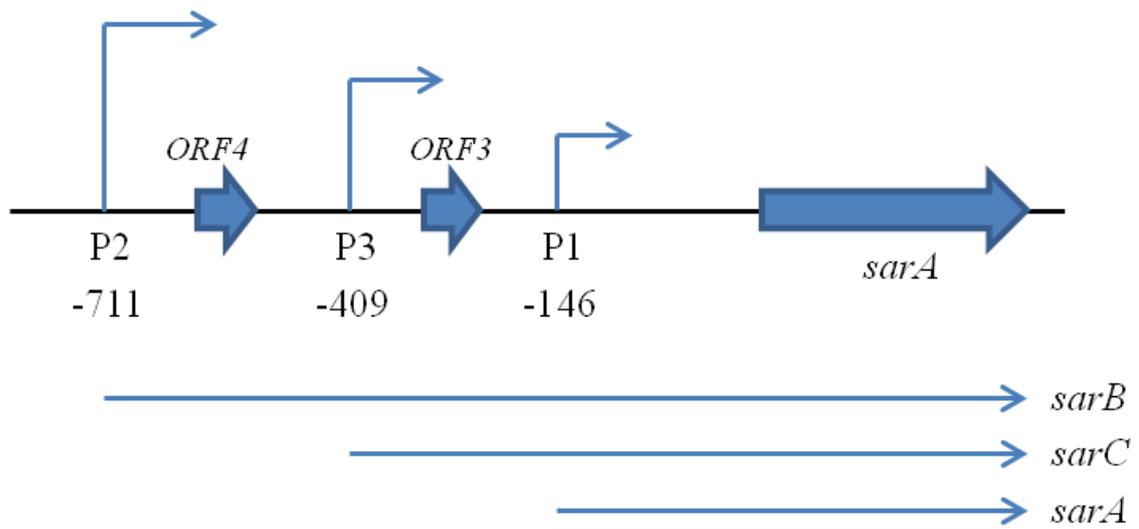


Figure 1.3 Schematic diagram of the *sarA* locus. The 3 promoters and their respective location upstream of the *sarA* are shown, along with the transcripts produced. All transcripts terminate at the same transcriptional terminator.

Regulator (Sar) and encodes a single DNA binding protein, SarA (Cheung et al., 1992; Cheung & Projan, 1994). The *sarA* locus contains 3 promoters which all initiate *sarA* transcription to produce three transcripts (*sarA*, *sarB* and *sarC*) that are preferentially expressed at different points during growth (Figure 1.3). The *sarA* and *sarB* transcripts are most abundant during early log phase whilst *sarC* is highest during late stationary phase (Heinrichs et al., 1996). Downstream of the distal promoters P2 and P3 are two open reading frames (ORF) ORF3 and ORF4 potentially encoding short peptides (Bayer et al., 1996). Their function is still unknown however they are required for full SarA protein expression (Chien et al., 1998).

Phenotypic analysis have revealed that SarA promotes expression of proteins for adhesion, such as fibronectin and fibrinogen binding proteins, and toxins whilst repressing Protein A and proteases (Cheung et al., 2004). As mentioned previously SarA regulates *agr* expression by binding to both *agr* promoters increasing the levels of both RNAII and RNAIII thereby affecting *agr* regulation of virulence factors (Cheung et al., 1997; Morfeldt et al., 1996). However SarA also appears to regulate exoprotein expression in an *agr*-independent manner (Cheung et al., 1997). The SarA protein binds a conserved sequence, homologous to the SarA binding site of the *agr* promoters, upstream of the -35 of several target genes (Chien et al., 1999). Although SarA regulates transcription by binding to promoters SarA has also been shown to be involved in post transcriptional regulation. SarA has been found to stabilise a total of 138 mRNA species including *spa* and *cna* (collagen binding protein) during exponential growth (Roberts et al., 2006; Morrison et al., 2012). Further investigation found SarA binds mRNA *in vitro* and *in vivo* indicating that these stabilisation effects are direct (Morrison et al., 2012).

A total of 11 SarA homologues have been identified in the N315 genome. Nearly all of these homologues have been characterised and are important in promoting virulence factors and many either regulate *agr* or are regulated by it. The SarA family also show a complex regulatory network between themselves indicating the complexity of the regulatory network (Table 1.1) (Cheung et al., 2008).

SaeRS two-component regulator

Another two-component regulator which has been found to be very important in virulence gene expression is Sae (Giraud et al., 1997; Giraud et al., 1999). Sae has been shown to upregulate α -haemolysin, β -haemolysin, Eap, Emp and FnbpA (Fibronectin binding protein A) and so is important for colonisation and toxicity (Giraud et al., 1997; Steinhuber et al., 2003; Harraghy et al., 2005; Liang et al., 2006; Johnson et al., 2008). The *sae* locus consists of 4 open reading frames; *saeP*, *saeQ*, *saeR* and *saeS* (Figure 1.4) (Giraud et al., 1999; Novick & Jiang, 2003). SaeRS make up the response regulator and sensor kinase respectively of a classical TCR (Giraud et al., 1999; Sun et al., 2010). The remaining ORFs SaeP and SaeQ have been proposed to encode a lipoprotein and membrane protein respectively. A recent study has indicated that these proteins form a complex with SaeS to activate the sensor kinase's phosphatase activity (Jeong et al., 2012). This activity is important to return SaeS to its pre-activation state so that it is not constitutively active. The *sae* operon is under the control of two promoters, P1 and P3, which are found upstream of *saeP* and within *saeQ* respectively (Figure 1.4) (Novick & Jiang, 2003; Geiger et al., 2008; Jeong et al., 2011). Transcription from these promoters results in four transcripts (Figure 1.4). Transcripts C and A are transcribed from P1 and P3 respectively, however transcripts B and D would seem to be the result of processing of the C transcript as there is no

Locus	Gene symbol	Putative function
SA0573	SarA	Activates surface and exoprotein genes via <i>agr</i> and <i>agr</i> independent pathways
SA2089	SarR	A negative regulator of <i>sarA</i> and <i>agr</i> (Cheung et al., 2004; Manna & Cheung, 2006b; Reyes et al., 2011)
SA0108	SarS (H1)	An activator of <i>spa</i> , <i>hla</i> , <i>hld</i> , <i>sspA</i> expression (Tegmark et al., 2000)
SA2286	SarT	An activator of <i>sarS</i> and a repressor of <i>hla</i> expression (Cheung et al., 2004)
SA2287	SarU	A positive regulator of <i>agr</i> (Cheung et al., 2004)
SA1583	Rot	A repressor of toxin synthesis, opposite to <i>agr</i> (Saïd-Salim et al., 2003)
SA0623	SarX	A negative regulator of <i>agr</i> and activated by MgrA (Manna & Cheung, 2006)
SA0641	MgrA (Rat/NorR)	A regulator of autolysis and <i>agr</i> , regulates <i>sarX</i> , <i>sarV</i> , <i>sarZ</i> (Truong-bolduc et al., 2003; Manna et al., 2004; Manna & Cheung, 2006a; Ballal et al., 2009)
SA2174	SarZ	A positive regulator of <i>hla</i> , <i>hnb</i> , <i>sspA</i> , <i>mgrA</i> and <i>agr</i> and negative regulator of <i>spa</i> and <i>sarS</i> (Kaito et al., 2006; Tamber & Cheung, 2009; Ballal et al., 2009)
SA2062	SarV	A regulator of autolysis repressed by SarA and MgrA (Manna et al., 2004)
SA2091	SarY	Function unknown

Table 1.1The SarA protein family (from N315 genome). Table adapted from Cheung et al. (2008).

promoter for the expression of B alone (Figure 1.4) (Steinhuber et al., 2003; Adhikari & Novick, 2008). However the mechanism of the processing of the C transcript is not yet known.

The *sae* operon is positively auto regulated by SaeR at the P1 promoter, however during later stages of growth *in vitro* SaeR has also been shown to repress expression from P3 (Novick & Jiang, 2003; Geiger et al., 2008). This switch is believed to occur in an Agr dependent manner (Novick & Jiang, 2003). The *sae* operon is also regulated by a number of other regulatory factors and environmental stimuli. The induction of this operon requires the regulatory RNAIII from the Agr system, SarA and Fur, whilst being repressed by Rot and SigB (Novick & Jiang, 2003; Li & Cheung, 2008; Johnson et al., 2011). The P1 promoter is reported to respond to environmental signals such as high salt, high glucose, low pH, α -defensins, H₂O₂ and high copper (Novick & Jiang, 2003; Geiger et al., 2008; Adhikari & Novick, 2008; Baker et al., 2010). Interestingly, activation of *sae* expression is observed when grown in sub-inhibitory concentrations of antibiotics (Novick & Jiang, 2003; Kuroda et al., 2007; Blickwede et al., 2005). TCR systems allow the modulation of gene regulation in response to different environmental stimuli. Therefore as a TCR the SaeRS system monitors the environment to induce virulence transcription when colonising a host. However direct ligand binding has not been determined and therefore it is not known which of these stimuli directly interact with SaeR.

Regulatory network of *hla* expression

In the sections above the action and regulation of the most well studied virulence regulations have been discussed. To show how these regulators can work together on a

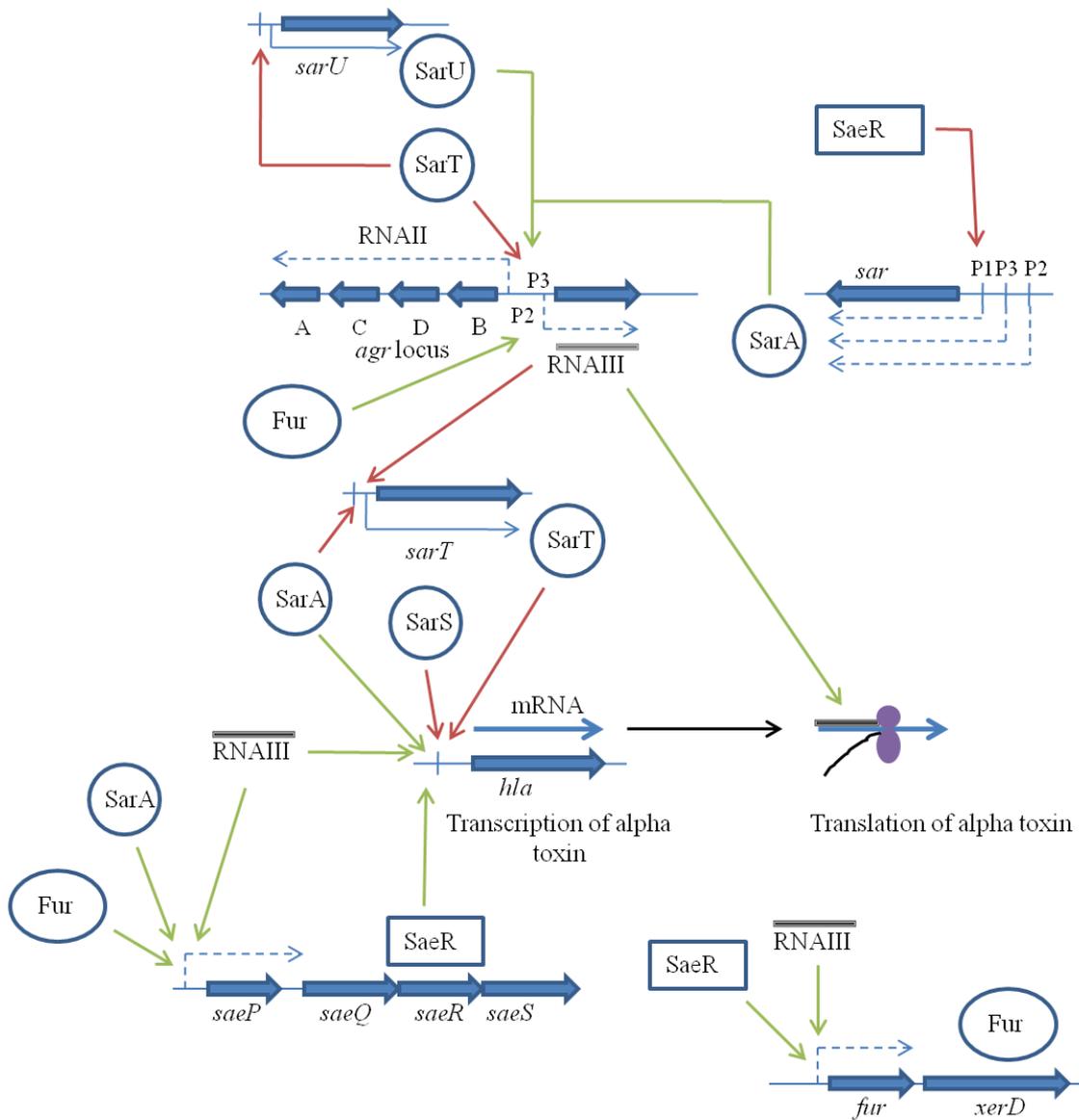


Figure 1.5 A schematic figure showing the regulation of *hla* (α -haemolysin) through interactions by the regulatory factors Agr, Sar and Sae. The *hla* gene is regulated by SarA, SarS, SarT, SaeR and RNAIII. But these regulatory factors also regulate each other making regulation more complex. Green and red arrows indicate positive and negative regulation respectively. Figure adapted from Bronner et al. (2004).

single virulence determinant and demonstrate the complexity of regulation, the regulation of *hla* by some of these factors is shown in Figure 1.5. *hla* transcription is directly controlled by SaeR, SarA, SarS, SarT and translation initiation regulated by RNAIII. These factors can also regulate each other to add another level of *hla* regulation through indirect interactions. It is imperative for bacteria to be able to sense the environment and modulate gene regulation so that nutrient acquisition and virulence factors are only expressed when required.

1.4 Iron regulation and Fur in *S. aureus*

One important environmental stress that pathogenic bacteria encounter is severe iron restriction. Iron is required as a cofactor for enzymes involved in cell proliferation, metabolism and DNA repair and so is essential for most prokaryotes and eukaryotes (Ganz & Nemeth, 2006). However, too much iron is toxic to cells due to the production of free radicals via the Fenton reaction shown below (Ganz & Nemeth, 2006).



Normal aerobic metabolism can generate mildly toxic products however when Fe is present it catalyses the production of the highly reactive hydroxyl radical (OH[·]). These highly reactive products can interact with carbohydrates, nucleic acids, amino acids and therefore lead to cell damage. In eukaryotes, free iron is sequestered by proteins such as haem and ferritin. This is not only to prevent iron toxicity but acts as an immune defence by limiting iron acquisition by bacteria. Therefore iron restriction is used by bacteria as a signal that they have entered a host organism. This leads to the expression

of iron acquisition systems and virulence factors required for colonisation and pathogenesis.

The majority of iron regulation occurs through the Ferric Uptake Repressor (Fur) in a number of bacteria. Fur was originally discovered in *Salmonella enterica* and studied in *Escherichia coli*, where it was found to be an iron dependent repressor of iron transport (Ernst et al., 1978; Hantke, 1984). *S. aureus* has three Fur homologues; Fur, PerR and Zur. Fur acts as a global iron regulator which represses genes encoding iron uptake proteins in high iron conditions (Xiong et al., 2000; Horsburgh et al., 2001b). It is also involved in the regulation of the oxidative stress response along with PerR, a manganese dependent repressor (Horsburgh et al., 2001b). Both proteins regulate the expression of *kata*, a catalase involved in oxidative stress, but do so in opposing ways. Fur positively regulates *kata* in high iron, to cope with the toxic effects of high iron conditions, whereas PerR represses *kata* in response to manganese (Horsburgh et al., 2001a; Horsburgh et al., 2001b). PerR has also been found to regulate Fur and ferritin, an iron storage protein, indicating a role in iron homeostasis as well (Horsburgh et al., 2001a; Morrissey et al., 2004). Due to their role in iron regulation and oxidative stress it is understandable that both of these proteins are important for virulence (Horsburgh et al., 2001b). Zur is a zinc dependent repressor which regulates the expression of zinc uptake pathways (Gaballa & Helmann, 1998; Lindsay & Foster, 2001). However, unlike Fur and PerR, no additional roles have been identified and it is not involved in virulence (Lindsay & Foster, 2001).

Fur classically functions to repress target genes when intracellular iron is abundant.

When iron is bound to the Fur protein this complex binds to a consensus DNA sequence

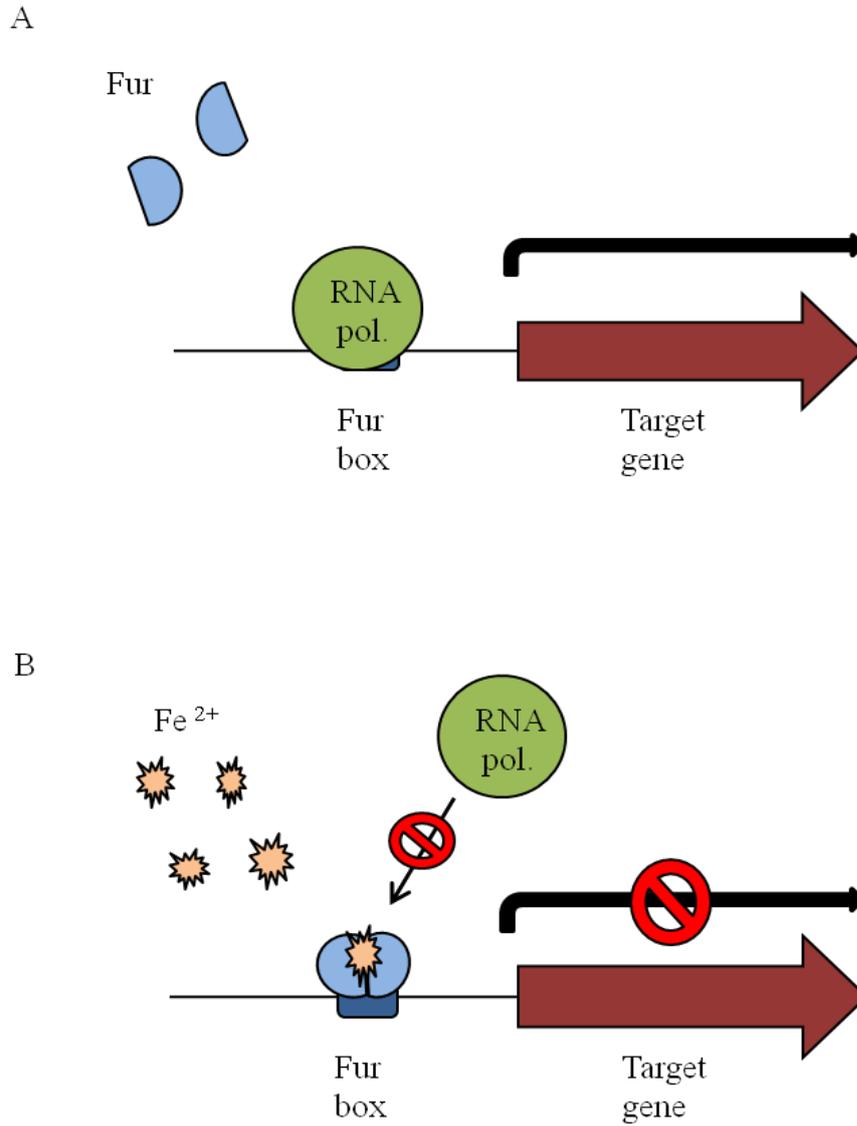


Figure 1.6 Classical Fe regulation by Fur. In (A) low Fe there is no Fur binding and the gene is transcribed. In (B) high Fe the Fur-Fe complex binds the Fur box therefore repressing transcription.

called a Fur box. The consensus sequence of these sites can differ between bacteria, although the Fur protein itself is highly conserved. Fur boxes are found close to promoter regions and so binding at these regions prevents polymerase binding, and therefore transcription (Figure 1.6) (Bagg & Neilands, 1987). However recently there have been examples of non-classical Fur regulation. In *S. aureus* Fur acts as a repressor of ferritin (*ftnA*) in low Fe conditions (Morrissey et al., 2004). Positive regulation of *kata*, *sodA* and *gapA* by *S. aureus* Fur has been identified in high Fe conditions (Horsburgh et al., 2001b; Purves, 2011). Whereas biofilm formation, through Eap and Emp, is positively Fur regulated in low Fe conditions (Johnson et al., 2005; Johnson et al., 2008). This regulation appears to be indirect through positive Fur regulation of *agr* and *sae* (Johnson et al., 2011). However it is still not clear how Fur exerts this non-classical, positive regulation in *S. aureus*.

In other bacteria there have been reports of Fur acting in a non-classical fashion to regulate transcription. In *H. pylori* apo-Fur was shown to directly bind *pfr* and *sodB* promoters to repress transcription (Delany et al., 2001; Ernst et al., 2005). Positive regulation by Fur-Fe binding DNA to activate transcription occurs in *Neisseria meningitidis*, *Helicobacter pylori*, *Pseudomonas aeruginosa* and *E. coli* (Delany et al., 2004; Grifantini et al., 2003; Alamuri et al., 2006; Gancz et al., 2006; Wilderman et al., 2004; Nandal et al., 2010). However for most of these examples the mechanism of regulation by Fur binding is not known. In *N. meningitidis* *in vitro* transcription assays revealed that Fur-Fe alone was sufficient to activate *norB* transcription (Delany et al., 2004). Whereas in *E. coli*, Fur-Fe acts as an anti-repressor. The histone-like nucleoid-associated protein (H-NS) binds the promoter of *ftnA* to repress transcription; this interaction is disrupted by the binding of Fur-Fe thereby allowing *ftnA* transcription

(Nandal et al., 2010). Activation by apo-Fur has been shown in *S. aureus* where apo-Fur directly binds a Fur box within the *norA* promoter to promote transcription (Deng et al., 2012). This indicates that this form of Fur regulation is possible in *S. aureus* and therefore further study is required. However, the most common mechanism for positive Fur regulation is through the action of regulatory RNA molecules.

1.5 Riboregulation

In recent years there has been a sharp increase in the number of studies investigating the role of riboregulation in stress resistance and virulence. Therefore the importance of this regulation is only now beginning to be appreciated. Several types of regulatory RNA have been identified including riboswitches, cis-encoded antisense RNAs and trans-encoded RNAs.

1.5.1 5' UTR Riboregulators

The 5' UTR of some mRNA molecules can be structured to allow or prevent translation in response to a number of signals. "RNA thermometers" are riboswitches found in the 5' UTR of temperature responsive genes and switch between "open" and "closed" RNA conformation in response to varying temperatures (Papenfert & Vogel, 2010). Two well-known examples of such regulation are found in *Yersinia pestis* and *Listeria monocytogenes*. The *lcrF* and *prfA* mRNA of *Y. pestis* and *L. monocytogenes* respectively show secondary structure of the 5' UTR which prevent translation at low temperatures (Figure 1.7). However, at higher temperatures, like those found within a mammalian host, there is a conformational change in this structure, revealing the RBS and allowing translation (Figure 1.7) (Hoe & Goguen, 1993; Johansson et al., 2002). Both of these genes encode transcriptional activators involved with virulence gene

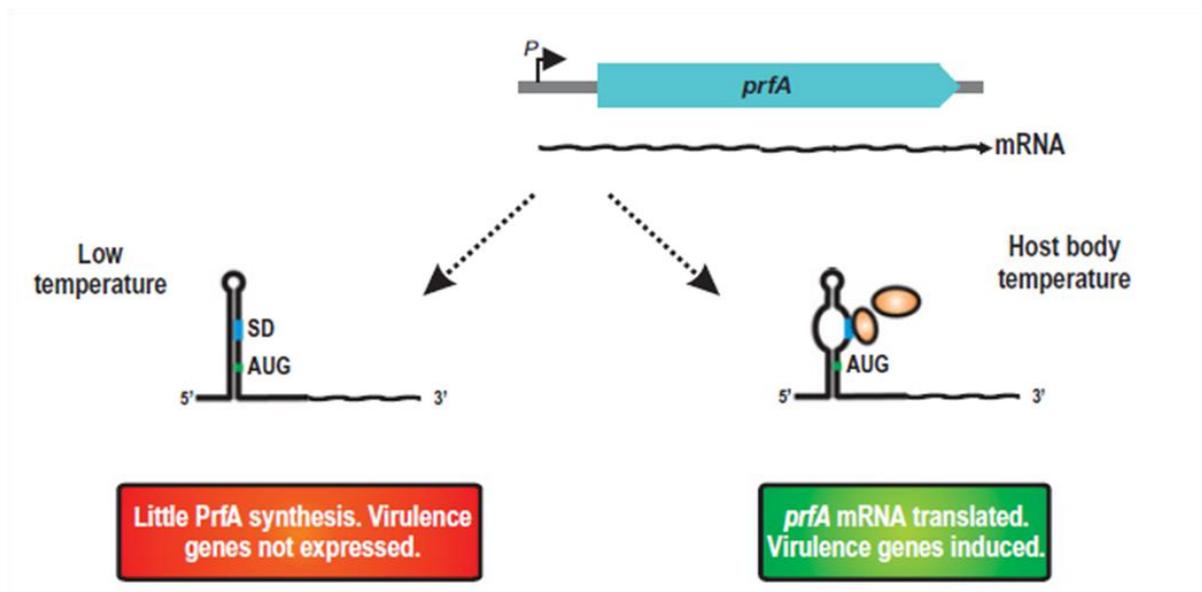


Figure 1.7 The effect of temperature on the *prfA* 5' riboswitch in response to **temperature**. Translation of *prfA* mRNA is regulated by an RNA thermometer in the 5' UTR. At lower temperatures, like those outside of a host, the secondary structure prevents translational initiation. At higher temperature like those inside a mammalian host the secondary structure changes allowing access to the RBS and translation initiation. Figure taken from Papenfort & Vogel (2010).

regulation showing that temperature can be used as an environmental stimulus for virulence gene induction. Further studies into bacterial riboswitches have found that they can also recognise a range of metabolites, metal ions and pH changes (Dambach & Winkler, 2010; Nechooshtan et al., 2009).

1.5.2 *cis*-encoded antisense RNA

Antisense RNA (asRNA) are encoded from the opposing strands of coding genes, as such they show substantial levels of complementarity with their targets. These asRNAs can affect expression at transcriptional and translational levels. Transcriptional control can occur through formation of transcriptional terminators and therefore prematurely stops transcription. In *Shigella flexneri*, the asRNA RnaG interrupts transcription of *icsA*, which encodes a protein required for epithelial cell invasion, by inducing a structural change producing a termination hairpin (Giangrossi et al., 2010).

Translational control can occur through asRNA-mRNA binding leading to changes in mRNA stability. The formation of an asRNA-mRNA duplex can lead to rapid degradation of both molecules as observed with the *isiA/IsrA* duplex in *Synechocystis* PCC6083 (Dühring et al., 2006). Conversely, duplex formation can also stabilise transcripts. In *E. coli*, the GadY asRNA stabilises the *gadX* and *gadW* transcripts by inducing the correct processing of the bicistronic *gadXW* message (Tramonti et al., 2008; Opdyke et al., 2011). In addition to altering stability, asRNA binding can also affect translation initiation. In *E. coli*, the SymR-*symE* interaction is an example of repression through translational regulation. SymR overlaps the 5' end of the *symE* mRNA covering the RBS and AUG start codon preventing ribosome binding. Although asRNA are completely complementary to their target the secondary structures of both RNA molecules are important. Single stranded or weakly structured regions are

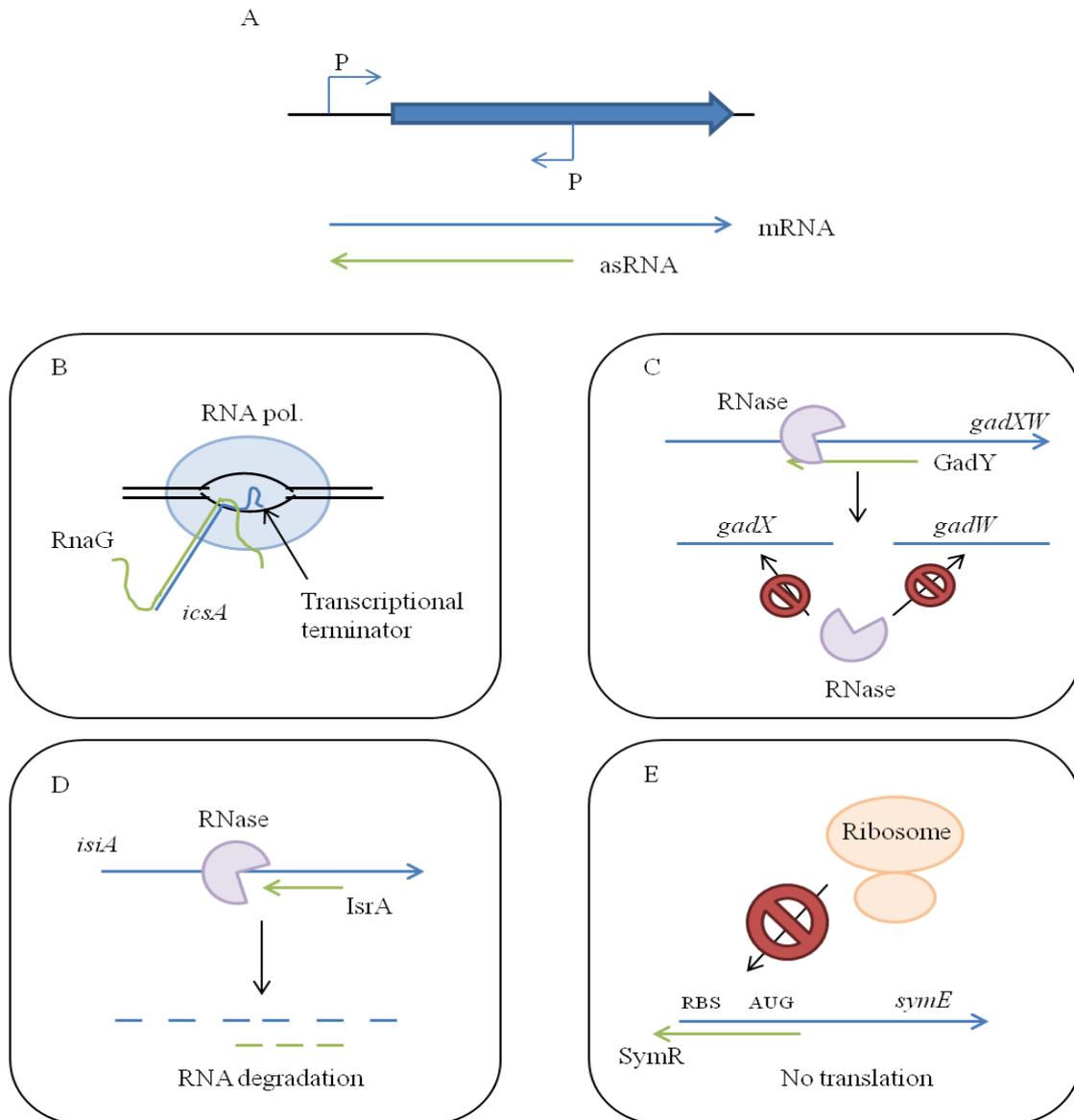


Figure 1.8 The possible effects of *cis*-encoded asRNA. (A) asRNA are encoded from the opposite strand of the gene they act upon. asRNA can be encoded anywhere along the opposite strand meaning that asRNA can bind different regions along the mRNA. (B) RnaG acts to stop *icsA* transcription by binding and causing the formation of a transcriptional terminator. (C) GadY initiates processing of the bicistronic *gadXW* mRNA into separate transcripts making them more stable. (D) IsrA binding to *isiA* induces degradation by ribonucleases. (E) SymR binds the 5'UTR of *symE* to occlude the RBS and AUG start codon preventing translational initiation.

required for initial binding but duplexes are very stable due to the extended complementarity (Georg & Hess, 2011). The number and importance of asRNA are only just being realised and further investigation is required to fully understand the extent of their regulation.

1.5.3 *trans*-encoded small RNA

Small RNA (sRNA) molecules are non-coding RNA molecules which range from 50 to 400 nucleotides in length (Guillier & Susan Gottesman, 2008). These sRNA function in similar ways to asRNA but obviously share less sequence complementarity to their targets. However this also means that sRNA are not restricted to a single mRNA target making them global regulators. sRNAs were first discovered in *E. coli* and it is thought that the *E. coli* genome contains at least 100 of these molecules. sRNAs have been identified in a number of bacteria; *Salmonella* contains at least 70, 17 have been identified in *P. aeruginosa* and 3 identified in *L. monocytogenes* (Sittka et al., 2008; Padalon-Brauch et al., 2008; Livny et al., 2006; Christiansen et al., 2006). sRNA are also the regulatory RNA molecules that have shown to be involved with positive Fur regulation.

sRNAs act at the post transcriptional level through several different mechanism to induce or repress translation and/ or degradation. The majority of sRNA-mRNA interactions negatively regulate mRNA by preventing translation initiation and/or promoting degradation (Figure 1.9). The DsrA, OxyS and RyhB sRNAs bind *hns*, *fhlA* and *sodB* respectively to block the RBS and prevent ribosome binding (Altuvia et al., 1998; Lease et al., 1998; Večerek et al., 2003). In the case of RyhB-*sodB* this also leads to degradation of the *sodB* mRNA through interactions with RNaseE and RNaseIII

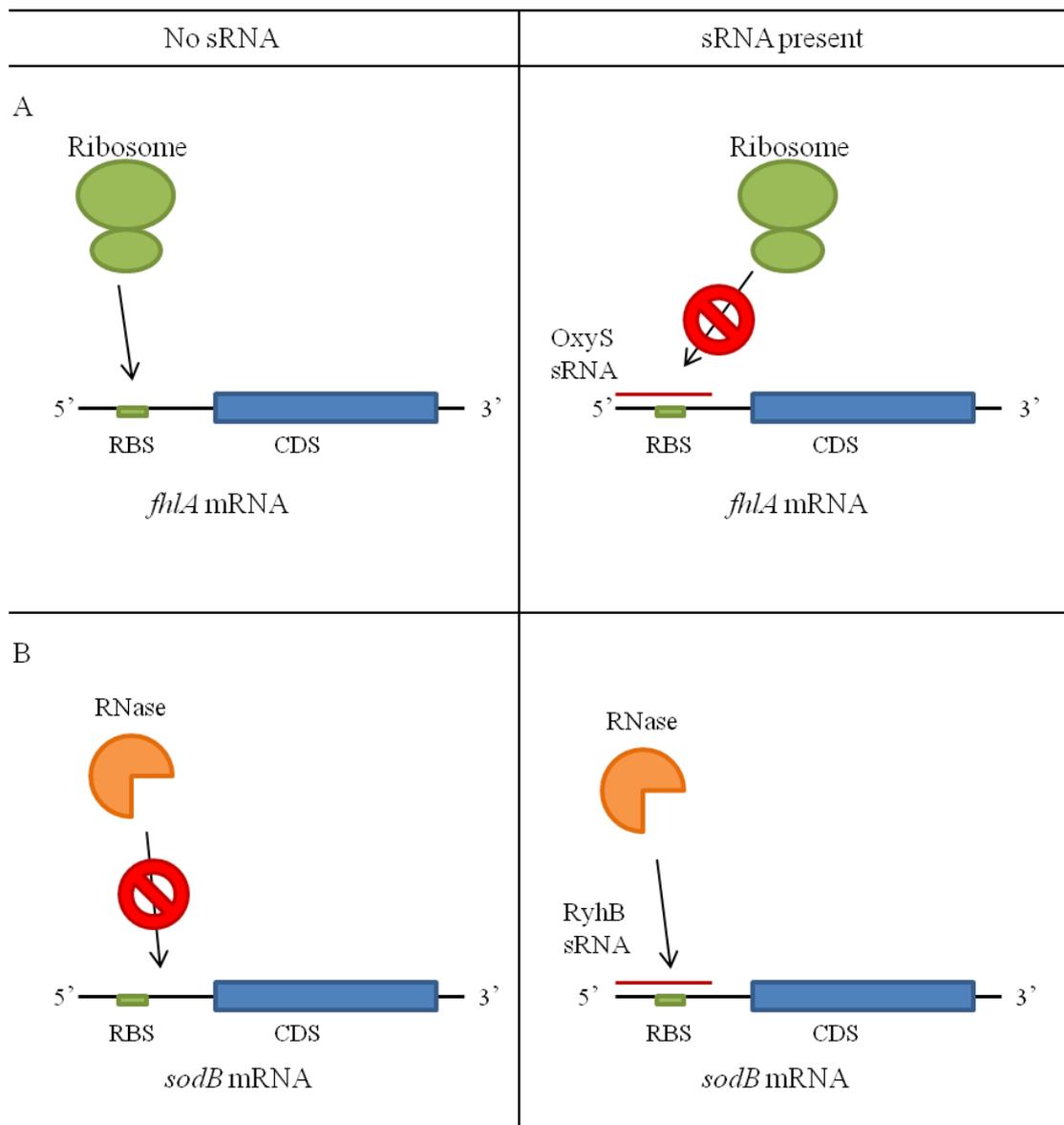


Figure 1.9 Examples of negative regulation by sRNA through (A) repression of translation or (B) induced degradation. sRNA (red) can repress translation by allowing or blocking ribosomal binding to mRNA (black & blue). sRNA can also affect stability by increasing availability of mRNA for ribonuclease attack.

(Morita et al., 2005; Afonyushkin et al., 2005).

Conversely, positive regulation by sRNA can occur through changes in secondary structure which reveal the RBS allowing translational initiation referred to as anti-antisense regulation (Figure 1.10.A). The DsrA and RyhB sRNAs can also work to increase translation of *rpoS* and *shiA* mRNA respectively (Lease et al., 1998; Sledjeski et al., 1996; Prévost et al., 2007). In both of these cases the sRNA binds to the mRNA to prevent the formation of an inhibitory structure in the transcript, therefore allowing ribosome binding and translation (Majdalani et al., 1998; Prévost et al., 2007). The RyhB and DsrA sRNA show that they can bind multiple targets and that their effect is dependent on the target they bind, showing the flexibility of these regulators. Recent studies have identified examples of stabilisation through sRNA-mRNA binding. In *C. perfringens* the 3' end of VR-RNA binds the 5' UTR of the *colA* mRNA which results in endonucleolytic cleavage just downstream of the duplex. The resulting mRNA is more stable and in addition the change in structure also reveals the RBS allowing translation initiation (Figure 1.10.B) (Obana et al., 2010). An example of direct stabilisation by sRNA-mRNA binding is seen in *Streptococcus*. The binding of FasX to *ska* produces a double stranded structure at the 5' end of the RNA resulting in stabilisation of the transcript (Figure 1.10.C) (Ramirez-Pena et al., 2010). A common feature of both of these stabilisation mechanisms is that it results in the removal of a single stranded protrusion at the 5' end which is thought to be a target for ribonucleases.

1.6 Involvement of Fur in sRNA regulation

Fur can exert positive regulation in iron replete indirectly via the regulation of sRNA molecules. A well-studied example of this is the *E. coli* sRNA RyhB which regulates

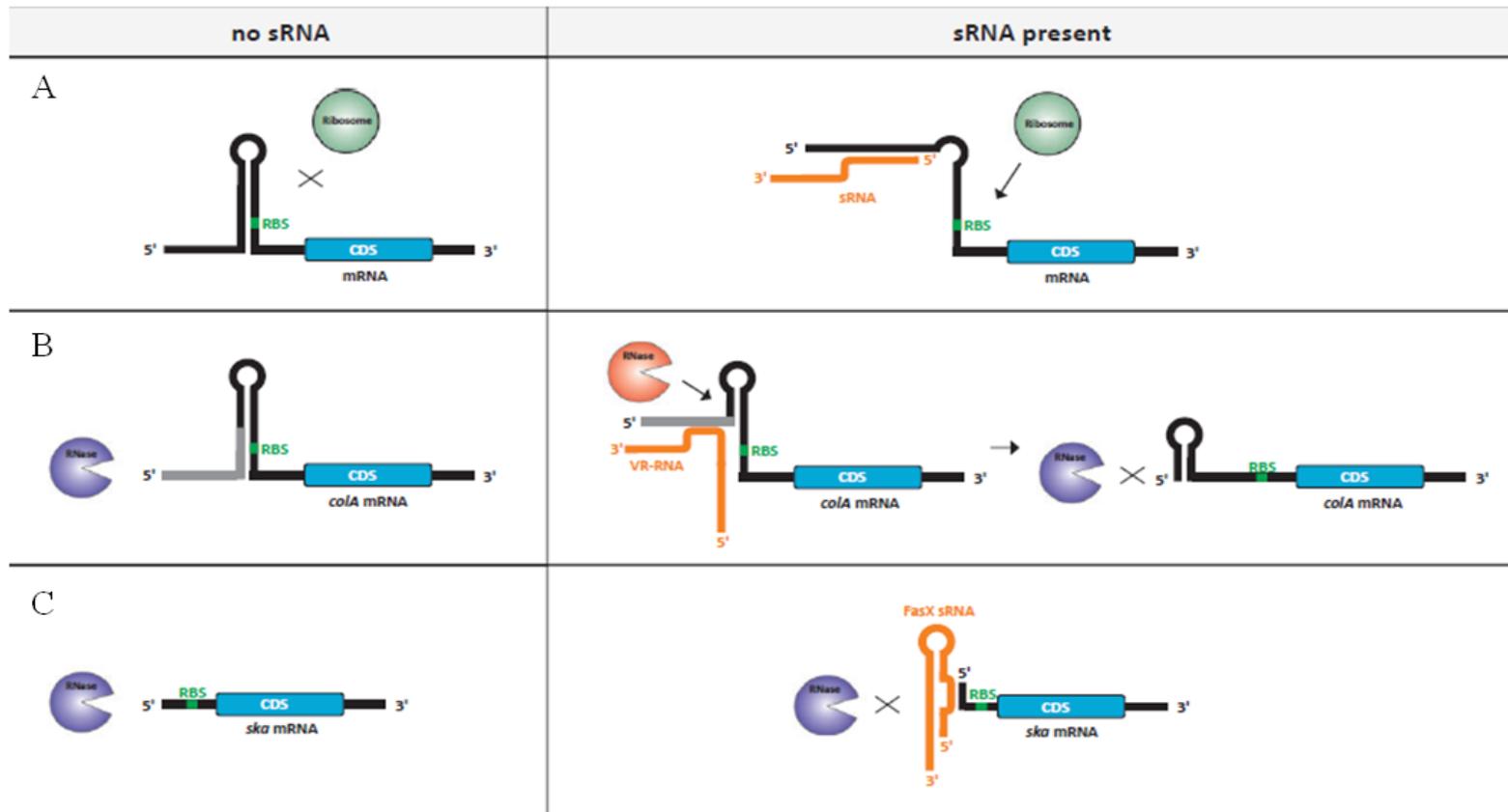


Figure 1.10 Possible positive effects of sRNA-mRNA binding. (A) anti-antisense mechanism where sRNA binding prevents formation of inhibitory structure and therefore allowing translational initiation. (B) VR-RNA binding to *colA* mRNA results in processing of the 5' end resulting in a more stable transcript. (C) FasX stabilises *ska* mRNA directly through blocking access to the ribonucleases. Figure taken from Podkaminski & Vogel (2010).

the expression of a number of proteins including SodB, bacterioferritins, aconitase and fumarase (Massé & Gottesman, 2002). As mentioned previously high iron concentration can become toxic by the production of superoxide species. Therefore it is important for bacteria to induce the oxidative stress response in high iron. Fur regulation is also important in the iron sparing response when iron availability is low. In this instance Fur can negatively regulate genes encoding non-essential iron containing proteins so that more iron is available for essential iron requiring proteins.

The expression of *sodB* was found to be positively affected by Fur, as the *sodB* mRNA was less stable in a *fur* mutant (Dubrac & Touati, 2000). Further study found this regulation to be indirect through the repression of the sRNA RyhB (Dubrac & Touati, 2002; Massé & Gottesman, 2002). RyhB acts by inducing degradation of the *sodB* mRNA therefore preventing translation of *sodB*. However when iron is present Fur acts to repress the transcription of RyhB, meaning that there is no degradation of *sodB* and so translation can occur (Massé & Gottesman, 2002). A similar mechanism is present in *N. meningitidis*, *P. aeruginosa* and *V. cholerae* which all have RyhB homologues involved in positive iron regulation (Metruccio et al., 2009; Wilderman et al., 2004; Davis et al., 2005). The binding of RyhB to *sodB* mRNA requires the action of the Hfq protein which stabilises this interaction.

1.7 What is Hfq?

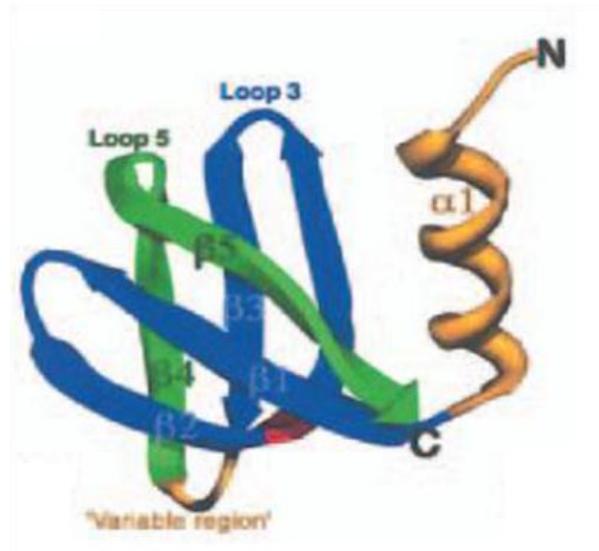
Hfq is a well characterised RNA binding protein found in most bacteria. Studies into this protein revealed its importance in sRNA-mRNA interactions for post transcriptional regulation. However the role of Hfq in regulation is broader than initially thought as Hfq can also bind mRNA alone and DNA, implicating it in transcriptional control as

well. In this section the structure and function of Hfq will be discussed in detail.

The Hfq protein was first identified in *E. coli* as a host factor required for phage Q β RNA replication. Hfq orthologues have been found in half of all sequenced Gram positive and negative bacteria. The family of Hfq proteins are thermostable and range from 70 to 110 amino acids in length. Their structure consists of an N-terminal α helix followed by five β strands forming a bent sheet (Brennan & Link, 2007). The functional protein is a hexamer and forms a circular structure with a central pore (Figure 1.11) (Zhang et al., 2002). This cyclic hexamer is formed by interactions between β -strand 4 and 5 (Brennan & Link, 2007). The three dimensional structure of Hfq from several bacteria have been resolved and show strong similarity between the bacterial species even though there can be variation in the amino acid sequence (Figure 1.12) (Sobrero & Valverde, 2012).

Structural analyses of Hfq revealed a strong similarity to eukaryotic Sm proteins. These proteins bind different RNAs and affect RNA metabolism such as splicing and mRNA decay (Møller et al., 2002). Sm proteins are characterised by Sm motifs, these are bipartite sequence motifs of highly conserved amino acid residues in a characteristic pattern of hydrophobic and hydrophilic residues. Sm proteins contain two conserved segments (Sm1 and Sm2) separated by a region of variable length (Branlant et al., 1982). It is the Sm motifs that dictate a common folding domain responsible for RNA binding. Hfq shows strong sequence similarity for a Sm1 motif which lies in the first three β - strands, but it seems to lack an obvious Sm2 motif (Brennan and Link, 2007). Sm proteins bind to U-rich sequences on RNA and it has been found that Hfq also binds similar sites. Hydroxyl radical footprinting was carried out with *E. coli* Spot42 RNA

A

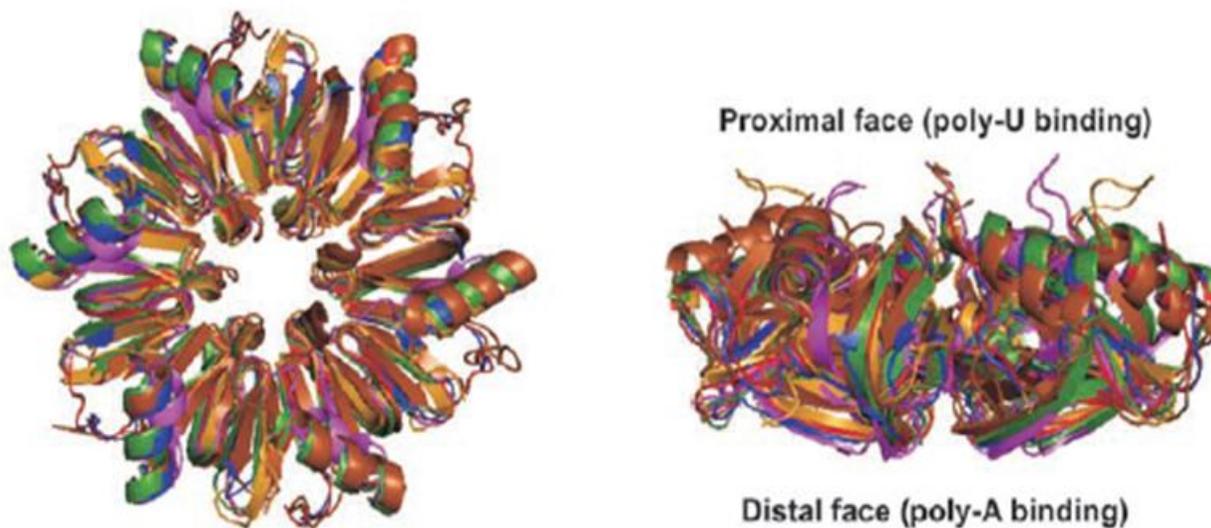


B



Figure 1.11 Structural diagrams of the Hfq protein (A) monomer and (B) hexamer. The Hfq monomer is made up of an α -helix followed by 5 β -strands forming a bent sheet. The functional hexamer forms a ring like structure with a central pore. Figure modified from Schumacher et al. (2002).

A



B

<i>Bacillus subtilis</i>	--GPLGSMKP INIQDQFLNQIRKENTYVTVFLLNGFQLRGQVKGFDNFTV-LLESEGKQQLI YKHAI ST FAPQKNVQLELE---
<i>Staphylococcus aureus</i>	-----MIANENIQDKALENFKANQTEVTVFLLNGFQMKGVIEEYDKYVV-SLNSQGKQHLYKHAI STYTVSTEGQASTESEH
<i>Escherichia coli</i>	-----MAKGQSLQDPPFLNALRRERVVPSIYLVNGIKLQGGIESFDQFVI-LLKN-TVSMVYKHAI STVV-SRPVSHHSNNAG
<i>Pseudomonas aeruginosa</i>	-----MSKGHSLQDPPYLNTRKERVVPSIYLVNGIKLQGGIESFDQFVI-LLKN-TVSMVYKHAI STVVP SRPVRLPSGDQ-
<i>Methanococcus jannaschii</i>	MNKPVKKQPKKVI PNFEYARRLNGKKVKIFLRNGEVLDAEVTGVSNYE I-MVKVGDRLNLLVFKHAIDY IEY-----
<i>Synechocystis sp.</i>	-----MSRFD SGLPSVRQVQLLIKDQT PVEIKLLTGDSLFGT IRWQTDGLGLVDDSERSTIVRLAAIAY ITPRR-----
:	: * : : * : : : . : : : . : : **

Figure 1.12 Comparison of Hfq (A) three dimension structure and (B) CLUSTAL-W sequence alignment. (A) Structural alignment of 6 Hfq homologs: *Escherichia coli*, *Staphylococcus aureus*, *Synechocystis sp.*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and the archeon *Methanococcus jannaschii*. (B) The sequence alignment was generated using the same strains. Figure taken from Sobrero & Valverde (2012).

and Hfq. The binding of Hfq led to the protection of three U-rich sequences. In particular Hfq binds to regions near structured areas of the RNA (Møller et al., 2002). The crystal structure of Hfq with an A/U oligonucleotide bound has revealed that RNA is bound in a circular unwound manner around the central core of the hexamer (Schumacher et al., 2002). The RNA binds at 6 separate but linked binding pockets which spiral around the core causing it to expand. Part of the bound oligonucleotide appeared to exit through the back of the pore, indicating that RNA can pass through the core as well as binding around it (Valentin-Hansen et al., 2004).

1.7.1 Interactions between Hfq and RNA

The predominant function found for Hfq is to enhance interactions between sRNA-mRNA. Although sRNA-mRNA interactions can occur on their own, the rate of these interactions is too slow to be biologically relevant *in vivo* and so require the action of Hfq (Vanderpool & Gottesman, 2004; Masse et al., 2005; Kawamoto et al., 2006; Soper & Woodson, 2008). Therefore Hfq can act positively or negatively depending on the action of the sRNA. As discussed previously in section 1.5.3, this can result in promoting or repressing translation or degradation (Figure 1.13A-C).

The architecture of the Hfq hexamer suggests two mechanisms by which it functions. The first is that binding of Hfq unwinds the RNA causing destabilisation of RNA structures and therefore allowing new RNA: RNA interactions (Valentin-Hansen et al., 2004). Significant changes were revealed in *sodB* mRNA in the presence of Hfq when compared to without Hfq (Geissmann & Touati, 2004). This restructuring occurred in the region of complementarity with RyhB facilitating the exposure of this sequence and allowing the formation of the Hfq-sRNA-mRNA complex (Geissmann & Touati, 2004;

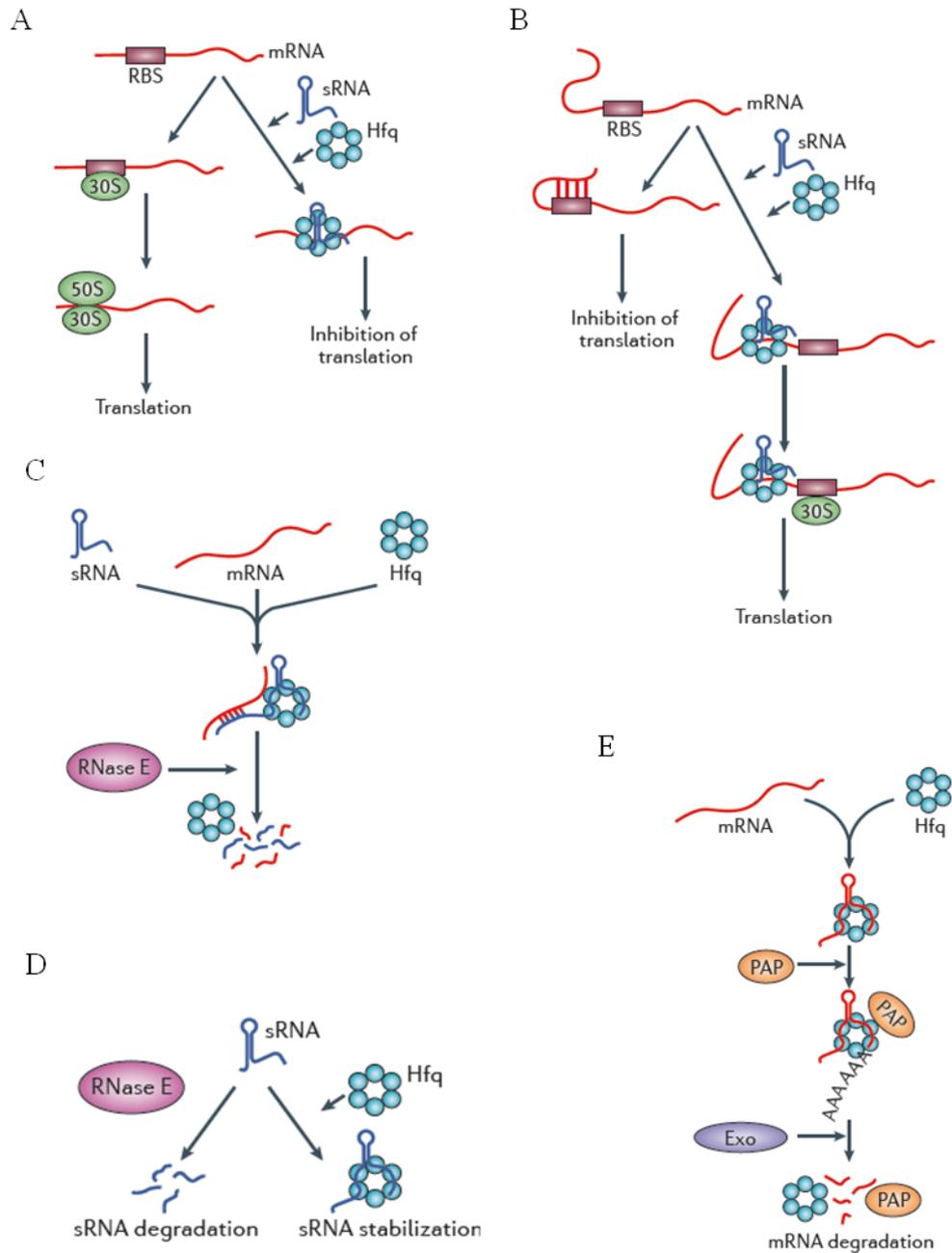


Figure 1.13 Reported modes of Hfq activity on mRNA and/or sRNA. (A) Hfq represses translation by aiding binding between sRNA and 5' mRNA preventing ribosome binding. (B) Conversely Hfq can activate translation through sRNA-mRNA binding to disrupt inhibitory secondary structures. (C) Hfq-sRNA-mRNA complex can result in degradation of the mRNA and sRNA molecules. (D) Hfq can prevent ribonuclease cleavage by RNaseE. (E) Hfq can directly destabilise mRNA through promoting poly-adenylation. Figure adapted from Vogel & Luisi (2011).

Afonyushkin et al., 2005). Another example of this mechanism is *rpoS* which is also restructured by Hfq to enhance DsrA binding (Soper et al., 2011). Although required for the initial binding, Hfq is not needed to maintain the interaction as the duplex remained intact even when Hfq was removed (Moll et al., 2003b; Zhang et al., 2002; Kawamoto et al., 2006). This provides evidence of a chaperone activity for Hfq as it is required for bringing the RNA molecules together but can then be released without affecting the formed RNA duplex.

The second is that the repetition of identical binding pockets implies that Hfq can bind more than one RNA target allowing simultaneous binding of two RNA strands enhancing interaction (Valentin-Hansen et al., 2004). The *fhlA* mRNA and OxyS sRNA have been found to simultaneously bind Hfq, as *fhlA* can be co-purified with preformed Hfq-OxyS complexes (Salim & Feig, 2010). Simultaneous binding has also been shown with *rpoS* and DsrA (Soper & Woodson, 2008). As mentioned previously binding of *rpoS* to Hfq alters structure, implying that the two mechanisms of Hfq action are not mutually exclusive.

In addition to its role in sRNA-mRNA regulation Hfq can act directly on mRNA to affect stability. RNase E recognition sites are A/U rich sequences adjacent to stem loop structures in RNA, which as mentioned previously is also the binding site for Hfq. This suggests that binding of Hfq may prevent binding of RNase E and therefore increase stability. The mRNAs, *ompA*, DsrA and RyhB all showed reduced stability in *hfq* mutant strains, whereas stability was increased in an RNaseE (*rne*) mutant suggesting Hfq protects against RNaseE cleavage (Figure 1.13.D) (Moll et al., 2003a). SsrA and SsrS, which do not bind Hfq, were used to show this interaction was specific. The SsrA

sRNA was also included as a control for RNaseE specificity, as SsrA is only degraded by RNaseE. Further evidence for this RNaseE protection was revealed in a study on *cspA* mRNA, when *in vitro* binding of Hfq to *cspA* resulted in near complete inhibition of ribonuclease cleavage by RNaseE (Hankins et al., 2010).

Conversely Hfq can directly bind mRNA to destabilise the transcripts. The addition of poly-A tails on the 3' end of mRNA by Poly-A polymerase (PAP) is an important part of the mRNA turnover pathway (Hajnsdorf et al., 1995; O'Hara et al., 1995; Mohanty & Kushner, 1999). In *hfq* mutants the level of polyadenylation of *rpoS* and *cspA* by PAP was reduced. Hfq was also found to bind the 3' end of the RNAs, suggesting that Hfq binding is required for PAP activity. The addition of poly-A tails to RNA molecules results in 3'-5' degradation by exoribonucleases, and therefore binding of Hfq stimulates mRNA decay (Figure 1.13.E) (Hajnsdorf & Régnier, 2000; Le Derout, 2003; Mohanty et al., 2004; Folichon et al., 2005; Hankins et al., 2010).

Finally, Hfq has been implicated in the correct processing of tRNA in *E. coli*. Zhang et al. (2002) identified tRNAs bound to Hfq whilst searching for sRNA-Hfq interactions. Further study confirmed that Hfq can bind tRNA at the same site as sRNA and with similar efficiencies to that of sRNA molecules (Lee & Feig, 2008). In an *hfq* mutant there is a significant decrease in translational fidelity which may be due to improper or incomplete tRNA modification. Unmodified or hypomodified tRNA molecules can result in mischarging of the tRNAs or misreading of mRNAs leading to “mutant” proteins (Lee & Feig, 2008). However it is still not known how Hfq may affect tRNA processing.

1.7.2 Interactions between Hfq and DNA

Although primarily an RNA binding protein Hfq has also been found to bind DNA (Takada et al., 1997; Updegrove et al., 2010; Geinguenaud et al., 2011). In *E. coli* Hfq was abundant in the nucleoid accounting for 24% of the total proteins bound to genomic DNA (Sobrero & Valverde, 2012). Studies into Hfq-DNA binding have shown differing Hfq binding sequences. Updegrove et al. (2010) found that DNA fragments co-purified with Hfq were predominantly those that encoded for membrane proteins and shared a common motif, (A/T)T(A/G)TGCCG. However a later study has shown that Hfq binds DNA sequences enriched in A/T which coincide with promoter sequences (Geinguenaud et al., 2011a). There are also reports that there may be a structural component to DNA binding as Hfq appears to preferentially bind DNA with a higher helical curve (Azam & Ishihama, 1999; Updegrove et al., 2010a). Although there appears to be evidence for DNA binding it is still unknown as to how Hfq and DNA interact and the effect on regulation.

1.7.3 Interactions between Hfq and proteins

Another interesting property of Hfq that could affect riboregulation is its ability to bind proteins or form large protein complexes. In *E. coli* Hfq has been found to bind around 30 proteins mainly involved in transcription, translation, RNA metabolism and protein folding (Butland et al., 2005). However it is not known if many of these interactions are direct or indirect through RNA/DNA binding. Hfq protein binding has been shown to be important in Hfq dependent degradation of mRNA. The most well studied example of direct binding is the interaction with RNaseE in the degradosome (Morita et al., 2005). Hfq can also bind poly-A polymerase (PAP) to destabilise mRNA by enhancing polyadenylation and therefore promoting degradation (Mohanty et al., 2004; Folichon et

al., 2005; Hankins et al., 2010).

Hfq-protein binding has also been implicated in regulation through interactions with the RNA polymerase (RNAP) -S1 complex and Rho. Immobilised sRNA molecules were able to precipitate Hfq along with RNA polymerase β subunit and S1 ribosomal protein from *E. coli* extracts (Windbichler et al., 2008). Further investigation by Sukhodolets & Garges (2003) found that recombinant Hfq readily binds the RNAP-S1 complex but not the RNAP core region. This was confirmed by Vecerek et al. (2010) who found that Hfq did not bind S1 alone *in vitro*. Therefore it would seem that the RNAP-S1 complex needs to form before Hfq will interact, however it is still not known what function Hfq has in this complex. Hfq can also form a stable complex with the transcriptional termination factor Rho (Butland et al., 2005; Rabhi et al., 2011).

1.7.4 Importance of Hfq in virulence

The effects of *hfq* mutants have been investigated in a wide range of Gram-negative bacteria showing pleiotropic phenotypes affecting physiology and virulence. The wide range of factors affected demonstrates the importance and global nature of Hfq regulation. A detailed list of bacteria and *hfq* mutant phenotypes was recently presented in the review by Sobrero & Valverde (2012). Table 1.2 shows those Gram negative bacteria in which an *hfq* mutant results in growth defects, reduction in stress resistances (e.g. oxidative stress, osmotic stress, temperature) and reduced colonisation and virulence *in vivo*. Although Hfq shows an important role in virulence it does not have the exact same function for each bacterium. This could be due to the different sRNAs that are present or just the different ways each pathogen colonises or causes disease. In comparison to Gram negative bacteria very few studies have been conducted with

Phenotype	Bacteria	References
Reduced growth	<i>A. baylyi</i> <i>A. caulinodons</i> <i>B. burgdorferi</i> <i>E. coli</i> <i>F. novicida</i> <i>F. tularensis</i> <i>L. pneumonophila</i> <i>M. catarrhalis</i> <i>N. gonorrhoeae</i> <i>N. meningitidis</i> <i>P. aeruginosa</i> <i>V. cholerae</i> <i>V. parahaemolyticus</i> <i>Y. pestis</i>	Kaminski et al., 1994; Tsui et al., 1994; Drepper et al., 2002; Sonnleitner et al., 2003; Ding et al., 2004; Mcnealy et al., 2005; Nakano et al., 2008; Dietrich et al., 2009; Fantappiè et al., 2009; Schilling & Gerischer, 2009; Geng et al., 2009; Meibom et al., 2009; Bai et al., 2010; Lybecker et al., 2010; Chambers & Bender, 2011
Reduced stress tolerance	<i>B. abortis</i> <i>B. cenocepacia</i> <i>E. coli</i> <i>F. tularensis</i> <i>K. pneumonia</i> <i>M. catarrhalis</i> <i>N. meningitidis</i> <i>P. aeruginosa</i> <i>R. sphaeroides</i> <i>V. alginolyticus</i> <i>Y. pestis</i>	Robertson & Roop, 1999; Sonnleitner et al., 2003; Attia et al., 2008; Nakano et al., 2008; Kulesus et al., 2008; Fantappiè et al., 2009; Hansen & Kaper, 2009; Meibom et al., 2009; Geng et al., 2009; Mellin et al., 2010; Berghoff et al., 2011; Ramos et al., 2011; Chambers & Bender, 2011; Chiang et al., 2011; Liu et al., 2011
Attenuated colonisation and virulence	<i>B. burgdorferi</i> <i>B. cenocepacia</i> <i>B. cepacia</i> <i>E. coli</i> <i>F. tularensis</i> <i>K. pneumonia</i> <i>L. pneumonophila</i> <i>N. gonorrhoeae</i> <i>N. meningitidis</i> <i>P. aeruginosa</i> <i>S. enterica</i> serovar <i>Typhirium</i> <i>S. sonnei</i> <i>V. alginolyticus</i> <i>V. cholera</i> <i>Y. pestis</i> <i>Y. pseudotuberculosis</i>	Sonnleitner et al., 2003; Ding et al., 2004; Mcnealy et al., 2005; Sittka et al., 2007; Kulesus et al., 2008; Fantappiè et al., 2009; Dietrich et al., 2009; Mitobe et al., 2009; Meibom et al., 2009; Geng et al., 2009; Lybecker et al., 2010; Schiano et al., 2010; Sousa et al., 2010; Chiang et al., 2011; Ramos et al., 2011; Liu et al., 2011

Table 1.2 Gram negative bacteria in which *hfq* mutants cause defects in growth, stress resistance and virulence.

Gram-positive bacteria and therefore the role of Hfq in regulation is less understood. The requirement of Hfq for growth and gene expression makes it an important protein to study further to understand virulence.

1.8 Hfq and sRNA regulation in *S. aureus*

The presence of sRNA molecules in *S. aureus* was first demonstrated by Pichon & Felden (2005) who identified 12 sRNA molecules present in the *S. aureus* genome. Further investigation has revealed there are about 100 sRNA in the *S. aureus* genomes, similar to the numbers found in *E. coli* indicating that they are as important in *S. aureus* (Geissmann et al., 2009; Abu-Qatouseh et al., 2010; Bohn et al., 2010; Anderson et al., 2006; Roberts et al., 2006). The sRNAs identified can be found in the core genome but also in mobile accessory elements such as pathogenicity islands (Pichon & Felden, 2005; Abu-Qatouseh et al., 2010). Those sRNA on mobile genetic elements, such as SprA-G, may suggest that they are important for virulence factor regulation. It also raises the question as to whether the presence of some sRNA differs between strains and does this contribute to strain variation in regulation.

Although many sRNA have been identified, the function of only a few of these sRNAs has been shown. One of the first sRNA identified was RNAlII, the main regulatory molecule for the Agr system and one of the largest sRNA at 514nt (Pichon & Felden, 2005; Benito et al., 2000). As discussed in Chapter 1.3.3 this sRNA has been found to regulate mRNA translation and stability of a number of virulence factors. The SprD sRNA, encoded on a pathogenicity island, also affects virulence regulation. SprD was shown to regulate Sbi, a protein which interferes with the host's innate immune response (Chabelskaya et al., 2010). The expression of the Rsa family of sRNA is

induced by osmotic stress, oxidative stress and acidic pH, indicating they have roles in stress resistance (Geissmann et al., 2009). Investigation into RsaE suggests this sRNA has a role in the regulation of primary metabolism through interaction with *opp3A* mRNA. The *opp* operons encode ABC transporters involved with the uptake of small peptides, with *opp3* dedicated to nitrogen nutrition. In both of these cases the SprD and RsaE sRNA bind the 5' end of the mRNA occluding the RBS and preventing translation initiation (Geissmann et al., 2009; Chabelskaya et al., 2010). These examples show that *S. aureus* sRNA are involved with important processes such as metabolism and virulence.

The *S. aureus* genome contains an *hfq* gene encoding an Hfq protein whose crystal structure has been determined. The full length protein consists of 77 residues and the structure shows the same important motifs as seen in Hfq proteins from other bacteria. The C-terminus of the *S. aureus* is shorter than most Gram-negative Hfq proteins, but as the C-terminus is not thought to be involved with RNA binding, the smaller size should not affect RNA binding function. Crystallisation conducted with A/U rich ribonucleotide confirmed that the *S. aureus* Hfq protein bound RNA molecules in a similar manner to other Hfq proteins, with the RNA oligo being bound in a circular fashion around the central core (Schumacher et al., 2002).

However several groups have shown that unlike other bacteria, the *S. aureus* Hfq is not required for sRNA-mRNA interactions. The sRNA RNAIII is an important regulator of virulence and therefore the role of Hfq in this regulation has been studied. Huntzinger et al. (2005) found that RNAIII immunoprecipitated with Hfq from cell lysate. This binding was then confirmed using band shift experiments along with *spa* mRNA which

showed a lower affinity for Hfq than RNAIII. However these experiments showed that Hfq was not required for the formation of the RNAIII-*spa* complex. Further study supported these findings as Hfq was not required for RNAIII interaction between *rot* or SA1000 mRNA. In addition, the steady state levels of several mRNAs, including *rot*, and RNAIII were not affected in a *hfq* mutant indicating that Hfq is not needed for RNA stability (Geisinger et al., 2006; Boisset et al., 2007). This has also been shown with other sRNA molecules indicating that it is not just RNAIII that does not require Hfq. The binding and subsequent regulation of *sbi*, an immune evasion molecule, by the sRNA SprD was shown to occur in the absence of Hfq (Chabelskaya et al., 2010). Furthermore the steady state levels of the 11 Rsa sRNAs were also unaffected in a *hfq* mutant (Geissmann et al., 2009). Interestingly the *S. aureus hfq* gene appears to be transcribed at a much lower rate than in other bacteria (Geisinger et al., 2006; Bohn et al., 2007; Liu et al., 2010). In comparison to other bacteria the *S. aureus hfq* gene appears to be missing upstream sequences which may have included a strong *hfq* promoter (Geisinger et al., 2006). These results are remarkably different from those studies in Gram-negative bacteria and *L. monocytogenes* where Hfq is essential for sRNA-mRNA interactions. However, they do agree with studies in *Bacillus subtilis* in which Hfq is not required for sRNA stability nor sRNA-mRNA interactions (Heidrich et al., 2006; Heidrich et al., 2007). Therefore it would appear even though Hfq may still bind sRNA it is not required for sRNA-mRNA regulation and so may have a different function in *S. aureus*.

The role of Hfq in *S. aureus* virulence regulation is at the moment controversial. To date only a few papers have been published investigating phenotypes of an *hfq* mutant in *S. aureus* and they presented conflicting results. Several studies of the phenotypic analysis

of an *hfq* mutant revealed no differences in RNAPIII-regulated exoprotein expression, including haemolysins and Protein A or growth in a number of different conditions including rich and nutrient deprived media (Geisinger et al., 2006; Boisset et al., 2007; Bohn et al., 2007). Phenotype Microarray (PM) technology which can test many phenotypes simultaneously including growth using different sources of carbon, nitrogen, phosphorus and sulphur also showed no reproducible phenotypic differences between the *hfq* mutant and the wild type (Bohn et al., 2007; Boisset et al., 2007). In Bohn et al. (2007) the effect of an *hfq* null mutation on stress resistance and growth was examined. In other pathogenic bacteria Hfq has been responsible for resistance to different stresses. Under osmotic, oxidative stress and heat shock conditions the *S. aureus hfq* mutant grew as well as the wild type (Bohn et al., 2007). The lack of obvious phenotype is very unusual when compared to other bacteria which may indicate that Hfq functions differently in *S. aureus*.

Although Bohn et al. (2007) and others reported no phenotypic differences in an *hfq* mutant previous work in this laboratory did show expression changes in a *S. aureus hfq* mutant. Preliminary data in this laboratory has showed possible functions of Hfq in iron and Fur regulation. In our work the level of RNA was not only examined in the Newman strain and its isogenic *hfq* mutant strain but also *fur* and *hfq/fur* mutant strains. IsdA and IsdB are important virulence factors involved in the uptake of iron from heme (Mazmanian et al., 2003). Northern analysis with an *isdA* and *isdB* probe revealed that Hfq positively regulates expression of *isdA* and *isdB* (Figure 1.14). A more recent study by Liu et al. (2010) revealed many *hfq* mutant phenotypes similar to those seen with other bacteria. Microarray analysis revealed 116 genes with altered expression in the *hfq* mutant; 33 showing decreased expression and 83 showing increased, therefore

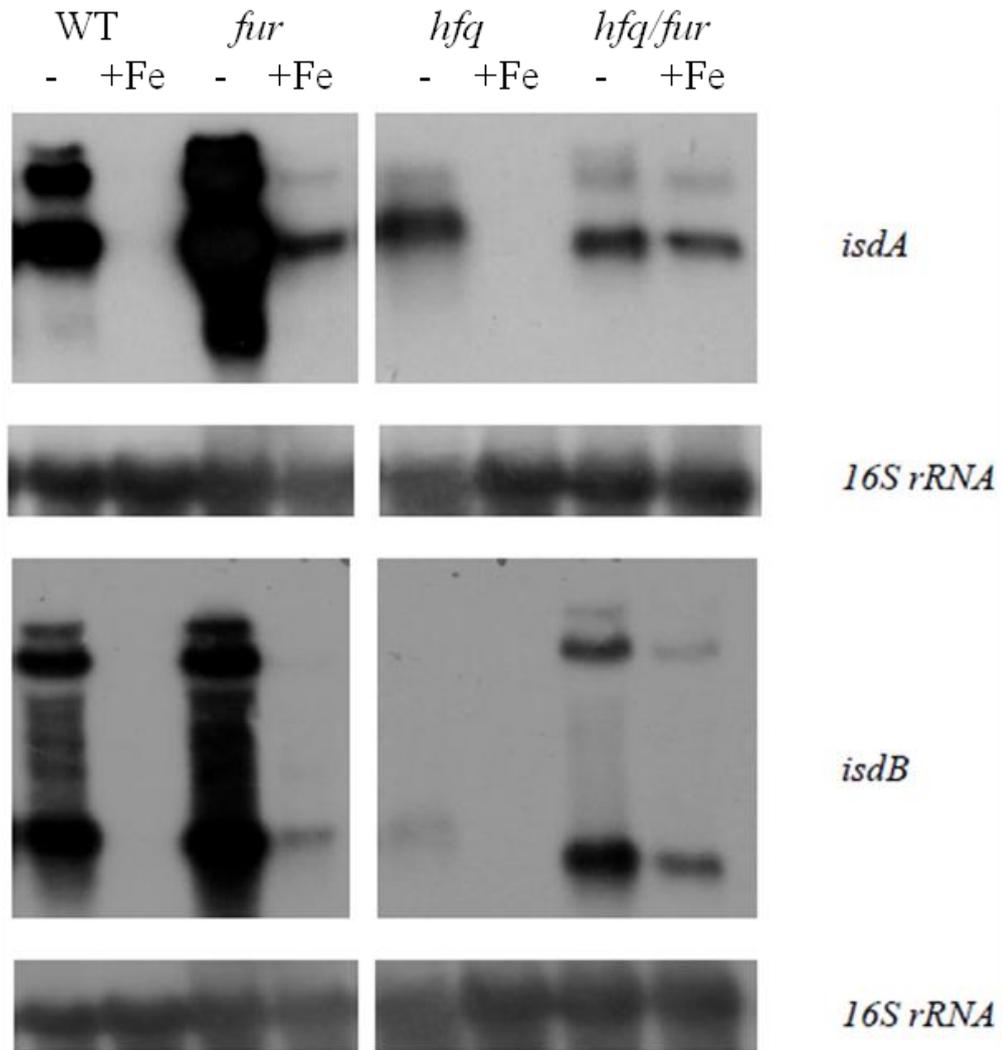


Figure 1.14 Northern blot analysis of *isdA* and *isdB* expression in Newman and its isogenic *hfq*, *fur* and *hfq/fur* mutant strains. Total RNA was extracted from 6 hour growth cultures in CRPMI \pm Fe as indicated. Blots were hybridised with DNA probes specific to *isdA* and *isdB*, stripped and reprobbed with *16S* rRNA probe as a control. Figure taken from Johnson (2008).

showing a mostly negative role as seen with other bacteria. The majority of these changes occurred with genes involved with stress resistances and pathogenicity. This was also seen phenotypically as the *hfq* mutant showed increased oxidative stress resistance but a reduction in pathogenicity. This clear difference in phenotypic results could be a result of the use of different strains between studies. Studies into Hfq protein level in Liu et al. (2010) showed that the Hfq protein is not present in some strains such as RN6390 and COL which were used in previous studies. Therefore it appears that Hfq does have a role in *S. aureus* regulation but further study is required to determine what this role is and how it differs between strains.

1.9 Aims and objectives

The acquisition of multiple antibiotic resistances make *S. aureus* infections very hard to treat. Originally a concern in the hospital setting, *S. aureus* has now been found to infect healthy individuals in the community. Therefore it is important to study *S. aureus* virulence to elucidate targets for antimicrobial development. The high number of virulence factors produced by *S. aureus* is tightly regulated and understanding how the expression of these factors is regulated could be crucial in identifying possible targets. The RNA binding protein Hfq is important for growth and virulence in many other bacteria. However in *S. aureus* the role and importance of this protein is not fully understood. The conflicting results published so far suggests that strain variation and growth conditions may heavily affect the role of Hfq.

The first aim of this study was to identify phenotypic differences in *S. aureus hfq*, *fur* and *hfq/fur* mutants to assess the role of Hfq and Fur in global regulation. In particular experiments will concentrate on protein expression, growth in different media and

resistance to different environmental stresses which have all been found to be important in virulence. The second aim of this project was to determine the mechanism of Hfq and Fur regulation. This involved investigating the role of each in transcriptional and post transcriptional regulation. In addition these experiments were carried out in a number of different strains to determine how the role of Hfq and Fur differs between them.

1.9.1 Objectives

- To determine any sequence variation of the *hfq* gene.
- To investigate phenotypes of *S. aureus hfq*, *fur* and *hfq/fur* mutants in protein expression, growth and stress resistance in different strains.
- To determine whether Hfq regulation occurs at the post transcriptional level.
- To determine whether Hfq stabilises target mRNAs.
- To investigate direct binding between Fur and the promoter of target genes.

Chapter 2 Materials and methods

2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are shown in Table 2.1 and 2.2 along with genotype and reference. Plasmid maps can be found in Appendix Figure A-1. The *hfq* mutant was constructed by the insertion of a kanamycin resistance cassette, contained a promoter but no terminator, in the *HindIII* site 132bp downstream of the start of the *hfq* gene. The mutation was sequenced before being transduced into test strains.

2.2 Bacterial storage and growth conditions

Bacteria were stored at -80°C in TSB with 20% (v/v) glycerol in a 1.5 ml screw capped tube. Unless otherwise stated all strains were plated onto Luria agar plates and incubated overnight at 37°C. Liquid cultures were produced in Luria broth and incubated shaking at 37°C. For iron restrictive conditions strains were streaked out on to horse blood agar and liquid cultures were produced in CRPMI (chelexed RPMI-1640 medium) with 10% (v/v) RPMI-1640 and grown statically at 37°C in 5% CO₂.

2.3 Growth media and supplements

All media were made up with dH₂O and sterilised before use by autoclaving or filter sterilisation. Autoclaved media were heated to 120°C at 15 pSI for 15 minutes. When filter sterilising large volumes (>50 ml) a Stericup vacuum driven disposable filtration system (Millipore) with a 0.22µm pore size was used. For smaller volumes (<50 ml) plastic syringes with 0.2µm Acrodisc membranes attached were used.

Table 2.1 Bacterial strains used in this study

Strain	Genotype	Reference
<i>S. aureus</i>		
BB	Bovine isolate	Dr. Alan Cockayne
C01865	Bovine isolate	Sung et al., 2008
C00595	Bovine isolate	Sung et al., 2008
C01719	Bovine isolate	Sung et al., 2008
C01771	Bovine isolate	Sung et al., 2008
Mn8	Clinical MSSA isolate	Schlievert & Blomster, 1983
Mn8 Δhfq	$\Delta hfq::kan$	This work
Mn8 Δfur	$\Delta fur::tet$	This work
Mn8 $\Delta hfq/\Delta fur$	$\Delta hfq::kan; \Delta fur::tet$	This work
MRSA PM64	MRSA252 clonal variant	Moore & Lindsay, 2002
MRSA-15 (PM25)	MRSA endemic isolate	Dr. Jodi Lindsay
Newman	Clinical MSSA isolate	Duthie & Lorenz, 1952
Newman Δhfq	$\Delta hfq::kan$	Dr. Julie Morrissey
Newman Δfur	$\Delta fur::tet$	Johnson et al., 2005
Newman $\Delta hfq/\Delta fur$	$\Delta hfq::kan; \Delta fur::tet$	Dr. Julie Morrissey
RF122	Bovine isolate	Herron et al., 2002
RN4220	Restriction deficient 8325	Laboratory stock
SH1000	8325-4 with <i>rsbU</i> mutation repaired	Horsburgh et al., 2002
SH1000 Δhfq	$\Delta hfq::kan$	This work
SH1000 Δfur	$\Delta fur::tet$	This work
SH1000 $\Delta hfq/\Delta fur$	$\Delta hfq::kan; \Delta fur::tet$	This work
38963	Bovine isolate	Sung et al., 2008
8325	NTCC8325	Novick, 1967
8325 $\Delta hfq1$	$\Delta hfq::kan$	This work
8325 $\Delta hfq2$	$\Delta hfq::kan$	This work

8325-4	8325 cured of prophages	Horsburgh et al., 2001a
8325-4 Δhfq	$\Delta hfq::kan$	Dr. Julie Morrissey
8325-4 Δfur	$\Delta fur::tet$	Horsburgh et al., 2001b
8325-4 $\Delta hfq/\Delta fur$	$\Delta hfq::kan; \Delta fur::tet$	Dr. Julie Morrissey
<i>E. coli</i>		
TOPO 10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsd RMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M/5 Δ lac-X74 <i>deoR</i> <i>recAI araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
Rosetta (DE3) pLysS	F ⁻ <i>ompT hsdS_B</i> (R _B ⁻ m _B ⁻) <i>gal dcm</i> λ (DE3 [<i>lacI lacUV5-T7 gene 1 ind1</i> <i>sam7 nin5</i>]) pLysSRARE	Novagen

Table 2.2 Plasmids used in this study

Plasmid	Description	Reference
pEAP-gfplux	<i>eap</i> translational reporter	Harraghy et al., 2005
pEAPs-gfplux	<i>eap</i> transcriptional reporter	This work
pCOLD1	<i>E. coli</i> based protein expression vector, induced by cold shock	Takara Bio Inc
pCOLD1- <i>hfq</i>	pCOLD1 containing the ORF for Hfq from Newman	This work
pCOLD1- <i>hfq</i> *	pCOLD1 containing the ORF for Hfq from Mn8	This work
pLEICS01	<i>E. coli</i> based protein expression vector, induced by IPTG	Protex, University of Leicester
pLEICS01- <i>fur</i>	pLEICS01 containing the ORF of Fur from Newman	This work

2.3.1 Luria-Bertani (LB) medium

LB broth contained 1% (w/v) Tryptone (Oxoid), 0.5% yeast extract and 0.5% NaCl dissolved in 400 ml dH₂O. The pH was corrected to 7.5 and autoclaved. For Luria-Bertani agar (LA) 1.5% Bioagar was added before being autoclaved.

2.3.2 LK broth and LK agar

LK media was made as described with LB however 0.7% KCl was added in place of NaCl.

2.3.3 Trypticase soy broth (TSB)

To make TSB 3% BBL Trypticase Soy Broth (BD Diagnostics Systems) was dissolved in 400 ml dH₂O and autoclaved.

2.3.4 Chelexed RPMI (CRPMI)

To a 500 ml bottle of RPMI-1640 (Sigma Ltd) 6% Chelex 100 (Sigma Ltd) was added and stirred overnight before being filter sterilised. Sterile CRPMI was stored at 4°C.

2.3.5 SMMP50 medium

Stock SMM buffer was made by adding 34.2% sucrose, 0.464% maleic acid and 0.38% MgCl₂ to dH₂O. The pH was corrected to 6.5 before being autoclaved. To make 20 ml SMMP50; 11 ml SMM buffer, 8 ml Pennesay broth (Difco antibiotic medium 3, BD) and 1 ml 10% (w/v) BSA were added together and then filter sterilised. The sterilised SMMP50 medium was stored at 4°C.

2.3.6 Brain Heart Infusion (BHI) broth

For BHI 3.7% (w/v) Brain Heart Infusion broth powder (Oxoid) was dissolved in dH₂O and autoclaved.

2.3.7 Blood agar

Blood agar was produced by dissolving 4% Blood agar base (Oxoid) powder in dH₂O before being autoclaved. The agar was allowed to cool to 60°C before 6% horse or rabbit blood was added. For sheep blood agar the agar base was replaced with Sheep blood agar base (Oxoid) and made as before.

2. 3.8 Antibiotic and metal supplements

As required Tetracycline (Tet), Erythromycin (Ery) and Chloramphenicol (Cm) were added to media at a final concentration of 10 µg/ml. Kanomycin (Kan) was added to 50 µg/ml and Ampicillin (Amp) to 100 µg/ml. These concentrations were used for both *S. aureus* and *E. coli* strains. For iron replete conditions 50 µM FeSO₄ was added to the media.

2.4 DNA preparations and manipulations

2.4.1 Staphylococcal chromosomal DNA extraction (Ausubel et al., 1995)

5ml of LB were inoculated with the desired staphylococcal strain and incubated shaking overnight at 37°C. Cells were harvested from 1.5 ml of culture by centrifugation at 13,000 rpm for 5 minutes. The resulting pellet was re-suspended in 250 µl of P1 buffer (QIAGEN DNeasy Blood & Tissue Kit) with 100µg/ml lysostaphin added. The cells were then incubated at 37°C until they had lysed and the mixture was clear and viscous. 2.5 µl of proteinase K (20 mg/ml) was added and mixed well before adding 27 µl of

10% (w/v) SDS. This was then incubated at 37°C for 20-30 minutes. 97 µl of NaCl (5 M) and 81 µl of preheated CTAB (cetyl trimethyl ammonium bromide) were added, mixed by inverting the tube and incubated at 65°C for 20 minutes. An equal volume of 24:1 chloroform:isoamylalcohol was added and then centrifuged at 10,000 rpm for 10 minutes. The aqueous upper layer was transferred to a new microcentrifuge tube before adding an equal volume of isopropyl-alcohol (IPA). The DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes. The samples were allowed to dry at room temperature over night and then re-suspended in 100 µl of sterile dH₂O.

2.4.2 Plasmid DNA preparation

5 ml of LB was inoculated and incubated shaking at 37°C overnight. Extraction was then carried out using the e.Z.N.A. Plasmid Mini Prep Kit I (Omega Bio-tek) following the protocol provided. For extraction from *S. aureus* cells, 100 µg/ml lysostaphin was added to Solution I and incubated at 37°C until the cells had fully lysed.

2.4.3 Gel electrophoresis

Agarose (Lonza) was added to 1xTAE (Tris-Acetate-EDTA) buffer to give a 1% (w/v) gel. The agarose was dissolved by heating and 5 µl ethidium bromide (10 mg/ml) added to every 100 ml of TAE. The melted agarose was kept at 55°C until needed. Samples were run alongside Hyperladder I (Bioline). Gels were run at 80V until the samples had moved a suitable distance down the gel. DNA samples on the gel were visualised using a UV transilluminator (Gene Genius Bio Imaging System, Syngene).

2.4.4 Restriction digests

Restriction digests were carried out in a final volume of either 30 µl or 50 µl depending

on the DNA to be digested. Digestion mixes were made up with 10% (v/v) buffer, 1% (v/v) BSA, a maximum of 10% (v/v) restriction enzyme and DNA to be digested (volume depends on concentration). dH₂O was then added to make up to the final volume. The PCR and plasmid digests were incubated at 37°C for 1-1.5 hours and 1.5-2 hours respectively.

2.4.5 Gel extraction

DNA samples were separated by gel electrophoresis, excised and purified using the Zymogen Gel Extraction kit. The manufacturers protocol was followed except that samples were centrifuged a second time after removal of the wash buffer to ensure removal of all alcohol. DNA samples were eluted in 15 µl 65°C, sterile dH₂O and left at room temperature for 1 minute before being centrifuged.

2.4.6 Ligations

Restriction digests were separated by gel electrophoresis and the required fragments extracted and purified using the Zymogen Gel Extraction kit. Ligation reactions were carried out at a 3:1 ratio of insert:vector. To the DNA samples 3 µl of T4 DNA ligase buffer and 1 µl T4 DNA ligase were added and the final volume made up to 30 µl using dH₂O. Ligation reactions were incubated overnight at 16°C.

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

This paper reported a new *in vitro* DNA recombinant method to simply combine long pieces of DNA in a single isothermal reaction (Figure 2.1). The DNA fragments are amplified with primers designed with specific overhangs complimentary to that of the next DNA piece to be joined. All the fragments are mixed with an enzyme mix of T5

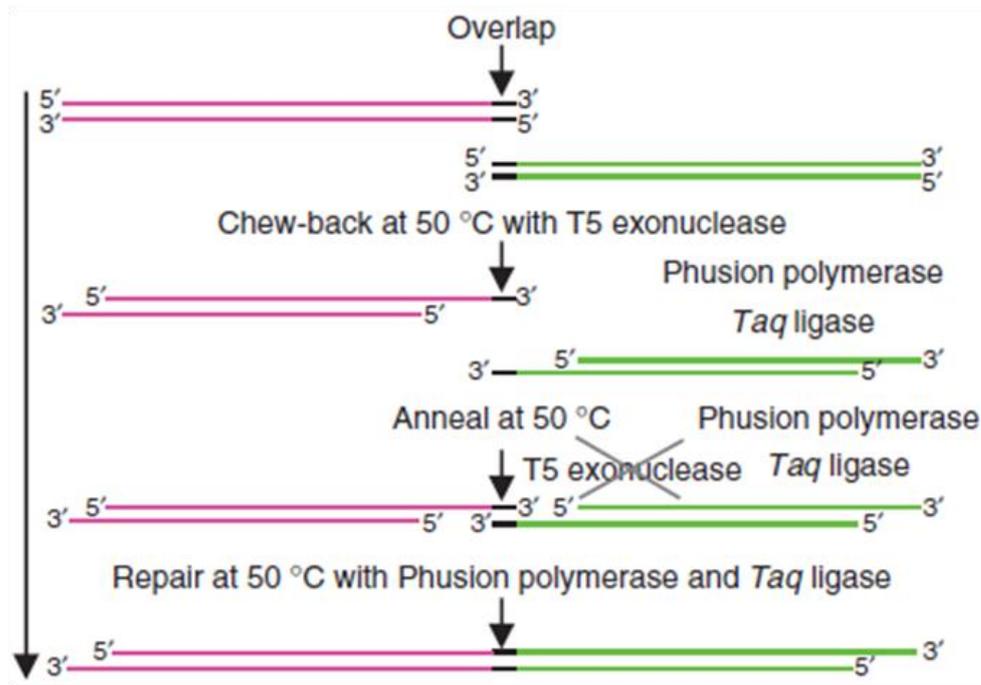


Figure 2.1 Schematic diagram depicting the stages of the isothermal reaction. Two DNA fragments (magenta and green) sharing terminal sequence overlaps are amplified. T5 exonuclease removes nucleotides from the 5' ends making complementary single-stranded overhangs, which then anneal. The T5 exonuclease is heat-labile and gets inactivated during the incubation. Phusion polymerase fills in the gaps between the fragments and *Taq* DNA ligase seals the nicks. Figure taken from Gibson et al. (2009).

exonuclease, polymerase and ligase. When incubated together the exonuclease starts digesting the 5' ends of the DNA fragments revealing the complimentary overlaps which then anneal. The DNA polymerase fills in the gaps between the annealed fragments and the ligase seals the nicks. The T5 exonuclease is heat labile and so activity does not continue to digest the fragments for long.

The DNA fragments to be joined together were amplified using the high fidelity polymerase Phusion (NEB). The reaction mix consisted of 4 µl 5x Phusion Buffer, 0.4 µl 10 mM dNTPs, 1 µl of each primer (10 µM stock), 2.5 µl DNA template and 0.2 µl Phusion. Reactions were used in the following PCR programme: 98°C for 30 seconds followed by 25 cycles of 98°C for 10 seconds, 50°C for 30 seconds, 72°C for 3 minutes before a final 5 minutes at 72°C. PCR fragments produced from plasmid DNA were digested with the restriction enzyme DpnI to digest the plasmid template. All fragments were cleaned via gel extraction and samples run on an agarose gel to determine concentration.

The isothermic reaction buffer (5x IRB) was made up of 25% PEG-8000, 500 mM Tris-HCl (pH 7.5), 50 mM MgCl₂, 50 mM DTT, 1 mM of each dNTP, 5 mM NAD in dH₂O. To 8 µl 5x IRB 0.8 µl T5 exonuclease (0.2 U/µl), 4 µl Taq ligase (40U/µl) and 0.5 µl Phusion (2U/µl) were added and made up to 40 µl with dH₂O. A 15 µl aliquot of this enzyme mix was added to 5 µl of DNA (containing all DNA fragments) and incubated at 50°C for 60 minutes.

2.5 PCR, primers and sequencing

2.5.1 Primers used in this study

Primers used in this study are listed in Table 2.4 and contain sequences and when the primer was used.

2.5.2 PCR recipe

A standard PCR reaction mix consisted of 5 µl of FailSafe PCR 2x Premix D (Epicentre Biotechnologies), 0.5 µl (2.5U) of Kapa Taq polymerase (Kapa Biosystems), 1µl of each primer (10 µM stock) and 2.5 µl of chromosomal or plasmid DNA. The reaction was made up to 10 µl with dH₂O. For products that were used for cloning the Kapa Taq polymerase was replaced with Bio-X-act DNA polymerase (bioline). This enzyme has 3'-5' proof reading activity to significantly reduce the error rate of PCR reactions.

2.5.3 PCR standard programme

DNA was initially denatured at 97°C for 2 minutes, followed by 30 cycles of a denaturing step at 97°C for 30 seconds, a 30 second annealing step at T_m°C (depending on primer), and a 30 second elongation step at 72°C. This was followed by a final elongation step of 72°C for 1 minute. This programme was used for amplicons up to 1kb in length, for larger amplicons the elongation step was increased by 30 seconds for every 1kb size increase.

2.5.4 Colony PCR

A colony PCR reaction mix was the same as the standard except that 2.5 µl dH₂O was used in place of DNA. A single *E. coli* colony was picked from an agar plate using a

Table 2.3 Primers used in this study

Primer name	Sequence 5'-3' (restriction sites)	Application
hfqB	CTCGCGAAGCGTATCAATG	<i>hfq</i> flanking primer
hfqSE	CATCTTGAGCATGTACACC	<i>hfq</i> flanking primer
hfqF	CGACAAATGACATGGTTC	<i>hfq</i> sequencing
hfqR	GTCGTATCATATATTGACTCG	<i>hfq</i> sequencing
pCOLD hfqF	GCGGGATCCATGATTGCAAACGAAAACAT	pCOLD/ <i>hfq</i> construction
pCOLD hfqR	GCGTCTAGATTATTCTTCACTTTCAGTAG	pCOLD/ <i>hfq</i> construction
pCOLDF	ACGCCATATCGCCGAAAGG	pCOLD/ <i>hfq</i> construction
pCOLDR	GGCAGGGATCTTAGATTCTG	pCOLD/ <i>hfq</i> construction
pGFPlux IF eap	AAGTCTGGCTATAATTAAGTCCCGGGTTAACAAGGAG	pEaps-gfplux reporter construction
pGFPlux IR	TTCGTAAC TTTCCAGCCGCCTCCG	pEaps-gfplux reporter construction
pGFPlux IIF	GCGGCTGGAAAAGTTACGAAAAAGA	pEaps-gfplux reporter construction
pGFPlux IIR eap	AAGGAGTGATAATGATGGTGCTGGCCGTCGTTTTACAA	pEaps-gfplux reporter construction
eap GFPlux F	CGTTGTAAAACGACGGCCACCATCATTATCACTCCT	pEaps-gfplux reporter construction
eap GFPlux R	TTCCTCCTTGTTAACCCGGGACTTAATTATAGCCAGACTT	pEaps-gfplux reporter construction
fur pLEICS F	TACTTCCAATCCTTGGAAGAACGATTAATCGCGTTAA	pLEICS- <i>fur</i> expression vector construction
fur pLEICS R	TATCCACCTTACTGCTATCCTTTACCTTTAGCTTGGCA	pLEICS- <i>fur</i> expression vector construction
fhuC promF	ATGGTATGAGCACACATAC	<i>fhuC</i> promoter for EMSA
fhuC promR	AATTTCCCTACTTTCAATAAA	<i>fhuC</i> promoter for EMSA
eap EMSAF	CACCATCATTATCACTCC	<i>eap</i> promoter for EMSA
eap EMSAR	AAATTATCTCTCCTTTTTTG	<i>eap</i> promoter for EMSA
saeP1 F	TTGGTACTTGTATTTAATCGTCTATC	<i>saeP1</i> promoter for EMSA
saeP1R	GTTGTGATAACAGCACCAGCTGC	<i>saeP1</i> promoter for EMSA
saeP3s F	GAGTGGTATAAGTGGTTTTTCG	<i>saeP3</i> promoter for EMSA
saeP3 R	TTCACCTCTGTTCTTACGACCTC	<i>saeP3</i> promoter for EMSA

Table 2.3 continued.

gyrRT F	GACTGATGCCGATGTGGA	<i>gyr</i> qRT-PCR (Harraghy et al., 2005)
gyrRT R	AACGGTGGCTGTGCAATA	<i>gyr</i> qRT-PCR (Harraghy et al., 2005)
eapRT F	AAGCGTCTGCCGCAGCTA	<i>eap</i> qRT-PCR (Harraghy et al., 2005)
eapRT R	TGCATATGGAACATGGACTTTAGAA	<i>eap</i> qRT-PCR (Harraghy et al., 2005)
saePRT F	CAAATCATCAAAAGGTCCAGA	<i>saeP</i> qRT-PCR
saePRT R	CCATTGCGATTTCTTTACCG	<i>saeP</i> qRT-PCR
saeRT F	AAACTTGCTTGATAATGCGCTAAA	<i>saeS</i> qRT-PCR (Harraghy et al., 2005)
saeRT R	GTTCTGGTATAATGCCAATACCTTCA	<i>saeS</i> qRT-PCR (Harraghy et al., 2005)
16s RT F	ACGGTCTTGCTGTCACTTATT	16s rRNA qRT-PCR (M. Wada et al., 2010)
16s RT R	TACACATATGTTCTTCCCTAATAA	16s rRNA qRT-PCR (M. Wada et al., 2010)

sterile pipette tip, patched onto fresh agar and added directly to the PCR mix. A 10 minute 97°C incubation was added to the beginning of the PCR programme to lyse the cells. The patch agar plate was incubated at 37°C overnight to produce a stock of every colony tested.

2.5.5 DNA sequencing

Each sequencing reaction was made up of 4 µl diluted Big Dye (1.75 µl dH₂O, 1.75 µl 5x sequencing buffer, 0.5 µl Big Dye V3.1(Applied Biosystems)), 1µl of 3.2 µM sequencing primer and DNA template (200 ng plasmid DNA, 20-40 ng PCR product). The reaction was made up to 10 µl using dH₂O. The reactions were run on the following PCR programme. An initial denaturing step of 10 seconds at 96°C, followed by 29 cycles of 10 seconds at 96°C, 10 seconds at 50°C, 4 minutes at 60°C, and a final elongation step of 10 minutes at 60°C. To clean the reactions SDS was added to a final concentration of 0.2% and heated to 98°C for 5 minutes. This was allowed to cool to room temperature before being purified using a Performa DTR Gel Filtration cartridge (Edge BioSystems). Sequencing gels and analysis was carried out by PNAACL (Protein Nucleic Acid Chemistry Laboratory, University of Leicester) using an Applied BioSystems 3730 sequencer.

2.6 Transformations and transductions

2.6.1 Electrocompetent *E. coli*

5 ml of LB were inoculated with *E. coli* TOPO10 and incubated overnight shaking at 37°C. 1 ml of this culture was transferred to 100 ml pre-warmed LB and incubated shaking at 37°C until it reached an OD₆₀₀ of 0.5. The culture was then rapidly chilled on ice and centrifuged at 4°C to pellet cells. The pellet was re-suspended in 10 ml of ice

cold dH₂O, and centrifuged at 4°C. The step was repeated and the cells re-suspended in 50 ml ice cold 10% (v/v) glycerol. The cells were again pelleted at 4°C and re-suspended in 5 ml ice cold 10% (v/v) glycerol. After a final centrifugation at 4°C the cells were re-suspended in 0.5 ml ice cold 10% (v/v) glycerol and stored at -80°C.

2.6.2 *E. coli* transformation with plasmid DNA

Plasmid DNA was dialysed for 30 minutes on a 0.02 µm 13 mm nitrocellulose disc (Millipore) floating on dH₂O in a petri dish. The dialysed DNA was then added to a 50 µl aliquot of competent cells and kept on ice. This mixture was then transferred to a 2mm electroporation cuvette (Geneflow) and given a pulse using a Biorad gene pulser at 1.5V, 200 Ω and 25 µF. 1 ml of LB was added immediately after and the cells transferred to a 15 ml Sterilin tube and incubated shaking at 37°C for 1 hour. 100 µl of culture were plated on replica LA plates containing antibiotics. 100 µl of each transformation was also plated out on LA to show that the *E. coli* cells survived electroporation.

2.6.3 Electrocompetent *S. aureus* cells

5 ml of TSB was inoculated with RN4220 and incubated overnight. The culture was diluted 1 in 50 into fresh TSB and incubated to an OD₆₀₀ of 0.4. Cells were pelleted at 4000 rpm for 10 minutes then resuspended in equal volume of ice cold 0.5M sucrose. This wash was repeated resuspending cells in half the volume of sucrose and incubated on ice for 30 minutes. After one final centrifugation step the cells were resuspended in 500 µl sucrose and stored at -80°C in 40 µl aliquots.

2.6.4 *S. aureus* transformation with plasmid DNA

Plasmid DNA was dialysed as described above and added to an aliquot of electrocompetent cells. The solution was incubated at room temperature for 30 minutes before being electroporated as before. Following this, 900 µl SMMP50 media was added and the solution transferred to a 15 ml Sterilin tube and incubated at 37°C for 3 hours. 100 µl of the transformation were spread onto replica LA plates containing selective antibiotics. A negative control was carried out with no DNA added to the cells.

2.6.5 *S. aureus* phage lysate production

The donor strain was used to inoculate 5 ml of LB and incubated at 37°C overnight. This was subcultured into 25 ml of fresh media to an OD₆₀₀ of 0.05. To this, 0.2 ml of 1M CaCl₂ and 1M MgSO₄ were added before being incubated at 37°C until an OD₆₀₀ of 0.2 was reached. 10 ml of this culture was subcultured into 25 ml fresh LB with the added salts. 1 ml of phage phi11 or phi85 was added and the culture incubated at 37°C for at least 4 hours until full lysis had occurred. Cell debris was pelleted by centrifugation at 4000 rpm for 10 minutes and the supernatant filter sterilised before being stored at 4°C. The phage lysate titre was checked by carrying out serial 10-fold dilution and spotting 10 µl of each onto a lawned plate of donor bacteria.

2.6.6 *S. aureus* phage transduction

The recipient strain was used to inoculate 20 ml of LK broth and incubated at 37°C overnight. The cells were pelleted by centrifugation at 4000 rpm for 5 minutes and resuspended in 1 ml of LK. A reaction tube and control were set up for each transduction.

Sample	Control
500 µl cells	500µl cells
1 ml LK + 10mM CaCl ₂	1.5 ml LK + 10 mM CaCl ₂
500 µl donor phage lysate	

Solutions were incubated in a 37°C water bath for 25 minutes then shaking at 37°C for 15 minutes. 1ml ice cold 1M Na citrate was added to each reaction before being centrifuged at 4000 rpm for 10 minutes. The pellets were resuspended in 1m ice cold 1M Na citrate and incubated on ice for 2 hours. 100 µl of each transduction were plated onto several replica LK plates containing 0.05% Na citrate and selective antibiotic. Plates were incubated at 37°C for 12-72 hours.

2.7 Growth assays

2.7.1 Growth curves taking hourly time points

Strains were grown overnight in 10 ml of CRPMI and then sub-cultured into 25 ml CRPMI to an OD₆₀₀ of 0.05. The required supplements (50 µM FeSO₄, 50 µM CuCl₂ or 1 M NaCl) were added and the cultures incubated at 37°C in 5% CO₂. The OD₆₀₀ of 1 ml of culture was measured at intervals of 1 hour for a total of 9 hours; a final reading was also taken at 24 hours.

2.7.2 Oxidative stress growth curves

For growth assays carried out in TSB strains were grown overnight in 10 ml of TSB and incubated shaking at 37°C. These cultures were then sub-cultured into fresh TSB to an OD₆₀₀ of 0.05 and H₂O₂ (0.0024%) was added if required. The OD₆₀₀ of 1 ml of culture was measured at intervals of 1 hour for a total of 7 hours. Percentage growth

was calculated to account for growth differences in TSB. To calculate this, the initial OD₆₀₀ reading was subtracted from all the time point readings. This value for the +H₂O₂ samples was divided by the value for the samples in TSB and multiplied by 100.

2.8 *S. aureus* protein extraction

20 ml CRPMI was inoculated and incubated statically at 37°C in 5% CO₂ for 16 hours. Cultures were centrifuged at 4,000 rpm for 10 minutes to pellet cells.

2.8.1 Whole cell

The pellet was transferred to a microcentrifuge tube and weighed. For every 10 mg of cell pellet 350 µl 80 µg/ml lysostaphin (10 mg/ml) and 1 mg/ml benzamidine in PBS were added and the cells resuspended. Samples were incubated at 37°C for 30 minutes. An equal volume of 2x Laemmli buffer was added and samples boiled for 5 minutes. Samples were chilled on ice then sonicated for 15 minutes.

2.8.2 Supernatant protein extraction

The supernatant was sterilised through a 0.2 µm membrane filter and 15 ml of the sterilised supernatant concentrated down through an Amicon Ultra Centrifugal Filter Device (Milipore). Cell pellets were weighed to equalise supernatant preparations.

2.8.3 SDS extraction of non-covalently bound surface proteins

The supernatant was removed and the pellet transferred to a microcentrifuge tube before being weighed. For every 10 mg of pellet 100 µl Tris-SDS (125mM Tris pH 7.5 with 2% SDS) were added and the cells resuspended. The samples were boiled for 3 minutes and centrifuged at 13,000 rpm for 1 minute. The supernatant was quickly removed and

placed in a new microcentrifuge tube.

2.8.4 Cell wall, membrane and cytoplasmic proteins

The supernatant was removed and the pellet transferred to a microcentrifuge tube before being weighed. 25 μ l of 80 μ g/ml lysostaphin, 1mg/ml benzamidine and 30% (w/v) raffinose in PBS were added for every 1 mg of cell pellet. The samples were vortexed to resuspend pellets and incubated at 37°C for 30 minutes. After centrifugation at 13,000 rpm for 5 minutes the supernatant was removed which contained the cell wall proteins.

The pellet contains protoplasts which need to be split to obtain the membrane and cytoplasmic proteins. The pellets were resuspended in PBS and equalised with the same volumes used previously. The samples were chilled on ice for 10 minutes and sonicated for 15 minutes. The protein fractions were separated by centrifugation at 13,000 rpm for 10 minutes. The supernatant was removed and placed in a new microcentrifuge tube; this fraction contains the cytoplasmic proteins.

2.9 SDS PAGE protein gels

2.9.1 10x Running buffer

0.25 M Tris, 1.92 M glycine and 0.035 M SDS were dissolved in 1 l of dH₂O. The pH of the solution was checked but not altered if found to be between 8 and 8.5. The buffer was stored at room temperature and diluted to 1x with water before use in electrophoresis.

2.9.2 Buffer A

0.75 M Tris and 0.007 M SDS was dissolved in 500 ml dH₂O and the pH adjusted to 8.8 with HCl. The buffer was stored at room temperature.

2.9.3 Buffer B

0.25 M Tris and 0.007 M SDS was dissolved in 500 ml of dH₂O and the pH adjusted to 6.8 with HCl. The buffer was stored at room temperature.

2.9.4 2x Laemmli buffer

The buffer was made up of 1 ml 1M Tris-HCl pH 6.8, 4 ml 10% (w/v) SDS, 1 ml 1% (w/v) Bromophenol blue and 2 ml glycerol. The buffer was stored at room temperature. Before use 200mM DTT in sodium acetate pH 5.2 was added. With DTT added the buffer must now be stored at -20°C.

2.9.5 Coomassie blue stain

0.25 % (w/v) Coomassie R250 (Sigma) was dissolved in 450 ml 50% (v/v) methanol and 50 ml glacial acetic acid. The stain was stored at room temperature.

2.9.6 Destain

Destain was made up of 7.5% (v/v) glacial acetic acid and 20% (v/v) methanol in dH₂O.

2.9.7 SDS PAGE gels

Unless otherwise stated proteins were separated on a 10% SDS gel by electrophoresis (SDS-PAGE). 25 ml of 10% (w/v) separating gel was made by mixing 12.27 ml buffer A, 8.32 ml 30% Protogel Acrylamide solution and 3.48 ml dH₂O. 864 µl 1% (w/v) APS

(ammonium persulphate) and 68.18 μ l TEMED (N,N,N',N'-tetramethylethylenediamine) were added just before pouring the gel. A layer of dH₂O was poured over the gel whilst it set, which was removed before the next gel was added. A 5% (w/v) stacking gel was poured over the separating gel and a comb inserted to create the wells. 7 ml 5% (w/v) stacking gel consisted of 3.5 ml buffer B, 1.12 ml Acrylamide mix and 2.16 ml dH₂O. 175 μ l 1% (w/v) APS and 14 μ l TEMED were added to the mix just before the gel was poured. Once set the comb was removed and the wells washed out with dH₂O. Protein samples were boiled with Laemmli buffer for 3 minutes then loaded onto the gel along with PageRuler prestained protein ladder (Fermentas). Electrophoresis was carried out in 1x SDS running buffer at constant 60mA for 1.5 to 2 hours. Protein bands were visualised by staining with Coomassie stain overnight. Non-specific staining was removed by sequential washes with destain. Bands of interest were excised and sequenced using the MALDI-TOF (Matrix assisted laser desorption ionisation- time –of-flight) mass-spec system by PNACL (Protein and Nucleic Acid Chemistry Laboratories) at Leicester University.

2.10 Exoprotein activity assays

2.10.1 Haemolysin assay using blood agar

Strains were grown overnight in 5 ml LB broth. 10 μ l drops of each strain were spotted onto rabbit and sheep blood agar plates. The drops were allowed to dry before the plates being incubated at 37°C for 24 hours. The diameter of bacterial growth and halo were measured and a picture taken of the plate. Agar plates were then stored at 4°C overnight and checked again.

2.10.2 Haemolysin assay using microtitre plate

Strains were grown overnight in 10 ml of LB broth. Supernatant protein preparations were carried out as described above. In a clear, round bottomed 96-well plate 50µl of PBS were added to each well (except first column which will contain blood only). 50µl of the protein preparation were added to the third column and a serial dilution carried out to a final dilution of 2^{-9} . 1ml of blood was centrifuged at 4000 rpm for 2 minutes and the resulting pellet resuspended in 5 ml of PBS. 50 µl of this blood preparation were added to all wells. The plates were incubated at 37°C for 30 minutes before taking a photograph of the plate and recording the dilution factors for total lysis.

2.10.3 Nuclease activity

Deoxyribonuclease test agar (Sigma) was made up following manufacturer's instructions. Bacterial strains were grown at 37°C overnight in 5 ml LB and 10 µl aliquot spotted onto the agar. The plates were incubated at 37°C for 24 hours before the surface was flooded with 1M HCl acid. This turns the agar opaque with clear halos around the bacteria that produce DNase enzymes.

2.10.4 Autolysin activity

Strains were grown overnight in 5ml BHI broth and sub-cultured into 50 ml BHI broth to an OD₆₀₀ of 0.05. These cultures were incubated shaking at 37°C to an OD₆₀₀ of 1 before being split into 2x 25ml. Cells were pelleted and washed with PBS twice before being resuspended in PBS or PBS + 0.02% Triton. Samples were incubated shaking at 37°C with OD₆₀₀ reading taken after 10 minutes, 30 minutes and at 30 minute interval thereafter for 4-5 hours. Autolysin activity was calculated by dividing the OD₆₀₀ of PBS plus Triton by OD₆₀₀ of PBS.

2.11 lux reporter assays

Reporter strains were streaked out onto LA plates containing appropriate antibiotics and incubated at 37°C overnight. 10 ml CRPMI (without phenol red) was inoculated with reporter strains and incubated statically at 37°C in 5% CO₂ overnight. Cultures were centrifuged at 4000 rpm for 10 minutes to pellet cells. The pellet was resuspended in 1 ml media and subcultured into 10 ml fresh CRPMI with antibiotics to an OD₆₀₀ of 0.1. Three 200 µl aliquots of each strain were transferred to a flat bottomed 96-well plate (Nunc) along with aliquots of CRPMI to be used as blanks. The plate was covered with a gas permeable membrane (Breathe easy sealing membrane) and placed into a FLUOstar Omega plate reader (BMG Labtech). Plates were incubated at 37°C with 5% CO₂. Absorbance (OD₆₀₀) and luminescence readings were taken every 30 minutes and averages recorded for each sample. Luminescence values were divided by the OD₆₀₀ values to calculate the relative light units (RLU).

2.12 Cloning and expression of recombinant protein

2.12.1 HIS-tagged Fur

The *fur* gene was amplified using the primers pLeics furF and pLeics furR using Bio-Xact short DNA polymerase. The PCR products were gel extracted and sequenced. The remaining product was sent to the Protein Expression Laboratory (PROTEX, University of Leicester) to clone into their pLeics01 expression vector. The resulting plasmid was sequenced again to ensure the sequence was still correct and in frame before being transformed into the Rosetta *E. coli* strain.

Expression strains were grown overnight in 5 ml LB. Cultures were subcultured into 50 ml of fresh media to an OD₆₀₀ of 0.05 and incubated to an OD₆₀₀ of 0.5-0.6. 1 mM IPTG

was added and the culture incubated at 30°C for 2 hours. After induction cells were pelleted at 4000 rpm for 5 minutes. The pellet was resuspended in 600 µl HIS binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH 7.5) with 100 µg ml⁻¹ lysozyme and incubated in a 37°C water bath for 30 minutes. Cells were then sonicated for 10 minutes on high with a 30 second on/off cycle. Cell lysates were stored at 4°C.

2.12.2 HIS-tagged Hfq

The *hfq* gene was amplified using the primers pCOLD *hfq*F and pCOLD *hfq*R containing *Bam*HI and *Xba*I sites respectively. The PCR products and pCOLD1 vector were digested using these enzymes and gel extracted. Ligation reactions were set up with a 3:1 ratio of insert:vector and incubated overnight before being transformed into the Rosetta *E. coli* strain. Transformations were plated onto LA containing 100 µg/ml Carbenicillin and incubated at 37°C. Colonies were tested via colony PCR using pColdF and pColdR primers. pCOLD1-*hfq* plasmid DNA was extracted from transformants and the *hfq* gene sequenced.

The Hfq expression strains was grown overnight in 10 ml LB. This was diluted 1 in 100 ml in 1 l of LB and incubated at 37°C until an OD₆₀₀ between 0.4-0.5 was reached. Cultures were incubated at 15°C for 30 minutes statically before 1mM IPTG was added. Expression was induced by incubating at 15°C for 24 hours. Cells were pelleted for 10 minutes at 4000 rpm and resuspended in 5 ml HIS binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH 7.5) containing a protease inhibitor cocktail (Roche) and 100 µg/ml lysozyme. This was incubated at 37°C for 30 minutes and then sonicated on ice on high for 10 minutes with a 30 second on/off cycle in a

Bioruptor sonicator. The soluble and insoluble protein fractions were separated by centrifugation for 30 minutes at 10000 rpm.

2.12.3 Protein purification and quantification

The tagged proteins were purified from the cell lysate by passing through a Histrap FF Crude column (GE Healthcare). The columns were equilibrated with binding buffer before adding the cell lysate. The column was washed with an appropriate volume of binding buffer and then proteins eluted over a gradient of imidazole concentrations (10 mM to 500 mM). Fractions were analysed on an SDS-PAGE gel and used in western analysis. The concentration of purified protein was calculated using the Bradford assay. 250 μ l Bradford reagent were added to 100 μ l purified protein and 100 μ l aliquots of BSA solution of known concentrations (0.25 – 1.4 mg/ml). Solutions were incubated for 30 minutes at room temperature and the absorbance measured at 595 nm. The BSA standards were used to create a standard curve to determine the concentration of sample proteins.

2.13 Western blotting

2.13.1 Transfer Buffer

0.096 M Tris and 0.077 M Glycine was dissolved in 500 ml dH₂O, before adding 200 ml 100% methanol and 3.7 ml 10% (w/v) SDS. This was then made up to 1 l with dH₂O and stored at 4°C.

2.13.2 TBS-T buffer

50 ml 1M Tris (pH 7.5), 30 ml 5M NaCl and 1 ml Tween 20 were combined and made up to 1 l using dH₂O. This was then stored at 4°C.

2.13.3 Western blot

Protein samples were boiled for 3 minutes with 2x Laemmli buffer and loaded across 2 mini SDS PAGE gel. Gels were run in 1x SDS running buffer at 30 mA for 1.5 hours. One gel was then stained with coomassie stain and destained as described previously. The second gel was used to transfer the proteins to a PVDF membrane (Immobilon P) at 150 mA for 1 hour in ice cold transfer buffer. The PVDF membrane was activated by wetting with 100% methanol, then washed with dH₂O before being left in ice cold transfer buffer for 10 minutes. The membrane was blocked with 5% (w/v) skimmed milk in 0.1% TBS-T shaking at room temperature for 1 hour and then at 4°C overnight. The membrane was incubated at room temperature for 1 hour before adding the primary antibody (anti-poly Histidine-HRP antibody, Sigma-Aldrich; anti-Hfq antibody, Huntzinger et al., 2005) diluted in 5% (w/v) skimmed milk in 0.1% TBS-T and incubating at room temperature for a further hour. The membrane was washed 3 times for 10 minutes with 0.1% TBS-T. If required, the secondary antibody (Protein A-peroxidase, Sigma-Aldrich) was diluted in 5% (w/v) skimmed milk in 0.1% TBS-T, added to the membrane and incubated at room temperature for 1 hour. The membrane was washed 3 times for 5 minutes, 2 times for 15 minutes and 3 times for 5 minutes with 0.1% TBS-T. The blot was developed using the Enhanced Chemiluminescence (ECL) kit from Amersham. 1.5 ml each of ECL solution 1 and 2 were warmed separately at room temperature before being combined and added to the surface of the blot. This was left for 1 minute before the excess liquid was poured off and the blot wrapped in cling film and placed in an autoradiograph cassette. Autoradiograph film (Medical X-ray film, Fuji Film) was placed in the cassette over the blot and allowed to expose at room temperature. The film was developed by submerging in developer fluid for 1 minute, followed by 30 seconds in neutraliser solution and finally 30 seconds in

fixer solution.

2.14 Electrophoretic mobility shift assay (EMSA)

2.14.1 10X TB buffer

0.89 M Tris and 0.89 M boric acid was dissolved in 1 l of dH₂O. The pH was corrected to 8 and the solution autoclaved. The solution was stored at room temperature.

2.14.2 Maleic acid buffer

0.1 M maleic acid and 0.15 M NaCl was dissolved in 1 l of dH₂O. The solution was corrected to pH 7.5 before being autoclaved.

2.14.3 Washing Buffer

Washing buffer was made as with maleic acid buffer with 3 ml Tween 20 added after being autoclaved.

2.14.4 10x Blocking solution

10% (w/v) blocking reagent (Roche) was made up in maleic acid buffer and heated at 65°C until dissolved. The solution was autoclaved and then stored at -20°C. Before use blocking solution was diluted to 1x with maleic acid buffer.

2.14.5 Detection solution

0.1 M Tris and 0.1 M NaCl was dissolved in 1 l dH₂O and corrected to pH 9.5. This was autoclaved and then stored at room temperature.

2.14.6 5x Binding buffer

20mM Bis-Tris (Sigma-Aldrich) solution was made up with dH₂O and corrected to pH7.6.

2.14.7 5x EMSA loading buffer

125 µl 10x TB buffer and 3 ml 100% glycerol were combined and made up to 5 ml with dH₂O.

2.14.8 DNA probe DIG labelling

Two 50 µl PCR reactions of the desired probe were pooled together and gel extracted. The DNA concentration was measured using a Nanodrop (ThermoScientific) and converted to pmol using the following equation:

$$\text{ds DNA (ng)} = \text{pmol} \times 0.66 \times \text{DNA length (bp)}$$

3.85 pmol of DNA were added to dH₂O to a final volume of 10 µl. 4 µl of 5x Terminal Transferase Buffer, 4 µl 25mM CoCl₂, 1 µl DIG-ddUTP and 1 µl Terminal Transferase were added to the DNA and incubated at 37°C for 15 minutes. The DNA probe was diluted to 1.55 fmol/µl before use.

2.14.9 EMSA

4µl 5x binding buffer, 2 µl 20 mM DTT, 2 µl 1 mg/ml BSA, 2µl 1mM MnSO₄, 2 µl 3 µg/ml Salmon sperm DNA and 1 µl fmol/µl DIG-labelled probe were added to an appropriate amount of purified protein and adjusted to a final volume of 20 µl. Samples were left on ice whilst the native gels were set up.

Samples were run on an 8% native PAGE gel made up of 4.1 ml dH₂O, 0.3 ml 10x TB buffer, 1.6 ml Protogel 30% acrylamide solution (Geneflow), 100 µl 10% (w/v) APS and 10 µl TEMED. The gels were pre-run for 15 minutes at 40V in 0.5x TB buffer. During this time the samples were incubated at room temperature to allow binding of the protein and DNA probe. 5 µl 5x EMSA loading buffer was added before being loaded on to the gel. Any empty wells were loaded with 5 µl 5x EMSA loading buffer. Gels were run at 80 V for 2 hours and then transferred to nylon membrane at 40 V for 1 hour in 0.5x TB buffer. The DNA was fixed to the membrane using a UV crosslinker at 700 kJ/cm² before being briefly washed with washing buffer. The membrane was incubated in 1x blocking solution for 30 minutes followed by incubation with 20 ml 1x blocking solution containing 1µl anti-digoxigenin-AP for 30 minutes. Unbound antibody was removed by washing the membrane twice with washing buffer for 15 minutes each. The membrane was next incubated in detection solution for 5 minutes before the excess solution was drained off and 1 ml of CSPD working solution (10µl CSPD in 1 ml detection buffer) was applied evenly to the membrane. This was left at 37°C for 10 minutes to enhance the CSPD chemiluminescence reaction. The working solution was drained off and the membrane wrapped in cling film and placed in an autoradiograph cassette. Autoradiograph film was added and exposed at room temperature for 1 hour. The film was developed as described for western blotting analysis.

2.15 qRT-PCR

2.15.1 Total RNA extraction

For iron limited conditions *S. aureus* cultures were grown in 10 ml CRPMI. These cultures were then resuspended in fresh 10 ml CRPMI to an OD₆₀₀ of 0.1 and incubated

for 6 hours until growth had reached late exponential phase. When this point had been reached cells were pelleted and resuspended in 1 ml RNAlater (Ambion) and stored at 4°C overnight. These suspensions were centrifuged and the supernatant removed before freezing the cells at -80°C for at least 1 hour. RNA was then extracted using the Total RNA Purification kit (Norgen) and following the manufacturer's instructions with the following addition. When lysing the cells 100 µg/ml lysostaphin was added to the lysozyme in TE buffer and incubated for 30 minutes at 37°C. Eluted RNA was stored at -80°C. To remove any DNA contamination the RNA preparations were treated with Turbo DNA-free (Applied Biosystems) using the manufacturer's protocol except that digests were left for an hour at 37°C. A second digest was carried out repeating the same protocol except using 10x second digest buffer (200 mM Tris-HCl pH7.5, 10 mM MgCl₂, and 5 mM CaCl₂) instead of the provided buffer. The treated RNA was stored at -80°C.

2.15.2 Reverse transcription (RT)

The RNA was converted to cDNA using the Omniscript RT kit (Qiagen) and following the manufacturer's instructions. The concentration of DNase treated RNA was measured using a Nanodrop and these values used to calculate the volume required to obtain 2 µg RNA per RT reaction. The RNase inhibitor used was diluted to 10 U/µl using 1x RT buffer provided with the kit.

2.15.3 qRT-PCR reaction

1.65 µl aliquots of template cDNA were transferred to separate microcentrifuge tubes for each primer set. Each PCR reaction contained:

Power SYBR green PCR master mix (Applied Biosystems)	10 μ l
Forward Primer (10 uM)	0.5 μ l
Reverse Primer (10 uM)	0.5 μ l
dH ₂ O	9 μ l

A master mix for each primer set. The SYBR green is photosensitive and therefore only used in the dark. An endogenous control is required for each qRT-PCR, for this the house keeping gene gyrase was used. 64.35 μ l of mastermix were added to each template sample and three 20 μ l aliquots transferred to a 96-well PCR plate (Star Labs). When all samples have been loaded plates were sealed with StarSeal Advanced Polyolefin Film (Star Labs) and wrapped in foil. The qRT-PCR reaction and analysis were carried out using the ABI7300 Real-time PCR system.

2.16 mRNA stability

10 ml media were inoculated and incubated at 37°C overnight. Cells were harvested by centrifugation at 4000 rpm for 10 minutes and resuspended in 1 ml fresh media. This was then subcultured into 15 ml media to an OD₆₀₀ of 0.1. Cultures were incubated at 37°C 4 hours. After 4 hours the OD of each culture was recorded and 300 μ g/ml rifampicin added. Immediately after addition of the rifampicin 1ml of culture was removed and spun down and the remaining culture returned to the incubator. The pellet was resuspended in RNA later (Ambion) and stored at 4°C. This was repeated at set time points. Total RNA was extracted and treated as described previously and then used to carry out qRT-PCR.

2.17 *Galleria mellonella* infection model

Galleria mellonella larvae were purchased from Livefood UK Ltd. and stored at 4°C. Before use larvae were left at room temperature overnight. Bacterial strains were grown overnight in 5 ml LB at 37°C. Cells were harvested by centrifugation at 4000 rpm for 10 minutes, washed 3 times with phosphate buffered saline (PBS) and resuspended in 10 ml PBS to an OD₆₀₀ of 0.1. Larvae were swabbed with 70% ethanol to sterilise them before injection. 20 µl bacterial suspensions was injected into the larvae between the 4th set of pro-legs using a syringe with a 29 gauge needle and a Stepper Repetitive Pipette (Tridak). A negative control group was injected with PBS and a second negative control group were sterilised but not injected at all. Groups of 10 larvae were used for each condition. They were incubated at 37°C and viability determined after every 24 hours for 96 hours.

2.18 Statistical analysis

To determine the statistical significance of data points between strains the unpaired t-test equation was used.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{X_1X_2} \cdot \sqrt{\frac{2}{n}}} \quad \text{where} \quad S_{X_1X_2} = \sqrt{\frac{1}{2}(S_{X_1}^2 + S_{X_2}^2)}$$

Here, X bar is the mean for sample one and two and n is the number of repeats within each sample. S_{X₁X₂} is the combined standard deviation of the samples.

Chapter 3 Phenotypic analysis of a *Staphylococcus aureus hfq* mutant

3.1 Introduction

Our hypothesis is that Fur positive regulation occurs through post transcriptional regulation involving the protein Hfq. Originally identified in *E. coli*, *hfq* homologs have been identified in a wide range of bacterial taxa. The role of Hfq in virulence has been studied in a range of pathogenic bacteria. The deletion of *hfq* in these pathogens results in pleiotropic phenotypes affecting the fitness and virulence of the bacteria. In *S. aureus* the role of Hfq appears to be more complicated and conflicting results have been published. In 2007 Bohn et al. published a study reporting no phenotypic effects of a *hfq* knockout in strains Newman, COL and RN6390. This is interesting considering in all other bacteria tested Hfq plays a major role in regulation. Northern blot analysis and RT-PCR in *S. aureus* showed a very low level of *hfq*. This is in contrast to *E. coli* Hfq which has been observed to be highly expressed and may account for the lack of phenotype in *S. aureus*. However, in 2010 Liu et al. published data in which an *hfq* mutant in *S. aureus* 8325-4 showed reduced virulence, increased resistance to oxidative stress and altered regulation of a number of genes. The level of Hfq protein expression was reported as differing between strains which may explain the difference in results. Prior to these publications preliminary work in this laboratory found that in *S. aureus* Newman, Hfq positively regulated expression of the Fe regulated cell wall proteins *isdA* and *isdB*. This was not observed in the Bohn et al. paper and could be due to the different growth conditions used. Therefore the first objective of this study was to carry out further phenotypic analysis with Newman to identify any more *hfq* mutant phenotypes. This was then continued in two other strains to investigate the effect of

strain variation on Hfq regulation.

3.2 Sequence variation of *hfq* in different *S. aureus* isolates

The variation in Hfq function could be due to sequence differences as different *S. aureus* strains can demonstrate a high degree of sequence variation. As discussed in Chapter 1.3.1 about 25% of the genome consists of accessory genes which are or were mobile genetic elements. The moving of these elements allows different strains to express a variety of virulence factors and resistances. But even in the core genome which encodes important genes required for growth and regulation there is also a level of diversity. Small variations in these genes can cause differences in expression and protein function. When these changes occur in regulators it can have important global effects on virulence. Two well known examples of such variations are the Sae and Agr regulators. The Newman *saeS* gene has a single point mutation translating to a L18P amino acid substitution. This single change causes constitutive kinase activity and decreased SaeS protein stability (Adhikari & Novick, 2008; Jeong et al., 2011). In the case of Agr variable regions in *agrC*, *agrD* and *agrB* have led to distinct AIP and sensor structures (McDowell et al., 2001; Dufour et al., 2002; Zhang & Ji, 2004). Therefore to identify any variations in Hfq we compared the sequence of the *hfq* gene from all the published *S. aureus* sequences along with those from nine additional infectious human and bovine strains.

To analyse the *hfq* sequences in the unpublished genome isolates, the *hfq* gene was amplified from *S. aureus* strains using flanking primers hfqB and hfqSE. These products were then sequenced using nested primers hfqF and hfqR. Although their genomes are already published, sequencing was carried out with Newman, PM64

Newman	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
C00595	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
C01865	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
E15	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
BB	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
C01719	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
C01771	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
RF122	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
38963	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
PM64	ATGATTGCAAACGAAAACATCCAAGACAAAGCACTAGAGAATTTTAAAGCAAACCAAACCTGAAGTAACTGTATTCTTTCTAAACGGTTTCCAAATGAAAGGTGTTATT
Newman	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTATACAGTAGAAAACCTGAAGGTCAAGCATC
C00595	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTATACAGTAGAAAACCTGAAGGTCAAGCATC
C01865	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTATACAGTAGAAAACCTGAAGGTCAAGCATC
E15	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTACACAGTAGAAAACCTGAAGGTCAAGAATC
BB	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTACACAGTAGAAAACCTGAAGGTCAAGAATC
C01719	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTACACAGTAGAAAACCTGAAGGTCAAGAATC
C01771	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTACACAGTAGAAAACCTGAAGGTCAAGAATC
RF122	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTATACAGTAGAAAACCTGAAGGTCAAGAATC
38963	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTATACAGTAGAAAACCTGAAGGTCAAGAATC
PM64	GAAGAATACGACAAGTATGTCGTAAGCTTAAATTCTCAAGGCAAACAACACTTGATTTACAAACATGCGATCAGCACTTATACAGTAGAAAACCTGAAGGTCAAGAATC
Newman	TACTGAAAGTGAAGAATAA
C00595	TACTGAAAGTGAAGAATAA
C01865	TACTGAAAGTGAAGAATAA
E15	TACTGAAAGTGAAGAATAA
BB	TACTGAAAGTGAAGAATAA
C01719	TACTGAAAGTGAAGAATAA
C01771	TACTGAAAGTGAAGAATAA
RF122	TACTGAAAGTGAAGAATAA
38963	TACTGAAAGTGAAGAATAA
PM64	TACTGAAAGTGAAGAATAA

Figure 3.1 Alignment of the nucleotide sequences of *hfq* gene in various *S. aureus* strains. The *hfq* gene was amplified from a number of *S. aureus* isolates using *hfqB* and *hfqSE* primers. The PCR products were then sequenced with *hfqF* and *hfqR* primers. The nucleotides highlighted in red show the silent point mutations, those in yellow show point mutations causing an amino acid change.

(MRSA252) and RF122. This was to ensure that our stocks showed the same nucleotide sequences. Comparison of the *S. aureus hfq* gene sequence revealed that the majority of the genomes analysed had an identical sequence to Newman. However seven of the strains investigated showed 3 conserved nucleotide changes, 2 of which result in amino acid changes (Figure 3.1).

The strains E15, BB, C01719 and C01771 all have two point mutations resulting in one amino acid change, A71E. RF122 and 38963 have one point mutation resulting in the same amino acid change. The genome sequences of PM64, Mn8 and TCH60 showed the same A71E change but also contained an additional G69A mutation. Therefore the A71E change is conserved between different strains.

The lineages of the strains used were identified to determine if there was a correlation between *hfq* sequence and lineage. Multi locus sequence typing (MLST) is used to define lineages by the comparison of sequences of 7 housekeeping genes (*araC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*). At each locus a variant allele is given a number and this is compiled from all loci to create a profile. The closer these profiles are between strains the more likely they are to be related (Enright et al., 2000). These sequence types (ST) are grouped into Clonal Complexes (CC) by their similarity to allelic profiles. The ST's of a group must have at least 5 common MLST alleles with at least one other member of the CC. This can help identify ST's that have common ancestors. Interestingly strains from the same lineage showed the same *hfq* sequence indicating that these mutations are maintained (Table 3.1). By comparing a neighbour joining tree of *S. aureus* strains we can see how closely these changes are grouped together (Figure 3.2). The C104A base change could have been picked up at point 1 (Figure 3.2) as all the strains sequenced

Strain	ST	CC	Hfq amino acid changes
Newman 8325-4 USA300	8	8	-
C00595	97	97	-
C01865	1	1	-
Mu50 Mu3 N315	5	5	-
JH1, JH9	105	5	-
RF122 38963	151	151	A71E
E15 (MRSA PM25)	22	22	A71E
BB C01719 C01771	133 771	133	A71E
PM64 (MRSA 252) TCH60 Mn8	36	30	G69A, A71E

Table 3.1 Staphylococcal strains of known lineage and their changes in *hfq* sequence.

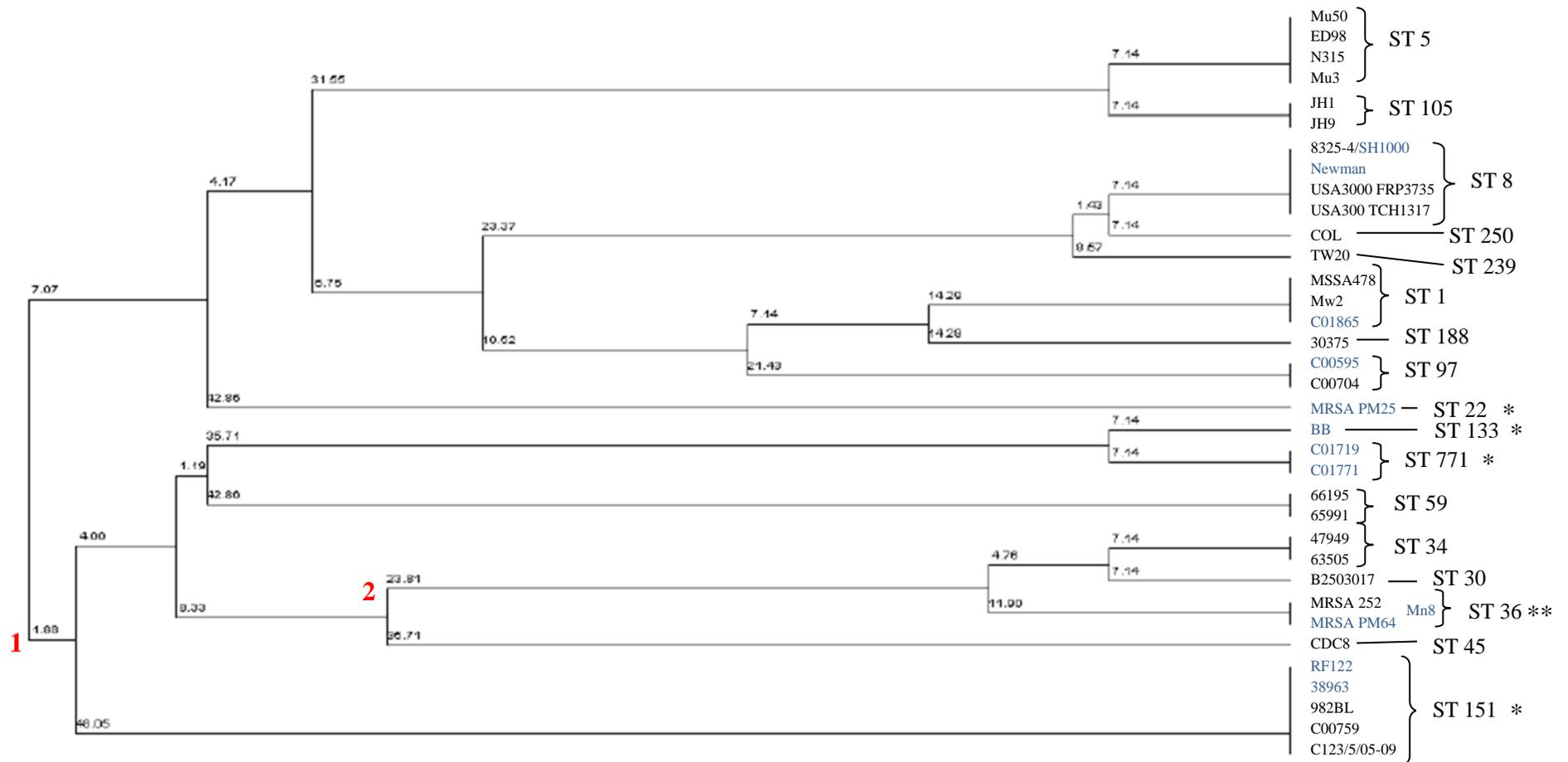


Figure 3.2 Neighbour-joining tree of *S. aureus* strains based on MLST. The tree above was derived from the MLST profiles of *S. aureus* strains.

The blue text indicates the strains whose *hfq* genes were sequenced in this study. The red numbers indicate possible points at which base changes were acquired.* indicates presence of A71E change, ** indicates presence of A71E and G69A changes. Figure was adapted from Purves (2011).

from here have this substitution. At point 2 (Figure 3.2) we see another branching where the top branch picked up the silent T81C substitution, whereas the lower branch picked up the G98C substitution. The only strain that does not agree with this theory is E15 (MRSA PM25) which appears to have acquired the C104A change later. This tree shows that these changes have come from a common ancestor and therefore may suggest they are somehow advantageous to the bacteria. Possible differences in expression and regulation by Hfq due to the amino acid changes were investigated during this project.

3.3 Phenotypic analysis of *Staphylococcus aureus* Newman *hfq* and *fur* mutants

3.3.1 Hfq and Fur do not affect growth in excess Fe, Cu or under osmotic stress

Previous studies investigating Hfq function in other bacteria have found reduced growth rates and increased sensitivities to oxidative and osmotic stress in the *hfq* mutant. In other bacteria both Fur and Hfq regulate resistance to oxidative stress by preventing Fe toxicity and through regulation of stress resistance genes (see Section 1.6.2). To investigate whether *hfq* and *fur* mutants showed any effect on growth rate the strains were grown in different growth conditions. Strains were grown in CRPMI to investigate the effect on growth in low nutrient and metal ion levels reflective of conditions found *in vivo* (Morrissey et al., 2002). Resistance to oxidative stress was investigated by the addition of H₂O₂, Fe or Cu to growth media. Fe and Cu are important metal ions required as cofactors for many proteins but high levels can become toxic. If too much of these metal ions are present they catalyse the production of superoxide species which leads to cell damage (Ganz & Nemeth, 2006; Samanovic et al., 2012). Finally, osmotic stress resistance was investigated by the addition of NaCl.

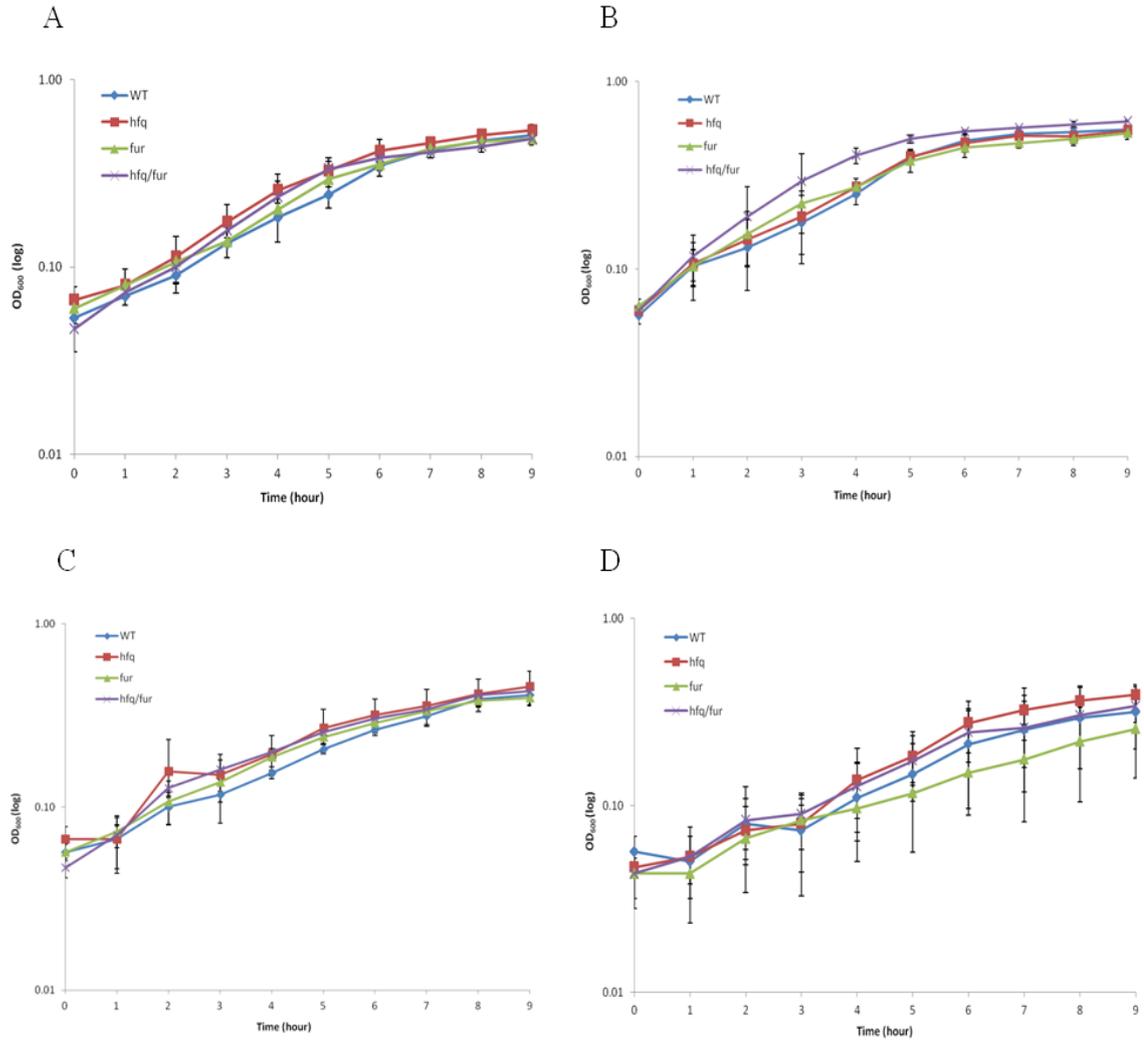


Figure 3.3 Comparison of growth of Newman wild type and mutant strains in (A) CRPMI, (B) + 50 μM FeSO₄, (C) + 50 μM CuCl₂ and (D) + 1M NaCl. Overnight cultures were used to inoculate 25 ml fresh media to OD₆₀₀ 0.05. The Fe, Cu or NaCl was added and cultures incubated at 37°C in 5% CO₂ for 9 hours. The OD₆₀₀ of each culture was measured every hour. The data presented is an average of three independent experiments with error bars showing the standard deviation of each data point.

To investigate whether Hfq or Fur are involved in bacterial growth under these conditions, 9 hour growth curves were carried out on strains Newman, Newman *hfq*, Newman *fur* and Newman *hfq/fur* in CRPMI alone or with the addition of 50 μ M FeSO₄, 50 μ M CuCl₂ or 1M NaCl. This concentration of Fe has been shown to improve growth in wild type strains and also is high enough to repress Fe regulated genes showing it is sufficient to change iron homeostasis regulation (Johnson, 2008). At this Cu concentration growth of Newman is significantly reduced indicating that it is stressing the cell (Baker et al., 2010). A range of NaCl has been used previously when investigating osmotic stress in *L. monocytogenes* and *S. aureus hfq* mutants (Christiansen et al., 2004; Bohn et al., 2007). The 1M concentration was chosen after comparison of the concentrations used in these publications and their results.

Figure 3.3.A showed that there was no difference in growth rate between the wild type and mutant strains in any of the conditions tested. When the bacteria were grown in media supplemented with Fe, there was an increase in overall growth in Newman as reported previously (Johnson, 2008) (Figure 3.3.B). However the double mutant does show an increased growth rate in Fe replete conditions compared to wild type during exponential growth ($P < 0.005$) (Figure 3.3.B). Addition of Cu or NaCl reduced growth for all strains (Figure 3.3.C & D). Unlike in other bacteria (see Table 1.2) there was no statistically significant difference between the growth of the *S. aureus* wild type and mutant strains in any of the conditions tested. The exception being the double mutant which interestingly showed increased growth in +Fe.

3.3.2 Hfq and Fur are involved in growth in rich media and resistance to oxidative stress

Oxidative stress assays were carried out in a rich medium, TSB, as previous results performed in this laboratory with CRPMI were found to be non-reproducible. Strains were grown in TSB and TSB + H₂O₂ for 7 hours with OD₆₀₀ readings taken every hour. There was no difference in growth between the wild type and *hfq* mutant in TSB (Figure 3.4.A). However the *fur* mutant shows a significant decrease in growth when compared to the wild type ($P < 0.001$). Interestingly the double mutant shows a significant increase in growth compared to the *fur* mutant ($P < 0.01$) suggesting that the absence of *hfq* compensates for the *fur* mutation (Figure 3.4.A). In the presence of H₂O₂ no growth was seen in the *fur* or double mutant, whereas the *hfq* mutant shows a reduction in growth during the late exponential growth phase ($P < 0.05$) (Figure 3.4.B). Statistical analysis was carried out for the mid-exponential phase (3 hours) and early stationary phase (6 hours) of the TSB and TSB plus H₂O₂ growth curves respectively. This was to confirm whether the differences observed here are statistically significant. To take into account the growth differences between strains when grown in TSB alone the percentage growth was calculated and plotted on a graph (Figure 3.4.C). This directly compares growth with H₂O₂ to growth without H₂O₂ for each strain at each time point. Unlike in other bacteria (see Table 1.2) the *hfq* mutant only shows a slight reduction in oxidative stress resistance in late exponential growth (Figure 3.4.C). The *fur* and double mutant still show no growth in H₂O₂.

Since the *fur* mutant already shows a growth defect in TSB adding H₂O₂ at the beginning of the assay causes too much stress to allow the cells to grow. Therefore another oxidative stress assay was carried out where H₂O₂ was added during

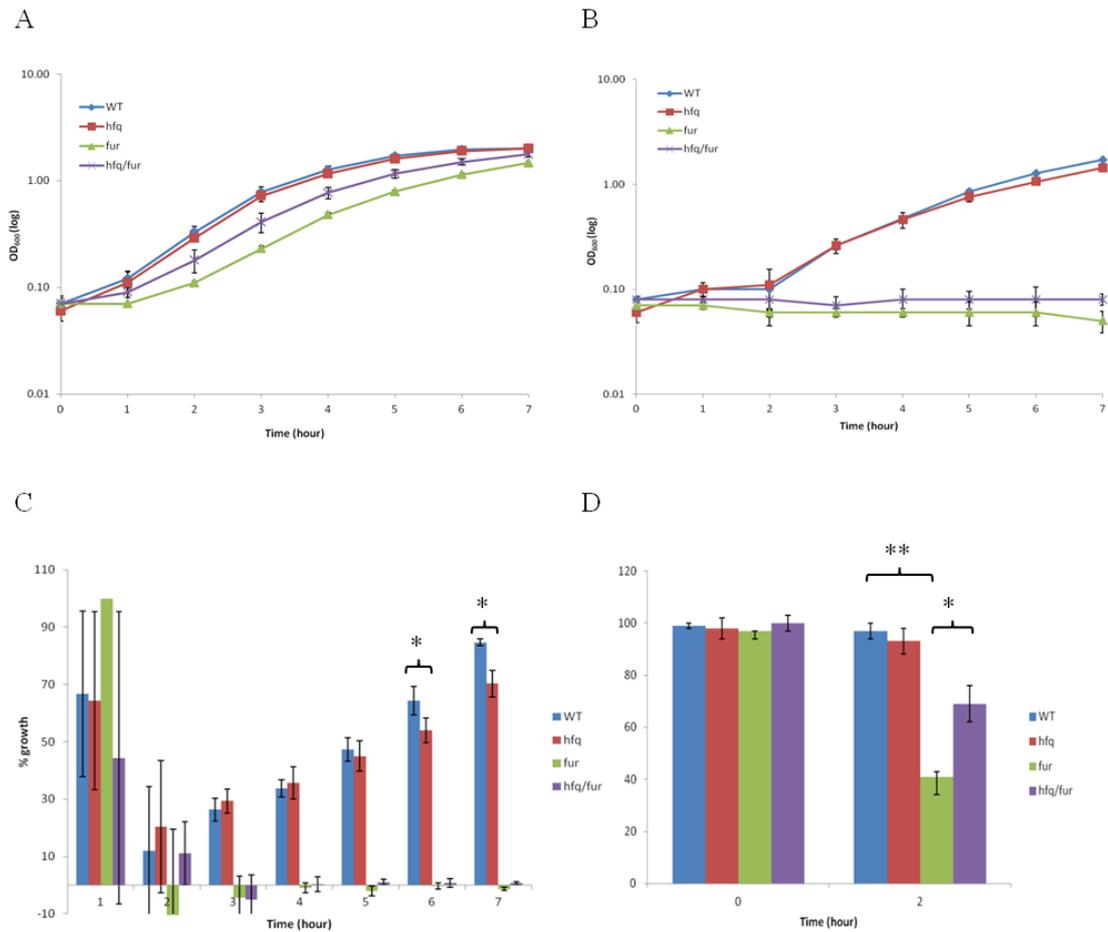


Figure 3.4 Comparison of growth of Newman wild type and mutant strains in (A) TSB and (B) TSB plus H₂O₂. Overnight cultures were used to inoculate 25 ml fresh media to OD₆₀₀ 0.05 and 0.0024% H₂O₂ added when required. Cultures were incubated at 37°C and OD₆₀₀ taken at hourly intervals for 7 hours. (C) Percentage growth difference in TSB with or without H₂O₂. (D) Percentage growth difference when H₂O₂ was added during exponential growth and OD₆₀₀ readings taken for 2 hours. The data presented is an average of three independent experiments with error bars showing the standard deviation of each data point. * indicates a P-value < 0.05 and ** indicates a P-value < 0.001

exponential growth. This was to reduce the effect of the growth defect due to the TSB and allow the H₂O₂ sensitivity to be seen. This time there was no significant difference in percentage growth between the *hfq* mutant and wild type strains. The *fur* mutant still shows a substantial growth defect under oxidative stress (P < 0.001). In TSB the double mutant showed an increase in growth compared to the *fur* mutant (P < 0.05) and this was seen again in the presence of H₂O₂ (Figure 3.4.D). This suggests that both Hfq and Fur are involved in the regulation of the oxidative stress response and that Hfq does indeed have a role in *S. aureus* adaptive stress response.

3.3.3 Both Hfq and Fur are required for full induction of Eap and Emp

Previous studies in other bacteria (see Table 1.2) have found that deletion of *hfq* causes changes in the regulation of a number of proteins. To investigate the global effect of a *hfq* knockout on protein expression and to identify any Hfq and/or Fur regulated proteins the supernatant, SDS, cell wall, cell membrane and cytoplasmic proteins were compared in the Newman wild type, *hfq*, *fur* single and double mutant strains.

Our previous studies have shown that cell wall proteins IsdA (FrpA) and IsdB (FrpB) are classically Fur regulated. These proteins are expressed in low Fe and constitutively expressed in the *fur* mutant (Morrissey et al., 2002). We have also shown examples of non-classically regulated Fur proteins such as Eap and Emp. As with classically regulated proteins, Eap and Emp are induced in low Fe, however in a *fur* mutant the levels of these proteins are decreased and not constitutive (Johnson et al., 2008). In Figure 3.5 the Isd proteins can be seen in the cell wall fraction (B) and show the same regulation as previously seen. The Eap and Emp proteins can be seen in the cell surface (A) and cell wall (B) fractions and show the non-classical Fur regulation previously

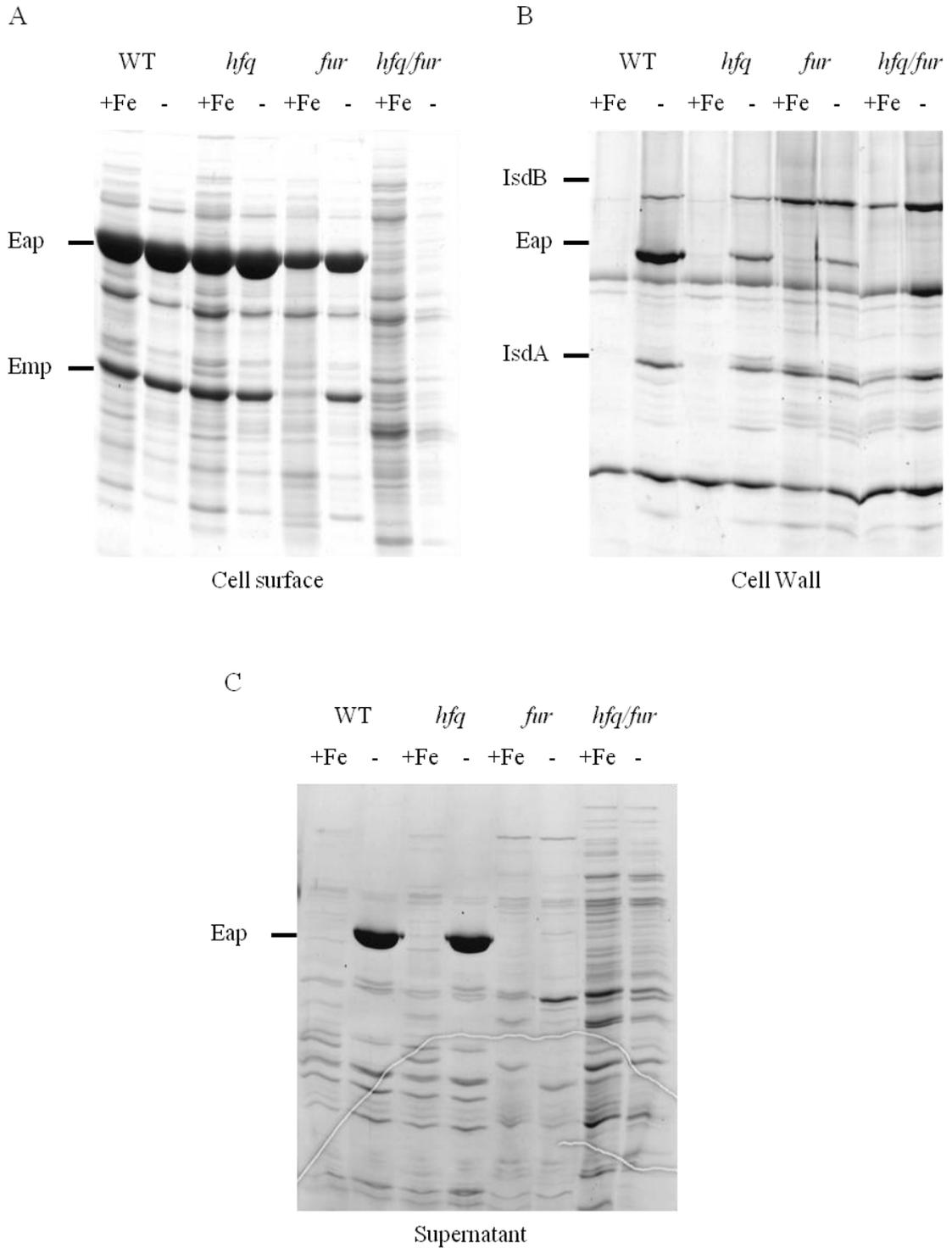


Figure 3.5 Protein profiles of Newman wild type and mutant strains. Protein extracts were obtained from cells grown for 16 hours in CRPMI \pm 50 μ M Fe. Gel pictures are representative of three independent experiments.

reported. Whilst Hfq does not appear to affect Isd expression, there is a decrease in Eap levels in the cell wall indicating that Hfq may be involved in the regulation of Eap and Emp (Figure 3.5.B). This is supported by the complete loss of Eap and Emp in all fractions in the double mutant. Therefore both Hfq and Fur are required for full expression of these important virulence factors. Interestingly, the Eap protein is not observed in the cell wall or supernatant fractions when grown with Fe. This could indicate that there is also Fe regulation of the export of Eap.

Hfq and Fur are both needed for the correct expression of a number of proteins as there are also an increased number of proteins present in the supernatant and SDS extracts in the double mutant in high Fe. However we do not see global proteins changes in the single *hfq* mutant as reported in other bacteria (see Table 1.2).

3.3.4 Clp and aconitase positively regulated by Hfq and Fur

The Isd, Eap and Emp proteins had previously been identified in Newman protein profiles and are abundant so easy to identify. The gels were also examined to find unknown proteins showing differential regulation in the mutants compared to the wild type. Protein bands that consistently differed between the wild type and *hfq* mutant strains or between *fur* and the double mutant were chosen to be identified by peptide mass fingerprinting (PMF). Protein bands were excised from the gel and digested using trypsin to produce a number of peptides. The peptide masses were measured by mass spectrometry and compared with theoretical digest of all *S. aureus* proteins in a database. Statistical scoring algorithms were used to measure the digested peptides against theoretical peptides to obtain identification. PMF was carried out by PNACL (University of Leicester).

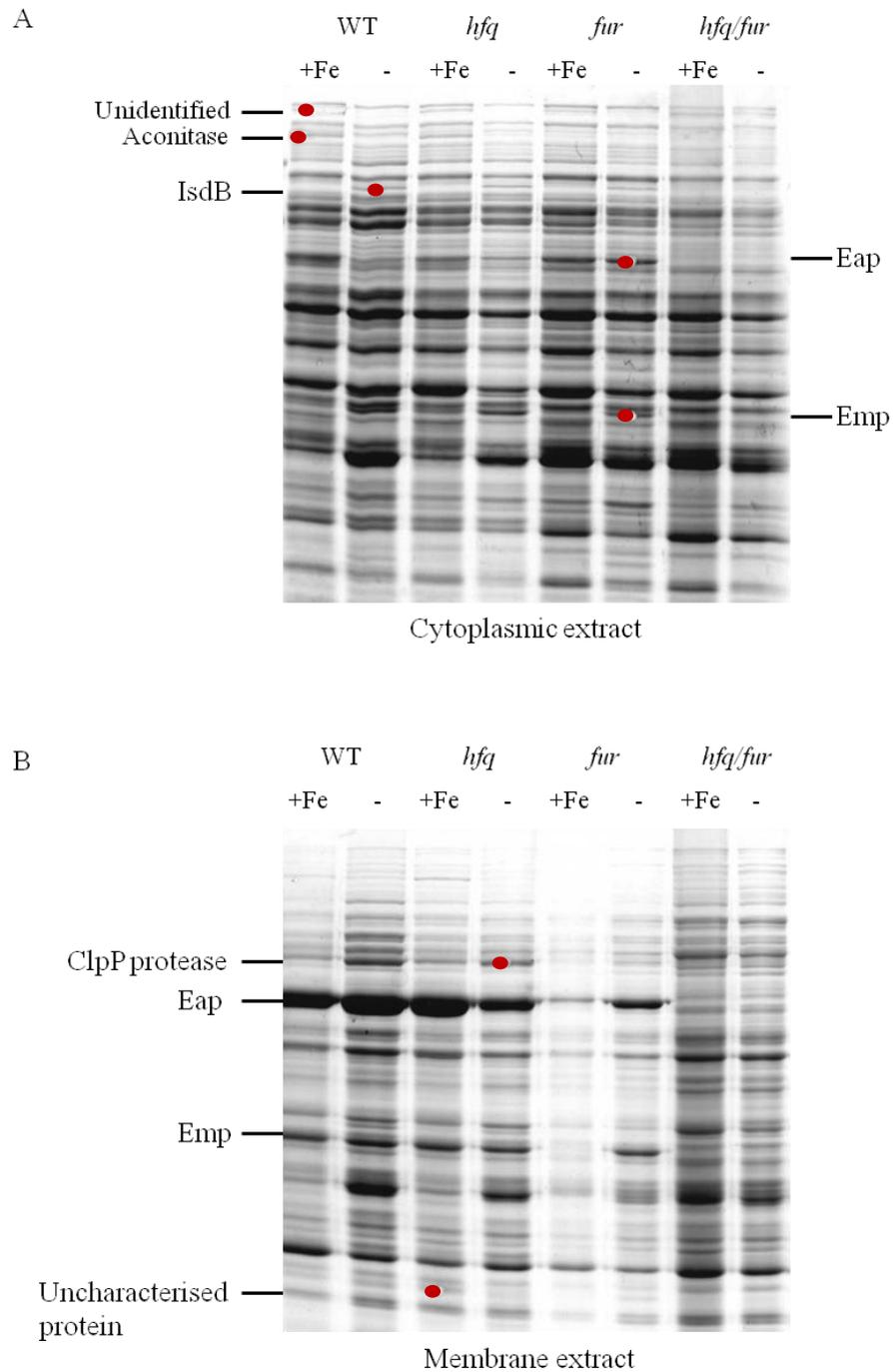


Figure 3.6 Cytoplasmic and membrane protein profiles used for protein sequencing. Protein extracts were obtained from cells grown for 16 hours in CRPMI \pm 50 μ M Fe. Red dots indicate bands sampled for sequencing and the corresponding result at the side of the gel. Gel pictures are representative of at least three independent repeats.

Four cytoplasmic and two membrane protein bands were chosen for protein identification (Figure 3.6). Two of the cytoplasmic proteins were identified as Eap and Emp confirming what was observed in the cell surface fractions. In this fraction IsdB was also identified, this band was present in the single mutants but not in the double mutant indicating that Hfq may also positively regulate IsdB expression. The presence of cell wall proteins in the cytoplasmic fraction could be from newly synthesised proteins before they are exported to the cell wall or there could be slight carry over of proteins between fractions.

Aconitase was also identified from the cytoplasmic extract. This protein appears to be Fe regulated and induced under high Fe conditions. In the *fur* mutant this regulation is lost and there is a reduction in protein level indicating that the Fe regulation is through Fur and that Fur positively regulates expression. The *hfq* mutant still shows the Fe regulation however there is less protein compared to the wild type indicating positive Hfq regulation. The protein is completely lost in the double mutant showing synergistic positive regulation by Hfq and Fur. In *B. subtilis* studies on aconitase have found it to be an RNA binding factor and regulated by Fur which supports our findings (Alén & Sonenshein, 1999; Gaballa et al., 2008).

From the membrane extract the ClpP protease was identified. This protein is Fe regulated and is induced under high Fe conditions. In the *fur* mutant the protein still appears Fe regulated but the protein level has decreased. This suggests that Fur is positively regulating ClpP but the Fe regulation is not due to Fur. In the *hfq* mutant there is a reduction in the protein level indicating positive regulation. The protein is lost in the double mutant showing synergistic positive regulation by Hfq and Fur. The Clp

family of proteins is important for the response to a variety of stresses by degrading misfolded proteins, along with an involvement in intracellular replication and biofilm formation (Frees et al., 2004). ClpP specifically has been found to positively regulate exoprotein expression and virulence in a mouse model (Frees et al., 2003). It has also been shown to be involved with Sar and Agr regulation indicating it has an important role in global regulation (Frees et al., 2005). Coincidentally aconitase has been found to be regulated by Clp proteins and involved in oxidative stress resistance (Chatterjee et al., 2005).

The identification of these novel Hfq regulated proteins supports our findings of a role for Hfq in the oxidative stress response and exoprotein expression. And through aconitase is also linked to metabolism, indicating Hfq does have a subtle yet global role in *S. aureus* regulation.

3.3.5 The *hfq* and *fur* mutants do not show increased sensitivity to detergents or autolysin activity

The surface protein profiles, especially in the SDS and supernatant fraction, revealed an increased number of surface proteins obtained from the *hfq/fur* double mutant. One reason for this could be that the culmination of both mutations has affected the cell wall integrity and therefore cells lyse more easily. To investigate this, Newman wild type and mutant strains were subjected to the detergents SDS (10% w/v) and Triton X-100 on agar plates. A Triton induced autolysin assay was also conducted to determine whether regulation of autolysin (*atl*) is affected. Autolysin is important for the correct turnover of cell wall peptidoglycan and a null mutant was found to have a rougher cell surface and show increased resistance to penicillin (Takahashi et al., 2002). Previous

studies have also indicated a role of Atl in protein-mediated biofilm formation, this could be through lysis of bacterial cells releasing proteins and eDNA required for biofilm formation (Houston et al., 2011). In the mutant strains, *atl* could be up regulated causing changes in protein levels on the surface or actually causing increased lysis of these cells.

For the plate assays, strains were spread onto LA plates with discs containing 10% SDS or Triton X-100. Plates were incubated at 37°C then the zones of lysis measured. For autolysin induced lysis, Newman wild type and mutant strains were grown to exponential phase, washed and then resuspended in PBS ± 0.02% Triton X-100. Cultures were incubated shaking at 37°C and the OD₆₀₀ measured over time. Those samples in PBS only were to account for any differences in optical density that occur without the addition of Triton. The Triton agent should induce production of autolysin causing the cells to lyse and the optical density to drop over the course of the assay. The plate assays showed no difference in lysis between the strains with SDS or Triton X-100 (data not shown). There were no significant differences seen between the strains in the Triton X-100 induced autolysis (Figure 3.7) indicating that autolysin production isn't affected by the *hfq* and *fur* mutations. These results suggest that the increased numbers of proteins in the double mutant are due to regulation changes and not a result of increased lysis.

3.3.6 Hfq and Fur are both required for full haemolysin and nuclease activity

Our protein work shows that Hfq and Fur regulate Eap and affect surface protein expression. Fur has been found to positively regulate a number of exoproteins in addition to Eap which have an important affect on virulence

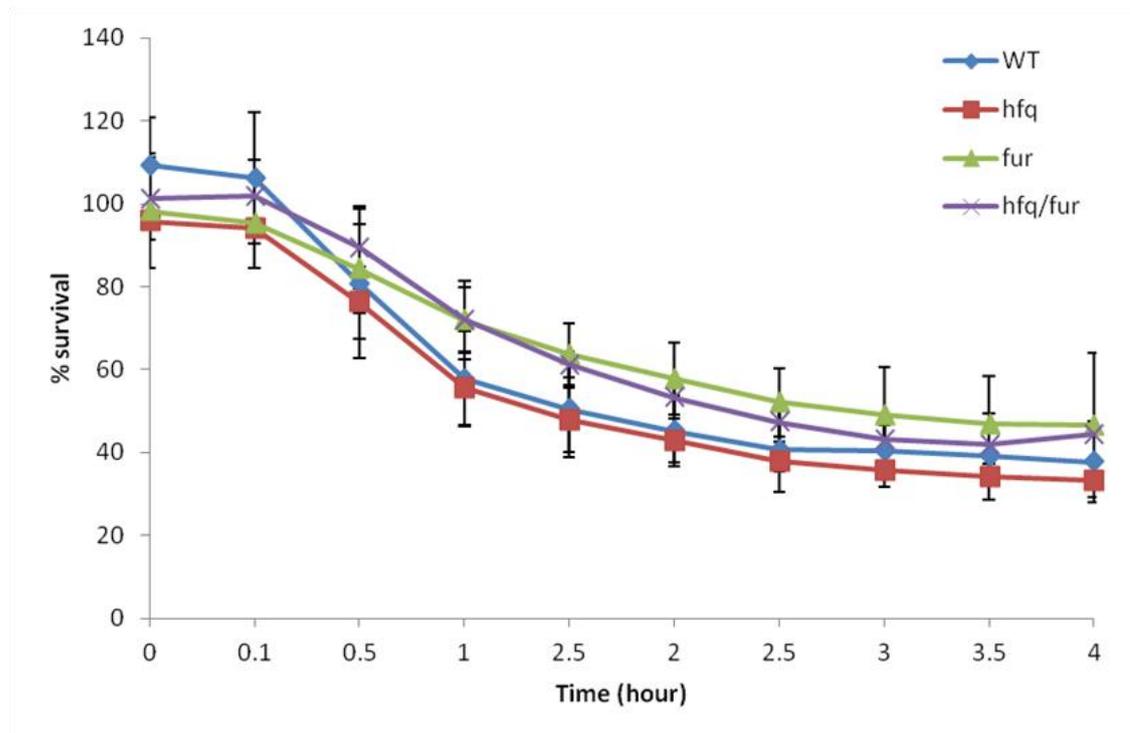


Figure 3.7 Comparison of Triton-X100 induced lysis in Newman wild type and mutant strains. Cells from exponential phase cultures were washed then incubated in PBS or PBS + 0.02% triton-X100. The OD₆₀₀ of each sample was measured after 10 minutes, 30 minutes and 30 minute intervals thereafter. The graph represents data from three independent experiments and error bars show the standard deviation for each time point.

(Johnson et al., 2008; Johnson et al., 2011; Torres et al., 2010). Therefore Hfq may also be involved in the regulation of exoproteins. To investigate the effect of Hfq and Fur on specific exoprotein activity plate assays were conducted to show the presence of haemolysin and nuclease activity in the wild type and mutant strains.

Cultures of each strain were spotted on to agar plates containing rabbit, sheep or horse blood to examine haemolysin activity. Most *S. aureus* strains contain 4 different haemolysins; α , β , δ and γ . The use of different animal blood has been used to differentiate between the activities of the haemolysins. α -haemolysin has found to be particularly effective in lysing rabbit erythrocytes, whereas β -haemolysin will lyse sheep blood but not rabbit (Dinges et al., 2000). The activity of γ -haemolysin will not be shown on the blood agar plates as agar appears to have inhibitory effects (Dinges et al., 2000). The horse blood plates (not shown) showed little visible haemolysis. The rabbit and sheep blood plates showed the same pattern of haemolysis between the strains. The *hfq* mutant showed little difference in haemolysis compared to the wild type strain (Figure 3.8.A). Whereas, the *fur* mutant showed a clear decrease in lysis compared to wild type but the colony size was also reduced which may account for this. However, as with Eap protein expression, the double mutant showed no lysis indicating that Hfq and Fur both act positively on haemolysin activity (Figure 3.8.A).

A second quantitative haemolysin assay was subsequently carried out to determine exactly how much effect each mutation has on haemolysis. This would also account for the growth difference seen in the *fur* mutant in rich media. Supernatant protein preparations were made from each strain and equalised based on bacterial pellet weight. Serial dilutions of these proteins were added to liquid blood and incubated for 30

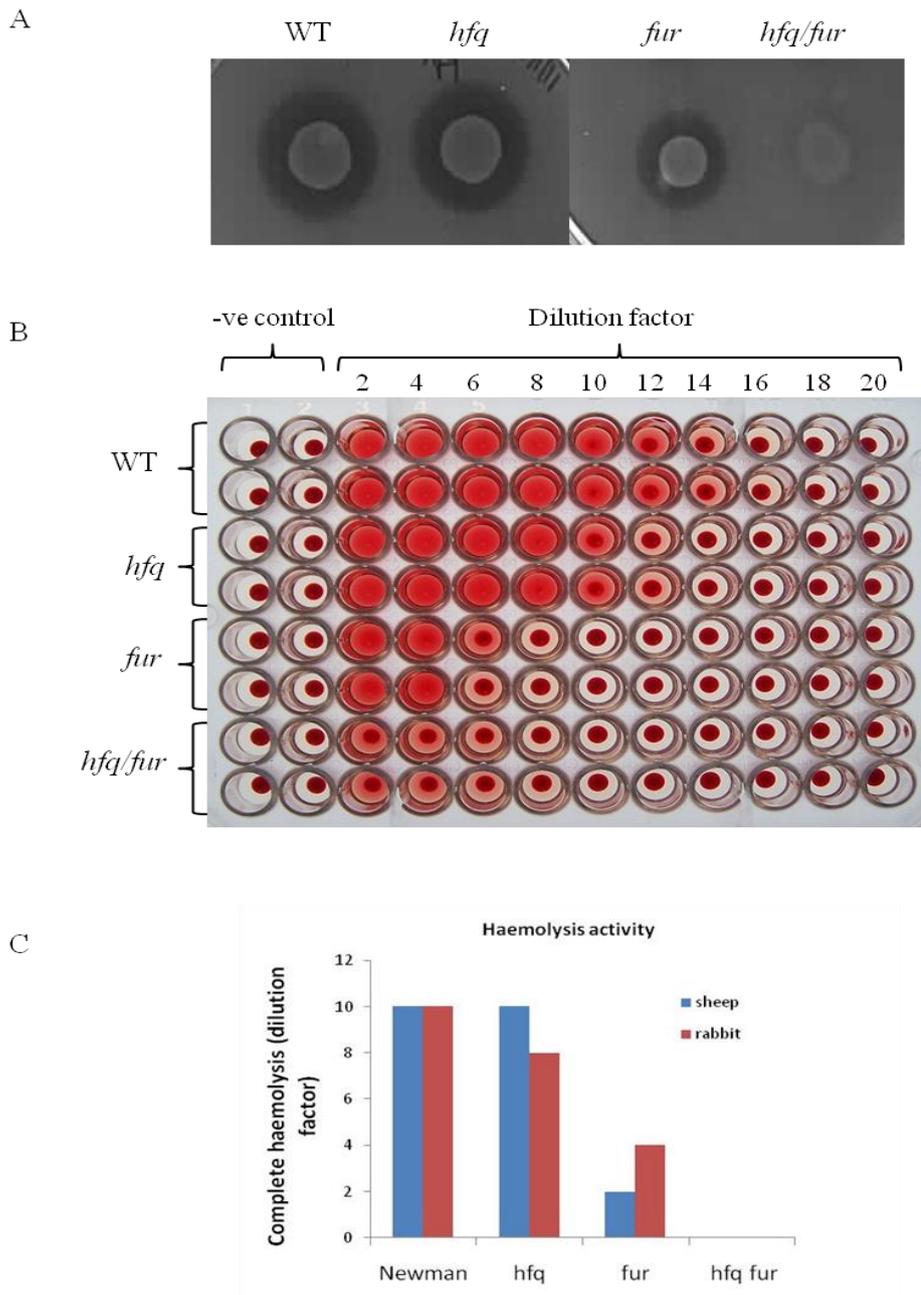


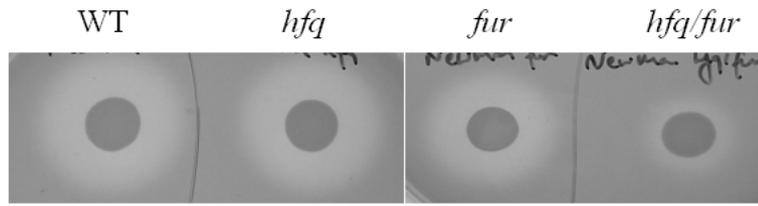
Figure 3.8 Haemolysin activities in Newman wild type and mutant strains. (A) shows results from strains spotted onto rabbit blood agar. (B) is an example of the quantitative assay with rabbit blood. (C) is a graphical representation of the quantitative blood assay results with rabbit and sheep blood. All data presented is representative of at least three independent experiments.

minutes. This assay was conducted using rabbit and sheep blood.

As Figure 3.8.B shows haemolysis decreases as the protein preparation is diluted. The dilution factors compared are the highest dilution factor that still causes total lysis, as partial lysis can be subjective. With sheep blood there was no difference between the *hfq* and wild type strains, with both strains showing complete lysis up to dilution factor 10. The *fur* mutant still shows a significant decrease in lysis (dilution factor 4) whilst the double showed no complete lysis indicating that Hfq, together with Fur is acting positively on haemolysin activity (Figure 3.8.C). In rabbit blood there is a decrease in total lysis in the *hfq* mutant (dilution factor 8) compared the wild type (dilution factor 10). The *fur* mutant shows a decrease in haemolytic activity (dilution factor 4) however this is 2 dilution factors greater than in sheep blood. The double mutant still shows no total lysis (Figure 3.8.C). These data agree with the previous plate assays and shows that the reduction in haemolysis activity in the *fur* mutant was not due to reduction in growth and therefore both Fur and Hfq positively regulate haemolysin expression. The difference in lysis of the *hfq* mutant between rabbit and sheep blood may indicate that Hfq has a greater regulatory role on *hla* expression.

The DNase activity plates showed a similar pattern to that of the blood plates. The *hfq* mutant showed little change in activity compared to the wild type (1.3 mm) (Figure 3.9). The *fur* mutant showed a significant decrease in activity (4 mm) and there was a greater decrease in the double mutant (11 mm) (Figure 3.9). Therefore Hfq and Fur positively regulate DNase activity. Together these data suggest that Hfq is involved in positive Fur regulation of exoprotein expression.

A



B

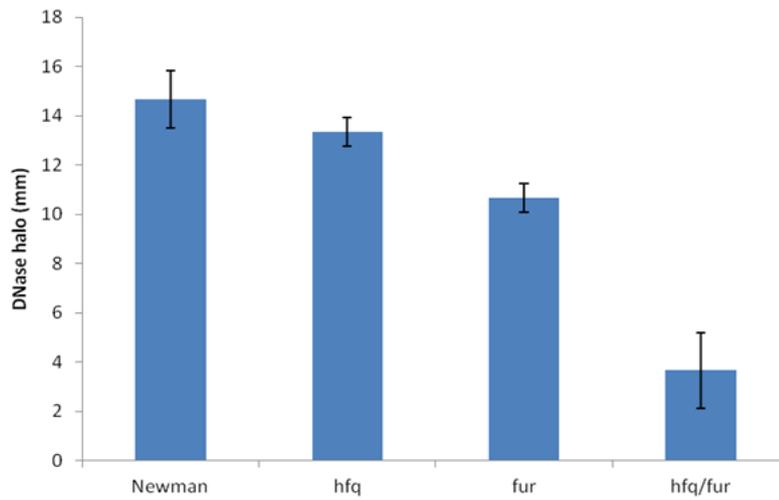


Figure 3.9 DNase activities in Newman wild type and mutant strains. (A) shows an example of plate results. (B) is a graphical representation of the halo diameters of each strain. The data shown is the average of three independent experiments with error bars showing standard deviation for each data point.

3.4 Phenotypic analysis of *hfq* and *fur* mutants in SH1000 and Mn8

Although we do show subtle phenotypes in the *S. aureus hfq* mutant it does not show the obvious phenotypes seen in other bacteria. This is consistent with Bohn et al. (2007) who also reported none of the phenotypes found in other bacterial species; however these results do contradict the study published by Liu et al. (2010) who found clear differences in their *hfq* mutants. These varying results could be due to the different strains used. *S. aureus* is highly adaptable and this is highlighted by the large number of different strains with various phenotypes. Also growth conditions can greatly affect gene regulation and can lead to differences between studies. Therefore the assays in this study were repeated with the *hfq* and *fur* mutants in different strain backgrounds in order to identify any differences between them. The two strains chosen were SH1000 and Mn8. Newman has a point mutation in *saeS* which makes it constitutively active. For a wild type *saeRS* strain SH1000 was chosen. This is a *rsbU* repaired version of 8325-4 which is from the same sequence type as Newman. Mn8 was chosen as it is *rsbU* and *saeS* wild type and contains the variant Hfq (G69A, A71E) and had the antibiotic sensitivities required to transduce the mutations.

3.4.1 Hfq and Fur are involved in growth in rich media and resistance to oxidative stress in multiple strains

To investigate any strain differences in oxidative stress resistance the 7 hour and 4 hour oxidative resistance assays were repeated with Mn8 and SH1000 strains and compared to Newman. Looking at Mn8 results we found no significant difference between the wild type strain and the *hfq* mutant when grown in rich medium consistent with Newman (Figure 3.10.A). The *fur* growth defect ($P < 0.005$) and partial rescue in the double mutant ($P < 0.01$) was also found with the Mn8 strains as well (Figure 3.10.A).

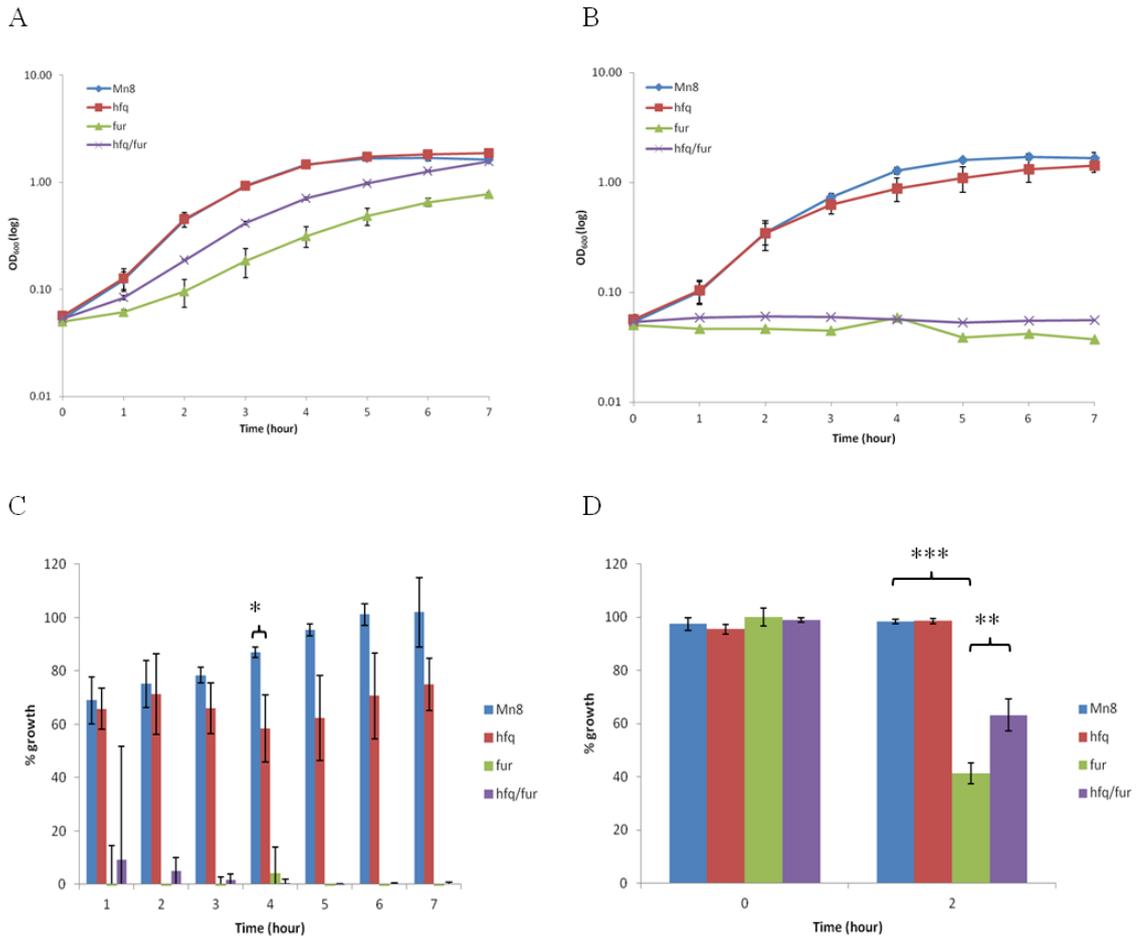


Figure 3.10 Comparison of Mn8 wild type and mutant strains grown in (A) TSB and (B) TSB plus H₂O₂. Overnight cultures were used to inoculate 25 ml fresh media to OD₆₀₀ 0.05 and 0.0024% H₂O₂ added when required. Cultures were incubated at 37°C and OD₆₀₀ taken at hourly intervals for 7 hours. (C) Percentage growth difference in TSB with or without H₂O₂. (D) Percentage difference when H₂O₂ was added during exponential growth and OD₆₀₀ readings taken for 2 hours. The data presented is an average of three independent results and error bars show the standard deviation for each time point. * indicates a P-value < 0.05, ** indicates a P-value < 0.01 and *** indicates

In the presence of H₂O₂ the Mn8 strains also followed the same pattern as Newman. The Mn8 wild type and *hfq* mutant show no difference in oxidative stress resistance until late exponential phase ($P < 0.05$), whilst the *fur* and double mutants showed no growth (Figure 3.10.B/C). When H₂O₂ was added during exponential growth there was no significant difference between the wild type and *hfq* mutant. Whilst the *fur* mutant showed a significant growth decrease ($P < 0.0001$) the double mutant showed that the *hfq* mutation partially recovers resistance to oxidative stress ($P < 0.01$) (Figure 3.10.D). The similarity of these results with Newman provides more evidence that Hfq is involved in oxidative stress resistance in *S. aureus*.

When these experiments were carried out with the SH1000 strains the results were different from that of Newman and Mn8. As with the previous strains the wild type and *hfq* mutant showed no significant differences in growth when grown in rich media (Figure 3.11.A). The *fur* mutant still shows a significant decrease in growth ($P < 0.005$), although the decrease in growth observed is less than in Newman and Mn8. The double mutant does not show the improved growth as seen with the previous strains but this may be due to the reduced defect in SH1000 compared to the other strains (Figure 3.11.A). This indicates that Hfq is not involved with growth in rich medium in SH1000. However under oxidative stress the results were the same as those found with Newman and Mn8. When H₂O₂ was added the *hfq* mutant showed no difference in oxidative stress resistance compared to the wild type. As with Newman and Mn8 the *fur* and double mutants did not grow under oxidative stress (Figure 3.11.B). When H₂O₂ was added during exponential growth the *fur* mutant showed a significant decrease in growth ($P < 0.0001$) which again was partially recovered in the *hfq/fur* ($P < 0.005$) (Figure 3.11.D). This shows that although we do not see the same pattern of growth in

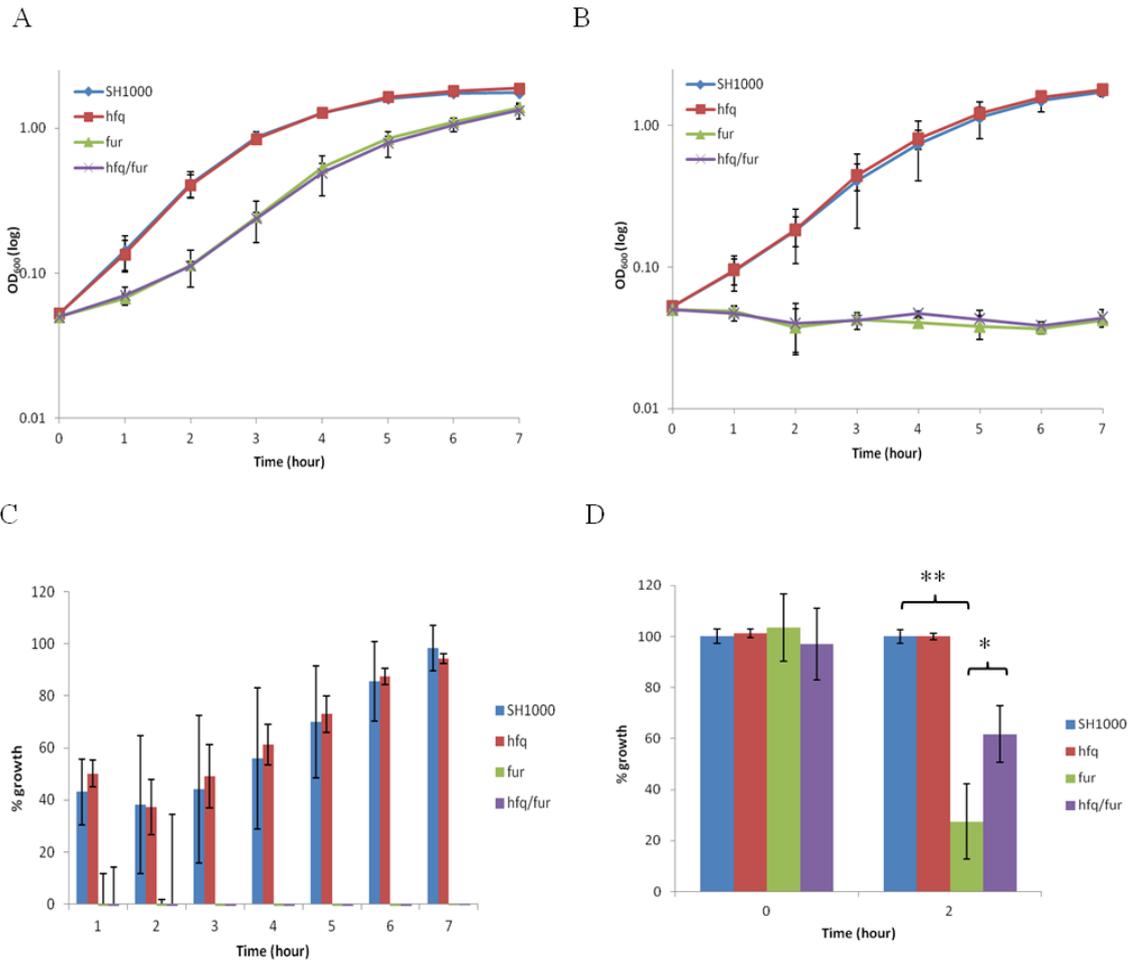


Figure 3.11 Comparison of growth of SH1000 wild type and mutant strains in (A) TSB and (B) TSB plus H₂O₂. Overnight cultures were used to inoculate 25 ml fresh media to OD₆₀₀ 0.05 and 0.0024% H₂O₂ added when required. Cultures were incubated at 37°C and OD₆₀₀ taken at hourly intervals for 7 hours. (C) Percentage growth difference in TSB with or without H₂O₂. (D) Percentage growth difference when H₂O₂ was added during exponential growth and OD₆₀₀ readings taken for 2 hours. The data presented is an average of three independent experiments with error bars showing the standard deviation of each data point. * indicates a P-value < 0.005 and ** indicates a P-value < 0.0001

TSB compared to Newman and Mn8, Hfq is still involved in oxidative stress resistance in SH1000.

3.4.2 SH1000 and Mn8 do not show loss of Eap protein in the double mutant

Protein profiles carried out with Newman strains showed that both Hfq and Fur positively regulate Eap, and that both are required for expression (Figure 3.5). To determine whether this regulation is the same across strains the protein profile experiments were repeated with Mn8 and SH1000. The first difference observed between Mn8 and SH1000 was the size of Eap. In SH1000, as in Newman, Eap runs to just below the 70kDa marker. However in Mn8 Eap runs at the same level as the 70kDa marker. This band was sequenced and confirmed to be Eap. The Mn8 Eap sequence does not appear to be longer than Newman and therefore it is unknown why Eap runs higher on a SDS gel. The protein profiles of Mn8 and SH1000 showed the same positive regulation of *eap* in low Fe, and showed positive regulation by Fur as protein levels decreased in the *fur* mutant (Figure 3.12). However, unlike Newman, there was no loss of Eap protein in the double mutant in either Mn8 or SH1000. In Mn8 *hfq* there is a decrease in Eap protein level which is not seen in SH1000 *hfq*. Therefore in Mn8 and SH1000 regulation of Eap occurs differently than in Newman.

3.4.3 Mn8 and SH1000 mutants showed significantly different haemolysin and DNase activities than Newman

In Newman Hfq and Fur both positively regulated haemolysin and DNase activity. When spotted onto sheep or rabbit blood agar the Mn8 wild type strains shows a clear halo indicating haemolytic activity but this activity was lost in both the *hfq* and *fur* single mutants (Figure 3.13.A). This indicates that Hfq and Fur are essential for

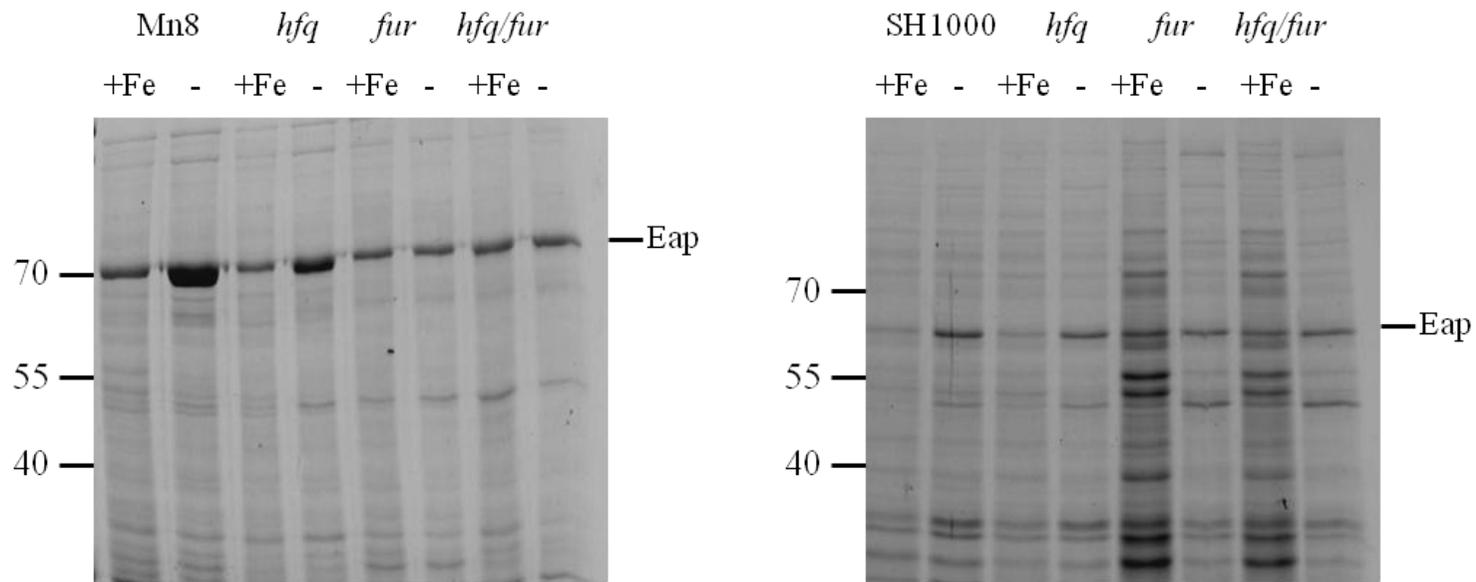


Figure 3.12 Non-covalently bound protein profiles of Mn8 and SH1000 wild type and mutant strains. Protein extracts were obtained from cells grown for 16 hours in CRPMI \pm 10 μ M Fe. Gel pictures are representative of three independent experiments.

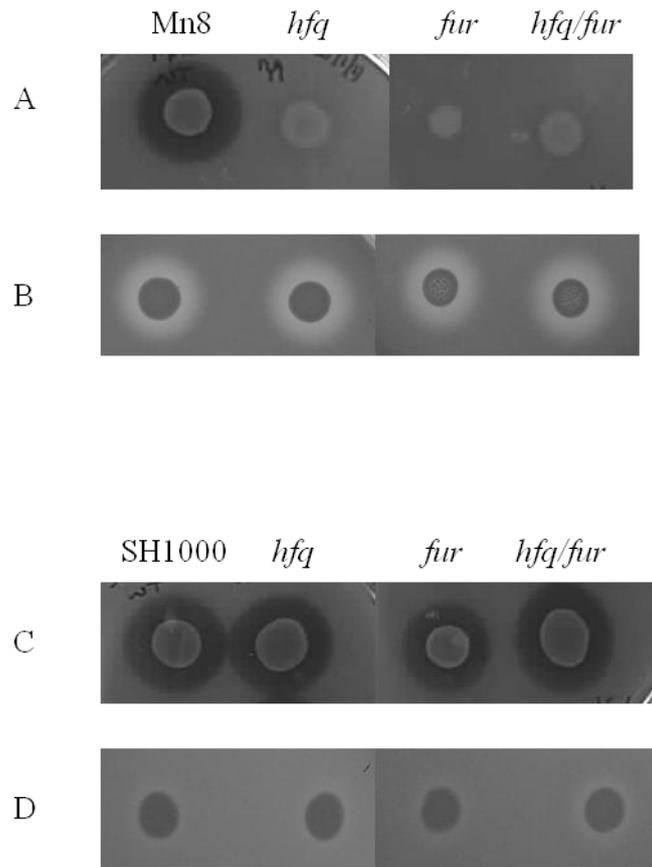


Figure 3.13 Haemolysin and DNase plate assays for Mn8 and SH1000 wild type and mutant strains. (A) and (C) show the strains grown on rabbit blood agar. (B) and (D) show strains grown on DNase plates. Pictures are representative of three independent experiments.

haemolysin activity in Mn8. SH1000 showed incomplete lysis on sheep and rabbit agar meaning only a faint halo is present (Figure 3.13.C). This suggests that SH1000 may not express all the different haemolysins or that their activity is reduced. β -haemolysin is also known as the hot-cold toxin as it does not lyse sheep erythrocytes until incubated at 4°C. Therefore after incubation at 37°C the blood plates were incubated at 4°C for 16 hours. Although the edges of the halo became more distinct there was still incomplete lysis indicating that there is β -haemolysin activity but this was not the reason for the incomplete lysis in SH1000.

To investigate nuclease activity strains were spotted onto DNase agar plates. Unlike Newman there was no difference in DNase activity in the Mn8 or SH1000 mutant strains compared to the wild type (Figure 3.13.B). There was a very faint halo seen with SH1000 indicating very little DNase activity (Figure 3.13.D). These results show the variation in exoprotein regulation between strains and also show that within the same strains not all exoproteins are regulated the same way. In Mn8 haemolysin activity is regulated by Hfq and Fur whilst DNase activity is not.

3.4.4 Newman and Mn8 *hfq* mutants do not affect virulence in the *Galleria mellonella* infection model

In Gram negative bacteria the virulence of *hfq* mutants has been found to be reduced in a variety of models (See Chapter 1.7.4). Liu et al. (2010) investigated virulence of their *S. aureus hfq* mutant using a murine peritonitis model. The results showed a significant decrease in the survival of the infected mice indicating Hfq was required for virulence in *S. aureus*. To determine whether virulence is affected in our *hfq* mutant strains the *Galleria mellonella* virulence model was used. Larvae of the wax moth *G. mellonella*

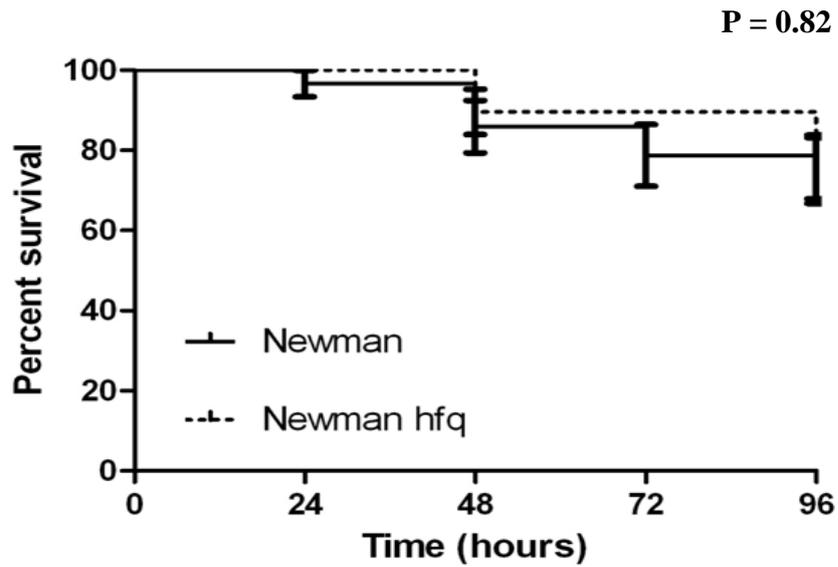
have been used to investigate the virulence of a number of organisms including *S. aureus* (Peleg et al., 2009; Purves et al., 2010; Desbois & Coote, 2011; Kelly & Kavanagh, 2011). These insects share many common aspects to mammalian innate immune systems and have consistently shown comparable results to similar experiments conducted with mammalian models (Jander et al., 2000). Therefore our results with this model can be compared to those of Liu et al. (2010).

G. mellonella larvae were injected with Newman and Mn8 wild type and *hfq* mutant strains and incubated for 4 days and the number of dead larvae counted every 24 hours. Groups of larvae were injected with PBS or not injected at all to act as negative controls. These controls showed 100% survival during each assay. Neither strain showed a significant difference in virulence between wild type and *hfq* mutant nor was there a difference in virulence between Mn8 and Newman wild type strains (Figure 3.14). These results show that loss of Hfq alone is not enough to reduce virulence in this model.

3.5 8325-4 *hfq* phenotypic results did not correlate with those from Liu et al. (2010)

During the course of this study Liu et al. published a paper reporting the importance of Hfq to gene expression and virulence in *S. aureus* 8325-4. Transduction of the *hfq* mutation into this strain resulted in an increased yellow colour of colonies and increase in oxidative stress resistance. RT-PCR showed an increase in expression of the carotenoid pigment which is responsible for the yellow colouring and has also shown to have antitoxic properties which could explain the increased resistance. Microarray analysis revealed 116 genes that showed altered expression in the *hfq* mutant, including

A



B

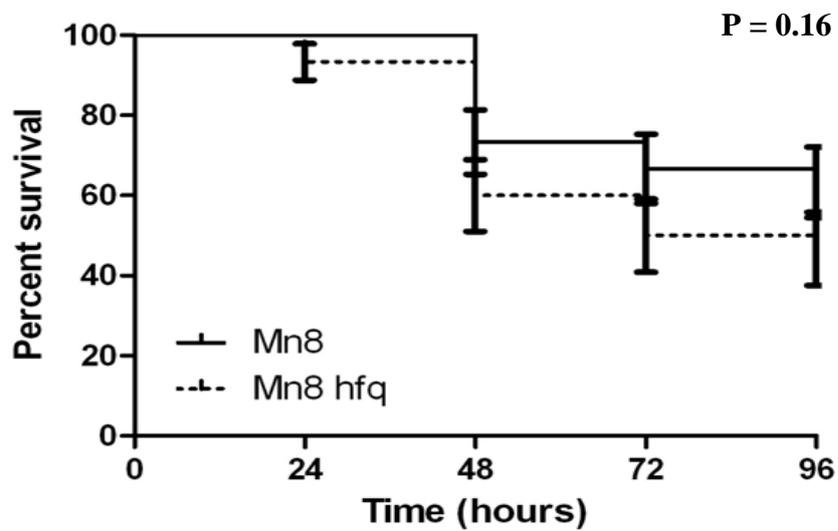


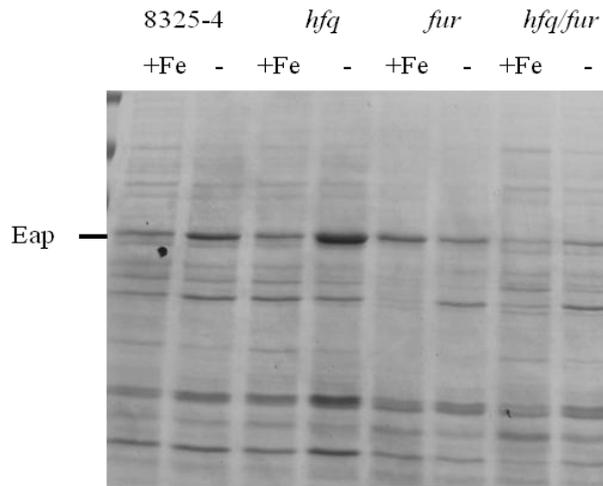
Figure 3.14 Pathogenesis of Newman and Mn8 wild type and hfq mutant strains in the *Galleria mellonella* infection model. The graph shows % survival of the *Galleria mellonella* larvae at 24, 48, 72 and 96 hours post inoculation with *S. aureus*. The data shows the average of three independent experiments and error bars show standard error. P-values comparing mutant and wild type are shown on the graphs.

Eap which we observed in our studies. However we did not observe any of these phenotypes in SH1000, the *rsbU* repaired 8325-4 strain. Therefore we investigated pigment production, oxidative stress and global protein expression in 8325-4 and *hfq*, *fur* and double mutants. We saw no colour change between our 8325-4 wild type and mutant strains or an increase in oxidative resistance. In fact we saw a significant decrease in oxidative stress resistance in the *hfq* mutant (Figure 3.15.B). The exoprotein protein profiles showed no changes in expression, not even with Eap which is consistent with our SH1000 results (Figure 3.15.A). There are clearly significant differences between these studies which are not understood.

The 8325-4 *hfq* mutant in the Liu et al. paper was also referred to as Δhfq -8325. This may just be a shortening of 8325-4 but 8325 is the phage positive strain from which 8325-4 is derived (Herbert et al., 2010). To investigate whether the differences between the *hfq* mutants were actually due to differing strains the *hfq* mutation was transduced into strain 8325. During these transductions different coloured colonies were produced (Figure 3.16), both of which were *hfq* mutants confirmed via PCR. The pale colour of 8325 is attributed to the *rsbU* mutation which in turn reduces carotenoid production (Kullik et al., 1998) therefore the *rsbU* gene in both colour variants were sequenced to see if this accounted for the colour change. The sequence data revealed that in the yellow variant the *rsbU* mutation had been repaired. Phenotypic analysis was carried out with both *hfq* variants to see if they also showed differing phenotypes. From here on *hfq*(W) refers to the white colour variant and *hfq*(Y) refers to the yellow colour variant.

As with the 8325-4 strains we investigated oxidative stress resistance, virulence factor expression and haemolysin activity of the 8325 wild type and both *hfq* mutant variants.

A



B

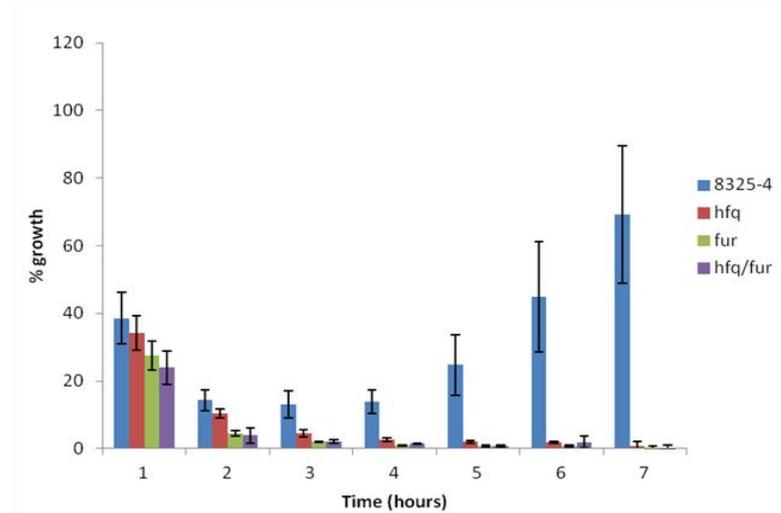


Figure 3.15 (A) Non-covalently bound protein profile and (B) oxidative stress resistance of 8325-4 wild type and mutant strains. Proteins were extracted from cells grown for 16 hours in CRPMI \pm 10 μ M Fe. The gel picture shown is representative of at least three independent experiments. For oxidative stress assays overnight cultures were used to inoculate 25 ml fresh media to OD₆₀₀ 0.05 and 0.0024% H₂O₂ added. Cultures were incubated at 37°C and OD₆₀₀ taken at hourly intervals for 7 hours. The data is shown as % growth comparing growth with H₂O₂ to that in TSB alone. The data presented is an average of at least three independent results and error bars show the standard deviation for each time point.

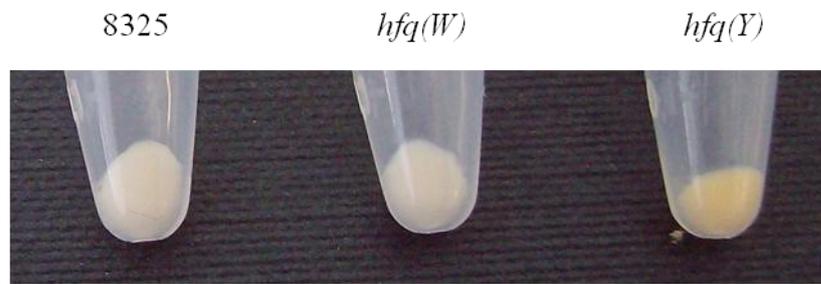


Figure 3.16 Pigmentation variations between 8325 wild type and *hfq* mutant strains. Strains were grown in BHI for 16 hours and cells pelleted.

We found that the yellow *hfq* variant showed no difference in oxidative stress resistance compared to the wild type, whereas the white variant shows decreased resistance ($P < 0.05$) (Figure 3.17.A). There was no difference in cell wall protein expression or haemolysin activity between wild type and *hfq(W)*. However *hfq(Y)* showed decreased cell wall protein expression and increased haemolysin activity which is consistent with the repaired *rsbU* phenotype (Figure 3.17.B/C). None of the phenotypes found are consistent with those of Liu et al. (2010) therefore other factors in their strain 8325-4 must be involved.

3.6 Investigation into the level of Hfq protein in our experimental strains

It has been shown by Northern blotting and RT-PCR that *hfq* is expressed in *S. aureus* COL, RN6390 and Newman strains but at much lower levels than in other bacteria (Bohn et al., 2007). Further to this Liu et al. (2010) conducted Western analysis to determine whether this mRNA is translated into Hfq protein. The results revealed that the protein is not detected in some strains, including COL and RN6390 which were used in the Bohn et al. (2007) paper. Western analysis was conducted to investigate the levels of Hfq protein in our experimental strains to determine whether this accounts for differing *hfq* regulation across strains.

3.6.1 Over expression of the Hfq protein

Before carrying out the Western analysis a positive control was needed, therefore the Hfq protein was over expressed and purified from *E. coli*. The expression of Hfq was not as straight forward as with Fur (See Chapter 4) and three different expression vectors were tried. Initially *hfq* was amplified and cloned into pLEICS01 and pLEICS02

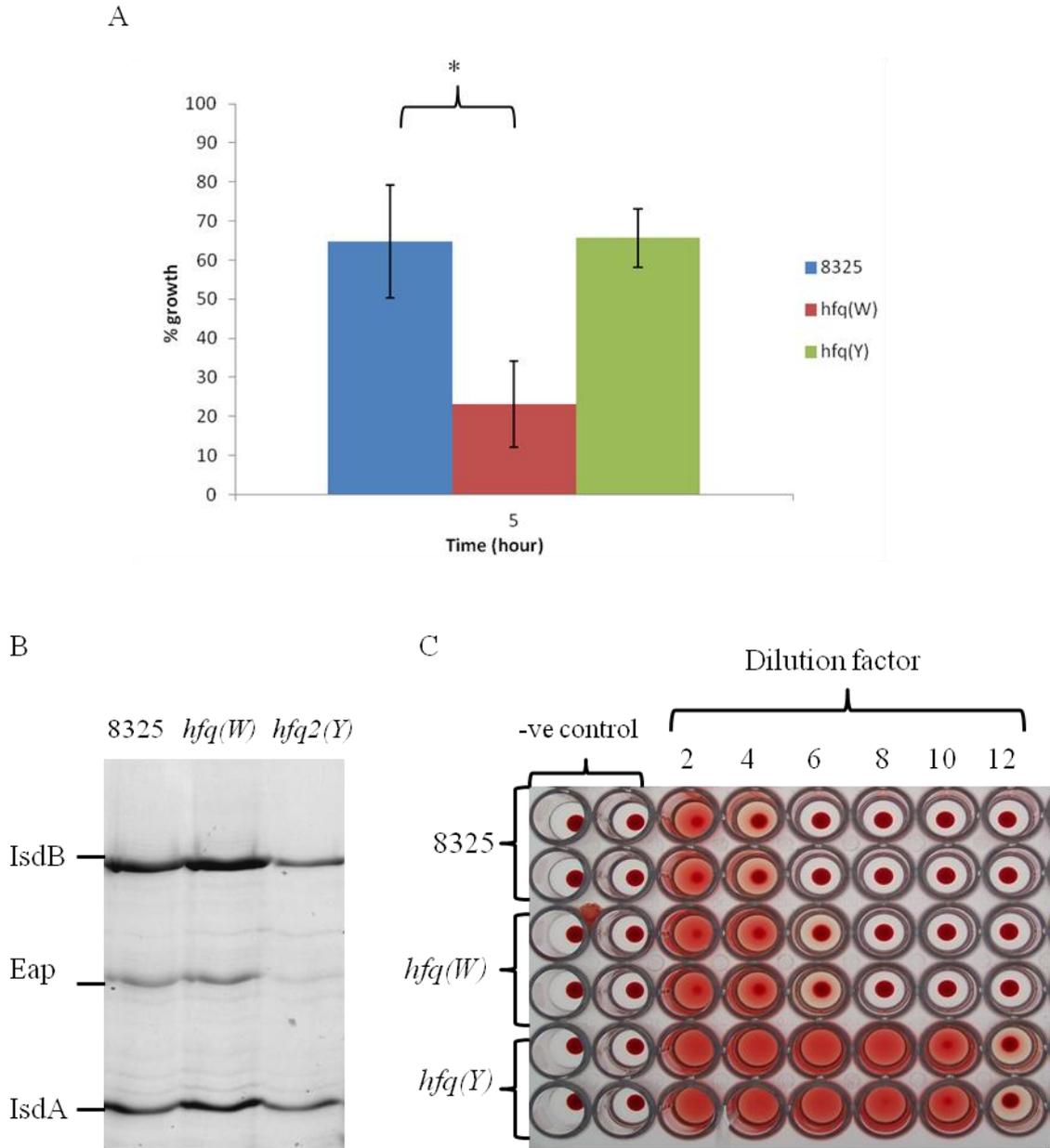


Figure 3.17 Phenotypic analysis of the 8325 and the *hfq(W)* and *hfq(Y)* mutants.

(A) Single time point of an oxidative stress assay. The graph is an average of three independent experiments and error bars show standard deviation. * indicates a P-value < 0.05 (B) Cell wall protein profile from cells grown for 16 hours in CRPMI. (C) Quantitative haemolysin assay with sheep blood. Gel and haemolysin plate pictures are representative of at least three independent experiments.

by PROTEX (University of Leicester). The Hfq proteins expressed from these plasmids contain a C-terminal HIS₆ tag and GST tag respectively. After sequencing to ensure the *hfq* gene sequence was correct the plasmids were transformed into Rosetta *E. coli*. Initially strains were grown in LB and expression induced with 280 μM IPTG at 30°C for 1 hour. Bacterial cells were lysed and centrifuged to separate the soluble and insoluble fractions. Samples were separated on 16% acrylamide gels and Western analysis carried out with anti-HIS and anti-GST antibodies (data not shown). No band was seen with the Hfq-HIS blot, but a band was seen on the GST blot. However the size of the Hfq-GST band seemed to equate to the GST tag only. The Hfq protein is only small (~8kDa) and so there may not be an obvious size difference on a gel. Samples were purified and treated with TEV protease to remove the tag and analysed on an acrylamide gel. The protein appeared to be the same mass and no smaller protein was seen suggesting that the Hfq protein is not present. Further investigation with the Rosetta Hfq-HIS strain was carried out using different concentrations of IPTG and different incubation times. Samples were also run on a higher percentage gel and transferred onto PVDF-P^{SQ} (Millipore) which has a smaller pore size in case the protein was being lost during transfer. The blots still did not show any bands equivalent to the size of Hfq.

The third expression vector used was pCOLD1 which produces a protein with a N-terminal HIS₆ tag and is induced by cold shock. The *hfq* gene was amplified using primers pCOLD hfqF and pCOLD hfqR, digested and ligated into the pCOLD1 vector. The plasmid was then transformed into Rosetta *E. coli* as before. A smaller volume of 50ml was used first to test the expression. Expression was induced as described in the pCOLD1 protocol (see section 2.12.3) and cells lysed as before. Western analysis with

these samples showed a faint band of ~15kDa which is larger than Hfq. The *S. aureus* Hfq protein had been expressed by Huntzinger et al. (2005), personal communication with this group indicated that Hfq tends to naturally form stable hexamers which require a longer heating time with loading buffer. They also used a much larger culture volume for protein expression. Therefore, as per their advice, a 1l culture volume was used and samples were heated to 90°C for 20 minutes before loading. Induction and cell lysis was carried out as before. The induced Hfq protein was difficult to see on the acrylamide gel indicating a low expression yield (Figure 3.18.A). The large band that appears at about 14kDa was seen in all gels and therefore is not induced protein but may be lysozyme which was used to lyse the cells. Western analysis of these samples gave strong bands of ~55kDa meaning that even after the extended heating the Hfq proteins were still forming hexamers (Figure 3.18.A). These samples were passed through an affinity column to purify the Hfq protein, which eluted in buffer containing between 350mM and 500mM imidazole (Figure 3.18.B). These two elution samples were combined and concentrated using an Amicon Ultra Centrifugal Filter Device (Milipore). Bradford assay results determined the concentration of the Hfq protein to be 0.124 mg ml⁻¹. Peptide mass fingerprinting was used to confirm that the purified protein contained Hfq.

3.6.2 *Staphylococcus aureus* Hfq Western analysis

Western blots were developed using anti-Hfq antibodies (Huntzinger et al., 2005) followed by anti-rabbit antibodies, the secondary antibody was later replaced with Protein A peroxidase (Sigma Aldrich) to reduce background noise and produce more distinct bands. Cells were grown in CRPMI and BHI in case there were differences in expression due to the media. Total cell protein extracts and cytoplasmic extracts were examined. Hfq should be located in the cytoplasm so these extracts should contain Hfq

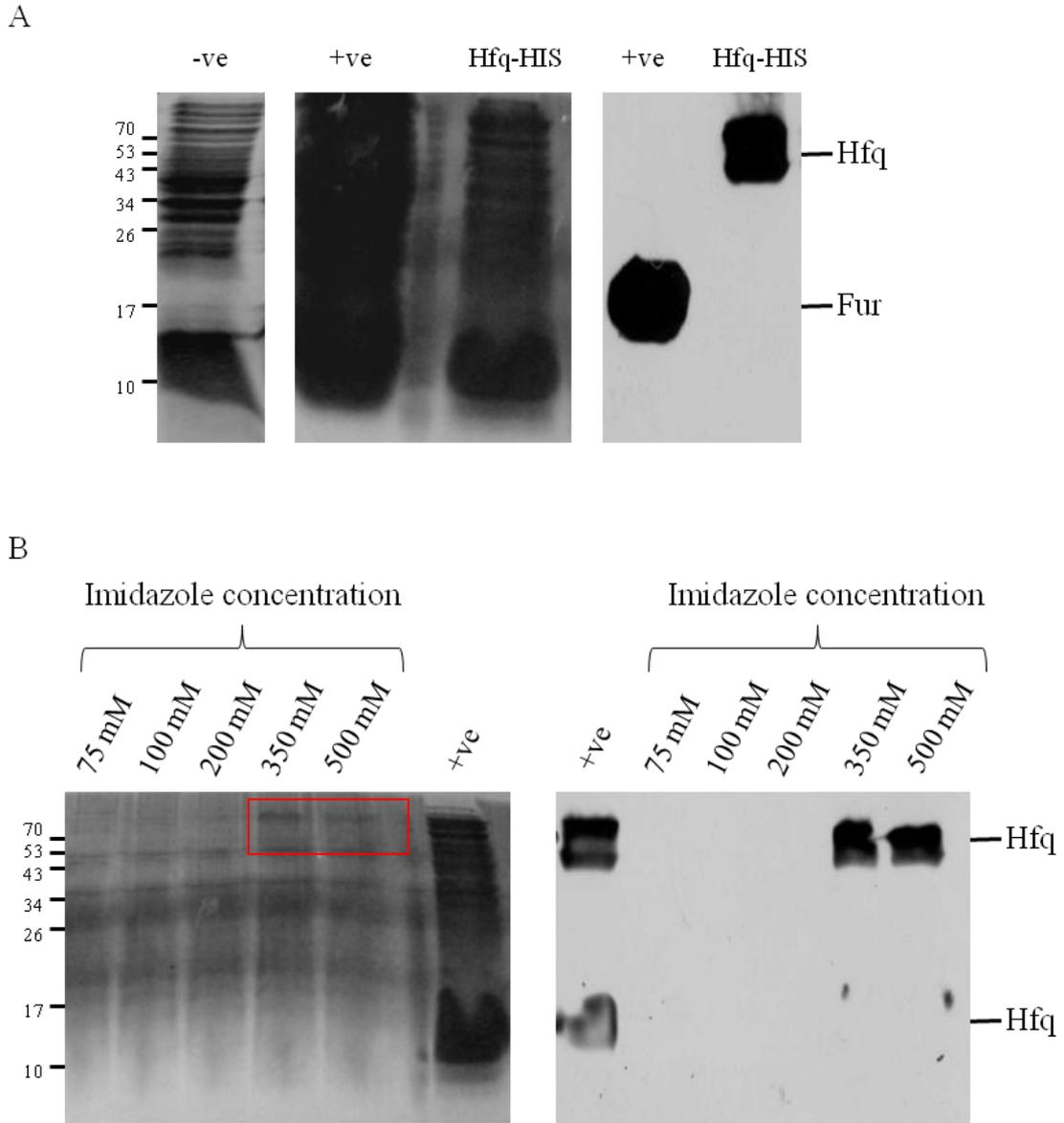


Figure 3.18 Hfq expression from pCOLD1 and purification. (A) Acrylamide gel (16%) and Western blot of soluble *E. coli* extract. The negative control is lysate from *E. coli* containing an empty pCOLD1 vector. The positive control is *E. coli* cell lysate containing Fur-HIS protein. (B) Western blot of the elution samples from the purification column. The positive control is a sample of *E. coli* lysate containing Hfq-HIS. The smaller protein observed in this sample could be Hfq break down products.

but reduce the number of other proteins that may cause unclear blots. The protein extract protocol in Liu et al. (2010) was also repeated to try and replicate their western results. A band correlating to Hfq could not be identified in any of these blots. Several bands could be seen but none of these were the correct size for the Hfq monomer or match the multimers seen with the purified protein control. The bands that were present were not always consistent between blots. Diluted samples of the purified Hfq protein was loaded alongside the *S. aureus* samples, however the signal from this control affected visualisation of other bands. However the use of this protein did confirm that the anti-Hfq antibody was functional. Also *hfq* mutant strains were used as negative controls, in these lanes bands were still present indicating that they are not Hfq. Protein A is known to bind antibodies which means that any protein A present in a blot will also show up. To try and remove any Protein A, samples were treated with agarose-IgG beads (Sigma Aldrich). 40 µl of the agarose-IgG solution was added to protein extracts and incubated at 4°C for 1 hour. The beads were separated from the protein extract by centrifugation. Protein A should bind to the IgG and therefore be removed along with the beads. An 8325-4 *spa* mutant was also looked at to see if we could identify the Protein A band. There was no notable difference in bands between the *spa* mutant and other strains tested or when treated with the IgG agarose (Figure 3.19). Therefore it was unclear what these bands represent.

3.7 Discussion

The aim of the work in this chapter was investigate the role of Hfq in *S. aureus* and its possible co-regulatory role with Fur. Our phenotypic experiments showed positive regulation of important exoproteins by Hfq and Fur and that both are required for maximal expression of these proteins. This is the first experimental evidence of

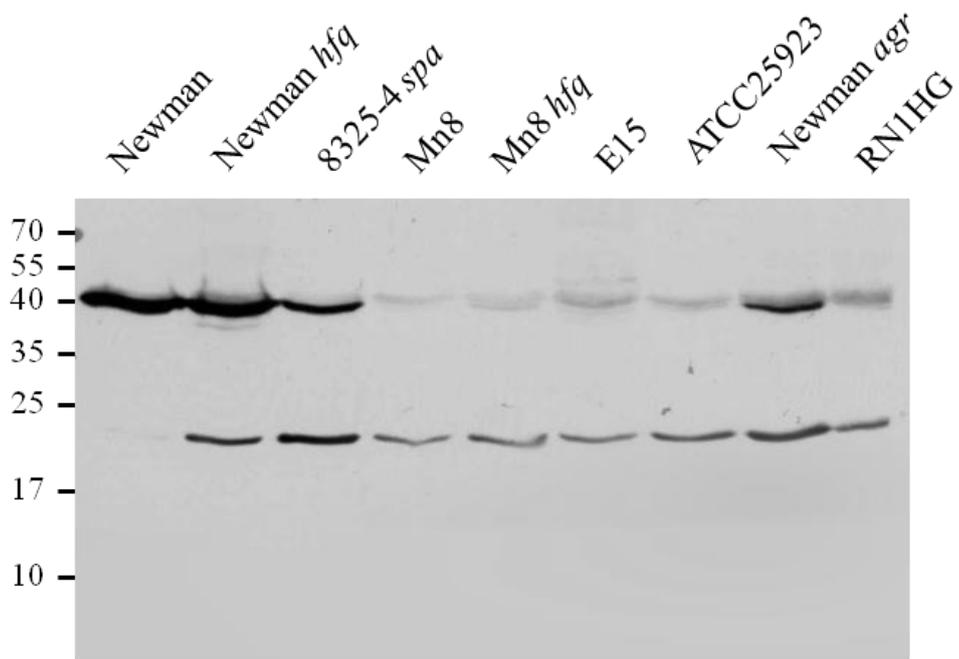


Figure 3.19 Example of Western analysis to identify Hfq protein in different *S. aureus* strains. Bacterial strains were grown in BHI for 6 hours and the cytoplasmic proteins extracted. Extracts were treated with agarose-IgG beads before being loaded onto a 10% protein gel.

regulatory interaction between Hfq and Fur in *S. aureus* although this appears different to that in other bacteria. These results also show that this interaction differs between strains suggesting that other factors may contribute to Hfq and Fur regulatory effects.

3.7.1 Positive protein regulation by Hfq and Fur

All of the proteins identified during phenotypic analysis were found to be positively regulated by Hfq and Fur. This is unusual as in other bacteria Hfq most commonly regulates negatively. In Newman, Hfq and Fur regulation was also found to be synergistic, which is not seen with Hfq and Fur regulation in other bacteria. To confirm these changes are due to the *hfq* and *fur* mutations complementation plasmids should be constructed and transformed into the mutant strains. However, these mutations have been transduced into multiple colonies and show the same phenotypes. Also they do not appear to be polar effects as the *hfq* gene is the last in the operon and previous studies with the *fur* mutant have shown that downstream *xerD* expression is not disrupted (Johnson, 2008). Therefore it can be safely assumed that the observed phenotypes are not due to polar effects or secondary mutations.

Although less common than negative regulation, positive Hfq regulation can occur through sRNA-mRNA binding to either initiate translation or stabilise mRNA transcripts (Soper et al., 2010). However studies so far into sRNA regulation have found that Hfq is not required in *S. aureus* indicating that Hfq may function differently in *S. aureus* (Boisset et al., 2007; Geissmann et al., 2009; Chabelskaya et al., 2010; Bohn et al., 2010). Although predominantly a RNA binding protein involved in sRNA:mRNA interactions Hfq has been found to function in other ways. In *E.coli* Hfq can stabilise mRNA transcripts through inducing poly-A polymerase I activity and binding poly-A

tails to protect them from degradation (Hajnsdorf & Régnier, 2000; Folichon et al., 2005). Another method of stabilisation can occur through binding to nascent transcripts. In *E. coli rpsO* mRNA was found to be directly stabilised through Hfq binding. It was suggested that Hfq binds the nascent transcripts during transcription to overcome transcription pauses or prevent preliminary transcript release (Jacques Le Derout et al., 2010). In addition to binding sRNA and mRNA Hfq has also been found to bind tRNA with high affinity. It has been suggested that Hfq is involved in the processing precursor into mature tRNA molecules and tRNA modification (Zhang et al., 2003; Lee & Feig, 2008). An *E. coli hfq* mutant was found to have reduced translation fidelity resulting in variant peptides, which could result in non-functional proteins (Lee & Feig, 2008). If this occurs in a regulator then it could result in changes in regulation of other genes. Although not fully understood Hfq has also been found to bind DNA, which could affect transcription initiation (Updegrave et al., 2010b; Geinguenaud et al., 2011a). These alternate functions of Hfq can all result in positive regulation of targets.

Looking at studies into other bacteria positive Fur regulation could be due to regulation of a sRNA or through a direct interaction causing transcriptional activation. The most common mechanism of positive Fur regulation is indirectly through classical regulation of a sRNA. However there are examples of Fur directly binding to target genes to activate transcription (Delany et al., 2004; Nandal et al., 2010). In *N. meningitidis in vitro* transcription analysis revealed that addition of Fur directly activated *norB* transcription (Delany et al., 2004). In *E. coli* Fur shows positive regulation of *ftnA* independently of RyhB. The histone-like nucleoid protein (H-NS) acts as a direct repressor of *ftnA* transcription, which is displaced by Fur thereby allowing transcription (Nandal et al., 2010). Further study is required to determine whether positive Fur

regulation occurs directly or indirectly in *S. aureus*. Determining the mechanism of Hfq and Fur positive regulation is important to further the understanding of how virulence is regulated.

During our protein analysis we identified two more proteins, aconitase and ClpP, which were also positively regulated by Hfq and Fur. These proteins are an interesting discovery as they are both involved in regulation and therefore could have downstream effects. Looking at what these proteins regulate may explain some of the regulation we see by Hfq and Fur.

Aconitase is a well known enzyme responsible for the conversion of citrate to isocitrate in the citric acid cycle (Beinert et al., 1996). However further study has revealed that aconitase is a dual function protein depending on whether Fe is present. In the presence of Fe aconitase acts as the citric acid enzyme but in low Fe conditions has been found to function as a RNA binding protein (Tang & Guest, 1999; Alén & Sonenshein, 1999; Banerjee et al., 2007). This RNA binding activity has been demonstrated in *E. coli*, *B. subtilis* and *M. tuberculosis* although the extent of aconitase's role in post transcriptional regulation is not fully understood (Tang & Guest, 1999; Tang et al., 2002; Alén & Sonenshein, 1999; Banerjee et al., 2007). In *E. coli* aconitase appears to be involved with the oxidative stress response through post transcriptional regulation of *sodA*. AcnA was found to bind *sodA* and enhance stability of the transcript (Tang et al., 2002). Aconitase expression was induced under oxidative stress conditions in *H. pylori* indicating that this could be a common role for aconitase across species (Huang & Chiou, 2011). Our results showing positive Fur regulation of aconitase agrees with previous studies in other bacteria that show induction of aconitase by Fur (Gruer &

Guest, 1994; Gaballa et al., 2008). Phenotypic analysis with an aconitase mutant could be conducted to determine the importance of aconitase in *S. aureus* regulation. The ability of aconitase to directly bind to potential target genes could then be confirmed by EMSA with RNA.

The Clp family of proteins are a family of molecular chaperones that are involved in the degradation of misfolded proteins due to stress. These proteins have been shown to be required for the resistance to high temperature, high osmolarity and oxidative stress (Frees et al., 2004). ClpP is a protease and can complex with other Clp proteins to form a proteolytic complex similar to the eukaryotic proteasome (Kessel et al., 1995; Msadek et al., 1998; Frees & Ingmer, 1999; Gaillot et al., 2000). In *S. aureus* ClpP is also involved in the regulation of virulence factors and a *clpP* mutant shows reduced virulence (Frees et al., 2003). In a 8325-4 *clpP* mutant strain there was a reduction in key exoproteins such as haemolysin (α and β), proteases and autolysin, however there was a general increase in the number of extracellular proteins (Frees et al., 2003). Interestingly this is similar to our findings with the Newman *hfq/fur* double mutant, which showed loss of several important factors (Eap, Emp, α -haemolysin, nuclease) but an overall increase of proteins seen in the supernatant and SDS extracts (Figure 3.5.A/C). Further study revealed that ClpP is involved in the regulation of several global virulence regulators including RNAlII, SarS which may account for ClpP regulation of extracellular proteins (Frees et al., 2003; Frees et al., 2012). However proteomic analysis of the *clpP* mutant does not show differential expression of Eap or Emp (Frees et al., 2012). This indicates that the Hfq and Fur regulation of these proteins observed is not simply through ClpP regulation of other global regulators.

3.7.2 Regulation of oxidative stress resistance by Fur and Hfq

Both Hfq and Fur were found to regulate oxidative stress resistance, as in the double mutant there is an improvement in stress resistance suggesting that the *hfq* mutation compensates for the *fur* mutation. In *S. aureus*, Fur has been found to positively regulate oxidative stress genes however the mechanism is not known (Horsburgh et al., 2001; Morrissey et al., 2004). In *E. coli*, Fur positively regulates SodB, an Fe containing protein involved in the oxidative stress response, through the action of the sRNA RyhB (Dubrac & Touati, 2002; Massé & Gottesman, 2002). Under normal growth conditions the RyhB sRNA binds to the *sodB* mRNA in the presence of Hfq to block translation and degrade the RNA molecules which results in no SodB expression (Figure 3.20.A). However under oxidative stress Fur represses *ryhB* expression meaning that *sodB* mRNA is not degraded and gets translated (Figure 3.20.B). In a *fur* mutant RyhB is constitutively expressed so preventing *sodB* translation leading to a loss of oxidative stress resistance. However in an *E. coli hfq* or *hfq/fur* double mutant *ryhB* is derepressed but can no longer efficiently bind to the RyhB sRNA or *sodB* mRNA allowing translation resulting in oxidative stress resistance (Geissmann & Touati, 2004).

Similar results were found when investigating oxidative stress resistance in the *S. aureus hfq* and *fur* mutants, indicating that oxidative stress resistance by Fur may occur through sRNA-mRNA interactions that are yet to be found. However our *S. aureus hfq/fur* double mutants only showed partial recovery of oxidative stress resistance. Investigation with an *E. coli hfq/fur* double mutant has not been carried out however using the model in Figure 3.20 we can predict the results. In the absence of Fur RyhB would be transcribed, however without Hfq it would not interact with *sodB* and therefore would allow translation. This would suggest that the bacteria would still be

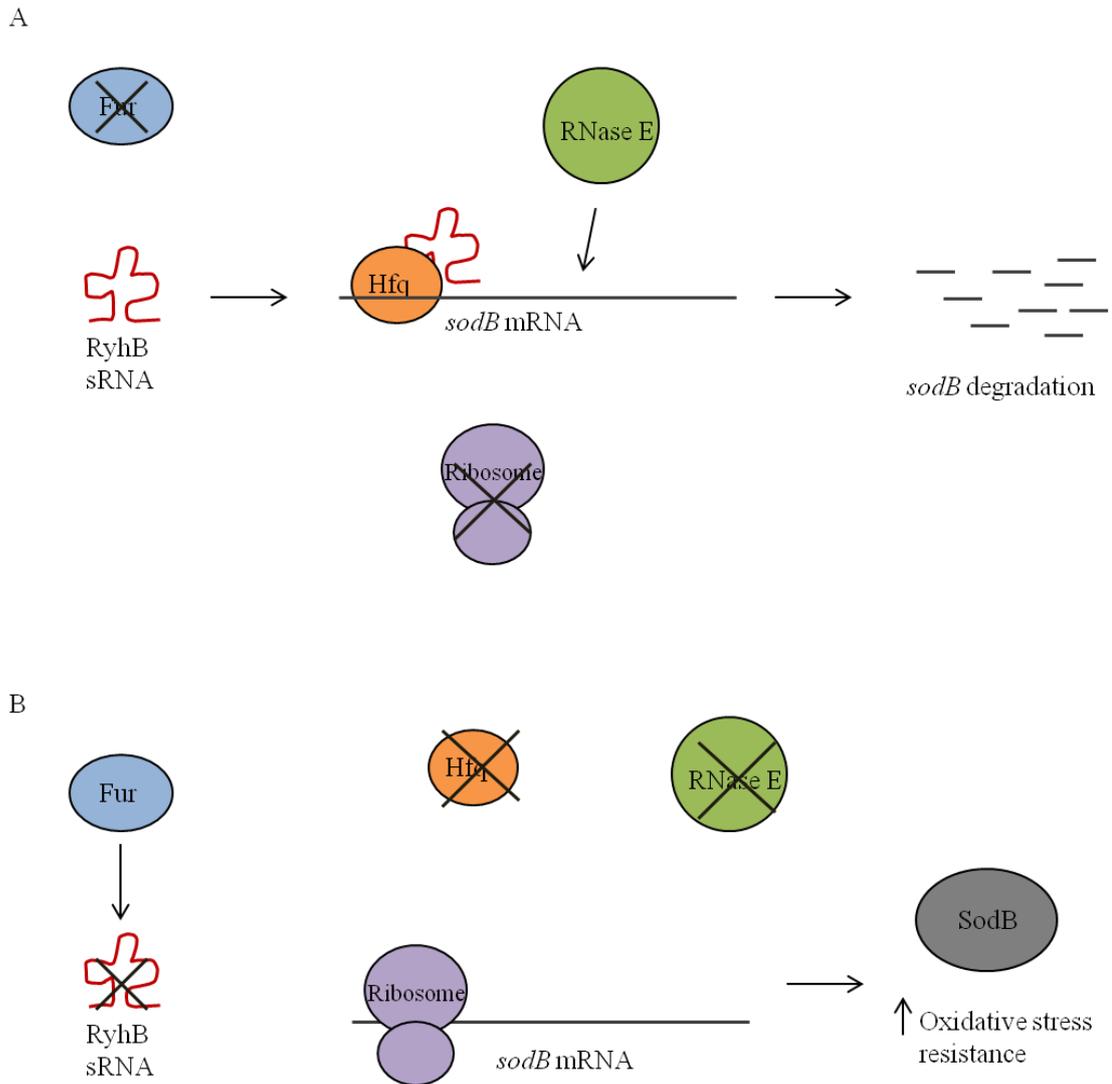


Figure 3.20 Model of SodB regulation through Fur and RyhB under (A) normal growth/low Fe conditions and (B) under high Fe/oxidative stress conditions. Under normal growth conditions Fur is inactive allowing the transcription of the sRNA RyhB. Hfq binds RyhB and stimulates interactions with *sodB* mRNA leading to RNaseE binding and degradation of *sodB*. Under high Fe/oxidative stress conditions Fur is active and represses expression of the RyhB sRNA. The Hfq and RyhB duplex no longer binds *sodB* mRNA allowing ribosome binding and therefore translation.

able to resist oxidative stress. However we do not see complete recovery of oxidative stress resistance indicating a slight difference in regulation between *S. aureus* and *E. coli*.

Our results showed that the *fur* mutant demonstrated a significant growth defect in rich media as found previously by Horsburgh et al. (2001a). One explanation could be the de-regulation of iron uptake in the *fur* mutant. When wild type strains are grown in rich media Fe uptake systems are repressed by Fur to limit the amount of Fe being taken into the cell. However in the *fur* mutant Fe uptake systems are constitutively expressed causing toxic levels of Fe and oxidative stress. This growth defect was partially repaired in the *hfq/fur* double mutant. Previous studies have found that Hfq is required for maximal Fe transporter expression (Johnson, 2008). Therefore in the double mutant there is de-repression of Fe uptake by the *fur* mutation but the additional *hfq* mutation results in reduced transporter expression. This may account for the partial recovery of growth in rich media by the double mutant. However this was not seen when the strains were grown in CRPMI plus 50 μM Fe. It could be that 50 μM Fe is not sufficient to see this effect as the Fe content of TSB has been found to be much higher (~13mM) (Moreira et al., 2003). The differing nutrients available between the two media would affect the metabolism of the bacterium; therefore another possible explanation for the defect is that Fur and Hfq are involved in metabolism. To further investigate the role of Hfq and Fur in oxidative stress resistance the regulation of the oxidative stress genes *ahpC*, *ahpF*, *sodA* and *sodM* was examined using Northern analysis (data not shown). However results were not reproducible and therefore no firm conclusions could be made and this needs to be investigated further.

As mentioned above Hfq and Fur were found to regulate aconitase and ClpP which, in other bacteria, have been shown to be involved in oxidative stress resistance. The loss of stress resistance in the *fur* mutant could be indirect through resistance gene regulation via aconitase activity. However aconitase regulation does not explain the partial recovery of stress resistance in the *hfq/fur* double mutant. This suggests that if aconitase does regulate resistance genes this is not the only regulator acting on them. Therefore investigation into aconitase regulation of resistance genes such as *ahpC/F*, *sodA/M* and *kat* is required to determine whether aconitase is involved. Proteomic analysis of a *clpP* mutant showed that the levels of SodA, AhpC and AhpF were altered (Frees et al., 2012). However only SodA showed a decrease in expression and therefore increased oxidative stress sensitivity is unlikely to be due to loss of resistance gene expression.

3.7.3 Strain variation of Hfq and Fur regulation

Conducting the assays with different strains showed similarities and differences in exoprotein regulation between the strains. Mn8 showed positive Hfq and Fur regulation of haemolysin activity and Eap expression as seen with Newman, however there was no total loss of Eap in the double mutant in Mn8 compared to Newman. Whereas in SH1000 there was no Hfq regulation of Eap and no Hfq or Fur regulation of haemolysis. Our 8325-4 and 8325 *hfq(W)* results are also consistent with SH1000 indicating that in these strains Hfq seem to have little effect on regulation. This raises the question: Why does Hfq and Fur regulation change between strains?

Does Hfq C-terminal variation affect function?

Previous studies have shown that naturally occurring sequence variation in SaeS and Agr alter function. Our investigation into Hfq strain variation revealed two amino acid

changes in the C-terminus (G69A, A71E) which were found to be conserved within clonal complexes. The conservation of these changes could indicate that they cause a functional change that is selected for. Comparisons of Hfq sequences from different bacteria have shown that the majority of the protein is highly conserved to maintain the Sm motifs important for structure and function (Sobrero & Valverde, 2012). However the C-terminus of the Hfq proteins show more variability between bacteria and vary in sequence and length. The C-terminus radiates out from the central core of the monomer and out away from the distal face of the Hfq hexamer (Figure 3.21). There have been conflicting reports on the function of the C-terminus on riboregulation. Vecerek et al. (2008) reported that truncating the *E.coli* Hfq C-terminus led to loss of RyhB and DsrA mediated regulation. *In vivo* studies also showed truncated *E.coli* Hfq proteins failed to bind two complementary RNA substrates whereas the full protein showed duplex formation (Beich-Frandsen et al., 2011). Conversely Olsen et al. (2010) and Updegrove & Wartell, (2011) found that truncated *E. coli* Hfq proteins are sufficient for riboregulation of a number of well studied examples, indicating little function for the Hfq C-terminus. Although different RNA targets were looked at between studies differing results were found even when looking at the same targets, showing that the conflicting results were not just due to the RNA investigated. Another possible function for the C-terminus is that it affects the stability of the protein or complex, although Vecerek et al. (2008) reported that loss of function in the truncated protein was not due to decreased protein stability in *E. coli*. However another study comparing *E. coli* and *V. cholerae* Hfq proteins revealed that the C-terminus may in fact play a role in stability (Vincent et al., 2012). The *E. coli* Hfq was found to be more stable than that from *V. cholera* and truncation of the *E. coli* Hfq caused a reduction in stability. Also swapping the C-terminus of the two proteins affected the level of stability (Vincent et al., 2012).

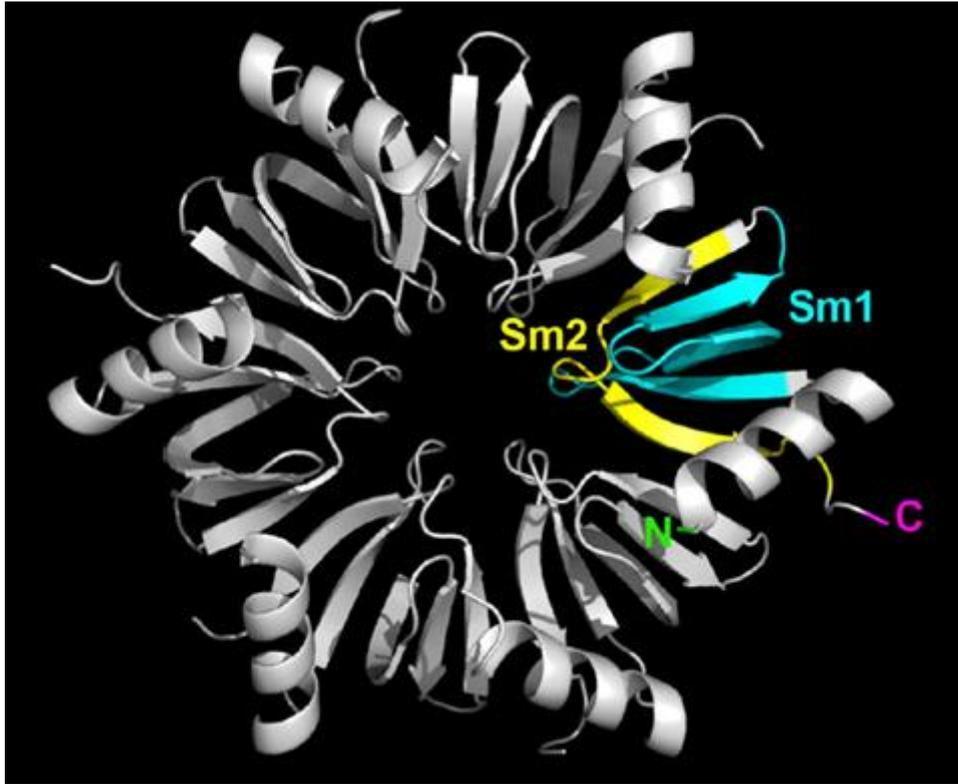


Figure 3.21 Ribbon diagram of the *E. coli* Hfq hexamer. The view shows the proximal side. The Sm1 and Sm2 motifs of one monomer are highlighted in blue and yellow respectively. The N-terminus and C-terminus are highlighted in green and purple respectively. Figure taken from Olsen et al. (2010).

Although the function of this region has not been confirmed it would seem that the C-terminus is involved in Hfq regulation and therefore the changes identified between *S. aureus* strains may have an effect on stability or function.

To determine whether these changes affect Hfq function both the Newman and Mn8 Hfq variant should be used in nucleic acid binding experiments. This may reveal differences in target binding between the Hfq variants. To investigate a global affect of the Hfq variants the Newman *hfq* mutant could be complemented with the Mn8 variant and vice versa. Phenotypic analysis would then show whether the swapped Hfq proteins could restore the wild type phenotype. The affect of the amino acid changes on Hfq stability can be investigated using protein stability assays from extracts taken from Newman and Mn8 as conducted with SaeS by Jeong et al. (2011). Using Western analysis and a Hfq specific antibody the amount of Hfq protein can be measured over time to determine whether one variant degrades faster than the other.

Does Hfq expression vary between strains?

In a number of *S. aureus* strains the level of *hfq* transcription appears to be considerably lower than in other bacteria such as *E. coli* (Geisinger et al., 2006; Bohn et al., 2007; Liu et al., 2010). Although transcription does not vary between *S. aureus* strains, Western analysis by Liu et al. (2010) revealed that there is variation in Hfq protein level, with some strains apparently lacking Hfq completely. The protein level in different strains from our lab were investigated to determine whether there was any correlation between Hfq level and the differing phenotypic results.

Unfortunately the Western analysis to measure Hfq protein levels in our strains did not

produce conclusive results. After trying several protocols including that from Liu et al. (2010) we could not obtain a single Hfq band shown in the Liu et al. (2010) paper. The pure protein showed that the Hfq preferentially forms hexamers which are extremely stable therefore it is difficult to identify the Hfq band by size. A recent paper by Vincent et al. (2012) showed that the *V. cholerae* and *E. coli* Hfq showed that the proteins stayed hexameric unless heat treated for 5 minutes in 1% SDS. We heated the protein for longer and in 1.25% SDS so we would expect the protein to be monomeric but it still remained hexameric. This could mean that the *S. aureus* hexamer is more stable. Liu et al. (2010) did not state how they treated their extracts before loading. There may also be other proteins that the antibody is binding causing multiple bands. Protein A is an example of such which was countered by using a *spa* mutant to identify the band and removing it with agarose-IgG but this did not reduce the number of bands. The *hfq* mutant strains were used as a negative control however the *hfq* mutation is an insertion mutant therefore the N-terminus of the Hfq protein may still be translated which may be enough to bind the Hfq antibody. A deletion insertion mutant may need to be constructed to ensure that the antibody is not binding to any Hfq fragments still being translated. The Hfq protein needs to be reliably identified on a Western blot before any comparison between strains can be carried out. This is also the case to be able to conduct the protein stability assays mentioned previously.

Strain variation between other regulatory factors?

As discussed in section 1.3.3, *S. aureus* virulence factors are controlled by a complex regulatory network. A number of different global regulators can control the expression of a single gene. Therefore the regulation of the identified targets such as *eap*, *emp* and *hla* will be influenced by other factors. The Sae and Agr regulators are also very

important global regulators that affect virulence gene expression. Therefore any strain variation in the activity of these regulators will affect regulation of virulence factors. An example of this is the Newman SaeS protein which contains a mutation that results in constitutive activation (Adhikari & Novick, 2008; Mainiero et al., 2010). This mutation accounts for the abundance of Eap and Emp observed during SDS-PAGE. A Newman strain containing the repaired SaeS was constructed by Luong et al. (2011). Phenotypic analysis of the *hfq* and *fur* mutants in this strain and comparing it to our previous results would show the affect, if any, this had on Hfq and Fur regulation. Although this may explain some of the differences in Newman regulation it is not the sole reason as there were also differences seen between Mn8 and SH1000 which are both wild type SaeS. Determining the mechanism of Hfq and Fur regulation of exoproteins and the involvement of any other factors may show why regulation differs between strains. The possible mechanisms of Hfq and Fur regulation are addressed in the next chapter.

3.7.4 Conclusion

Our phenotypic results demonstrated that Hfq does have a function in *S. aureus* and that it is involved with positive Fur regulation. These results show some similarities and differences to previous studies into *S. aureus* Hfq regulation. Comparing the phenotypic results from a variety of strains have shown clear differences in regulation by Hfq and Fur which could explain why such different results have been reported. This chapter identified a number of targets of Hfq and Fur regulation that can be used to begin to elucidate the mechanism. To do this a number of questions need to be addressed. At what level do Hfq and Fur function, transcriptional or post transcriptional? Are these interactions direct or through another factor? How does the synergistic regulation of Hfq and Fur occur?

Chapter 4 Mechanism of *eap* regulation by Hfq and Fur in

S. aureus

4.1 Introduction

Previous work in other bacteria has found Hfq primarily functions at the post transcriptional level by mediating sRNA-mRNA interactions (See Chapter 1.7.1). However it does not appear that Hfq is required for sRNA function in *S. aureus* the same way as in *E. coli*. So how is Hfq functioning in *S. aureus*? Our studies have shown that Hfq acts as a positive regulator of a number of virulence factors along with Fur. These results are unusual as in other bacteria Hfq predominantly acts negatively on expression, through mRNA destabilisation or by preventing translation (See Chapter 1.7.1). Although not involved in sRNA-mRNA interactions, *S. aureus* Hfq could still regulate expression post-transcriptionally. Hfq could be binding mRNA to stabilise the transcripts or alter structure to allow translation. In other bacteria Hfq can affect transcription through interactions with DNA and therefore this may be happening in *S. aureus* (Updegrave et al., 2010a; Geinguenaud et al., 2011b). Work by Liu et al. (2010) indicated binding of Hfq to several mRNA targets, including *eap*, supporting our hypothesis that Hfq binds mRNA to positively regulate gene expression in *S. aureus*. In this chapter we begin to elucidate how Hfq positively regulates and whether Fur is involved in this regulation.

4.2 Positive Hfq regulation of *eap* occurs post transcriptionally

The regulation of Eap was investigated further as positive Hfq and/or Fur regulation was seen in all strains tested. Firstly, the level of regulation was determined through the use of *lux* reporter assays. The reporters were transduced into *hfq* and *fur* single and double mutants. Figure 4.1 shows a schematic diagram of the *eap* sequences contained within the transcriptional (Figure 4.1.B) and the 5' UTR (Figure 4.1.A) reporters. The transcriptional reporter contains sequence upstream of *eap* which contains all promoter regions, such as -10, -35 and the conserved octanucleotide sequence (COS), so transcription factors that bind these sequences to regulate *eap* expression will also regulate *lux* expression. The COS has been found just 5' to the -35 box of several virulence factors and is required for transcription (Harraghy et al., 2008). If Hfq or Fur directly or indirectly affects *eap* transcription there will be a difference in *lux* expression between wild type and mutant strains. The 5' UTR reporter contains the same sequence as the transcriptional reporter plus the 5' UTR of the *eap* gene. Therefore this reporter shows both transcriptional regulation along with any regulation due to the 5' UTR of the mRNA. Any differences observed in the translational reporter only, show that this additional sequence is required for regulation.

Our hypothesis was that Hfq acts post transcriptionally on *eap* mRNA to positively regulate, and therefore we would expect to see a decrease in *lux* expression from the translational reporter in the *hfq* mutant. Fur is a well known transcriptional regulator and so should show a decrease in *lux* expression from the transcriptional reporter in the *fur* mutant compared to wild type. Therefore in the *hfq/fur* double mutant there would be a decrease at the transcriptional level and a further decrease in the 5' UTR reporter, showing the synergistic regulation by Hfq and Fur as seen at the Eap protein level.

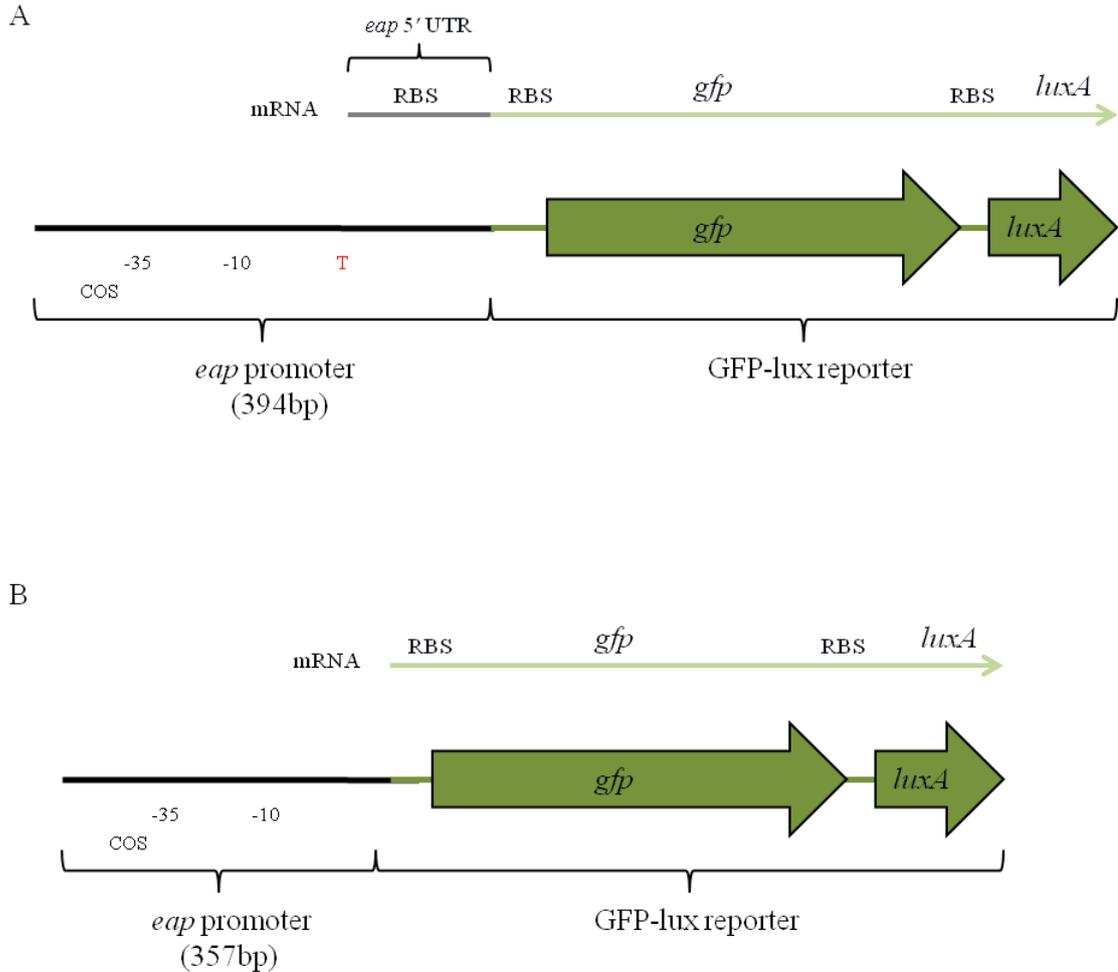


Figure 4.1 Diagrammatic representations of the (A) 5' UTR and (B) transcriptional *eap* reporters. The 5' UTR reporter made by Harraghy et al. (2008) consists of promoter DNA including COS, -10, -35 and RBS sequences up to the start codon of the gene. The red T represents the mapped transcriptional start site. The transcriptional reporter (this work) has the same sequence minus the 5' UTR which includes the RBS. The plasmid carries its own RBS sequence so that translation can still occur.

These hypotheses are in relation to regulation observed in strain Newman. However Hfq and Fur mediated protein expression differed between strains; therefore reporters were compared in all strains to identify any changes in transcriptional and translational regulation between them.

An *eap lux* reporter had already been made and used previously by Harraghy et al. (2005) (Figure 4.1.A). This construct (5' UTR reporter) contains all upstream promoter sequences required for transcription up to the start codon of the gene and therefore includes the 5' UTR. To make a transcriptional reporter the DNA sequence encoding the 5' UTR needed to be removed so that only the DNA promoter was present (Figure 4.1.B). Previous studies have found the transcriptional start site of *eap* and therefore the sequence that needed to be deleted was determined from this (Harraghy et al., 2008). Construction of the transcriptional reporter plasmid was achieved via the one step DNA assembly method described by Gibson et al. (2009). The nucleotide sequence of the transcriptional and 5' UTR reporter plasmids are shown in Appendix A-2 and A-3 respectively.

The plasmid backbone, minus the *eap* promoter, was amplified in two pieces from pEap-GFPlux using primer sets eapGFPlux IF/pGFPlux IR and pGFPlux IIF/eapGFPlux IIR (Figure 4.2.A). The new promoter region was amplified from Newman DNA using primers GFPlux eapF and GFPlux eapR (Figure 4.2.B). Each fragment contained a short sequence at their upstream and downstream ends complementary to the fragment they fuse with (Figure 4.2.C/D). The plasmid backbone PCR products were digested with *DpnI* to digest any template plasmid to ensure no plasmid template was carried over to the transformation. All fragments were then purified via gel extraction and incubated

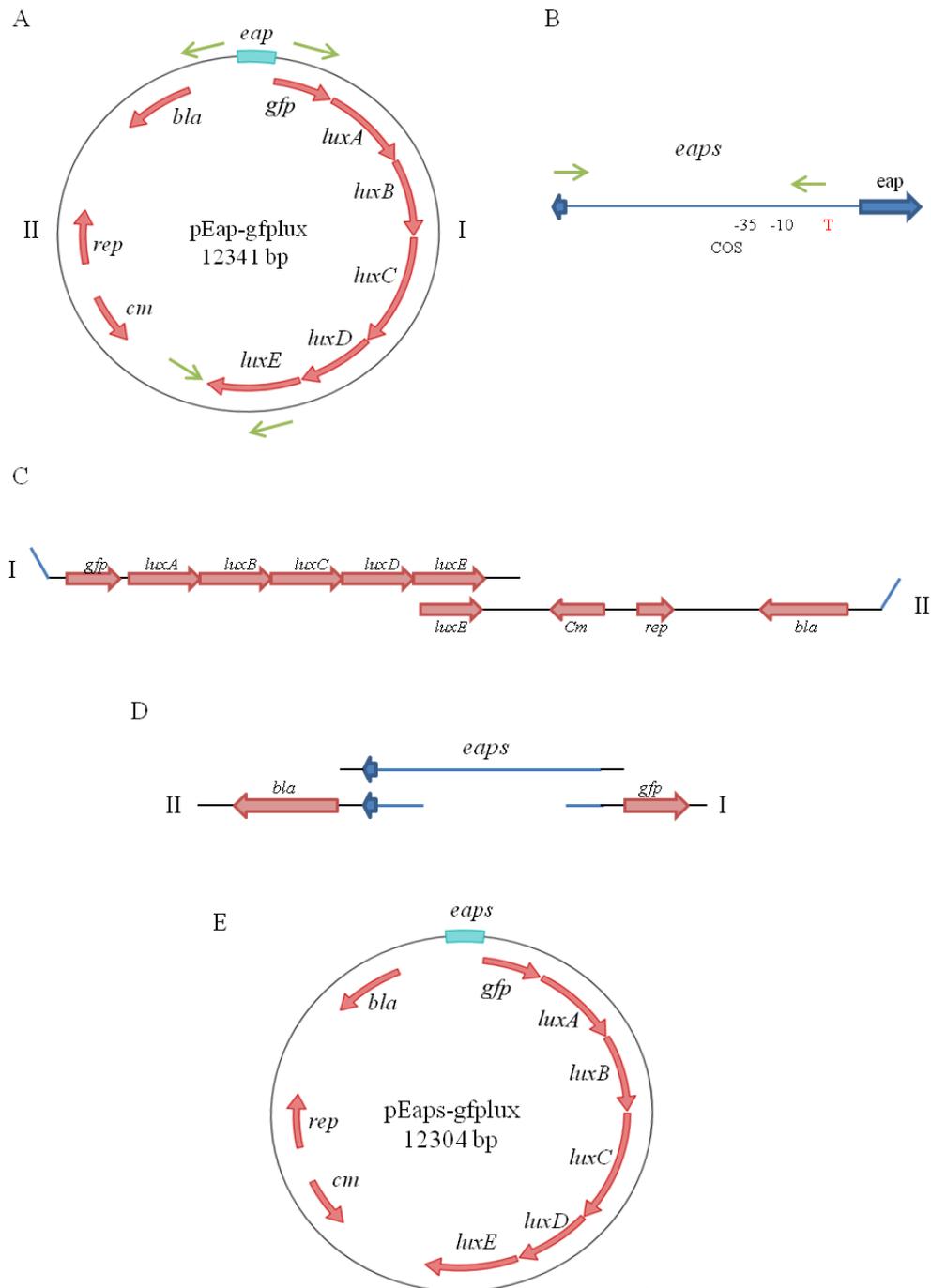


Figure 4.2 Schematic diagrams showing the construction of pEaps-gfplux. (A) The plasmid backbone was amplified in two fragments (I & II). (B) The replacement *eap* promoter fragment (*eaps*) was amplified from Newman chromosomal DNA. (C) The complementary ends allow annealing of the two backbone fragments. (D) The complementary ends on the *eaps* fragment and those of the backbone allowing annealing of the *eaps* to the plasmid. (E) Ligase repairs the nicks to form the new reporter plasmid. Green arrows represent primer binding and orientation.

together with the enzyme mix at 50°C for 1 hour. After incubation the reaction mix, containing the newly formed plasmid (Figure 4.2.E), was used to transform electrocompetent Topo10 *E. coli* cells. Colony PCR was used to identify transformants using primers GFPlux F and pGFPlux IR which should give a product size of ~6kb. Plasmid DNA was extracted from these transformants and sequenced to ensure the *eap* promoter was the correct sequence and it was fused with the GFP gene correctly. The plasmid was then transformed into RN4220 and phage lysate produced from this strain to transduce the plasmid into Newman, Mn8, SH1000 wild type and mutant strains.

To carry out the assay, reporter strains were grown overnight in CRPMI. These were then subcultured into fresh CRPMI to an OD₆₀₀ of 0.1 and grown for 24 hours taking OD₆₀₀ and luminescence readings taken every 30 minutes. For every time point the luminescence reading was divided by the OD₆₀₀ reading to calculate the relative light units (RLU) which take into account any growth differences. The luminescence values varied between experiments making it difficult to directly compare values, however the pattern of *lux* expression was consistent and therefore these were compared.

The transcriptional reporter showed that in all strains Hfq does not regulate *eap* expression at the transcriptional level as there was no difference in RLU seen in the *hfq* mutant compared to wild type (Figure 4.3). In all strains the *fur* mutant showed a decrease in reporter expression demonstrating positive transcriptional regulation of *eap* as seen previously with Northern blot analysis (Figure 4.3) (Johnson et al., 2008). Interestingly in the Newman double mutant there was minimal transcription indicating that Hfq and Fur are acting synergistically on transcription (Figure 4.3). However it is not clear why this regulation is not observed in the *hfq* mutant. It could be that in the *hfq*

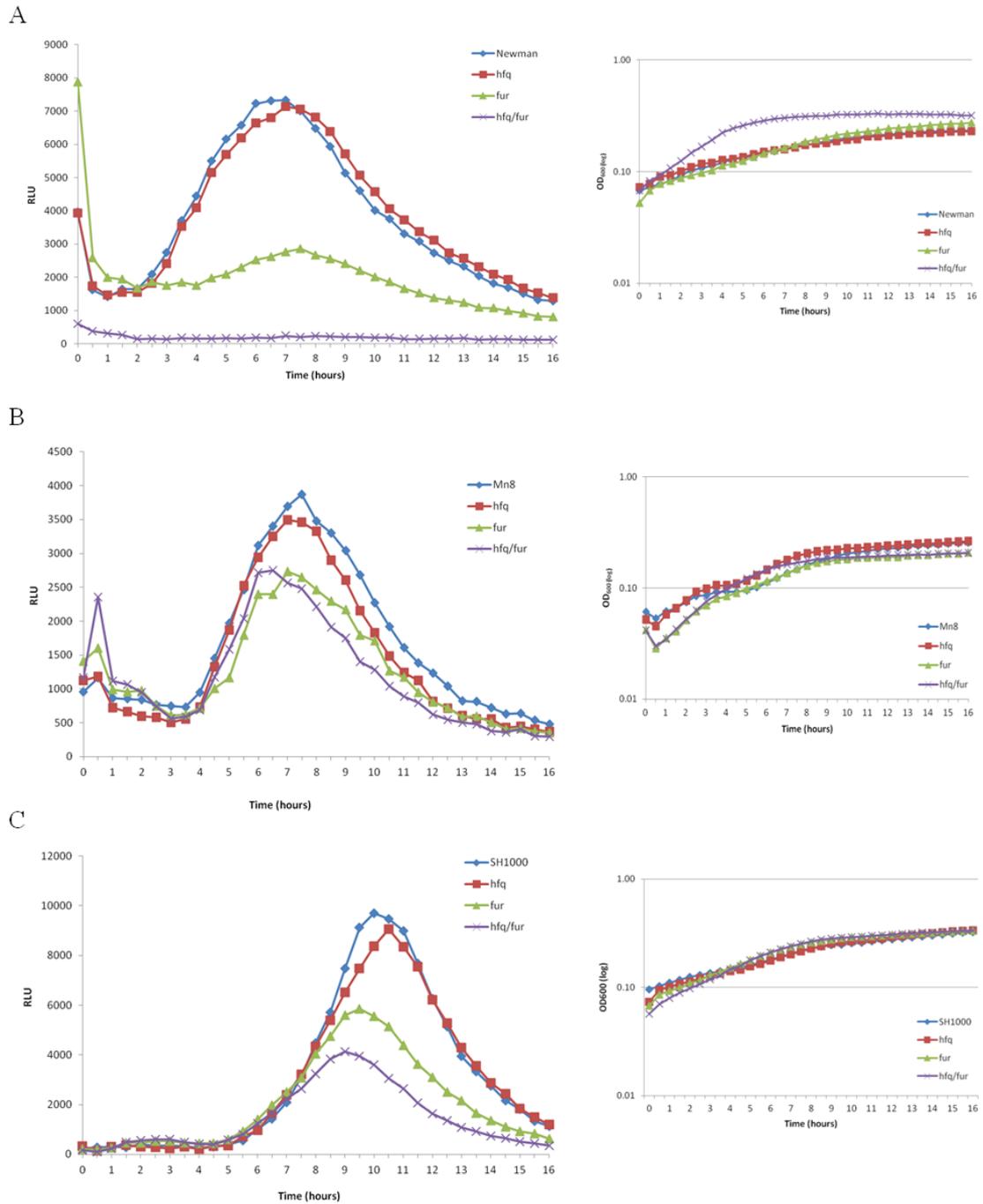


Figure 4.3 The RLU and OD₆₀₀ results of the transcriptional reporters in (A) Newman, (B) Mn8 and (C) SH1000 wild type and mutant strains grown in CRPMI. Relative light units (RLU) were calculated from luminescence readings divided by OD₆₀₀ reading for each time point. Graphs are averages calculated from at least three independent experiments.

mutant the presence of Fur compensates for the loss of Hfq transcriptional regulation. However this loss of transcription is not seen in the SH1000 and Mn8 double mutants, which agrees with our previous protein analysis, indicating Hfq and Fur act differently in these strains (Figure 4.3).

In all strains, Hfq positively regulates *lux* expression in the *hfq* mutant 5' UTR reporter (Figure 4.4). As Hfq is an RNA binding protein, and has been found to bind *eap* mRNA *in vitro*, this would strongly suggest that Hfq is acting on the 5' UTR of the mRNA to confer post transcriptional regulation (Liu et al., 2010). This would also indicate that the 5' UTR is sufficient for Hfq binding and regulation. Interestingly, the presence of the 5' UTR also affects Fur regulation of *eap* as a decrease in reporter expression is observed in the *fur* and double mutant strains compared to the transcriptional reporter (Figure 4.4.A/B). This could indicate that this region of DNA is involved in transcriptional regulation or Fur too is involved in post transcriptional regulation of *eap*. This effect is seen in Newman and Mn8 and SH1000 double mutant but surprisingly is not observed in the SH1000 *fur* mutant (Figure 4.4.C). This reporter also shows a significant difference in the time point of maximal expression in SH1000 (11 hours) compared to Newman and Mn8 (7 hours) as seen in the transcriptional reporter (Figure 4.4). However this does not appear to be due to any growth differences as SH1000 as the OD₆₀₀ graphs show similar growth curves to the other strains (Figure 4.4).

Together, these data indicates that Hfq positively regulates *eap* expression at the post-transcriptional level in all strain backgrounds tested. Newman reporter analysis also revealed that Hfq acts synergistically with Fur at the transcriptional level, as the double mutant showed very little transcription compared to the single mutant strains. This was

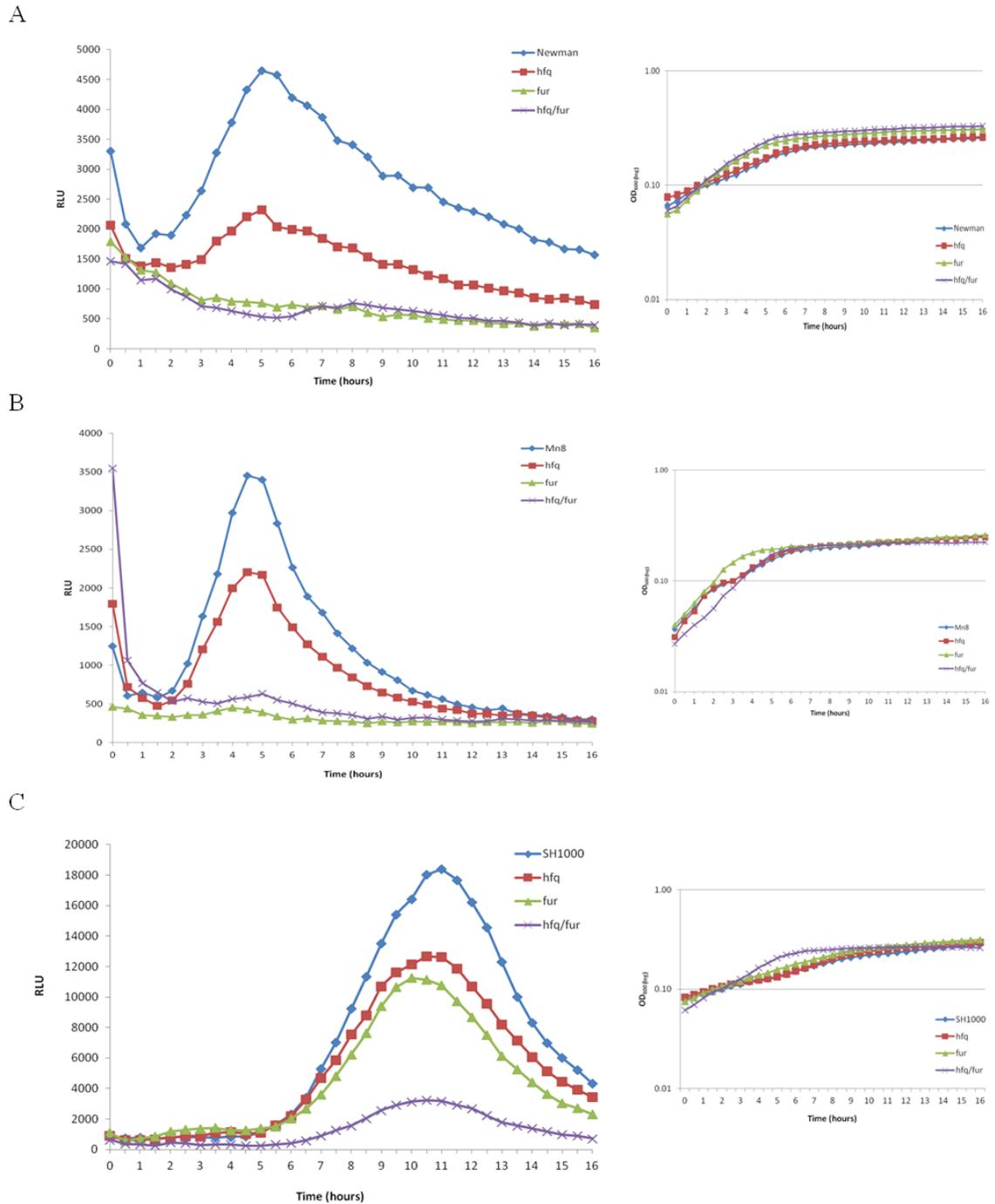


Figure 4.4 The RLU and OD₆₀₀ results of the 5' UTR reporters in (A) Newman, (B) Mn8 and (C) SH1000 wild type and mutant strains grown in CRPMI. Relative light units (RLU) were calculated from luminescence readings divided by OD₆₀₀ reading for each time point. Graphs are averages calculated from at least three independent experiments.

not observed in Mn8 and SH1000 indicating that Hfq and Fur co-regulation differs between strains and may involve other factors.

4.3 Hfq and Fur both have a positive effect on *eap* mRNA levels

Alongside the reporter assays qRT-PCR was used to measure the level of *eap* mRNA in Newman wild type and mutant strains. This was used as a control to show that the differences seen with the reporters was not a result of Hfq or Fur regulation of the reporter plasmid copy number or due to multicopy titration of regulator proteins.

Newman wild type and mutant strains were grown statically in CRPMI at 37°C for 6 hours, as the reporters showed the highest *eap* expression and the differences between the mutants was most obvious at this time point. Total RNA was extracted from cells and used in RT-PCR reactions. Levels of *eap* mRNA were detected using primers *eap*RTF and *eap*RTR and the gyrase (*gyr*) gene was used as the endogenous control using primers *gyr*RTF and *gyr*RTR. All results were calibrated to Newman and show the fold difference compared to this strain.

The results from these RT-PCR experiments show the same pattern as detected with the reporters indicating that the results were real and not an artefact due to plasmid copy number. The *hfq* and *fur* mutants both show a decrease in *eap* mRNA level, however usually a 2 fold or greater difference is taken as significant, which means that the decrease in the *hfq* mutant is not deemed significant (Figure 4.5). The 742 fold decrease in the double mutant though does confirm that Hfq and Fur are acting positively on *eap* as seen with the reporters (Figure 4.5).

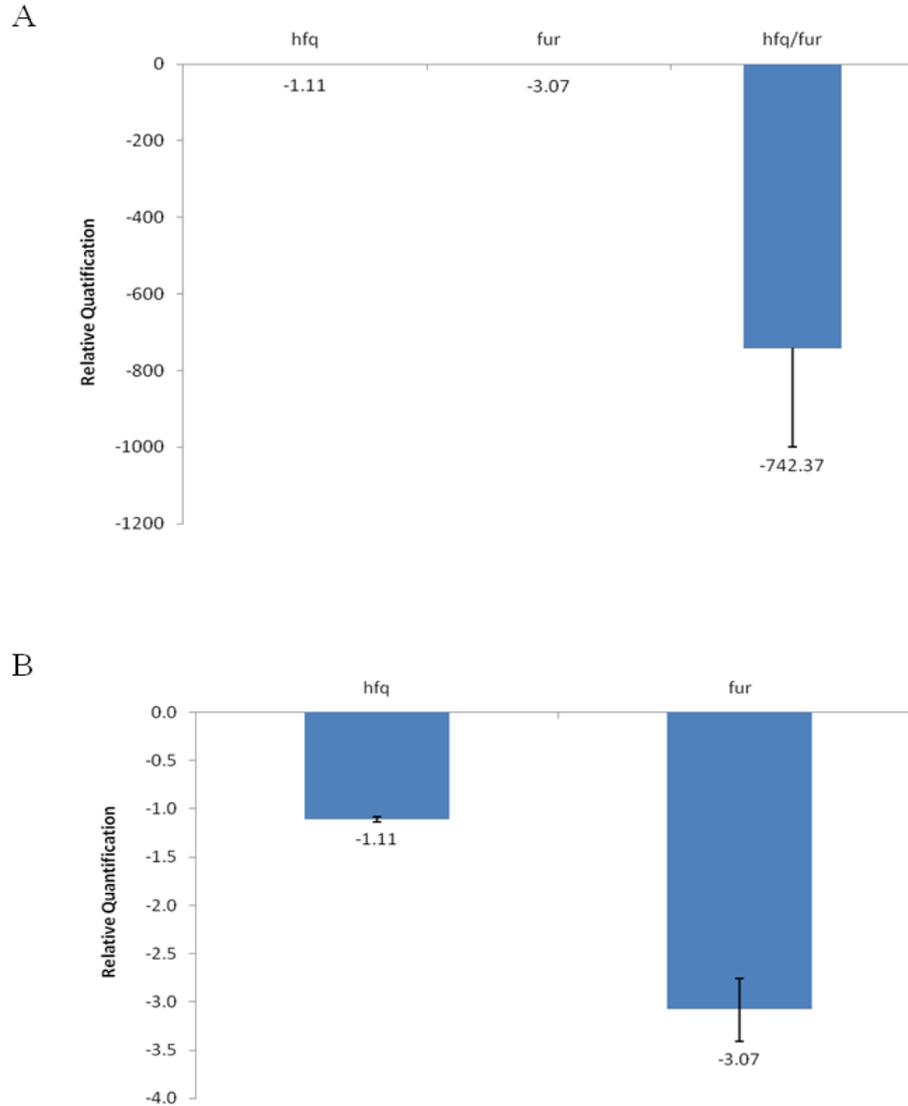


Figure 4.5 RT-PCR results measuring *eap* mRNA levels in Newman, *hfq*, *fur* and *hfq/fur* mutant strains. RNA was extracted from cells grown for 6 hours in CRPMI and used in RT-PCR experiments using *gyrase* as an endogenous control. Results are shown when compared to Newman with values indicating the fold decrease. Graphs are an average of two independent experiments with error bars showing standard error. (A) shows all strains whereas (B) focuses on *hfq* and *fur* mutant only.

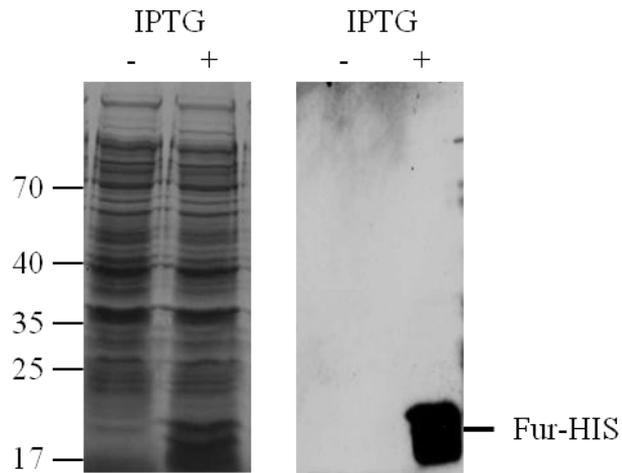
4.4 Hfq does not bind *eap* promoter DNA

Although the reporter analysis with the *hfq* mutant strain showed post transcriptional control the double mutant data also suggested that there may be a level of transcriptional control by Hfq. To investigate this DNA electrophoretic mobility shift assays (EMSA) were used with the *eap* promoter region used in the transcriptional reporter. This was carried out with the Newman variant of the Hfq protein (Hfq) and the Mn8 variant (Hfq*) to see if these changes alter binding capabilities. The *fur* mutant also showed positive transcriptional control of *eap* but the mechanism is not known. To determine whether this regulation was direct through Fur binding *eap* promoter DNA, EMSAs were also carried out with Fur.

4.4.1 Overexpression and purification of Hfq* and Fur

The Hfq* protein was expressed in *E. coli* as described in Chapter 3.6.1 for the Hfq protein except that the gene was amplified from Mn8 chromosomal DNA. For expressing the Fur protein the *fur* gene was amplified from Newman chromosomal DNA. Only one Fur protein was required as the *fur* gene shows 100% sequence identity between strains. The PCR product was purified and sent to PROTEX (University of Leicester) for cloning into the pLEICS01 vector. The *fur* gene of the resulting vector was sequenced to ensure that the sequence was correct. The pLEICS-*fur* vector was transformed into Rosetta (DE3) *E. coli*. The resulting strain was grown in LB and protein expression induced by IPTG for 3 hours (Figure 4.6.A). Cell lysate was passed through an affinity column and eluted in buffer containing 200 mM and 350 mM imidazole (Figure 4.6.B). Peptide mass fingerprinting confirmed that the purified proteins contained Hfq* and Fur. The protein concentration for Hfq* and Fur were found to be 0.75 mg ml⁻¹ and 1.2 mg ml⁻¹ respectively.

A



B

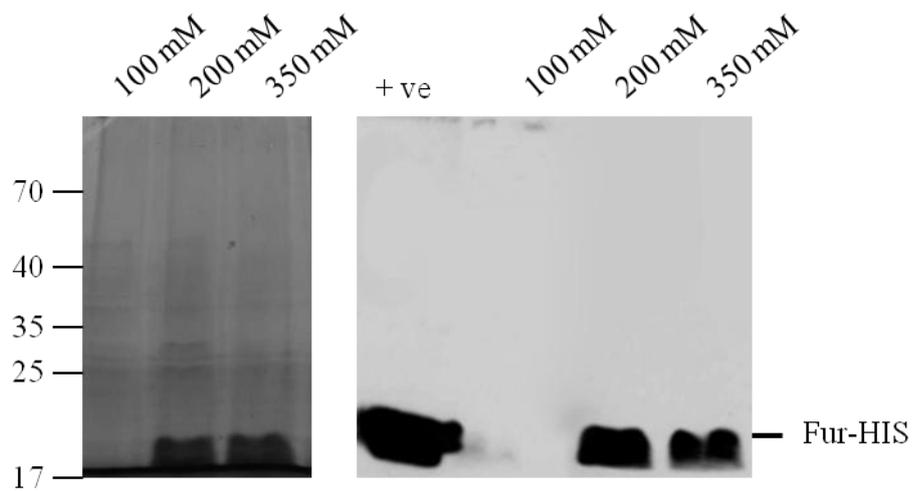


Figure 4.6 Expression and purification of Fur-HIS. (A) shows an acrylamide gel of the cell lysate from uninduced (-) and induced (+) cells showing the induction of Fur. (B) shows an acrylamide gel and Western blot of the purified Fur-HIS eluted in imidazole. The positive control (+ve) is a sample of cell lysate containing Fur-HIS.

4.4.2 Hfq, Hfq* and Fur showed no binding to *eap* DNA

The EMSA binding assays were initially carried out with Fur and the *fhuC* gene positive control to confirm the experimental protocol. *fhuC* is a classically regulated Fur gene and has been used previously in EMSA assays (Xiong et al., 2000). The *fhuC* probe was amplified from Newman chromosomal DNA with primers EMSA *fhuCF* and EMSA *fhuCR*. The *eap* probe was amplified using primers *eapEMSA F* and *R* from Newman chromosomal DNA. This region of DNA is the same as that present in the transcriptional reporter and showed transcriptional regulation by Fur and Hfq. The probes were purified and labelled, then incubated with increasing concentrations of Fur protein before being loaded onto a native protein gel. The range of Fur concentrations used were the same as those in Xiong et al. (2000) and so should be sufficient to see a shift.

The Fur-*fhuC* positive control produced a shift with just 0.1 µg of Fur protein confirming the assay is functioning and confirming Fur binding of the *fhuC* promoter (Figure 4.7.A). The *fhuC* probe was added in all Fur gel shift experiments as a positive control. Interestingly Fur does not appear to directly regulate *eap* expression by binding the *eap* promoter region, as no shift was detected when *eap* promoter DNA was incubated with any concentration of Mn-loaded Fur protein (Figure 4.7.B). In *S. aureus*, Fur has been shown to positively regulate in low Fe conditions; therefore to investigate whether apo-Fur binds the *eap* promoter, 2 mM EDTA was added to the reaction mix to remove all metal ions from the Fur protein. With the *fhuC* control, addition of EDTA showed a loss of shift as expected as Fur-Fe repressor binds the *fhuC* promoter. This also demonstrated that the concentration of EDTA is sufficient to remove metal ions from the Fur protein (Figure 4.7.C). However there was still no shift with the *eap* DNA

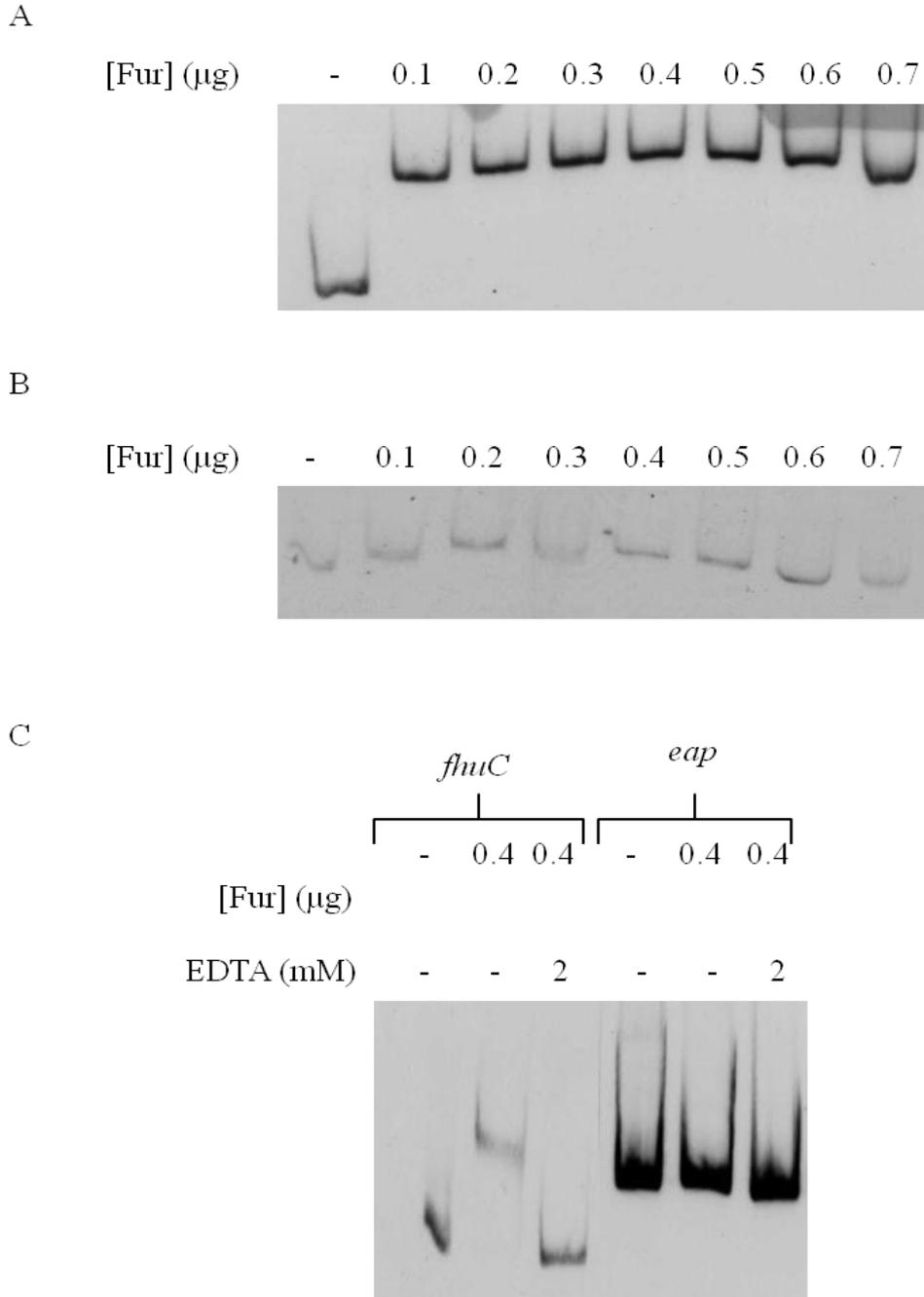


Figure 4.7 EMSA with Fur-HIS and (A & C) *fhuC* or (B & C) *eap* DNA. (A) *fhuC* DNA with increasing concentrations of Fur-HIS. (B) *eap* DNA with increasing concentrations of Fur-HIS. (C) *fhuC* or *eap* DNA with Fur-HIS \pm EDTA. EMSA pictures are representative of at least three independent experiments.

suggesting that even apo-Fur does not bind the *eap* promoter (Figure 4.7.C). When the EMSA were carried out with the Hfq proteins and *eap* promoter DNA no shift was seen indicating no direct Hfq transcriptional control of *eap* (Figure 4.8).

4.5 Hfq and Fur positively regulate *saeRS* expression

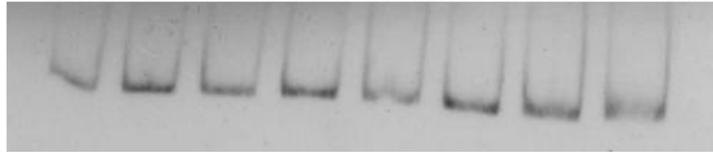
Transcriptional control of *eap* does not occur through direct binding of Fur or Hfq to *eap* promoter DNA. This suggests that the Hfq and Fur positive regulation is indirect and through another regulator. The two component regulator SaeRS is required for exoprotein expression including those we found regulated by Hfq and Fur (e.g. *eap*, *emp*, *hla*, *nuc*) (Giraud et al., 1997; Harraghy et al., 2005). The SaeRS regulator is essential for Eap expression as no *eap* transcripts are detected in a *saeRS* mutant (Harraghy et al., 2005; Johnson et al., 2008). Previous work has also shown that Fur positively regulates *saeRS* expression but it is not known how Fur achieves this (Johnson et al., 2011). Therefore regulation of SaeRS by Hfq and Fur was investigated to see if this is responsible for the transcriptional regulation of *eap*.

4.5.1 Hfq and Fur show positive regulation on *sae* mRNA

To determine whether Hfq and Fur affects *sae* transcription RT-PCR was conducted to measure *sae* transcripts in Newman wild type and *hfq*, *fur* and double mutant strains. Strains were grown in CRPMI for 6 hours and the total RNA extracted. The RNA was then used in RT-PCR reactions. Primer sets *saeRTF/saeRTR* and *saePRTF/saePRTR* were used to measure the levels of *saeS* and *saeP* transcripts respectively. The expression pattern of *sae* shows that there are four transcripts expressed from the two promoters (Figure 4.9) (Adhikari & Novick, 2008). The *saeP* primers will pick up transcripts C and D whereas the *saeS* primers will pick up all but D. Both primer sets

A

[Hfq] (μg) - 0.1 0.2 0.3 0.4 0.5 0.6 0.7



B

[Hfq*] (μg) - 0.1 0.2 0.3 0.4 0.5 0.6 0.7

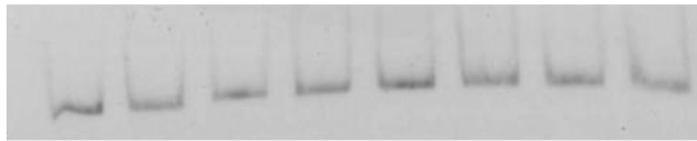


Figure 4.8 EMSA with (A) Hfq-HIS or (B) Hfq*-HIS on *eap* DNA. *eap* promoter DNA incubated with increasing concentrations of Hfq-HIS.

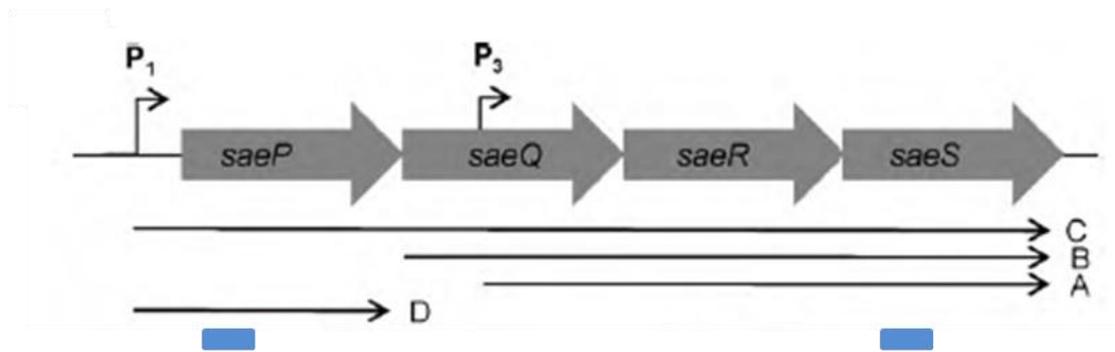


Figure 4.9 Diagram of the *sae* locus with all possible *sae* transcripts underneath.

Blue boxes represent the area amplified by *sae* RT-PCR primers. Figure adapted from Johnson et al. (2010).

were used in case there were differences in the levels of the different transcripts.

Using the *saeP* primers we can see that the C and D transcripts show a 5.61 fold decrease in the *hfq* mutant compared to Newman (Figure 4.10.A). The *fur* mutant does show a decrease in transcript levels although only by 1.57 which is not significant (Figure 4.10.A). However as with *eap* we see a 111.12 fold decrease in the double mutant indicating that Hfq and Fur both positively regulate *sae* (Figure 4.10.A). We also see positive Hfq and Fur regulation with the *saeS* primers however the *hfq* and *fur* single mutants only show 1.25 and 1.04 fold decreases respectively (Figure 4.10.B). The double mutant still showed a 107.93 fold decrease indicating again that both Hfq and Fur are required for expression (Figure 4.10.B). These results demonstrate that Hfq and Fur synergistically positively regulate *sae* expression, a major virulence gene regulator.

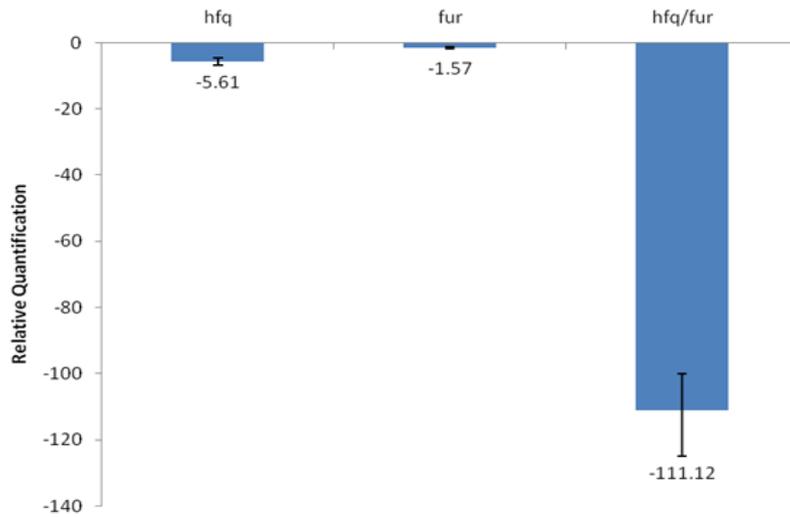
4.5.2 Fur positively regulates *sae* by direct binding to P1 and P3 promoters

We have shown that Hfq and Fur synergistically regulate *sae* transcription, to determine whether this is a direct interaction EMSA was carried out using *sae* promoter DNA. The *sae* operon has two main promoters, P1 and P3 (Figure 4.9), so both of these were used in the assay (Geiger et al., 2008b). The sequences used were taken from *sae* reporters used in Sun et al. (2010) and Jeong et al. (2011). These promoter regions contained -10, -35 and RBS sequences but no apparent Fur box consensus binding site.

The *saeP1* and *saeP3* promoters were amplified from Newman chromosomal DNA using primer sets *saeP1F/saeP1R* and *saeP3F/saeP3sR*.

EMSA with both Hfq variant proteins and the *sae* promoters did not show a shift indicating they do not bind to the *sae* DNA promoters (Figure 4.11). Unexpectedly, Fur

A



B

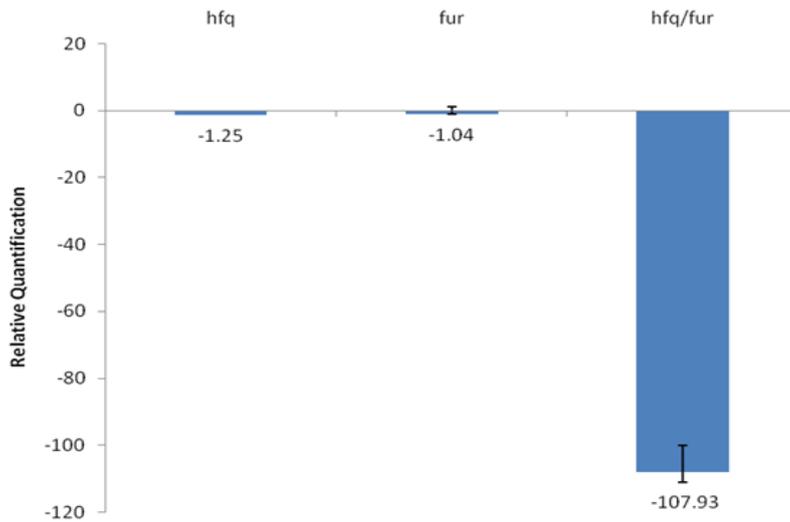


Figure 4.10 RT-PCR results measuring (A) *saeP* and (B) *saeS* mRNA levels in Newman, *hfq*, *fur* and *hfq/fur*. RNA was extracted from cells grown for 6 hours in CRPMI and used in RT-PCR experiments using gyrase as an endogenous control. Results are shown when compared to Newman with values indicating the fold decrease. Graphs are an average of two independent experiments with error bars showing standard error.

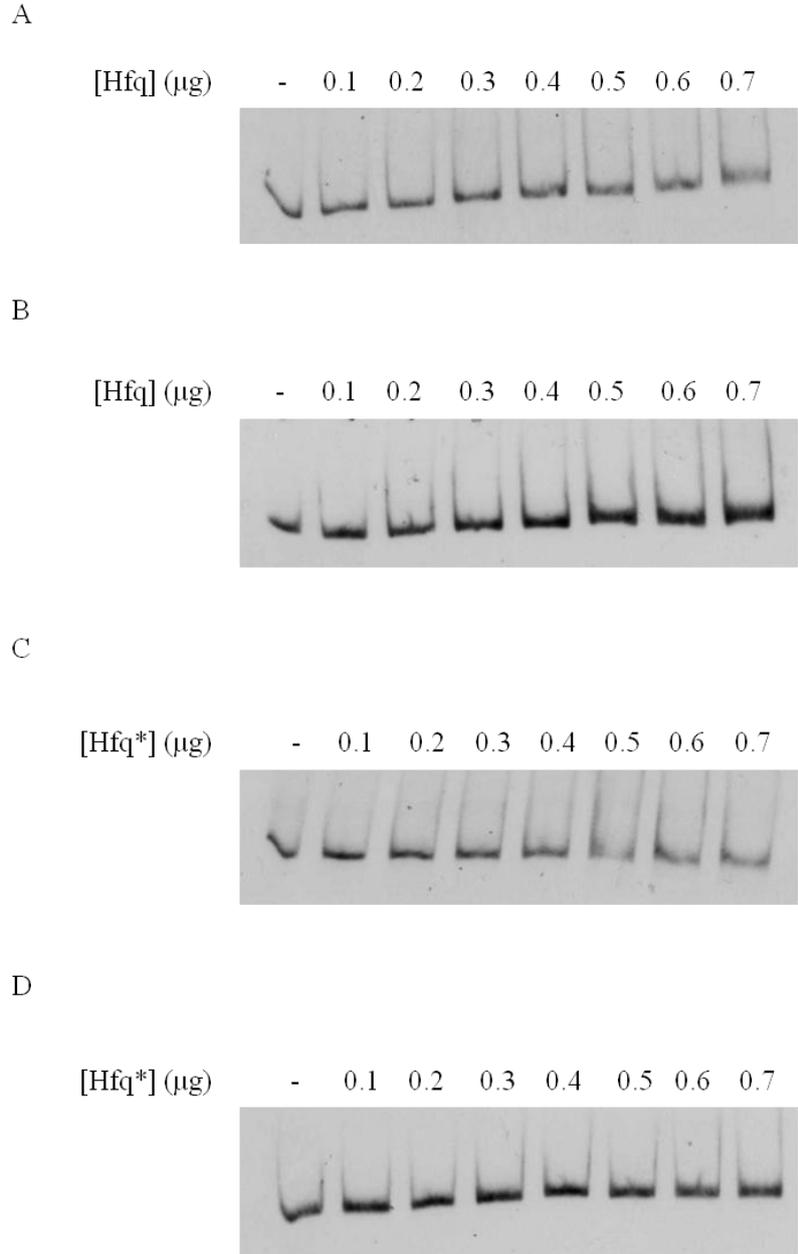


Figure 4.11 EMSA with Hfq-HIS and Hfq*-HIS on *saeP1* and *saeP3* promoter DNA. (A) *saeP1* and (B) *saeP3* promoter DNA incubated with increasing concentrations of Hfq-HIS. (C) *saeP1* and (D) *saeP3* promoter DNA incubated with increasing concentrations of Hfq*-HIS.

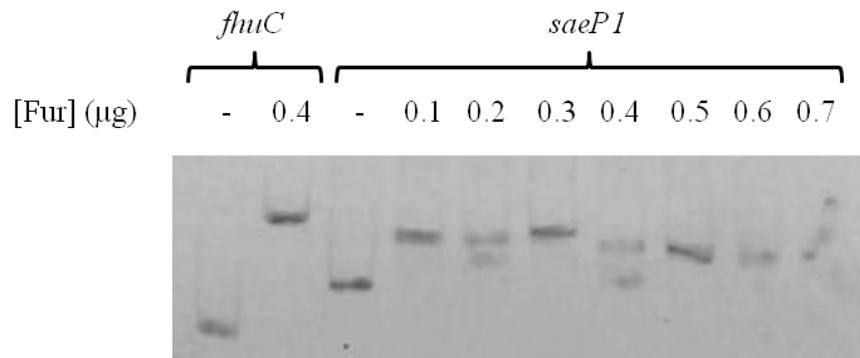
does bind the *saeP1* promoter and with the same efficiency as *fhuC*, only needing 0.1 µg of protein for a shift (Figure 4.12.A). Fur also appears to bind the *saeP3* promoter but this effect is only seen with higher Fur concentrations (Figure 4.12.B). To determine whether this binding requires Fe the shift was repeated with the presence of EDTA. As seen previously with *fhuC* in the presence of EDTA there is a loss of shift (Figure 4.12.B) indicating that this binding would occur in high Fe conditions *in vivo*.

Together these results show for the first time that Fur directly binds the promoters of the *sae* operon, an essential global virulence gene regulator, in high iron. Interestingly this causes positive regulation instead of repression like classically Fur regulated genes. This suggests that Hfq and Fur regulation of exoproteins is partially due to Fur acting directly on *sae* expression.

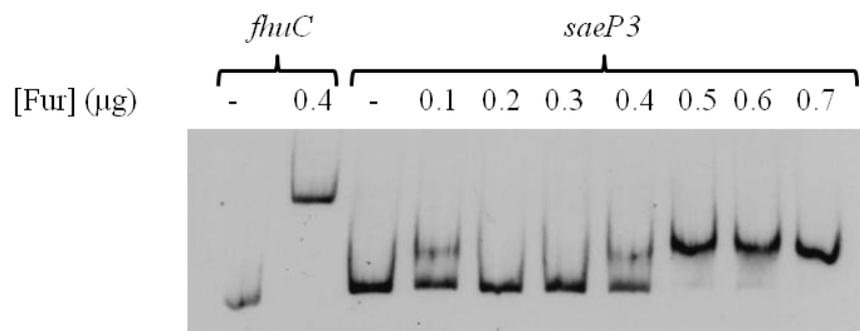
4.6 Hfq does not appear to stabilise mRNA

The reporter analysis with the *hfq* mutant indicated that Hfq is acting on the *eap* mRNA and that there is a decrease in expression when *hfq* is knocked out. Although sRNA molecules have been identified in *S. aureus* it appears that Hfq is not required for their regulatory activity (Boisset et al., 2007; Geisinger et al., 2006; Geissmann et al., 2009; Chabelskaya et al., 2010; Bohn et al., 2010). Therefore it is unlikely that Hfq is acting with a sRNA on the *eap* mRNA. It was hypothesised that Hfq could be acting to stabilise the mRNA and so deletion of *hfq* would lead to destabilisation and a reduction in expression. This was investigated using mRNA stability assays. Strains were grown to late exponential phase (4 hours) then treated with rifampicin to stop transcription. At set time points post treatment, samples of the cultures were removed and stored in RNA later. Total RNA was extracted from these samples and used in RT-PCR reactions. The

A



B



C

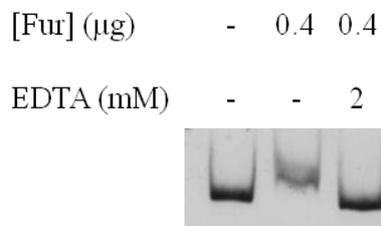


Figure 4.12 EMSA of Fur with (A) *saeP1* and (B) *saeP3* promoter DNA. *sae* promoter DNA was incubated with increasing concentrations of Fur-HIS protein. *fhuC* ± Fur-HIS was added at the beginning of each gel as a positive control. (C) *saeP1* EMSA repeated with 0.4 μg Fur-HIS ± 2mM EDTA.

levels of *saeP*, *saeS* and *eap* mRNA were measured using 16S rRNA as the endogenous control. Results are shown as a percentage compared to 16S rRNA levels, then each time point as a percentage of the initial point.

As a control for the experimental protocol, stability assays for *sae* were initially carried out in TSB, as in the paper by Jeong et al. (2011). Data from Jeong et al. (2011) showed that the time taken for a 50% reduction in *saeS* mRNA level in Newman is 18 minutes. Although the graph does not show the mRNA degrading to 50% if the graph is extended the data line would cross 50% at approximately 18 minutes (Figure 4.13.A). The graph of our *saeS* results demonstrated the same half life of 18 minutes confirming those findings of Jeong et al. (2011) (Figure 4.13.B). In addition to measuring the half life of *saeS* in Newman wild type, mRNA stability was also investigated in the Newman *hfq* mutant, and with *eap* mRNA in Newman and Newman *hfq*. In the *hfq* mutant, the *saeS* transcripts appear to be more stable however this difference is not statistically significant (Figure 4.13.B). The *eap* transcript shows greater stability than *saeS* in the wild type strain, with a half life of approximately 25 minutes (Figure 4.13.C). The *hfq* mutant shows no significant difference in stability compared to the wild type (Figure 4.13.C). Therefore in these conditions Hfq is not involved in the stabilisation of *eap* mRNA. As we had observed positive regulation of *eap* and *saeS* by Hfq it was expected that in the *hfq* mutant there would be a decrease in transcript stability. However, these preliminary results may indicate that Hfq acts via a different mechanism.

Our previous transcriptional analysis was carried out in CRPMI, therefore to ensure the growth conditions did not affect the results we carried out the stability assay with strains grown in CRPMI. However, growth in CRPMI is limited and would not allow

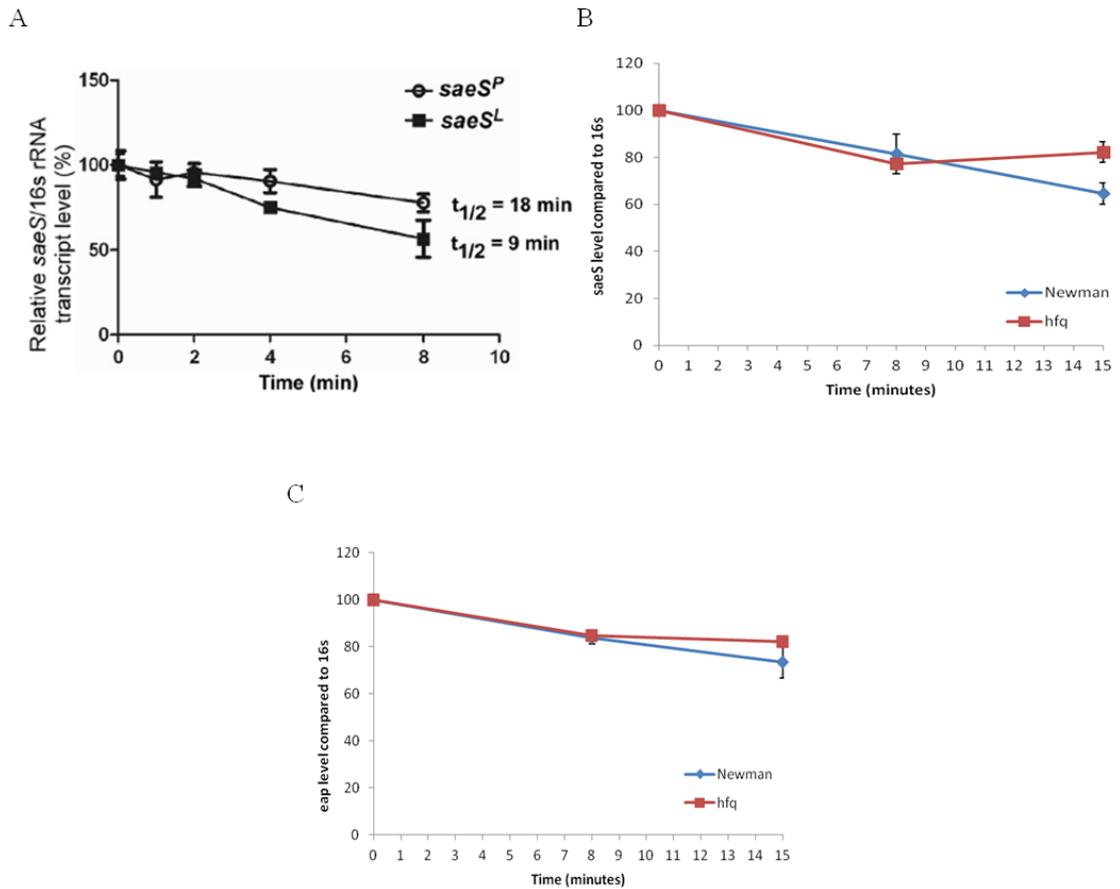


Figure 4.13 mRNA stability assays of (A/B) *saeS* and (C) *eap* mRNA in Newman and *hfq* mutant grown in TSB. (A) Figure taken from Jeong et al. (2011) presenting the stability of *saeS* transcripts. *saeS*^P shows the half life of Newman *saeS*. Newman (B) *saeS* and (C) *eap* mRNA stability assay results from this work. RNA was extracted from cells grown for 4 hours in TSB and used in RT-PCR using 16s rRNA as an endogenous control. RT-PCR data was extrapolated to show decreased stability as a percentage. Graphs show average results of two independent experiments and error bars show standard deviation.

collection of enough cells per time point without taking a larger sample or pooling cultures, both of which would both increase variation in results. Therefore 1% casamino acids were added to the medium to increase growth. Reporter assays and RT-PCR of *eap* transcript levels were carried out first to ensure that addition of casamino acids did not affect the results seen previously (data not shown). Due to time restraints the following results are preliminary as only one experiment was carried out. The *saeS*, *saeP* and *eap* transcript levels were analysed in Newman and the *hfq* mutant. There appears to be little difference in stability levels of the transcripts between wild type and mutant strains (Figure 4.14). These experiments would need to be repeated to draw any firm conclusions. These repeats would also need to contain more time points. Firstly to show the degradation until 50% RNA degradation, and secondly to determine whether the plateau effect is due to experimental error. However, these preliminary results suggest that Hfq does not act to stabilise these transcripts as in other bacteria which means that Hfq may have a novel mechanism in *S. aureus*.

4.7 Discussion

Phenotypic analysis of multiple strains showed positive Hfq and Fur regulation of surface and exoproteins, although this regulation differed slightly between Newman, Mn8 and SH1000 strains. The mechanism of this regulation was the main focus of the work in this chapter, to understand how Hfq and Fur regulate these proteins and to help explain the differences between strains. Eap was the target chosen for further study as it is an important virulence factor involved in colonisation and immune evasion and showed positive regulation by Hfq and Fur but the mechanism for regulation is unknown.

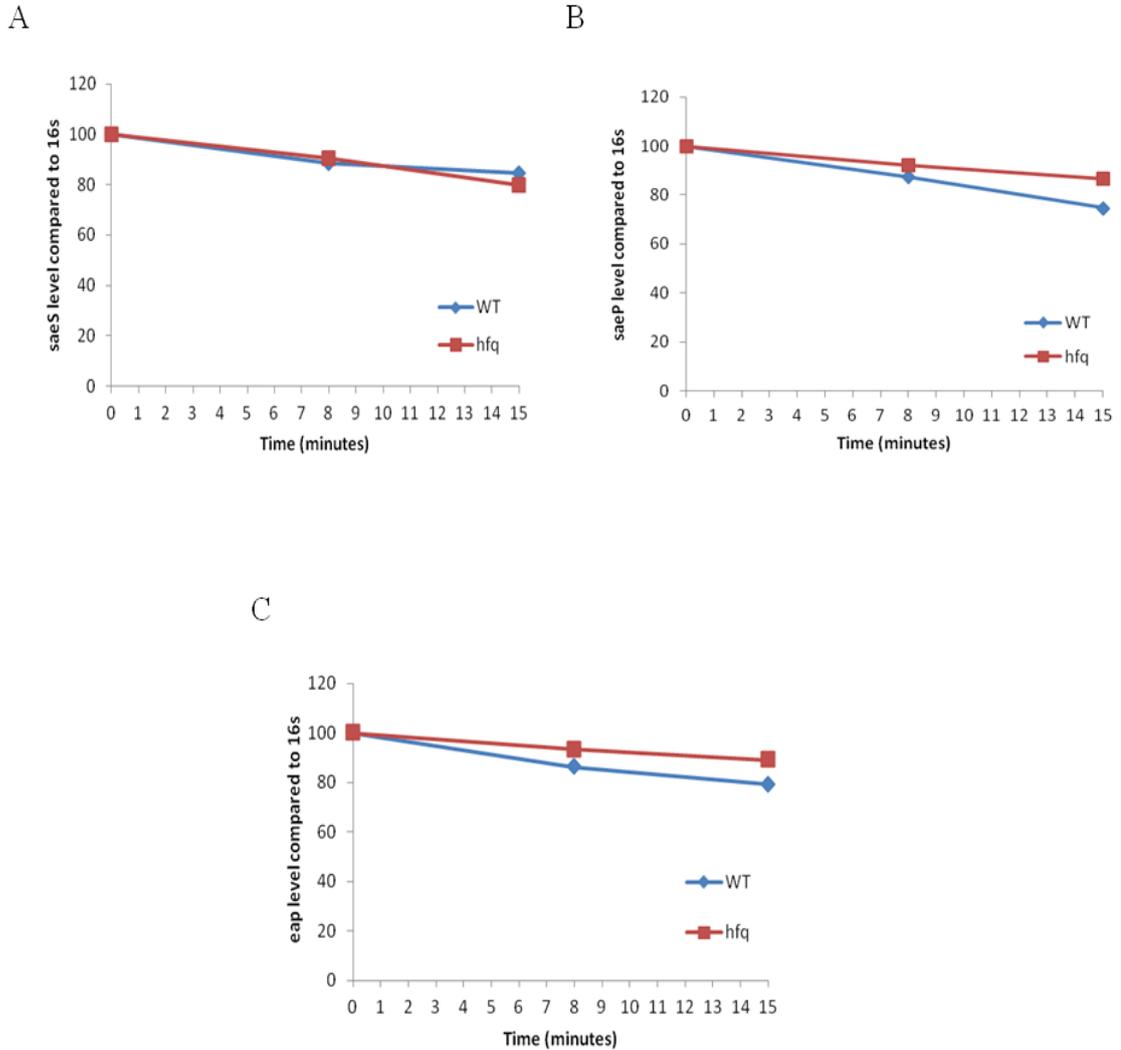


Figure 4.14 mRNA stability of (A) *saeS*, (B) *saeP* and (C) *eap* in Newman and *hfq* mutant grown in CRPMI + 1% casamino acids. RNA was extracted from cells grown for 4 hours in CRPMI + 1% casamino acids and used in RT-PCR using 16s rRNA as an endogenous control. RT-PCR data was extrapolated to show decreased stability as a percentage.

4.7.1 Hfq positively regulates at the post transcriptional level

In other bacteria Hfq is a well characterised RNA binding protein, therefore we hypothesised that Hfq would bind *eap* mRNA to positively regulate at the post-transcriptional level. Our reporter analysis supported this hypothesis as the *hfq* mutant only showed reduced expression in the 5' UTR reporter suggesting that *eap* mRNA is required for Hfq regulation. This post-transcriptional regulation by Hfq was seen in all strains tested indicating that Hfq acts on *eap* mRNA in all these strains. These results are supported by Liu et al. (2010) who found that *eap* mRNA immunoprecipitated with Hfq. To investigate the possible role of Hfq in *eap* translation, a translational lux reporter could be constructed. This reporter would contain *eap* coding sequence and the plasmid RBS would need to be removed. Differences in *lux* expression between the 5' UTR and translational reporter could suggest a role for Hfq in translation initiation. To confirm that Hfq does directly bind to *eap* mRNA the purified Hfq proteins would be used in RNA EMSA. RNase footprinting would also confirm Hfq-*eap* mRNA binding and would show exactly where Hfq binds to the *eap* mRNA.

In Gram-negative bacteria post transcriptional regulation by Hfq is usually negative through the inhibition of translation and promoting degradation of mRNA molecules. However there are examples of positive Hfq regulation in *E. coli*. Hfq can stabilise mRNA through preventing RNaseE binding and degrading the RNA (Moll, Afonyushkin, et al., 2003; Hankins et al., 2010). Interactions between RyhB-*shiA* mRNA and DsrA-*rpoS* mRNA lead to structural changes which reveal the ribosomal binding site to allow translation initiation. In both cases Hfq is required for this interaction (Prévost et al., 2007; Soper et al., 2010). Therefore there are three possible mechanism of post transcriptional control by which Hfq is positively regulating in *S.*

aureus. The first is through stabilisation of the transcript, the second is by promoting sRNA-mRNA interaction to initiate translation, and finally by Hfq altering the mRNA to allow translational initiation.

Does Hfq affect *eap* mRNA stability?

To begin to determine the mechanism of positive Hfq regulation, mRNA stability assays were carried out with *eap* mRNA to see whether loss of Hfq destabilised the mRNA. Although only preliminary, our results did not show a significant loss of stability indicating that positive Hfq regulation is not through mRNA stabilisation. However there were limitations to these experiments. The RNA degradation was shown to 20% and the graph theoretically extended to 50% to obtain the half life. To confirm these results the time course would need to be extended until the RNA has reached 50% degradation. The second limitation is that only two time points are shown, to obtain a more accurate graph showing the degradation more samples would need to be analysed. This would also confirm whether the plateau observed with the hfq mutant is accurate or whether it is due to experimental error. These assays with the protocol amendments mentioned above would need to be repeated to see if the results are consistent before drawing firm conclusions. However it would appear that Hfq does not drastically affect mRNA stability as this would still be observed with the limitations mentioned above.

Does Hfq promote interactions between sRNA and mRNA?

In *S. aureus* the sRNA RNAIII has been shown to bind and regulate *rot*, *spa*, *hla* and *eap* expression (Morfeldt et al., 1995; Huntzinger et al., 2005; Boisset et al., 2007; Geisinger et al., 2006; Liu et al., 2011). RNAIII positively regulates *hla* by binding to the mRNA and altering the secondary structure to promote translation, however the

mechanism of *eap* regulation by RNAIII is not known (Morfeldt et al., 1995; Liu et al., 2011). Hfq has been shown to bind RNAIII, *spa* and *eap* and therefore Hfq regulation through sRNA-mRNA interactions is possible. However, studies investigating the role of Hfq in sRNA-mRNA interactions have shown that binding between RNAIII and *spa* or *hla* occurs even in the absence of Hfq. This would indicate that even though Hfq does bind RNAIII and mRNA it does not seem to enhance the interaction between them. Interestingly, RNAIII binds the 5' UTR of *eap* mRNA, which is where the reporter analysis indicated that Hfq functions (Liu et al., 2011).

Does Hfq alter the mRNA to promote translation?

Another possibility may be that Hfq is altering mRNA secondary structure to promote translation. In *E. coli* Hfq was found to alter the conformation in *sodB* mRNA through RNase footprinting (Geissmann & Touati, 2004). mRNA is treated with ribonucleases with and without the presence of Hfq. If Hfq binds and changes mRNA structure this reveals or hides nuclease sites resulting in different sized fragments and therefore a differing gel pattern. Conducting this experiment with *eap* mRNA and Hfq would determine whether Hfq is altering secondary structure. If a change in structure is seen this could mean that translation of *eap* is being affected. To determine whether Hfq affects Eap translation *in vitro* translation assays could be conducted in the absence and presence of Hfq. An increase in Eap protein with the addition of Hfq would suggest that it promotes translation.

4.7.2 Positive transcriptional regulation by Fur

As predicted, reporter analysis showed that Fur positively regulates *eap* transcription in all strains. EMSAs with *eap* promoter DNA showed that Hfq and Fur regulation was not

due to direct binding to the *eap* promoter. This suggests that transcriptional regulation of *eap* occurs through another regulator. SaeRS is known to be required for exoprotein expression (Giraud et al., 1997; Harraghy et al., 2005) and has been found to be positively regulated by Fur (Johnson et al., 2008; Johnson et al., 2011). Therefore EMSAs were conducted with *sae* promoters to determine whether positive regulation of *sae* was direct. The results of these assays showed that Fur does bind the *saeP1* and, to a lesser extent, the *saeP3* promoter regions in high Fe conditions. This is the first example of direct Fur-Fe binding to positively regulate in *S. aureus*.

Recently in *S. aureus* apo-Fur has been shown to activate *norA* transcription by directly binding the promoter (Deng et al., 2012). DNase footprinting experiments revealed a novel Fur box in the promoter indicating that Fur may bind different sequences depending on the type of regulation (Deng et al., 2012). The *S. aureus sae* promoters do not contain a Fur box to which Fur usually binds nor does it contain the novel Fur box identified by Deng et al. (2012). DNase footprinting experiments with *sae* promoter DNA would confirm Fur-Fe binding and also reveal the binding site. This sequence can then be compared to the already identified Fur box sequences to determine any similarities or whether each binding site is distinct.

As with our results, Fur-Fe has also previously been shown to be an activator of transcription in *E. coli* and *N. meningitidis* (Delany et al., 2004; Nandal et al., 2010). In *E. coli* Fur acts as an anti-repressor to the Histone-like nucleoid-associated protein (H-NS). H-NS directly binds to multiple sites within, and upstream of, the *ftnA* promoter to repress transcription. When Fur is complexed with Fe it binds an extended Fur box upstream of the *ftnA* promoter displacing H-NS and allowing transcription (Nandal et

al., 2010). *S. aureus* does not have H-NS but this regulation could occur through the displacement of another DNA binding protein. Fur can also act as a direct activator, as seen with *norB* in *N. meningitidis* (Delany et al., 2004). *In vitro* transcription assays showed that the addition of Fur-Fe induced transcription of *norB*. This assay could be conducted with Fur and *sae* promoters to determine whether Fur can directly activate *sae* transcription. If transcription is not induced this could suggest that another factor is required for activation by Fur.

Post transcriptional regulation by Fur?

Unexpectedly the reporter analysis may also indicate some post transcriptional control of *eap* by Fur. Although different regulation from DNA binding has been reported Fur has not been found to bind RNA, indicating that this regulation is indirect through another factor. Fur has been found to positively regulate the global regulator Agr which also regulates *eap* (Johnson et al., 2011). The RNAlII transcript has been shown to positively regulate *eap* expression and found to bind the 5'UTR of *eap* mRNA (Liu et al., 2011). Therefore post transcriptional regulation of *eap* by Fur could be indirect through RNAlII.

Another possible explanation for the post transcriptional regulation observed could be due to another putative RNA binding protein, aconitase. During our phenotypic analysis we identified that Fur positively regulates aconitase. Although originally identified as a TCA cycle enzyme aconitase has been proposed to be multifunctional. In *E. coli* and *B. subtilis* aconitases have shown RNA binding properties and regulate expression post transcriptionally (Alén & Sonenshein, 1999; Tang & Guest, 1999; Tang et al., 2002). Aconitase regulation is closely linked with iron metabolism and considering that

virulence expression is also Fe regulated it is possible that aconitase may act on these targets as well. To determine whether aconitase is involved in the post transcriptional regulation of *eap*, EMSA can be carried with the aconitase protein and *eap* mRNA.

4.7.3 In *S. aureus* Newman, Hfq shows positive transcriptional regulation of *eap* gene expression.

Comparison of the transcriptional reporter analysis in Newman *fur* and *hfq/fur* double mutants showed a level of transcriptional control by Hfq. The *fur* mutant showed a reduction in *lux* expression whereas very little expression was seen in the double mutant, showing that the addition of the *hfq* mutation affects transcription. EMSAs with *eap* promoter DNA and Hfq suggested that this transcription regulation was not due to direct binding of *eap* promoter DNA. However, as there is no positive control for *S. aureus* Hfq and DNA binding, the possible lack of Hfq functionality cannot be ignored. As the Hfq protein appears to be purified as a multimer this would indicate that the Hfq is forming the hexamer it needs to function. To confirm that the purified Hfq protein is functional, EMSA could be carried out with RNA probes that have been previously shown to bind Hfq. Further investigation revealed that Hfq also positively regulates *sae* which is a positive regulator of *eap* and would account for the transcriptional regulation.

The decrease in *sae* transcript levels in the *hfq* mutant could indicate that Hfq acts to stabilise the transcripts. To determine whether Hfq does stabilise *sae*, mRNA stability assays were conducted with Newman and the Newman *hfq* mutant. The qRT-PCR was carried out with primers for *saeP* and *saeS* to determine whether there were any differences in stability between the transcripts. Again, these preliminary results showed that Hfq does not function to stabilise *sae* mRNA but these experiments would need to

be repeated to confirm these findings. However, using these primers sets cannot reveal the levels of each individual transcript. mRNA stability assays would need to be repeated using Northern analysis to show the levels of each individual transcript.

To confirm direct Hfq binding to *sae* transcripts, EMSAs using the Hfq protein and *sae* mRNA could be conducted. Subsequently, RNase footprinting could be conducted to determine exactly where Hfq binds. Previous studies have shown that *sae* transcription produces four different transcripts expressed from two promoters suggesting that the transcripts undergo some form of processing (Adhikari & Novick, 2008). The transcripts C and A are transcribed from the P1 and P3 promoters respectively (Figure 4.10). Conducting the footprinting assays on both of these transcripts may help identify the role of Hfq. Binding sites at the 5' end of the mRNAs would indicate a role in translational initiation, however binding sites within the transcripts may indicate a role in processing.

The mechanism of *sae* processing or the effect of unprocessed *sae* transcripts is not yet known. A recent study into transcript processing revealed that the *saePQRS* operon also encodes an antisense molecule (Lasa et al., 2011). This same study revealed that RNaseIII is important in the cleavage of sense-anti sense duplexes formed with asRNA regulators or from overlapping transcription (Lasa et al., 2011). Therefore the processing of the *sae* transcripts could be through cleavage by RNaseIII after the formation of a duplex with the asRNA. To determine whether Hfq is also involved in this process, Northern analysis with multiple *sae* probes could be conducted which would show changes in level of each individual transcript between wild type and *hfq* mutant strains. These experiments would then be followed by western analysis using

anti-SaeR/S antibodies to determine whether loss of *hfq* causes reduction in Sae expression.

4.7.3 Strain variation of Hfq and Fur regulation between strains

In Newman, phenotypic analysis revealed a synergistic regulatory relationship between Hfq and Fur for exoproteins such as Eap, Emp, haemolysins and nuclease. This relationship on Eap expression was confirmed by the reporter analysis. However this was not seen in SH1000 or Mn8, even though reporter analysis showed that Hfq and Fur acted at the post transcriptional and transcriptional level respectively. This suggests that regulatory interaction between Hfq and Fur is different in these strains.

Mn8 *eap* promoter sequence differences

The *eap* sequence for the transcriptional and translational reporters were amplified from Newman and 8325-4 genomic DNA respectively. The *eap* promoter sequence is identical between Newman, 8325-4 and SH1000; however the recently published Mn8 genome sequence revealed an 11bp difference (Figure 4.15). The majority of these changes occur further upstream of the transcriptional promoters and so the Mn8 COS, -35 and -10 promoter sequences are all identical to Newman. There are 3bp differences in the 5'UTR which may affect Hfq binding as the reporter analysis revealed that Hfq acts on this region. However it still appears that Hfq does bind this region in Mn8 as there is an observable decrease in Eap protein level in the Mn8 *hfq* mutant. The reporter plasmids would need to be remade with Mn8 sequence to assess whether these changes affect regulation. However, as reporter expression, using the Newman promoter sequence in a Mn8 strain background, follows the same pattern in both Newman and

Newman	1	CACCATCATTATCACTCCTTTTATATAGCTTACAACAAAATAGATGCAAAATTGATTAAC	60
Mn8	1745458	CACCATCATTATCACTCCTTTTATATAGCTTACAACAAAATAGATGCAAAATTGATTAAC	1745517
Newman	61	TAAATTTAAATTAATTATTTAAATAGAATTTATATTTTTAAGTAAATGTTTTTGAGTATA	120
Mn8	1745518	TAAATTTAAATTAATTATTTAAATCGAATTTATATTTTTGAGTAAATGTTTTTGAGTATA	1745577
Newman	121	AAGATGCTGTAATATAATCGACAAATCTAATCGGAATTAAGAAATGAATGTTGTTATGA	180
Mn8	1745578	AAGATGCTGTAATATAATTGACAAATGTAATCGGATTTAAGAAATGAAGGTTGTAATGA	1745637
Newman	181	CATTTAAGTTTGAAGCTTGGTATAAACAAAACAAACAACATTTTAGGTATTGTAATTA	240
Mn8	1745638	CATTTAAGTTTGAAGCTTGGTATAAACAAAACAAACAACATTTTAGGTATTGTAATTA	1745697
Newman	241	ATTAGTTAATCAAATTTAAAAGAACTTTTAAACAGTGTTAAAAAATTATTGTTAAAGATAA	300
Mn8	1745698	ATTAGTTAATCAAATTTAAAAGAACTTTTAAACAGTGTTAAAAAATTATTGTTAAAGATAA	1745757
Newman	301	TTTATTATTAATATTCAGTTAATTCAAAAATAGAGAAAGTCTGGCTATAATTAAGTTGC	360
Mn8	1745758	TTTATTATTAATATTCAGTTAATTCAAAAATAGAGAAAGTCTGGCTATAATTAAGTTGC	1745817
Newman	361	AAATACGAATTACACAAAAAGGAGAGATAATTT	394
Mn8	1745818	AATCACGAATTACATAAAAAAGGAGAGATAATTT	1745851

Figure 4.15 Sequence alignment of the *eap* promoter from Newman and Mn8. The two sequences show 11bp difference (97% identity) indicated by yellow letters. The green letters show the end of an upstream gene. The purple and blue text highlights the COS and -35/-10 sequences respectively. The single red T shows the transcriptional start site and the red text shows the RBS.

Mn8 *hfq* and *fur* single mutants it would suggest that any regulatory differences are due to varying regulator level or activity rather than changes in binding efficiencies.

Do Hfq and Fur regulate *sae* expression in Mn8 and SH1000?

In this chapter we show that, in Newman, transcriptional regulation by Fur and Hfq was through regulation of *sae*. In SH1000 and Mn8 there appears to be little contribution by Hfq to transcriptional regulation suggesting that Hfq regulation of *sae* may be different.

To determine whether Hfq and Fur both positively regulate *sae* expression in SH1000 and Mn8, qRT-PCR needs to be repeated using RNA extracted from these strains.

However, EMSAs showed that Fur binds to the *sae* p1 and P3 promoters in Newman and SH1000 as the *sae* sequence is identical. The Mn8 *sae* P1 and P3 sequences contain 13bp and 1bp differences respectively. As with the *eap* sequence, the majority of the base differences occur upstream of the -35 and -10 promoter sequences (Figure 4.16). The single base change in P3 occurs after the transcriptional start site and so is unlikely to affect Fur binding to the promoter. Therefore Fur does appear to bind *sae* P3; however the EMSA needs to be repeated with P1 DNA fragments amplified from Mn8 to confirm binding here. As previously mentioned SaeS contains a missense mutation that affects protein function. Therefore this mutation along with any variations in *sae* regulation by Hfq and Fur could account for some of the strain variation in *eap* regulation.

Does the Agr system differ between strains?

Our reporter analysis revealed an interesting difference in SH1000, the maximal expression of *eap* at a later time point than in Newman and Mn8. The Agr system

Newman	1	TTGGTACTTGTATTTAATCGTCTATCTTATTTTCTTGTTGTTTTTATTGTGGCAAAAGGT	60
Mn8	208812	TTGGTACTTGTATTTAATCGTCTATCTTATTTTCTTGTTGTTTTTATTGTGGCAAAAGGT	208871
Newman	61	TTATAAATTTTAATACCAAACTA TTAAACACTTCTGATATTCTTAGTTC AAAATATCAG	120
Mn8	208872	TTATAAATTTTAATACCAAACTA AAAAACACTTCTGATATTCTTAGTTC GCTATATCAG	208931
Newman	121	AAGTGTTTTATAGTG TTA TCTAGTT CAGATAAAATATTTCTT ACTTAAAAAA ACGCCCTC	180
Mn8	208932	AAGTGTTTTATAGTG A A ACTAGTT TAGATAAAATATTT CTC ACTTAAAAAA CCGCCCTC	208991
Newman	181	CTCTTATTTTGAC CCCTATTTATTTAAATCAGACAATTATTTTCATTTTCAAATTATTCT	240
Mn8	208992	CTCTTATTTTGAC T CCCTATTTATTTAAATCAGACAATTATTTTCATTTTCAAATTATTCT	209051
Newman	241	TTCTTCAATATTAGTTAAGCGATATTTAAACGAAGTTAAGAATTAGTTA ATGGCATATTA	300
Mn8	209052	TTCTTCAATATTAGTTAAGCGATATTTAAACGAAGTTAAGAATTAGTTA ATGGCATATTA	209111
Newman	301	TTTGCCTTCATTT TAAACTTAA C TTATCAAATTGAAGAAATGAGGAGTTAGCATGAATAC	360
Mn8	209112	TTTGCCTTCATTT TAAACTTAA C TTATCAAATTGAAGAAATGAGGAGTTAGCATGAATAC	209171
Newman	361	AAAATATTTTTTAGCAGCTGGTGCT G TTATCACAAC	396
Mn8	209172	AAAATATTTTTTAGCAGCTGGTGCT A TTATCACAAC	209207

Figure 4.16 Sequence alignment of the *sae* P1 from Newman and Mn8. The Mn8 sequence shows 13 bp differences to the Newman sequence (97% identity) indicated by the yellow letters. The -35 and -10 promoters are highlighted in blue and the transcriptional start site in red.

positively regulates exoprotein expression in a growth phase dependent manner. Although there is no difference in growth rate of SH1000 compared to the other strains, *agr* expression may still be different which would affect the exoprotein expression. Reduced sensitivity to the auto-inducing peptide (AIP) or reduced expression of RNAIII would delay the positive regulation of RNAIII on its target genes. Adding extracellular AIP to SH1000 cultures would induce expression of RNAIII, if this does not occur it could suggest that SH1000 shows insensitivity to AIP. To determine whether the expression of RNAIII differs between strains, RNAIII transcription reporter analysis could be conducted in all strains.

Does SarA differ between strains?

Previous studies in this lab have shown that SarA positively regulates *eap*, *emp* and, to a lesser extent, *sae* expression (Johnson, 2008). Therefore differences in SarA expression between strains may affect regulation of Eap and Emp. As discussed in Chapter 1.3.3 SarA is a well known DNA binding protein, however a recent study has shown possible RNA binding to stabilise transcripts. Conducting reporter analysis in a *sarA* mutants will determine at what level SarA regulates Eap expression. EMSA with *eap* DNA or RNA would then reveal if this regulation is direct. It is also possible that Hfq and Fur regulate *sarA* expression, which can be investigated using qRT-PCR.

Are there differences in Fur protein level?

In Chapter 3.7.3 variations in Hfq protein level and sequence changes were discussed in relation to how this may affect function between strains. The *fur* sequence shows no variations between strains; however differences in transcript level have been previously shown (Figure 4.17) (Purves, 2011). Northern analysis or qRT-PCR would be

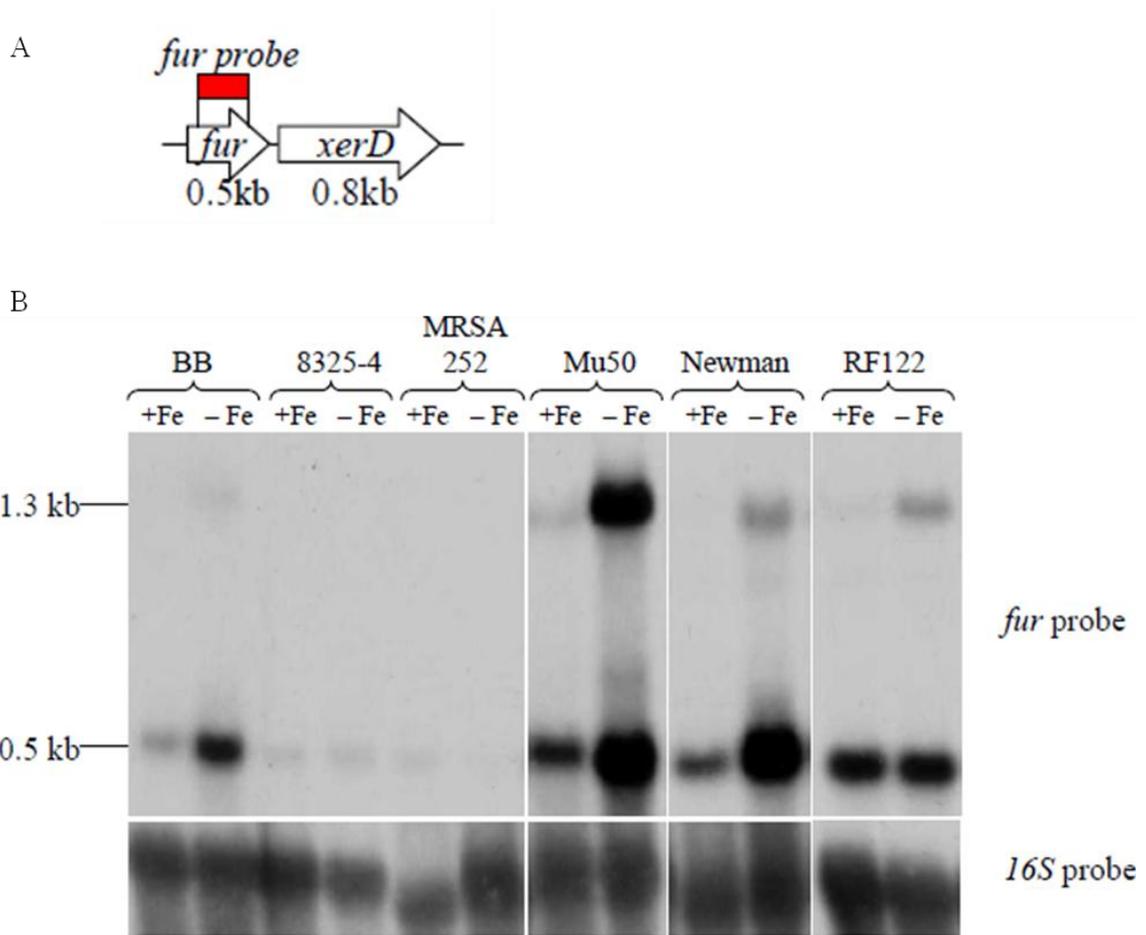


Figure 4.17 Northern analysis showing *fur* transcript level between different *S. aureus* strains. (A) Schematic representation of *fur/xerD* operon as determined by Johnson et al. (2010) and the binding area for the *fur* probe. (B) Northern blot showing *fur* transcript levels in strains BB, 8325-4, MRSA252, Mu50, Newman and RF122. Total RNA was extracted from cells grown in CRPMI \pm 50 μ M Fe₂SO₄ to exponential phase. The *16s* rRNA probe is included to show equal loading of RNA between lanes. This blot is representative of two biological repeats. Diagram taken from Purves (2011).

conducted to determine the level of *fur* transcripts in the strains used in this study. To determine whether the Fur protein shows the same variation, western analysis would be carried out. The previous Northern analysis showed that Newman has a higher *fur* transcript level than most of the other strains. If this is also true with the strains used here it could begin to explain the difference in Fur regulation.

4.7.4 Conclusion

These results show that positive Hfq regulation of *eap* occurs at both the transcriptional and post transcriptional levels. In Newman the transcriptional control occurs indirectly through positive regulation of *sae*, an important regulator of virulence genes, showing that is involved in global regulation. Expression of *sae* is also positively regulated by Fur, which begins to explain the synergistic regulation of exoproteins by Hfq and Fur. For the first time, Fur regulation of *sae* was found to be direct through Fur-Fe binding at the P1 and P3 promoters. Preliminary experiments suggest that post transcriptional regulation by Hfq is not by stabilisation of transcripts. Further investigation is needed to determine the mechanism of Hfq regulation, which could be through either transcript processing or inducing translational initiation. Together these results allow us to begin to build a model of *eap* regulation by Hfq, Fur and Sae (Figure 4.18). However there are other factors involved such as RNA III and their role also needs to be investigated for a complete model. As observed in the phenotypic analysis, the mechanism of regulation also shows strain variation. Therefore a complete model would also help identify possible points of variation in regulation.

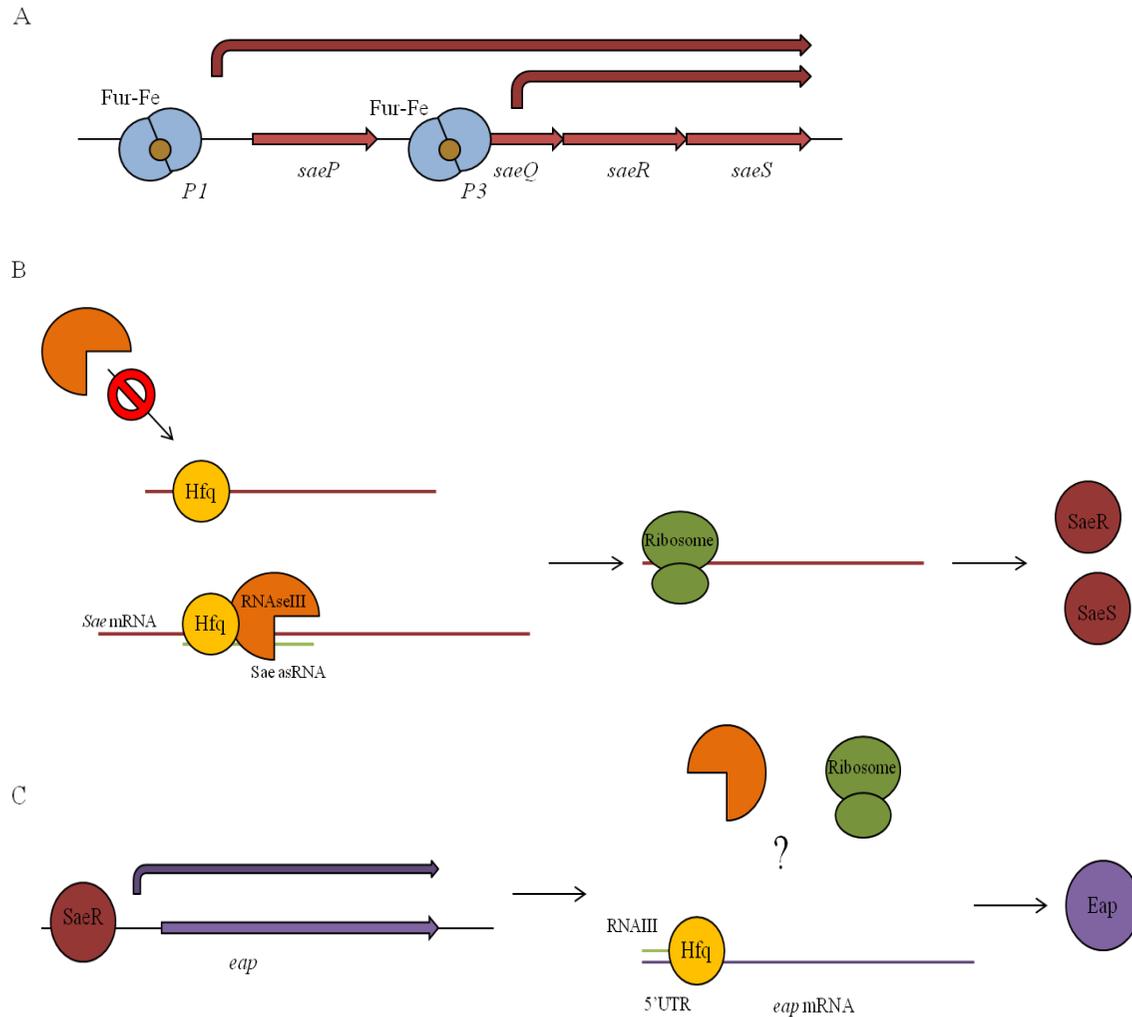


Figure 4.18 Schematic diagrams showing possible regulatory model of *eap* expression by Hfq, Fur and Sae. (A) Fur-Fe binds to *sae* P1 and P3 promoters to activate transcription. (B) Hfq may act on *sae* mRNA to stabilise transcripts (preliminary work needs confirming) or to process *sae* transcripts along with as RNA and RNaseIII to promote translation. (C) SaeR activates *eap* transcription. Post transcriptional regulation of *eap* by Hfq appears to involve RNaseIII binding at the 5'UTR to stabilise (preliminary work needs confirming) or activate translation.

Chapter 5 Final Discussion

There is now extensive evidence of the importance of Hfq and sRNA in post transcriptional gene regulation in a number of Gram negative bacteria. Post transcriptional regulation by sRNA has been shown in *S. aureus*; however the role of Hfq in this regulation remains unclear. The first aim of this thesis was to phenotypically analyse a *S. aureus hfq* mutant alongside a *fur* mutant and an *hfq/fur* double mutant to identify genes regulated by Hfq and to determine any relationship with Fur regulation. The second aim of this thesis was to determine the mechanism by which Hfq and Fur regulate gene expression.

The results of this study showed that Hfq and Fur positively regulate the expression of a number of important cell surface and exoproteins as well as resistance to oxidative stress. Further investigation into the mechanism of *eap* regulation showed direct and indirect regulation by Hfq, whereas Fur showed indirect transcriptional regulation. The indirect regulation by both proteins was suggested to be partly through co-regulation of the global regulator system, *sae*. The direct interaction between Fur and *sae* is the first time *S. aureus* Fur has been shown to bind in high Fe to positively regulate gene expression. The regulation of such an important virulence gene regulator by Hfq and Fur shows that both have a role in the *S. aureus* global regulatory network, which has previously been largely overlooked.

Although the work in this thesis begins to demonstrate the importance of Hfq and Fur in virulence gene expression, there are still a number of unanswered questions. These questions will be addressed in this chapter.

5.1 Where do Hfq and Fur fit into the regulatory network?

As discussed in Chapter 1 the vast numbers of virulence factors in *S. aureus* are controlled by an increasingly complex regulatory network. Each virulence factor is under the regulation of several global regulators which in turn are also tightly regulated and influenced by an array of temporal, internal and environmental signals. The results of this study allow us to build upon current models of virulence regulation to include Hfq and Fur (Figure 5.1).

Although Fur was initially identified as a regulator of Fe homeostasis, it is also essential for the expression of a number of virulence factors. But how does Fur fit into the regulatory network? Further study showed that Fur positively regulates the global virulence regulators Sae, Agr and Rot (Johnson et al., 2011) (Figure 5.1). However how Fur positively regulated these global regulators was unknown. Work in this study confirmed the positive Fur regulation of *sae* reported by Johnson et al. (2011) and for the first time showed that this regulation was direct. Phenotypic analysis in this study has shown positive Hfq regulation of a number of exoproteins. Reporter analysis showed Hfq was regulating at multiple levels, acting directly on target mRNA molecules but also indirectly through the regulation of *sae* (Figure 5.1). The involvement of Hfq in the regulation of an important virulence regulator indicates that Hfq has a global role in regulation.

5.2 What are the global targets of Hfq and Fur?

The results of this study show that Hfq and Fur positively regulate *sae* expression and Fur had been found to regulate other global virulence regulators such as Rot and Agr. Does Hfq also regulate these global regulators? Investigating the role of Hfq in the

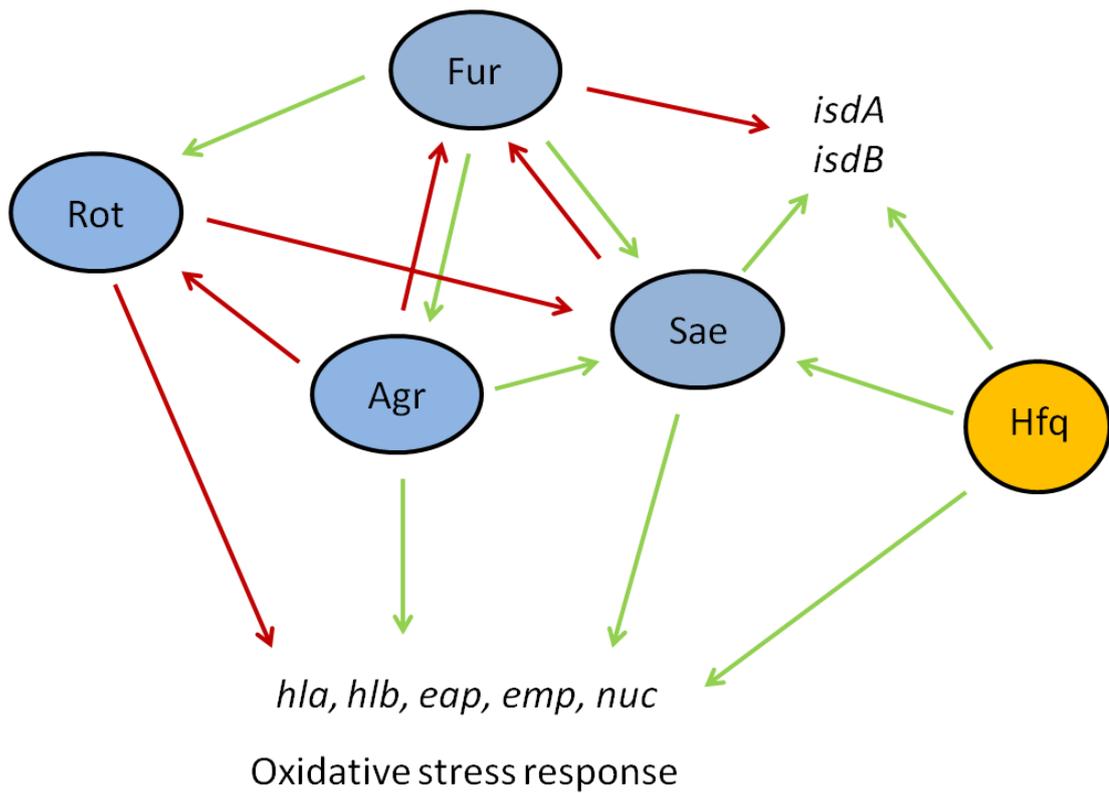


Figure 5.1 Model of regulatory network showing how Hfq and Fur interact with other global regulators such as Sae, Agr and Rot. Positive and negative regulatory pathways are indicated by green and red arrows respectively.

regulation of other global regulators will demonstrate the importance of Hfq in the regulatory network. If Hfq does regulate other global regulators then Hfq shows regulation of gene expression at multiple levels indicating an important global role. Also by regulating global virulence factors then it greatly increases the number of factors Hfq can regulate. Throughout our phenotypic analysis we observed a regulatory relationship between Hfq and Fur and showed that they acted on the same targets. Reporter analysis revealed that Hfq acts directly on targets and indirectly through Sae. However it is also possible that Hfq indirectly regulates through the regulation of *fur* expression. If Hfq is required for full expression of Fur then this will affect all regulated Fur genes. As well as virulence factor expression, Hfq was also found to be involved with the regulation of oxidative stress response. The resistance to oxidative stress has been shown to be regulated by SigB, PerR and Fur. The alternate sigma factor σ^B is a well known stress response factor required for resistance to a number stresses including oxidative stress (Kullik et al., 1998; Giachino et al., 2001). Fur and PerR positively and negatively regulate important oxidative stress response genes such as *sodA* and *kata*. Therefore Hfq regulation of any of these oxidative stress response regulators could explain the changes in stress resistance seen.

To determine the global roles of Hfq and Fur in the regulatory network and also the effects on downstream gene regulation RNA-seq could be conducted with *hfq* and *fur* mutant strains. Traditionally, microarray analysis has been conducted to analyse changes in gene regulation. However, RNA-seq can also reveal changes in asRNA/sRNA levels and RNA processing.

5.2.1 Do Hfq and Fur regulate asRNA/sRNA expression?

Previous studies have suggested that Hfq is not required for sRNA regulation in *S. aureus*. However it is thought that *S. aureus* expresses an estimated 100 sRNA molecules and only a small number have been investigated (Felden et al., 2011). To date only the expression of RNAPIII and RsaA-K have been determined in a *hfq* mutant, which demonstrated that steady state levels of these sRNA are unaffected by Hfq. There are still many more uncharacterised sRNA that may be regulated by Hfq which would be identified by RNA-seq. As in other bacteria, positive Fur regulation can occur through sRNA, however Fur regulation of sRNA in *S. aureus* has not been investigated. A number of sRNAs have been characterised as being important in regulation of metabolism and stress responses (reviewed in Felden et al., 2011). Discovering sRNA molecules regulated by Fur would show whether Fur has further regulatory roles in metabolism and stress responses.

5.3 Does Hfq and Fur regulation occur through direct or indirect interactions?

The RNA-seq analysis will reveal how the RNome changes in the *hfq* and *fur* mutant strains, however it will not show whether these changes are due to direct or indirect interactions. To determine which of these interactions is direct; co-immunoprecipitation assays can be conducted which reveal *in vivo* protein-DNA interactions.

5.3.1 Identification of Fur binding through Chromatin immunoprecipitation (ChIP)

Direct interactions between Fur and DNA *in vivo* can be determined from chromatin immunoprecipitation (ChIP) techniques, which can be coupled with microarray chip

technology (ChIP-on-chip) to show Fur-DNA interactions across the whole genome. Results in this study show that positive Fur regulation of *sae* occurs directly in high Fe conditions, which is the first example of this type of regulation in *S. aureus*. Whereas, a recent paper has shown that in *S. aureus* apo-Fur bound the *norA* promoter to positively regulate expression (Deng et al., 2012). Therefore, ChIP-on-chip analysis would need to be carried out in high and low Fe conditions to identify Fur-Fe and apo-Fur interactions. The results from these assays compared with the results from RNA-seq would reveal whether Fur-DNA interactions results in positive or negative regulation. These experiments would broaden our understanding of Fur regulation by revealing all possible Fur-DNA interactions.

5.3.2 Identification of Hfq binding through RNA immunoprecipitation (RIP)

Direct interactions between Hfq and mRNA or sRNA *in vivo* can be detected through RNA immunoprecipitation (RIP). Previous work using *in vitro* techniques have revealed a number of RNAs that bind Hfq including RNAIII, *spa* and *eap* (Huntzinger et al., 2005; Liu et al., 2010). However the Hfq-RNAIII interaction is the only one that has been demonstrated with an *in vivo* technique (Huntzinger et al., 2005). A recent paper by Liu et al. (2010) used an *in vitro* RIP by passing total cell RNA over purified Hfq protein to identify interactions. Although this technique indicates that Hfq can bind the identified RNAs it does not show whether these interactions are occurring *in vivo*. Also the RNA was extracted from cells grown in BHI which is a rich medium. Previous studies in our lab have shown that virulence gene expression is induced in low Fe and nutrient conditions, which are more reflective of *in vivo* conditions (Morrissey et al., 2002). Therefore to understand how virulence is regulated *in vivo* the co-immunoprecipitation and RNA-seq techniques should be conducted on cells grown in a

restricted media.

5.4 Does Hfq interact with other proteins?

In other bacteria Hfq has been shown to interact with proteins such as ribosomal proteins, RNases and PAP1 which provide more evidence for its role in post transcriptional regulation (Morita et al., 2005; Sukhodolets & Garges, 2003; Kajitani & Ishihama, 1991; Mohanty et al., 2004). Two hybrid analyses with Hfq against a *S. aureus* library would determine which proteins interact with Hfq. As Hfq forms a stable hexamer, interactions between Hfq proteins can be used as a positive control.

Identifying interactions between Hfq and proteins in *S. aureus* may reveal functions of Hfq in post transcriptional regulation. For example if RNaseIII is identified then this indicates that Hfq is involved in mRNA processing or degradation. These assays may also reveal novel interactions indicating any possible novel mechanisms of Hfq regulation.

5.5 Regulation of *hfq* expression

In *S. aureus*, the expression of *hfq* is much lower than seen in other bacteria and in some strains the Hfq protein cannot be detected (Huntzinger et al., 2005; Bohn et al., 2007; Liu et al., 2010). So far no work has been conducted on how *hfq* expression is regulated or why translation is affected in some strains. Determining how *hfq* expression is regulated may reveal why this strain variation occurs. In *E. coli*, *hfq* has been found to be regulated in a growth rate and growth phase dependent manner similar to other protein components of transcriptional and translational machinery (Kajitani et al., 1994; Azam et al., 1999; Liveris et al., 1991). Induction of *E. coli hfq* expression in relation to cell growth would suggest that Hfq is important for this process, which is confirmed in

an *E. coli hfq* mutant that showed an altered growth rate (Tsui et al., 1994). In *S. aureus*, *hfq* does not appear to be involved in bacterial growth but has been shown to be important for virulence factor regulation. Therefore it is possible that Hfq is not growth phase dependent but is regulated by other global virulence regulators. To determine whether *hfq* expression in *S. aureus* is growth dependent or regulated by other factors, qRT-PCR with RNA taken from different time points during growth and in mutant strains (e.g. *fur*, *sae*, *agr*) could be conducted. A transcriptional *hfq* reporter would show how expression changes over time; however *hfq* appears to be transcribed as part of an operon with no clear promoter directly upstream of *hfq*. Therefore the transcriptional start site of *hfq* would need to be determined first through transcript mapping.

In *E. coli* the expression of Hfq is also under a degree of post transcriptional regulation. RNaseE has been found to regulate the level of *hfq* transcript levels with a 3 fold increase in *hfq* mRNA in an *rne* mutant (Tsui & Winkler, 1994). Auto regulation of translation by Hfq has also been reported in *E. coli* and *Sinorhizobium meliloti* (Vecerek et al., 2005; Sobrero & Valverde, 2011). In *E. coli*, Hfq binds to two different sites on its own mRNA which results in translational repression by occluding the RBS from translational machinery (Vecerek et al., 2005). Post transcriptional regulation of *S. aureus hfq* mRNA may begin to explain why the Hfq protein cannot be detected in some strains. Investigating the differences in post transcriptional regulators between strains may indicate as to what is regulating Hfq translation.

5.6 Conclusion

In conclusion this study has shown that Hfq does have a role in exoprotein expression and regulation of an important global regulator. In addition Fur has shown to positively

regulate by direct DNA binding in high Fe conditions. These results provide evidence for another level of regulation in the complex network of regulators that exists in *S. aureus*. Further investigation is required to understand the global role of these regulatory mechanisms and how they fit into the virulence regulatory network. Determining the complete pathways for virulence regulation can help identify common aspects present in this highly variable pathogen, and allow the development of effective antimicrobials.

Appendix

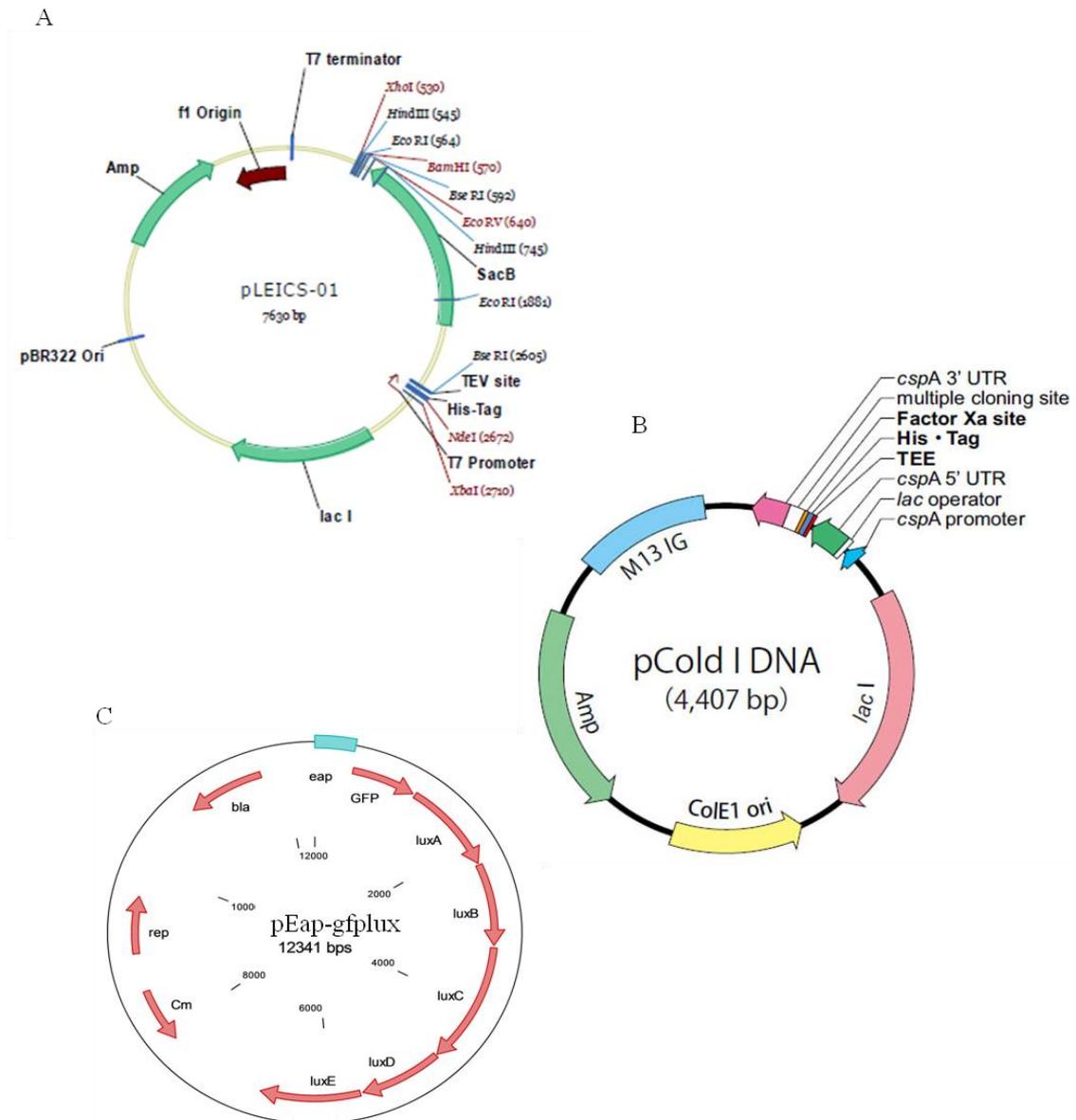


Figure A-1. Plasmids used in this study.

A) Protein expression vector pLEICS-01 with an IPTG inducible promoter and an Amp resistance gene. Figure supplied by PROTEX (University of Leicester).

B) Protein expression vector with a cold shock induced promoter and Amp resistance gene. Figure taken from pCOLD manual (Takara Bio Inc.)

C) pEap-gfplux reporter plasmid with Amp and Cm resistance genes. Map constructed from sequence requested from Harraghy et al. (2005) using Clone Manager software.

CACCATCATTATCACTCCTTTTATATAGCTTACAACAAAATAGATGCAAAATTGATTAAC
 TAAATTTAAATTAATTATTAATAGAATTTATATTTTTAAGTAAATGTTTTTTGAGTATA
 AAGATGCTGTAAATATAATCGACAAATCTAATCGGAATTAAGAAATGAATGTTGTTATGA
 CATTAAAGTTTGAAGCTTGGTATAAACAAAACCAACAACATTTTAGGTATTGTAAATTA
 ATTAGTTAATCAAATTA AAAAGAACTTTAACAGTGTTAAAAAATTATTGTTAAAGATAA
 TTTATTATTAATATTCAGTTAATTCAAAAAATAGAGAAAGTCTGGCTATAATTAAGTCCC
 GGGTTAACAAGGAGGAATAAAAAATGAGTAAAGGCGAAGAACTTTTCACTGGAGTTGTCC
 CAATTCCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGG
 GTGAAGGTGATGCAACATACGGAAAACCTTACCCTTAAATTTATTTGCACTACTGGAAAAC
 TACCTGTTCCATGGCCAACACTTGTCACTACTTTTCGGTTATGGTGTTCAATGCTTTGCGA
 GATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATG
 TACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCA
 AGTTTGAAGGTGATACCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAG
 ATGGAAACATTCTTGGACACAAATTGGAATACA ACTATAACTCACACAATGTATACATCA
 TGGCAGACAAAACAAAAGAATGGAATCAAAGTTAACTTCAA AATTAGACACAACATTGAAG
 ATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTG
 TCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACG
 AAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCA
 TGGATGAACTATACAAAATAAATGTGTCGACAGGAGGACTCTCTATG

Figure A-2 The nucleotide sequence of the transcriptional *lux* reporter plasmid up to the start of the *luxA* gene. The *eap* promoter sequence is written in red, *gfp* gene in green and the *lux* in purple.

CACCATCATTATCACTCCTTTTATATAGCTTACAACAAAATAGATGCAAAATTGATTAAC
 TAAATTTAAATTAATTATTAATAGAATTTATATTTTTAAGTAAATGTTTTTTGAGTATA
 AAGATGCTGTAAATATAATCGACAAATCTAATCGGAATTAAGAAATGAATGTTGTTATGA
 CATTAAAGTTTGAAGCTTGGTATAAAACAAAACCAACAACATTTTAGGTATTGTAAATTA
 ATTAGTTAATCAAATTA AAAAGAACTTTTAACAGTGTTAAAAAATTATTGTTAAAGATAA
 TTTATTATTAATATTCAGTTAATTCAAAAAATAGAGAAAAGTCTGGCTATAATTAAGTTGC
 AAATACGAATTACACAAAAAAGGAGAGATAATTTCCCGGGTTAACAAGGAGGAATAAAAA
 ATGAGTAAAGGCGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGT
 GATGTTAATGGGCACAAATTTTCTGTCAAGTGGAGAGGGTGAAGGTGATGCAACATACGGA
 AAACCTACCCTTAAATTTATTTGCACTACTGGAAAACCTACCTGTTCCATGGCCAACACTT
 GTCACTACTTTTCGGTTATGGTGTTC AATGCTTTGCGAGATACCCAGATCATATGAAACAG
 CATGACTTTTTCAAGAGTGCCATGCCCCGAAGGTTATGTACAGGAAAGA ACTATATTTTTTC
 AAAGATGACGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTT
 AATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAA
 TTGGAATACA ACTATAACTCACACAATGTATACATCATGGCAGACAAAACAAAAGAATGGA
 ATCAAAGTTAACTTCAA AATTAGACACAACATTGAAGATGGAAAGCGTTCAACTAGCAGAC
 CATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTAC
 CTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTT
 CTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAAATG
 TGTCGACAGGAGGACTCTCTATG

Figure A-3 The nucleotide sequence of the 5' UTR *lux* reporter plasmid up to the start of the *luxA* gene. The *eap* promoter sequence is written in red, *gfp* gene in green and the *lux* operon in purple.

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