Iron and copper homeostasis in *Staphylococcus aureus*

Thesis submitted for the degree of Doctor of Philosophy

at the University of Leicester

by

Jonathan Peter Baker BSc (Hons)

Department of Genetics

University of Leicester

September 2009

Abstract

Staphylococcus aureus is a pathogenic bacterium that causes a wide spectrum of human diseases and is a leading cause of nosocomial infection in the UK. Metal homeostasis is an important aspect of bacterial biology as transition metals such as copper and iron are required as enzyme cofactors but can also be toxic to cells at high concentrations. These metal homeostasis systems can be important for virulence. However, several important aspects of *S. aureus* metal homeostasis remain to be defined.

This project focuses on novel *S. aureus* iron/Fur gene regulation and copper homeostasis. Fur is a well-described DNA binding repressor protein, found in many pathogenic bacteria. In *S. aureus*, Fur has been seen to both activate and repress genes in iron replete and iron restrictive conditions, and there is also Fur independent iron regulation. However, the regulatory mechanisms involved remain undefined. This investigation into novel iron regulation identified a new *S. aureus* iron regulator, LysR. *lysR* expression was found to be auto-regulated and activated by Fur in low iron. Phenotypic analysis suggested a possible role for LysR in the control of genes of the histidine utilisation pathway, as well as oxidative stress resistance.

Two copper responsive operons have been found in *S. aureus*; *copAZ* and *copB/mco*. However, many important aspects of the *S. aureus* response to copper remain undefined. In this study, copper tolerance was shown to vary between strains and ATCC 12600 was identified as the first hyper copper-tolerant *S. aureus*, due to a transferable copper-resistance plasmid. A new *S. aureus* regulator, CsoR, was found to control the copper response of *copAZ* and both chromosomal and plasmid encoded *copB/mco*. Finally, this data shows that H_2O_2 scavenging is an essential *S. aureus* copper resistance mechanism and that extracellular surface copper toxicity is important in *S. aureus*.

Acknowledgements

Firstly I would like to thank my supervisor Dr Julie Morrissey for all her hard work and support during this project. I also thank Miranda for answering all manner of silly questions and for helping every time I managed to get stuck. Also the rest of our group; Jo, Emma and Geetha plus the rest of lab 121 who are great fun to work with and are always very helpful. In addition, I am grateful to the students that I have had the pleasure of teaching in the lab whose enthusiasm for the work always helped rekindle mine. I would also like to thank the many friends I have made in Leicester who have kept me sane during the thesis and helped me get through the tough times.

Finally I thank my parents and my sister, who have always been very supportive and helped me get this far! I hope I can return the favour when my sister writes her thesis in a few years time!

List of abbreviations

^{o}C	degrees Celsius
µF	microfarad
µg	microgram
µJ	microjoule
µl	microlitre
µm	micrometer
µmol	micromole
µM	micromolar
ABC	ATP binding cassette
ADP	adenosine diphosphate
APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pair
CA-MRSA	community acquired MRSA
cm	centimetre
CRPMI	chelexed RPMI (medium)
CTAB	cetyl trimethyl ammonium bromide
Cu(n)	n µm $CuCl_2$
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	di-ethyl-pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EMRSA	epidemic MRSA

ETEC	enterotoxigenic *Escherichia coli*
$FADH_2$	flavin adenine dinucleotide (reduced)
Fe(n)	n μm $Fe_2(SO_4)_3$
g	gram
GADPH	glyceraldehyde 3-phosphate dehydrogenase
h	hour
HA-MRSA	hospital acquired MRSA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMA	heavy-metal associated (protein domain)
IAA	isoamyl alcohol
kb	kilobase
kDa	kilodalton
kV	kilovolt
l	litre
LA	Luria Bertani agar
LB	Luria Bertani
LIP	labile iron pool
LTTR	LysR type transcriptional regulator
M	molar
mA	milliamps
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation - time-of-flight
Mbq	megabecquerel
MCO	multi-copper oxidases
mg	milligram
MGE	mobile genetic element
min	minute
ml	millilitre
mm	millimetre
mM	millimolar

MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	messenger RNA
MRSA	methicillin resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognising adhesive matrix molecules
NAC	nitrogen assimilation control
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NAP1	Newman with the ATCC 12600 plasmid (strain)
ng	nanogram
nm	nanometre
OD	optical density
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNACL	Protein and Nucleic Acid Chemistry Laboratories
PNAG	polymeric N-acetylglucosamine
PVL	Panton-Valentine leukocidin
RBS	ribosomal binding site
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute-1640 (medium)
rRNA	ribosomal RNA
SaPI	*Staphylococcus aureus* pathogenicity island
SCC	staphylococcal chromosomal cassette
SDS	sodium dodecyl sulphate
SMM	supplemented minimal medium (buffer)
SMMP50	supplemented minimal medium Penassay 50 (medium)
SOC	Super optimal broth with catabolite repression (medium)
SOD	superoxide dismutase

sRNA	small RNA
SSC	sodium chloride - sodium citrate
SSD	staphylococcal siderophore detection (medium)
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
TSB	Trypticase soy broth
TSST-1	toxic shock syndrome toxin-1
U	unit
UV	ultraviolet
V	volt
v/v	volume/volume
VISA	vancomycin intermediate-resistant *Staphylococcus aureus*
VRSA	vancomycin resistant *Staphylococcus aureus*
w/v	weight/volume
w/w	weight/weight
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
× g	times gravity

Table of Contents

Chapter 1. Introduction

This project aimed to investigate novel iron regulation and copper homeostasis in the pathogenic bacterium *Staphylococcus aureus*. The following introduction will explain why despite our many advances in antibacterial treatments, *S. aureus* remains an important disease causing agent. The genome of *S. aureus* will be discussed along with the types and mechanisms of staphylococcal infections and the new multi-resistant strains that have emerged. The significance of environmental regulation for the expression of virulence factors will provide a basis for the decision to examine *S. aureus* iron and copper homeostasis. The significance of iron and copper as biological metals will be summarised along with the mechanisms that bacteria have evolved for their uptake and utilisation. Both these metals can also be toxic for bacterial growth and this has led to the evolution of environmentally regulated systems. Our current understanding of the well studied iron systems and recently identified copper operons of *S. aureus* will be reviewed in order to illustrate the important questions that this project tried to answer.

1.1. The staphylococci and *S. aureus*

The *Staphylococcus* genus

Staphylococci are Gram positive spherical bacteria which are approximately 1 µm in diameter. They divide in more than one plane to produce clusters when viewed by microscopy. Staphylococci are found as either part of the natural human or environmental flora, depending on the particular species (Tuazon, 1984; Vaz-Moreira *et*

al., 2008). They typically produce the oxidative stress enzyme catalase and are consequently described as catalase positive. Most are facilitative anaerobes, able to utilise either respiration in the presence of oxygen or fermentation when oxygen is deficient in order to produce ATP. An additional characteristic feature of the staphylococci is their low genomic G/C content, which differentiates them from other Gram positive cocci species such as the micrococci (Woese, 1987). Many staphylococci species are linked to human infections/diseases and therefore are of interest to the clinician and the researcher. These include; *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, *S. cohnii*, *S. xylosus*, *S. capitis*, *S. warneri*, *S. hominis*, *S. simulans*, *S. saccharolyticus*, *S. caprae*, *S. lugdunensis* and *S. schleiferi* (Martins & Cunha, 2007). Of the all the staphylococci, it is *S. aureus* which is primarily associated with human disease and for this reason it is the most widely studied species. *S. aureus* infections vary significantly in their cause, severity, location and effective treatments. In addition, the emergence of new *S. aureus* strains which are multi-drug resistant and possess additional virulence factors, has ensured that this pathogenic bacterium is still of great interest to researchers and medical professionals alike.

1.2. Features of the *S. aureus* genome; variation and adaptability

The sequenced *S. aureus* strains

Due to its medical significance, *S. aureus* is one of the most sequenced bacterial species with the first completed in 2001 (Kuroda *et al*., 2001). Currently, there are 13 human *S. aureus* isolates and 1 bovine isolate which have been fully sequenced (Lindsay, 2008). These projects have been essential for the subsequent research into this important pathogen. This is particularly true with the sequencing of the new emerging strains,

which has allowed for strain comparisons that have shown significant genomic differences between the different *S. aureus* isolates.

The *S. aureus* genome is highly variable between different strains. Approximately 78% of *S. aureus* genes are conserved while 22% are accessory (Fitzgerald *et al*., 2001; Lindsay & Holden, 2004). The conserved genes primarily encode proteins essential for cell maintenance, although some do encode virulence factors which must be essential for *S. aureus* survival and pathogenesis (Fitzgerald *et al*., 2001). Accessory refers to genes that are entirely absent in different strains, giving each strain a specific gene profile. These tend to be non-essential and mainly encode proteins for the colonisation of specific strain niches (Fitzgerald *et al*., 2001; Lindsay & Holden, 2004).

Mobile genetic elements

Between 10-20% of the *S. aureus* genome is made up of mobile genetic elements (MGEs) which usually carry accessory virulence genes and are genetically highly mobile, thus allowing for easy dissemination of virulence factors (Lindsay & Holden, 2004; Lindsay & Holden, 2006; Lindsay, 2008). These MGEs primarily encode toxins, antibiotic resistances and immune evasion elements, and have facilitated the rapid spread of important virulence factors within the *S. aureus* population. Table 1.1 summarises the types of *S. aureus* MGEs with examples for each one described. All of these MGEs insert into the *S. aureus* chromosome at either specific or random sites. Plasmids are the exception, which self-replicate and are normally maintained at a variable copy number. However, these may occasionally insert into the chromosome (Holden *et al*., 2004). The number of these MGEs in a specific *S. aureus* strain can also vary.

Type	Description	Example	Reference
Plasmids	Self-replicating discrete circular pieces of DNA	pLW1043 - from a clinical vancomycin resistant *S. aureus* which encodes the vancomycin resistance gene *vanA*	Clark *et al*. (2005) and Weigel *et al*. (2003)
Transposons	Randomly inserting gene cassettes	Tn*554* - erythromycin and spectinomycin resistance genes	Murphy *et al*. (1985)
SCC elements	Staphylococcal Chromosomal Cassettes which integrate at a single chromosomal site	SCC*mec* - carries methicillin resistance and other virulence factors	Ito *et al*. (2003)
Prophages	Viral phage genome inserted into the chromosome	wPVL - Panton-Valentine leukocidin genes: *lukS-PV* and *lukF-PV*	Kaneko *et al*. (1998)
SaPIs	Large mobile transferable chromosomal DNA, which integrate at specific sites	SaPI1 – contains the toxic shock syndrome toxin-1 gene	Lindsay *et al*. (1998)
Insertion sequences	Transposons which only encode their own transposase	Function to disrupt gene transcription - a total of nine IS*1272*s in methicillin resistant *Staphylococcus aureus* strain 252 (MRSA252)	Holden *et al*. (2004)

Table 1.1. The types of *S. aureus* MGEs.

Strains will only normally contain one of five molecular types of a specific staphylococcal chromosomal cassette (SCC) element but may contain different numbers of the other types, such as MRSA252 which contains one SCC but has a total of three transposons, two prophages, four types of insertion sequences and an integrated plasmid (Holden *et al*., 2004). Therefore, although the sequenced *S. aureus* strains share many genetic similarities there are also substantive genomic variations due to past gene acquisition by MGEs. This has resulted in high strain diversity and has ensured the adaptability of *S. aureus* strains to better survive and cause disease.

1.3. *S. aureus* as a disease causing bacterium

Transmission and major diseases

S. aureus is an important pathogen which can cause both human and animal infections. Important animal infections include bovine mastitis, an infection of the udder which is a

significant problem for the dairy industry (Barkema *et al*., 2006). For humans, *S. aureus* primarily colonises the nasal passage as a commensal with a prevalence of between 30-70% in the population, although precise rates vary between different studies (Lindsay & Holden, 2004). Carriage is also dependent on various bacterial and host factors such as bacterial strain, host age and health status (Peacock *et al.,* 2001). Hospital colonisation by *S. aureus* is a consequence of its high prevalence in the population.

Human *S. aureus* infections include local to the more serious systemic diseases, dependant on the bacterial toxins produced, the host status and point of infection. *S. aureus* diseases can affect a wide range of tissues such as the skin, heart, lungs, muscles, bones, eyes and joints (Crossley & Archer, 1997). Infections include; septic shock, meningitis, pneumonia, endocarditis, osteomyelitis and many additional toxin mediated diseases such as the scalded skin syndrome, caused by the exfoliating scalded skin syndrome toxin (Iwatsuki *et al.,* 2006). It is this range of different diseases which has contributed to its significance as a human pathogen however, *S. aureus* bacteraemia remains the most common fatal manifestation of nosocomial infection.

S. aureus must usually breach the skin or a mucosal membrane in order to establish most of these diseases and infections are most commonly observed in the immune-compromised and post-surgical patients. Therefore, *S. aureus* is a particular problem in the modern hospital environment (Grundmann *et al.,* 2006). Medical treatments such as the fitting of prosthetic devices lead to an increase in the incidence of *S. aureus* infection, at a substantial cost to the healthcare system (Chu *et al.,* 2005). This

is because these devices provide an accessible point of entry into a host. Consequently, hospital patients with these *in vivo* foreign bodies, especially those on immune suppressants, are particularly vulnerable to *S. aureus* infection (Weems, 2001).

Treatments

As *S. aureus* has had a long history as an important nosocomial pathogen, numerous treatments and preventative measures have been developed (Lowy, 1998). However, these have had to be continually improved as *S. aureus* has adapted during their use to overcome these therapies. Treatment of a *S. aureus* infection is primarily a course of antibiotics. Penicillin or one of its many derivatives such as methicillin, nafcillin or oxacillin, which interfere with cell wall synthesis, is particularly effective although allergies to these penicillin-based drugs can present a problem during treatment. Also, drugs such as the tetracyclines, which disrupt protein synthesis, and erythromycin, which binds bacterial ribosomal RNA, are both effective in controlling *S. aureus* infections (Maltezou & Giamarellou, 2006). However, the development of resistance during drug therapy is a particular difficulty during *S. aureus* treatment and limits the single use of many antibiotics such as the fluoroquinolones, a group of synthetic antibiotics which interfere with DNA replication (Lowy, 1998). Therefore a combination of drugs is often used at the same time to both reduce the risk of resistance occurring during treatment and ensure that the antibiotics remove all the bacteria from the patient.

1.4. The emergence of new *S. aureus* strains

Methicillin resistant *Staphylococcus aureus*

A number of new *S. aureus* strains have appeared in the last 50 years and has meant that research into this pathogenic species remains important. The development of antibiotics and their subsequent widespread use has led to the emergence of *S. aureus* strains better able to resist antibacterial therapies. These antibiotic resistance factors are often encoded on MGEs. Methicillin resistant *Staphylococcus aureus* (MRSA) are strains that have acquired a specific SCC termed SCC*mec*, which can contain an assortment of virulence genes but importantly includes the *mecA* gene and its regulators (Ito *et al.*, 2003). The *mecA* gene codes for a β-lactamase that hydrolyses the β-lactam ring of penicillin based antibiotics, rendering them useless as bactericidal agents (Figure 1.1). Therefore, the resistance to methicillin provided by MecA also provides intrinsic resistance to all penicillin-based antibiotics. *S. aureus* resistance to methicillin was observed shortly after it became used as an antibacterial treatment and eventually spread throughout the *S. aureus* population (Crossley & Archer, 1997).

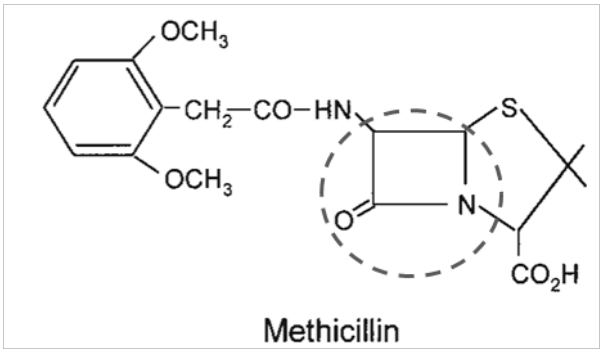


Figure 1.1. The structure of the β-lactam antibiotic methicillin. Highlighted is the β-lactam ring which is the target of the MecA resistance protein. Figure adapted from Stapleton & Taylor (2002).

MRSA have also acquired a multitude of extra virulence factors in addition to penicillin based antibiotic resistance, including antibiotic resistances to tetracycline and erythromycin as well as additional toxins (Ardic *et al.*, 2005). Although most isolates are still susceptible to a few of the previously used antibiotics, each isolate must be first tested for susceptibility to the available antibacterial drugs and this takes money and time which increases the risk to the patient and cost to the hospital.

The dominant hospital MRSA strains

The majority (> 95%) of UK nosocomial MRSA cases are solely due to two MRSA clonal types: epidemic MRSA-15 (EMRSA-15) and epidemic MRSA-16 (EMRSA-16) (Johnson *et al*., 2001; Moore and Lindsay, 2002). These two groups are classified by their set of core genes but vary in their accessory gene carriage (Moore and Lindsay, 2002). Of the two groups, the EMRSA-16s are more adaptable to survive in the hospital environment but it is not yet known what makes these two clonal types the dominant MRSAs and particularly, what allows for them to be such successful nosocomial pathogens. The number of MRSA cases has increased dramatically in the last 20 years, which made MRSA the most common cause of nosocomial infection in the UK (Johnson *et al*., 2001; Moore & Lindsay, 2002), although in more recent years incidence has started to fall as shown in Figure 1.2, which may be reflective of improved hospital practices or changes in reporting systems.

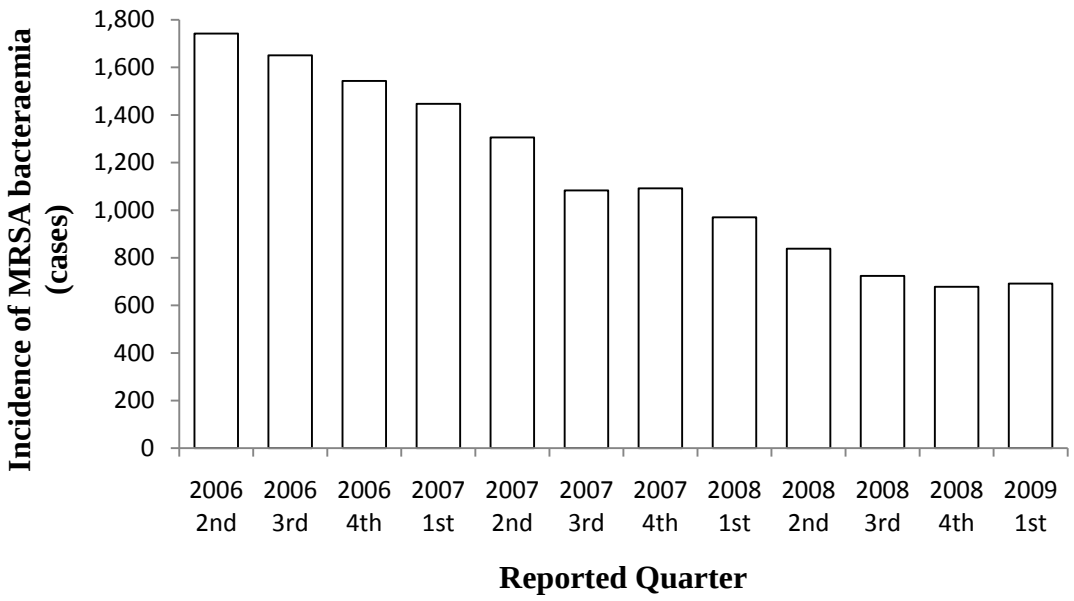


Figure 1.2. Mandatory reports of hospital MRSA bacteraemia from April 2006 to March 2009. Figure was created from Table 1 of the HPA UK hospital MRSA mandatory bacteraemia reporting scheme detailed on the HPA website.

Vancomycin resistance in *Staphylococcus aureus*

The effective drug for MRSA treatment is vancomycin as resistances have emerged for almost all of the previously used single and combined antibacterial therapies. However, the first partially resistant *S. aureus* (VISA) to vancomycin, Mu50, was isolated in 1996 from a surgical wound infection and is now a well studied sequenced *S. aureus* strain (Hiramatsu *et al.*, 1997). It is believed that this intermediate resistance is not provided by gene acquisition but occurs due to point mutations in the cell wall synthesis pathway which is the target of vancomycin. Also, the expression of a range of genes were observed to be altered in the VISA strain JH9 when compared to the vancomycin sensitive parent strain JH1, most of which are involved in cell wall synthesis and which suggests that intermediary vancomycin resistance may depend on more than one factor (McAleese *et al*., 2006).

Full vancomycin resistance (VRSA) was first observed for *S. aureus* in 2002 and subsequently several other strains have been isolated clinically (Sievert *et al*., 2008). Unlike the VISA strains, VRSA resistance is derived from the chromosomal transposon Tn*1546*, likely acquired from other vancomycin resistant species such as enterococci, but contained on plasmids in *S. aureus* (Courvalin, 2006). The genes encoded by the Tn*1546* element form an alternative pathway of peptidoglycan precursor biosynthesis, activated in response to vancomycin and which lowers the affinity of the antibiotic for its native target (Courvalin, 2006). VRSA strains are also often methicillin resistant and although currently rare, represent a potential future challenge for clinical *S. aureus* infection control.

Community acquired MRSA

A worrying development in addition to the new drug resistant *S. aureus* strains has been the emergence of MRSA which infect previously healthy individuals. The definition of these community acquired MRSA (CA-MRSA) refers to strains which are contracted outside of the nosocomial environment. Despite being a diverse group of *S. aureus*, CA-MRSA strains tend to possess a type IV SCC*mec*, which distinguishes them from the hospital acquired MRSA (HA-MRSA) strains which usually contain SCC*mec* type I, II or III (Okuma *et al*., 2002; Ito *et al.,* 2003). CA-MRSA strains may also carry additional virulence factors. Sequencing of the CA-MRSA MW2 found it contained many novel toxin genes when compared to the sequenced MRSAs; N315 and Mu50 (Baba *et al*., 2002). One of these is the pore forming Panton-Valentine leukocidin toxin, which is linked with necrotising pneumonia in the young and is found in the majority of CA-MRSAs (CDC, 1999; Lindsay, 2008). Therefore, MRSA strains appear to be

evolving separately in these two different environments; hospital and community, producing even more diversity in the *S. aureus* population. Potentially with the high level of MGEs in the *S. aureus* genome, new pathogenic strains may yet emerge. This means that research into *S. aureus* remains crucial, as this species is highly adaptable and frequently transfers virulence genes between strains.

Alternative and preventative measures

Due to the emergence of antibiotic resistance, other alternative therapies are being investigated to reduce *S. aureus* hospital infection rates, including vaccines, improved hygiene and anti-*S. aureus* materials. A polyclonal antibody preparation against the *S. aureus* protein adhesin ClfA (clumping factor A) has been investigated and mice vaccinated with anti-ClfA, anti-FnbA (fibronectin-binding protein A) and anti-FbnB (fibronectin-binding protein B) in combination, have been observed to show a reduction in colonisation by *S. aureus* (Hall *et al*., 2003; Arrecubieta *et al*., 2008). Also, a vaccine known as StaphVAX (a *S. aureus* polysaccharide conjugate vaccine) showed some promise in initial clinical trials but was eventually found to be ineffective, as was INH-A21, a human IgG preparation (Maltezou & Giamarellou, 2006; Schaffer & Lee, 2009).

Improved preventative measures are also being tested, such as better screening of hospital admitted patients and superior anti-*S. aureus* surfaces. Some recent studies involved the testing of copper as an antibacterial replacement for steel surfaces. In one report it was observed that compared to steel, copper surfaces significantly reduced MRSA prevalence and survival, including that of the two epidemic UK hospital strains

EMRSA-15 and EMRSA-16 (Noyce *et al*., 2006). Use of such surfaces has also observed a similar effect against *Pseudomonas aeruginosa*, *Escherichia coli* and *Clostridium difficile*, where copper surfaces were successful at killing the normally resilient *C. difficile* spores (Elguindi *et al*., 2009; Espírito Santo *et al*., 2008; Weaver *et al*., 2008; Wheeldon *et al*., 2008). However, other reports have found that increased bacterial killing by copper surfaces can be offset by an increased difficulty in removing bacteria and dirt when compared to the traditional steel fixtures (Airey & Verran, 2007). A clinical trial using copper surfaces to reduce the spread of *S. aureus* infections is currently being carried out at University Hospital Birmingham NHS Foundation Trust (Casey *et al*., 2008) and represents an exciting new method for reducing nosocomial *S. aureus* infections. However, not much is known about copper resistance in *S. aureus*.

1.5. *S. aureus* virulence factors

It is the vast range of virulence factors with diverse pathogenic functions which makes *S. aureus* such a successful and dangerous pathogen (Crossley & Archer, 1997). These can be classed by their function; attachment, host defence evasion/destruction and tissue invasion/damage through toxins and secreted factors. As previously described, these are often encoded on MGEs which allows for their horizontal transfer.

S. aureus express a range of cell wall proteins which bind host factors for adhesion and immune evasion. These belong to the MSCRAMM (microbial surface components recognising adhesive matrix molecules) family which are anchored to the cell wall by LPXTG motifs in their C-terminus by sortase proteins (Mazmanian *et al*., 2002). Members of this family include the fibrinectin binding proteins FnbA and FnbB, the

fibrinogen-binding proteins ClfA, ClfB and Efb, the collagen-binding protein Cna and the antibody binding protein A (Arciola *et al.,* 2005). Some of these have been shown to be directly required for virulence. This includes Cna, as when both a *cna*$^{+}$ and *cna*$^{-}$ *S. aureus* strain are used in an animal infection model, there is a significant increase in disease incidence from the *cna*$^{+}$ strain (Patti *et al.,* 1994). In addition, others have been found to aid immune evasion such as ClfA, which protects *S. aureus* against phagocytosis by macrophages (Palmqvist *et al*., 2004). Protein A is an important surface virulence protein and a superantigen, which means it directly activates T-cells which can lead to an unregulated immune response and subsequent inflammation and shock (Fraser & Proft, 2008). The cell wall anchored protein A also binds the Fc part of IgG and reduces both phagocytosis and opsonisation efficiency, to limit the immune system's effectiveness during host defence (Palmqvist *et al*., 2002).

Some important virulence factors include the variety of polysaccharides secreted by *S. aureus* which contribute to avoiding host immunity. These include the capsular polysaccharide and the polysaccharide polymeric N-acetylglucosamine (PNAG), encoded by the *ica* operon. Capsular polysaccharide is a large mass of macromolecules consisting of repeating carbohydrate subunits which surround the bacteria and help prevent phagocytosis (O'Riordan & Lee, 2004; Fattom *et al*., 1996). The best understood function of PNAG is its contribution to the formation of a biofilm (Cramton *et al*., 1999; Maira-Litrán *et al*., 2002). A biofilm is an aggregated mass of bacteria covered in a secreted polysaccharide that protects them from environmental stress and antibacterial compounds. The production of a biofilm is how this bacterium colonises medical devices and *S. aureus* is the leading cause of nosocomial prosthetic device infection (Lentino, 2003). Biofilm production is also a feature of the bovine strains

which cause intramammary infections (Fox *et al.,* 2005). However, some studies have found that *S. aureus* can produce a biofilm independently of PNAG and therefore the role of this polysaccharide in biofilm production is not fully defined (Toledo-Arana *et al*., 2005).

Several non-covalently attached cell wall proteins have been implicated in *S. aureus* virulence, such as Eap and Emp. Both these proteins are adhesins but lack the LPXTG motifs found in the MSCRAMMs. They have been found to bind numerous host factors including; prothrombin, elastin and the collagens (Harraghy *et al*., 2005; Hansen *et al.,* 2006). Both Eap and Emp have been implicated in the production of biofilm (Johnson *et al*., 2008) and also aid in immune evasion by aggregating host proteins to reduce phagocytosis efficiency (Hansen *et al.,* 2006).

S. aureus expresses many factors which are secreted to acquire nutrients, destroy host immune cells and cause disease. *S. aureus* produces a large assortment of toxins, many of which are the sole cause of a particular disease. This includes the toxic shock syndrome toxin (TSST-1), a superantigen which directly leads to the toxic shock syndrome disease (Crossley & Archer, 1997; Childs *et al.,* 1999). Also, the exfoliating toxins A and B cause the blistering skin disorder known as scalded skin syndrome that results in the separation of live and dead skin (Ladhani *et al.,* 1999). Another, enterotoxin A, is an intestinally released toxin and a superantigen, which causes vomiting and diarrhoea (Fraser & Proft, 2008). The remaining *S. aureus* secreted factors are harder to affiliate with a particular disease but act to damage host tissues and aid in invasion. These include lipases such Sal-1 and Sal-2 which disrupt host cell membranes

and the proteases such as the alpha/beta toxins and the haemolysins, which lyse host cells for the acquisition of *in vivo* nutrients and also function to kill host immune cells (Dinges *et al*., 2000; Burlak *et al*., 2007; Götz *et al*., 1998).

1.6. Regulation of virulence factors

Two component regulators

To ensure the appropriate expression of *S. aureus* virulence factors during infection, these genes are under the control of numerous regulators which can act in unison and in a hierarchical manner to coordinate gene transcription. There are two main families of *S. aureus* virulence factor regulators; the two component regulators (TCRs) and the SarA family, although there are numerous other regulators. The TCRs are organised as dual protein systems, which are often transcribed from a single operon. Their mode of action is illustrated below in Figure 1.3.

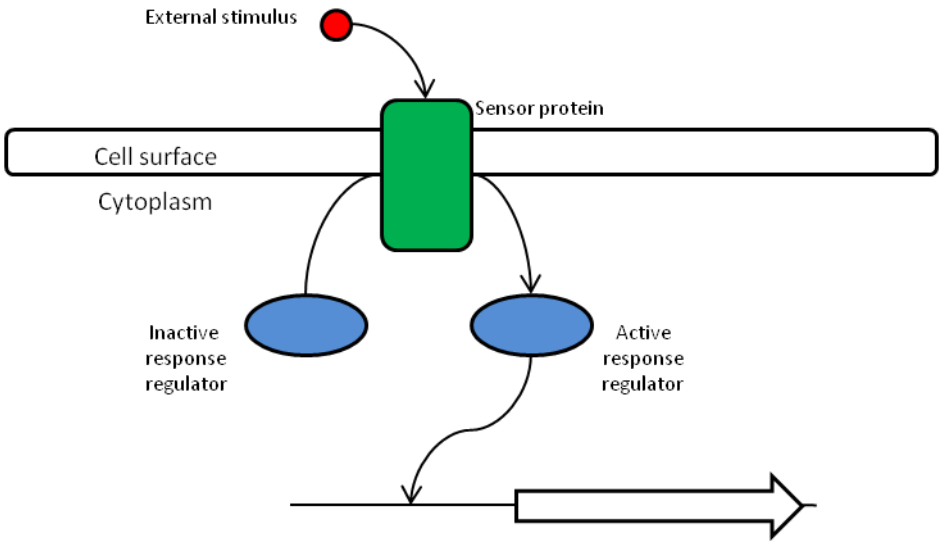


Figure 1.3. TCRs as transcriptional regulators. TCR systems consist of a cell surface sensor protein to detect a specific external stimulus and a cytoplasmic response regulator, which becomes modified by the sensor protein and subsequently affects target gene transcription. Created from information in Cheung *et al*. (2004).

One of the TCR proteins is a cell-surface attached sensor which detects a particular stimulus and modifies the second TCR protein; a cytoplasmic response regulator (Figure 1.3). This regulator becomes active and leads to an appropriate gene response through activating or repressing transcription directly or via effecter molecules such as non-coding regulatory RNAs (Cheung *et al*., 2004). There have been 16 TCRs identified in *S. aureus* N315 and many of these have been well characterised (Cheung *et al*., 2004). Examples include SrrAB, which responds to environmental oxygen levels to control TSST-1 and protein A expression, and SaeRS, which is a growth dependant regulator of the haemolysins, coagulase, protein A, Eap and Emp (Giraudo *et al*., 1999; Giraudo *et al*., 2003).

The SarA family

The second major group of virulence factor regulators are termed the SarA family which are small winged helix proteins that most likely bind DNA, or possibly RNA, to control gene transcription (Cheung *et al*., 2008). There are 11 members of this regulator family in *S. aureus* N315. These can directly control virulence factor transcription such as SarS, an activator of protein A synthesis, SarZ, which activates alpha toxin and Rot, which represses toxin synthesis (Tegmark *et al*., 2000; Kaito *et al*., 2006; Saïd-Salim *et al*., 2003). However, some Sar proteins regulate other members of the Sar family, such as SarR which negatively regulates SarA to control numerous *S. aureus* toxins such as TSST-1 and the haemolysins (Bronner *et al*., 2004; Cheung *et al*., 2004).

Hierarchical complexity of virulence factor regulation through the *agr* locus

The *S. aureus* regulatory families rarely function individually but operate together for a coordinated systematic response to the host environment. The best characterised example of hierarchical regulation involves the TCR AgrCA. This is a quorum sensing system that senses the local density of the *S. aureus* population via a secreted product of the *agr* regulon, AgrD, which is detected by the cell wall sensor protein, AgrC (Bronner *et al*., 2004). This controls two promoters; P3 for expression of the *agr* effecter molecule and P2 for the expression of the *agrBDCA* operon. The AgrCA TCR switches the *S. aureus* phenotype from cell wall anchored invasion proteins to secreted toxins during the post-exponential growth phase and also responds to *S. aureus* cell density (Bronner *et al*., 2004). This global regulator controls the transcription of 27 well characterised virulence genes through the expression of the AgrCA effecter, a RNA molecule termed RNAIII (Cheung *et al*., 2004; Bronner *et al.*, 2004). The *agr* locus has also been shown by microarray studies to up-regulate or down-regulate numerous genes in a cell density dependant manner, making it a truly global regulator (Dunman *et al.*, 2001; Ziebandt *et al*., 2004). RNAIII controlled genes are often also transcriptionally activated or repressed by additional regulator proteins, and an example of this hierarchical control is shown in Figure 1.4 for the expression of alpha toxin (*hla*). *hla* is activated by SaeR, SarA, and RNAIII but repressed by Rot, SarS and SarT. Therefore, the regulation of *S. aureus* virulence genes in response to entering the host can depend on several factors, which allows for precise control of virulence factor expression.

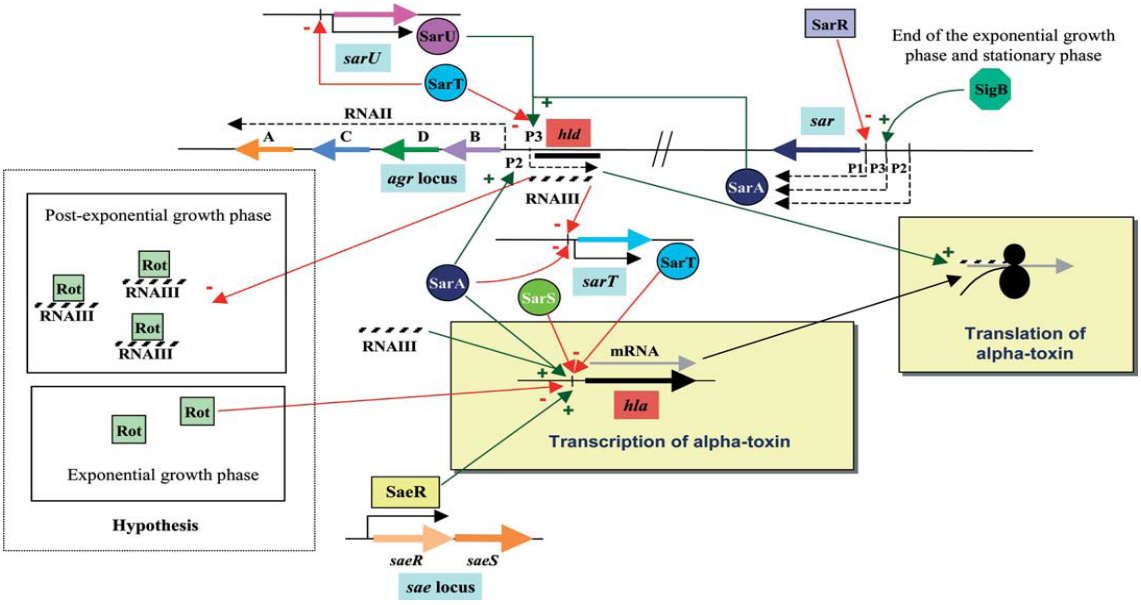


Figure 1.4. The interaction of the *agr* and *sar* loci for the regulation of alpha toxin (*hla*). Global regulators of the TCR and SarA family can act in unison through activation or repression, to regulate virulence gene expression. The complexity and hierarchical nature of these global regulators can be seen in the number of factors that are involved in the regulation of *hla*. These include parts of the *agr*, *sae* and *sar* loci. Figure taken from Bronner *et al*. (2004).

The significance of *S. aureus* environmental metal regulation

The previously described TCR and SarA protein families sense environmental stimuli for the regulation of virulence proteins. However, *S. aureus* also uses environmental signals for the control of non-virulence proteins to ensure the expression of required uptake/resistance systems and metabolic pathways for the efficient utilisation of local environmental nutrients, such as metals (Xiong *et al.*, 2000). The majority of this environmental sensing is through specific detection and regulatory systems which allow *S. aureus* to sense and respond to a range of environmental signals including; carbon sources, temperature and metal ion availability. Iron is essential for *S. aureus* growth but is kept restrictive in the host to both reduce metal toxicity and limit its use by pathogens. *S. aureus* uses the environmental iron concentration to increase the

expression of proteins for iron acquisition and as an indicator that it has entered a host and can switch phenotypes to express virulence genes (Allard *et al*., 2006). Therefore, characterisation of the *S. aureus* iron responsive genes and iron regulation is important for our increased understanding of how this pathogen responds to the *in vivo* environment during infection. However, although a large amount of research has been performed in this field there still remain several aspects of *S. aureus* iron homeostasis which are uncharacterised. Like iron, environmental concentrations of the transition metal copper must also be sensed by bacteria to induce the expression of proteins for its detoxification and/or uptake. Furthermore, whilst host copper concentrations are kept restrictive, pathogens can encounter elevated levels in specific host tissues. However unlike iron homeostasis, very little is known about the *S. aureus* copper response, which may also have links to virulence. Therefore, the characterisation of copper homeostasis could provide new insights into the *S. aureus* environmental response to the host.

1.7. The biological importance of iron and copper

The significant properties of iron and copper in living systems

Iron and copper are transition metals utilised by organisms for important biochemical reactions as cofactors for crucial enzymes and as an environmental signal for pathogens. Both can exist in a variety of oxidative states; iron potentially as one of six states from Fe^{2-} to Fe^{4+} but most commonly as Fe^{2+} and Fe^{3+}, and copper as one of three oxidation states; Cu, Cu^{1+} and Cu^{2+} (Crichton & Pierre, 2001; Stern *et al*., 2007). Ferrous iron (Fe^{2+}) is water soluble but is easily oxidised to insoluble ferric iron (Fe^{3+}) in the presence of oxygen (Arredondo & Núñez, 2005). Cupric copper (Cu^{2+}) is the most common form of copper present in biological systems and is soluble in water, while the

cuprous form (Cu^{1+}) is less soluble and also less abundant due to its readiness to become oxidised to the Cu^{2+} state (Linder & Hazegh-Azam, 1996; Arredondo & Núñez, 2005). Although reports of *in vivo* Cu^{3+} exist, it is thought that this oxidation state has no biological relevance (Crichton & Pierre, 2001). Both these metals are employed as metal cofactors by organisms due to their property of being able to switch between different oxidative states, which allows for the transfer/storage of energy and the reduction of substrates.

The iron redox potential covers almost the entire biologically relevant range of between -0.5 V and 0.6 V (Crichton & Pierre, 2001). It is the most important of the metals, utilised by almost all organisms for crucial biochemical reactions including; DNA biosynthesis, methanogenesis and respiration (Andrews *et al.,* 2003). However, there exist a few microorganisms which do not require iron for growth, including some lactobacilli species (Imbert & Blondeau, 1998). However, for most microorganisms iron is essential and iron availability is often the limiting growth factor *in vivo*. When compared to iron enzymes, copper enzymes typically possess higher redox potentials which allow them to oxidise certain substrates directly, such as superoxide by the copper containing superoxide dismutase (Crichton & Pierre, 2001; Rensing & Grass, 2003). Copper ions are used in redox reactions primarily in electron transfer and dioxygen transport/activation (Crichton & Pierre, 2001; Linder & Hazegh-Azam, 1996). Copper can also function as an essential structural component of macromolecules and is required for the maintenance of their structure, such as in the arsenate reductase, azurin (Pozdnyakova *et al*., 2001; Stern *et al*., 2007). As important metals that share some similar biochemical properties, there are often links between the uptake and utilisation of iron and copper in living systems such as human ceruloplasmin, a serum copper

containing oxidase with ferroxidase activity (Bull *et al*., 1993; Arredondo & Núñez, 2005). Therefore, it is sometimes difficult to separate these two metals when describing their importance in biology.

Iron as a protein cofactor

Proteins that incorporate iron as a cofactor can be classified into several distinctive groups based upon the type of iron centre they contain. Table 1.2 summarises the four classifications of iron proteins with some examples for each. There can be variations in the number of iron atoms associated with the centre, from one in the mononuclear non-haem proteins and haem proteins, two in the di-iron centre proteins and between one and eight in the iron-sulphur proteins (Table 1.2). These can be in coordination with nitrogen, oxygen or sulphur atoms or alternatively, with large molecules such as the organic porphyrin ring found in the haem proteins (Table 1.2).

Iron protein	Iron centre description	Examples	Reference
Mononuclear non-haem proteins	A single iron atom in coordination with combinations of sulphur, nitrogen or oxygen atoms	Amino acid synthases such as phenylalanine hydroxylase and detoxifying enzymes, including Fe/SOD	Li & Fitzpatrick (2008) and Esposito *et al*. (2008)
Di-iron centre proteins	Two iron atoms linked by several oxygen atoms	Hydroxylases and reductases such as methane monooxygenase and ribonucleotide reductase	Lange & Que (1998)
Iron-sulphur proteins	Combinations of iron atoms linked by sulphur, usually from cysteine residues. One to four iron atoms are the most common type but up to eight can be in a single cluster	Hydrogenases and the ferridoxins (electron transfer)	Frazão *et al*. (2008)
Haem proteins	A single iron atom buried in an organic "porphyrin" ring	Oxygen transport in haemoglobin, electron transfer in the cytochromes and catalysis in catalase	Emerit *et al*. (2001), Prince & George (1995) and Smulevich *et al*. (2006)

Table 1.2. Classification of iron proteins.

Copper as a protein cofactor

The proteins that contain copper as a cofactor can be classified into centre types by the number of copper atoms present and their arrangement in the quaternary protein structure, which are summarised in Table 1.3. Type I and type II copper centres contain a single copper atom which in the type I centres is bound to two histidines and one cysteine residue in trigonal planer coordination, and in type II centres with several potential amino acids ligands (Table 1.3). Type III centres contain two copper atoms each bound to three histidine residues and linked by dioxygen. Proteins can also contain both a type III and type II copper centre and are termed trinuclear centre proteins, often found in multi-copper oxidases (MCOs) such as the previously described human ferroxidase, ceruloplasmin (Table 1.3). There are three additional types of copper centre which are less frequent and found in only a few proteins. Copper A centres have two copper atoms ligated to two histidines plus a methionine and a protein bound oxygen, linked by two cysteine residues (Table 1.3). Copper B centres contain a single copper atom ligated to three histidines. Copper Z centres have four copper atoms ligated to seven histidine residues, linked by a sulphur atom (Table 1.3).

Copper centre	Description	Example	Reference
Type I (blue copper proteins)	Single Cu atom bound to two histidines and one cysteine residue. Additional copper bound ligand can vary between different proteins leading to three subclasses	Most common of the copper centres, involved in electron transfer in small electron transfer proteins or larger nitrite reductases and multi-copper oxidases	MacPherson & Murphy (2007) and Morozova *et al*. (2007)
Type II	Single copper atom and several potential amino acids ligands and coordinations. A coordination site for these centres can be vacant for substrate binding and subsequent protein activity in catalytic reactions	Oxidases, monooxygenases, dioxygenases, nitrate reductases (nitrite reductase) and dismutases (SOD)	MacPherson & Murphy (2007)
Type III	Contain two copper atoms each bound to three histidine residues and linked by dioxygen	Proteins which transport oxygen such as plant catechol oxidase	Klabunde *et al*. (1998)
Trinuclear centre	Contains a type II and type III copper centre	Multi-copper oxidases such as ceruloplasmin	Bento *et al*. (2007)
Copper A centre	Two copper atoms ligated to two histidines, a methionine plus a protein bound oxygen and are linked by two cysteine residues	Cytochrome *c* oxidase	Beinert (1997)
Copper B centre	Single copper atom ligated to three histidines	Cytochrome *c* oxidase	Kaila *et al*. (2009)
Copper Z centre	Four copper atoms ligated to seven histidine residues and linked by a sulphur atom	Nitrous-oxide reductase	York *et al*. (2008)

Table 1.3. Classification of copper protein centres.

1.8. Iron and copper toxicity

Although they are essential metals, both iron and copper can be toxic to eukaryotic and prokaryotic cells. This can occur through the induction of oxidative stress, peroxidation of cell membranes, protein misfolding and protein cofactor displacement, which will be subsequently explained in more detail by focusing on microorganism iron and copper toxicity. This has led to the evolution of homeostasis systems for these two metals to allow their use as cofactors whilst reducing their toxic effects.

Catalysis of redox stress

The primary reason for metal induced oxidative stress is cellular hydrogen peroxide, which is produced by the oxidation of NADPH and the subsequent activity of superoxide dismutase (SOD) (Nicholls, 2002; Nishiyama *et al*., 2001). Both Fe^{2+} and Cu^{1+} can react with this cellular hydrogen peroxide to produce reactive oxygen species (ROS) such as the harmful hydroxyl radical ($OH^{\bullet}$). This is known as the Fenton reaction, shown below (Touati, 2000).

$$Fe^{2+}/Cu^{1+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + OH^- + OH^{\bullet}$$

The hydroxyl radicals produced by the Fenton reaction are a highly reactive species which can directly damage DNA and macromolecules through the oxidation and subsequent disruption of their quaternary structures (Gaetke & Chow, 2003; Aruoma *et al*., 1991; Zastawny *et al*., 1995). Free Fe^{2+} for use in the Fenton reaction results from reduction of intracellular ferric iron. The *in vivo* reductant responsible for this is not wholly characterised but superoxide, $FADH_2$ and free cysteine have all been implicated (Woodmansee & Imlay, 2002; Park & Imlay, 2003). The actual *in vivo* reductant of cellular ferric iron may depend on several aspects of the cell status, such as oxygen availability. One possible source of Fe^{3+} for subsequent reduction and participation in Fenton chemistry are the Fe-S clusters of proteins such as dihydroxy-acid dehydratase. These can be inactivated by cellular superoxide which results in the release of cofactor iron (Flint *et al*., 1993). Also, hydrogen peroxide has been observed to induce the release of iron from cytochrome *c* which subsequently leads to DNA damage (Kim & Kang, 2006). Therefore, oxygen and oxygen metabolism products lead to the release of intracellular ferric iron from proteins and their subsequent reduction to ferrous iron by

reductants, promoting the creation of harmful ROS by Fenton chemistry which then leads to cellular toxicity.

When compared to other metals, significantly more research has been conducted into copper induced oxidative damage. Early reports using cell free systems showed that copper ions bound DNA directly and with hydrogen peroxide to produce various ROS at this site, resulting in DNA cleavage (Yamamoto & Kawanishi, 1989). In several bacterial species copper induces the oxidative stress response such as *P. aeruginosa*, where copper shock leads to the up-regulation of the oxidative stress resistance genes; *ahpF*, *sodM* and *katA* (Teitzel *et al*., 2006). However, a study using *E. coli* found that although inhibiting growth, excess copper did not directly lead to oxidative DNA damage (Macomber *et al*., 2007). Also copper and peroxide did not induce the up-regulation of the oxidative stress response in this species (Macomber *et al*., 2007). Furthermore, copper actually prevented hydrogen peroxide and iron induced oxidative stress (Macomber *et al*., 2007). It was proposed that an unknown cytoplasmic copper chelator/protein may prevent the short lived hydroxyl radical from being formed near enough to DNA to cause cleavage (Macomber *et al*., 2007). Potentially, it is the periplasmic accumulation of copper which leads to toxicity in this Gram negative strain (Macomber *et al*., 2007). These results contradict numerous other reports and have yet to be substantiated by other studies but suggest that the cause of copper induced oxidative stress may vary between bacterial species. Currently, the role of oxidative stress in copper toxicity in the Gram positive species, including *S. aureus*, is undefined.

Lipid peroxidation

Iron induced oxidative stress can also lead to lipid peroxidation where free radicals oxidise lipids and change their structure, which has been observed in both prokaryotic and eukaryotic organisms (Corson *et al*., 1999; Searle & Willson, 1983). This can result in the disruption of lipid membranes and the subsequent leakage of cellular solutes. Numerous studies into the effect of copper on the plasma membrane of *Saccharomyces cerevisiae* have found increased membrane permeability and disruption due to the presence of environmental copper ions, which is dependent on the lipid structure of the membrane (Ohsumi *et al*., 1988; Avery *et al*., 1996; Howlett & Avery, 1997).

Protein disruption

Copper ions have been observed to have other toxic effects in addition to oxidative stress induction. In *E. coli*, copper ions induce unwanted disulphide bond formation of periplasmic proteins under aerobic conditions (Hiniker *et al*., 2005). Furthermore, disruption of the periplasmic DsbC disulphide isomerisation pathway, which rearranges unwanted disulphide bonds, increases copper sensitivity which suggests a role for this system in *E. coli* copper tolerance (Hiniker *et al*., 2005). Copper can also displace metal cofactors from critical enzymes, such the zinc finger base excision repair protein Fpg. Cu^{2+} ions have been shown to disrupt Fpg enzyme activity 10-fold, which could not be reversed by a simultaneous or subsequent incubation with as much as 50-fold concentrations of zinc ions (Asmuss *et al*., 2000). This showed that the affinity of Fpg for copper was significantly higher than its affinity for its native cofactor; zinc (Asmuss *et al*., 2000). Also, copper has been shown to inhibit *E. coli* growth by inactivating

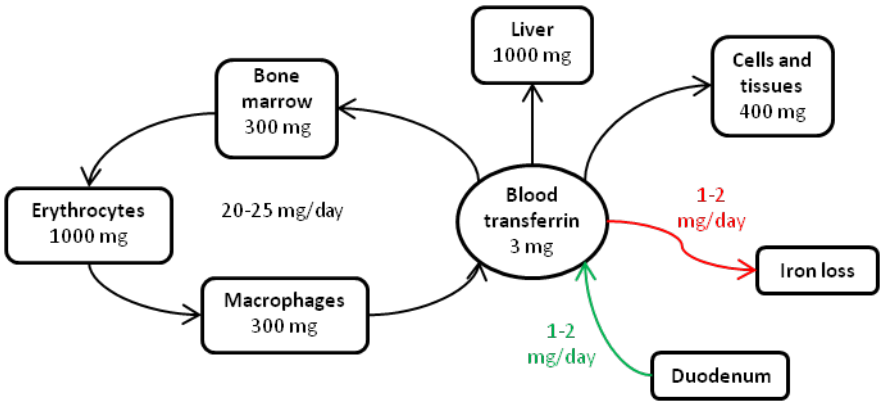
isopropylmalate dehydratase, an important amino acid synthesis enzyme (Macomber & Imlay, 2008). Therefore, both iron and copper can be toxic to eukaryotic and bacterial cells but the mechanisms of this toxicity may vary between different bacterial species.

1.9. Host iron and copper

Host iron and copper whole body metabolism

For pathogenic bacteria such as *S. aureus*, the environment during an infection will be a host. Therefore, the likelihood of microorganisms encountering growth limiting or toxic levels of either iron or copper will depend on the host metabolism and homeostasis systems for these two metals. Figure 1.5 summarises the tissues and routes of these metals *in vivo*. Both host iron and copper are obtained from the diet and absorbed in the intestine but host levels of iron are significantly higher than copper (Figure 1.5). Copper and iron are primarily stored in the liver but iron storage cells also include macrophages and enterocytes. Here iron is stored in ferritin, a protein capable of storing 4500 atoms of iron and which also has ferroxidase activity to oxidise cytoplasmic ferrous iron to ferric iron suitable for storage (Koorts & Viljoen, 2007).

A



B

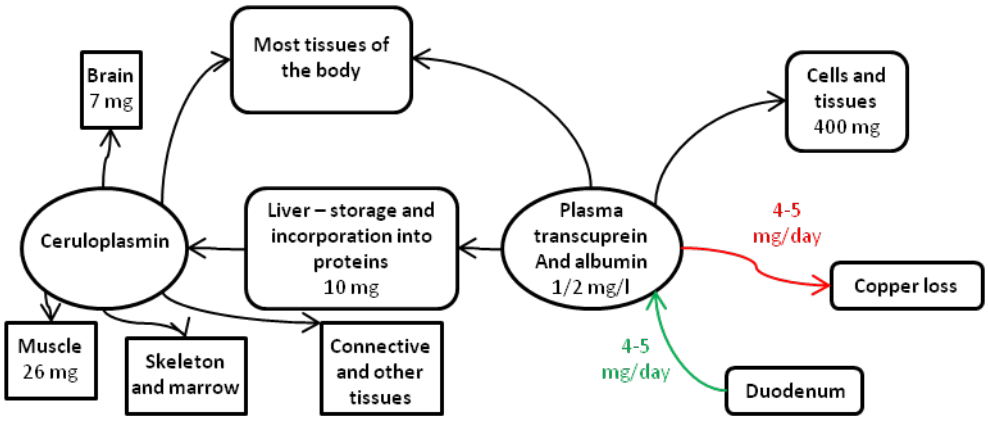


Figure 1.5. Host iron and copper metabolism.

(A) Iron is acquired from the diet and absorbed through the small intestine and either moved into the circulatory system or stored in ferritin. Serum iron is bound to transferrin. Iron is incorporated into haemoglobin in mature erythrocytes at the bone marrow, where 80% of the total host iron is localised. Liver hepatocytes, macrophages and enterocytes all store iron as ferritin and coordinate host iron metabolism through the release of this stored iron into the blood plasma. Figure adapted from Figure 3 of Hetnze *et al*. (2004).

(B) Copper is acquired from the diet and absorbed through the small intestine. Upon entering the blood via the intestinal wall, copper binds blood albumin and transcuprein and is deposited in liver hepatocytes. From here, it is incorporated into copper proteins including ceruloplasmin, which transports copper via the blood to the skeleton, brain, muscle and other tissues of the body to be incorporated as a cofactor in enzymes and electron transport proteins. Figure adapted from Linder & Hazegh-Azam (1996).

Availability of *in vivo* copper and iron for pathogens

Due to their toxicity and requirement by microorganisms, the free concentration of iron and copper is kept low *in vivo* through these metals being bound to high affinity proteins or chelators. Serum copper ions are bound to the MCO ceruloplasmin, albumin, transcuprein and to a lesser extent by small peptides and amino acids (Linder & Hazegh-Azam, 1996). Most tissues are low in copper but levels are greatest in the liver, brain, skeleton and muscle (Figure 1.5B). Lung tissue also contains high concentrations of copper due to its involvement as a cofactor in Cu/Zn superoxide dismutase (Catalani *et al*., 2008). Host cytoplasmic copper is mostly associated with protein chaperones and chelators which reduce intracellular toxicity by keeping copper ions safely bound to these high affinity proteins/molecules (Arredondo & Núñez, 2005).

Serum iron is mainly associated with two host proteins; the iron transporting transferrins and the oxygen transporter haemoglobin (Schaible & Kaufmann, 2004; Emerit *et al*., 2001). Haem is the most common host form of iron, principally found as haemoglobin in serum erythrocytes and levels in serum are usually low but can increase due to haemolytic diseases or bacterial haemolysin activity (Baker *et al*., 2003). In fact, serum host iron concentrations are lower than the concentration required for prokaryotic growth, which is an important part of the innate immunity (Litwin & Calderwood, 1993). Lactoferrin is a high affinity iron-binding protein found mainly in secreted fluids such as saliva, tears and milk as well as serum, which keep iron restricted in these fluids (Schaible & Kaufmann, 2004). Cytoplasmic iron from internalised transferrin can either join the labile iron pool (LIP) which is weakly bound iron mainly found in the Fe^{2+}

state, be assimilated for biological use or stored in iron storage proteins such as ferritin (Arredondo & Núñez, 2005; Nadadur *et al*., 2008).

Variation in the *in vivo* iron and copper concentration in response to infection

Both iron and copper concentrations can vary *in vivo* in direct response to infection, although the nature of this response is dissimilar for the two metals. Iron availability can be reduced by immune signals such as interferon-gamma which activates macrophages but also down regulates their transferrin receptor expression, which reduces iron availability for intracellular pathogens (Ryu *et al*., 2000). In addition, during inflammation and infection the levels of serum hepcidin, a liver secreted hormone of iron homeostasis, are increased by interleukin-6 (Nemeth *et al*., 2003; Nemeth *et al*., 2004). This leads to a reduction in iron release from the major iron storage cells; enterocytes, hepatocytes and macrophages and therefore further decreases serum iron levels for pathogens (Vyoral & Petrák, 2005). Conversely, *in vivo* serum copper levels become elevated rather than reduced during infection and inflammation (Arredondo & Núñez, 2005). Therefore, pathogenic microorganisms may encounter significantly varying iron/copper concentrations in the host during infection and must respond accordingly.

1.10. Bacterial adaptation to iron; acquisition

Microorganisms have developed several systems for the acquisition of iron in the metal restrictive environment of the host. This includes high affinity iron chelators known as siderophores and proteins for the liberation or direct acquisition of serum transferrin and

haemoglobin bound iron. Bacteria usually employ several of these systems for the greatest efficiency and adaptability of iron uptake.

Siderophores

Siderophores are small molecular weight iron chelators that are produced primarily by microorganisms. They have a high affinity for iron and scavenge ferric iron from low affinity chelators and host iron-proteins. Over 500 have been identified which can be classified according to the iron-binding ligand they contain; hydroxamates, catecholates, α-hydoxycarboxylates or a mixture of these ligands (Andrews *et al.*, 2003; Boukhalfa & Crumbliss, 2002; Winkelmann, 2007). Figure 1.6 shows two of the best studied siderophores; enterobactin, a catecholate and ferrichrome, a hydroxamate.

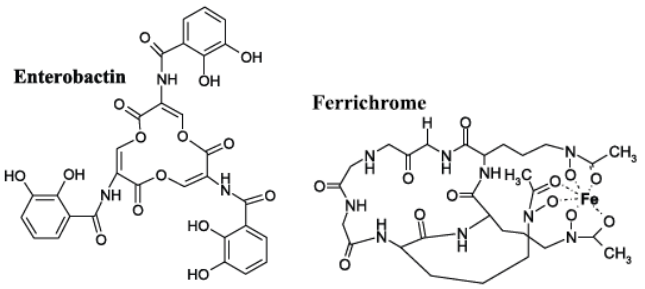


Figure 1.6. The siderophores enterobactin and ferrichrome. Ferrichrome is shown bound to iron using its iron binding hydroxamate ligand. Figure adapted from Figure 1.2 of Andrews *et al.* (2003).

Siderophore uptake

Siderophores are first excreted from the bacterial cell and once they acquire iron, the Fe-siderophore complexes are bound by surface receptors. It is common for a bacterial species to express many types of these receptors for both their own siderophores and

those produced by other bacterial species, which increases the effectiveness of iron uptake. In Gram negative bacteria, the siderophore receptors are outer membrane spanning 22-stranded β-barrels that include a "plug" to prevent unwanted periplasm exposure when not transporting siderophore complexes (Ferguson *et al*., 1998; Buchanan *et al*., 1999). *E. coli* has six such receptors and these siderophore uptake systems are shown in Figure 1.7. These are specific for single or groups of siderophores such as FecA, which is specific for ferric dicitrate and allows *E. coli* to use this as an iron source (Andrews *et al*., 2003).

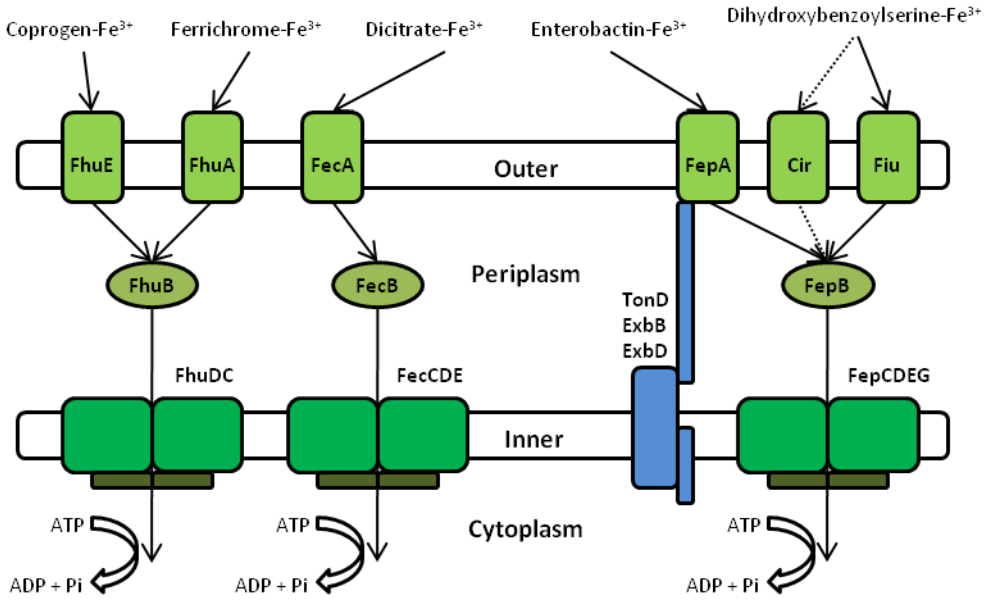


Figure 1.7. The siderophore uptake proteins of *E. coli*. This Gram negative bacterium expresses six outer membrane siderophore receptors for siderophore complexes; FhuA/E, FecA, FepA, Cir and Fiu. A ligand siderophore complex is shown for each receptor. These are taken into the periplasm by energy from the TonB/ExbB/ExbD system, only shown for FepA, which then bind periplasmic proteins. These have specific inner membrane transporters and the ATP driven hydrolysis of the cytoplasmic component translocates the siderophore into the cytoplasm. Figure adapted from Fig. 4 of Andrews *et al*. (2003).

The transport of Fe-siderophore complexes through these receptors requires energy, derived from the TonB-ExbB-ExbD system (Figure 1.7). TonB is a periplasmic spanning protein which links the outer membrane receptors to the cytoplasmic membrane to allow the cytoplasmic protein motive force to be transferred to the receptor for active Fe-siderophore uptake (Bradbeer, 1993; Braun *et al*., 1996; Wooldridge *et al*., 1992). ExbB and ExbD are cytoplasmic membrane proteins which interact with TonB to cycle TonB after energy transfer (Braun *et al*., 1996) and are required for TonB activity (Ahmer *et al*., 1995). The Fe-siderophore complexes then bind to free periplasmic proteins which deliver them to cytoplasmic membrane permeases for internalisation. Unlike the outer membrane receptors, these periplasmic binding proteins and cytoplasmic iron permeases are not specific for one or two siderophores but for particular siderophore groups. For example *E. coli* FhuB and FhuDC transport hydroxamates such as ferrichrome, FecB and FecCDE transport ferric dicitrate while FepB and FepCDEG transports the catecholate enterobactin (Figure 1.7) (Rohrbach *et al*., 1995). ATP driven hydrolysis of the cytoplasmic permeases translocates the siderophore into the cytoplasm (Schultz-Hauser *et al*., 1992). The cytoplasmic siderophore-Fe^{3+} complexes are then reduced by bacterial reductases and esterases to release the iron for utilisation. Gram positive bacteria do not require the outer membrane receptor, TonB system or free periplasmic binding proteins as they lack the large periplasmic space found in Gram negative bacteria. Instead, the exogenous siderophore complexes are taken up by cytoplasmic-membrane bound lipoprotein receptors, homologous to the free periplasmic binding proteins of the Gram negative systems, and moved into the cytoplasm by similar ATP permease proteins.

S. aureus siderophore uptake

S. aureus has been shown to produce four siderophores; staphyloferrin A, staphyloferrin B, staphylobactin and aureochelin, but can also utilise other bacterial siderophores, such as ferrichrome and enterobactin for its own iron requirements (Meiwes *et al*., 1990; Courcol *et al*., 1997; Dale *et al*., 2004; Cabrera *et al*., 2001). *S. aureus* has numerous putative uptake systems that are up-regulated under iron restriction (Allard *et al*., 2006), of which three have been described and are summarised in Figure 1.8. These are the Fhu complex for hydroxamate siderophores, the Sir complex for staphylobactin and the Sst complex, for which the siderophore target has not yet been identified (Figure 1.8) (Speziali *et al*., 2006; Sebulsky & Heinrichs. 2001; Morrissey *et al*., 2000; Heinrichs *et al*., 1999; Dale *et al*., 2004). The Sir system lacks an ATPase component and FhuC fulfils this role (Speziali *et al*., 2006).

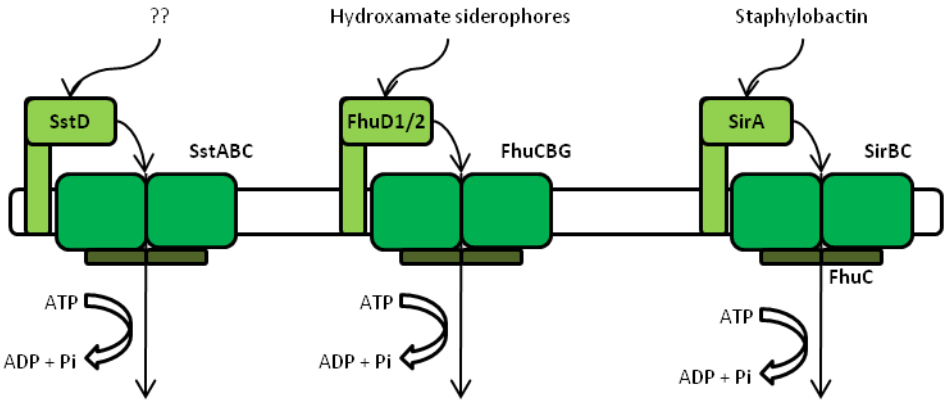


Figure 1.8. The defined *S. aureus* siderophore uptake systems. Shown are the three known siderophore uptake complexes of *S. aureus*; SstABCD, FhuCBGD1/2 and SirABC. The ATPase of the Sir system is provided by FhuC.

Ferrous iron uptake

Exogenous ferrous (Fe^{2+}) iron can also be directly obtained by bacteria without the necessity for secreted high affinity chelators. However as exogenous iron is often ferric, this requires either bacterial driven external iron reduction or anaerobic conditions, like those found in the mammalianintestine. Here, ferrous iron will be the dominant state. FeoAB of *E. coli* is a ferrous uptake system, where FeoB is a cytoplasmic membrane GTPase protein and FeoA is found in the cytoplasm (Kammler *et al*., 1993; Andrews *et al.*, 2003). *S. aureus* has a FeoB homologue which is up-regulated under iron restriction, however its exact role in iron acquisition is unknown (Allard *et al*., 2006). There also exist several Gram negative and positive ABC transporters which are specific for non-siderophore iron. The *S. epidermidis sit* operon encodes an ATP binding protein (SitA), a membrane protein (SitB) and a lipoprotein (SitC) which are iron induced and thought to be involved in the acquisition of metal ions (Hill *et al*., 1998). *S. aureus* possesses a homologue of this system, MntABC, which was found to be involved in manganese uptake and *mntABC* was repressed by manganese but not iron in *S. aureus* strain 8325-4 (Horsburgh[2] *et al*., 2002). However, later studies found that transcription of *mntABC* was repressed by iron and therefore the exact role of MntABC in iron homeostasis is still undefined (Ando *et al*., 2003).

Utilisation of the host iron proteins transferrin and lactoferrin

In addition to sequestering iron from host proteins by excreting siderophores, pathogens have developed systems to acquire host protein iron from the transferrins and haemoglobin during infection. Gram negative pathogens, including *E. coli* and *Neisseria* species, express separate systems for transferrin and lactoferrin utilisation; the

dual outer membrane receptors TbpAB and LbpAB respectively (Irwin *et al*., 1993; Pettersson *et al*., 1998; Bonnah & Schryvers, 1998). TbpA and LbpA are homologous in structure to the siderophore outer membrane receptors such as FhuA and also require energy from TonB (Boulton *et al*., 2000; Oakhill *et al*., 2005). Both TbpA and TpbB are involved in a four step process of transferrin binding, removal of iron, internalisation of iron and release of iron-free transferrin from the receptor (DeRocco *et al*., 2009). TbpB has been shown to have a high affinity for Fe^{3+}-transferrin (Renauld-Mongénie *et al*., 1998) and binds transferrin to bring it into contact with TbpA and is also required for the disassociation of iron free transferrin from TbpA (DeRocco *et al*., 2009). In *Neisseria* species, this periplasmic iron is then bound to the periplasmic ferric iron-binding protein A (FbpA) and transported to the cytoplasm via the cytoplasmic membrane iron permeases, FbpB, although this stage of transferrin iron uptake is still poorly defined (Ferreirós *et al*., 1999; Chen *et al*., 1993; Roulhac *et al*., 2008). An alternative mechanism for utilising transferrin bound iron involves the extracellular degradation of the transferrin protein by bacterial proteases, such as the extracellular alkaline protease produced by *P. aeruginosa*. This releases iron bound to external transferrin that is then taken up by *P. aeruginosa* siderophores and utilised for growth (Shigematsu *et al*., 2001; Kim *et al*., 2006).

S. aureus utilisation of transferrin

Although having been shown to bind transferrin, the receptor employed by the human *S. aureus* strains for transferrin utilisation is currently unknown, although it was originally thought to be facilitated by cell surface GADPH and later by the cell wall anchored protein StbA (also termed FrpA) (Modun *et al*., 1994; Modun *et al*., 1998;

Modun & Williams, 1999; Modun *et al*., 2000). However, StbA was found not to be the *S. aureus* transferrin-binding protein as it bound a peroxide conjugate non-specifically (Morrissey *et al*., 2002) which was used in the transferrin binding assay performed in the earlier study (Taylor & Heinrichs, 2002). Subsequently, StbA was found to be the haem binding protein IsdA (Morrissey *et al*., 2002; Mazmanian *et al*., 2002). The GADPH proteins GapB and GapC from bovine *S. aureus* strains have been shown to bind transferrin but this property has yet to be confirmed for their homologues in the human isolates (Goji *et al*., 2004). It has been reported that iron starved *S. aureus* produced more lactate, resulting in a medium pH change from 7.2 to 5.2 (Friedman *et al*., 2006). Transferrin bound iron is released under acidic conditions of between 5.6 and 6.0 (Sipe & Murphy, 1991) and therefore it has been proposed that *S. aureus* may acquire *in vivo* transferrin bound iron through inducing iron release by lowing extracellular pH under iron restriction. However, this hypothesis has yet to be confirmed as a method used by *S. aureus* for *in vivo* transferrin iron acquisition.

Utilisation of host haem

Another potential source of iron for pathogens during infection is haem, which represents the most common form of iron in the host. Bacteria can bind host haem containing proteins directly. For example, the *E. coli* outer membrane iron regulated ChuA protein has been shown to bind haemoglobin as well as haem itself (Torres & Payne, 1997). *E. coli* also expresses the Ton-B dependant haem uptake protein Hma and strains lacking both *hma* and *chuA* are severely compromised in their ability to utilise host haem as an *in vivo* iron source (Hagan & Mobley, 2009). The haem uptake systems are often similar in arrangement to the previously described transferrin uptake

complexes. For example, the *Vibrio cholera* HutABCD system comprises of a haem receptor (HutA), a periplasmic protein (HutB) and a cytoplasmic permeases (HutCD), which uses TonB derived energy for haem utilisation (Occhino *et al.,* 1998). Many pathogens can also excrete proteases which either release host haemoglobin from mature erythrocytes (haemolysins) for subsequent uptake or release haem from serum haem proteins. For example, Kgp is an excreted cysteine protease of *Porphyromonas gingivalis* which is required for the utilisation of haem and haemoglobin by this strain as an iron source for growth (Simpsonv *et al*., 2004).

S. aureus utilisation of haem

S. aureus can utilise haem-proteins, haemoglobin and myoglobin but not haemopexin as iron sources (Torres *et al*., 2006). The Isd system is thought to be required for haem uptake and consists of eight proteins expressed under iron restriction (Allard *et al*., 2006). Figure 1.9 shows the *S. aureus* utilisation of haem by the Isd proteins. IsdB and IsdH are cell wall anchored proteins which have been shown to bind haemoglobin and IsdB is required for virulence (Torres *et al*., 2006; Vermeiren *et al*., 2006; Torres *et al*., 2006). Haem is then passed from IsdB to IsdA and IsdC which then transports haem to IsdE (Zhu *et al*., 2008). IsdFD is a membrane transporter which moves haem into the cytoplasm (Skaar *et al*., 2004) where it is degraded to release iron by haem degradation enzymes such as IsdG and IsdI (Skaar *et al*., 2004). However, some recent studies dispute this model and have found that haem from IsdB and IsdH can move directly to IsdE and therefore this system is still not fully defined in *S. aureus* (Muryoi *et al*., 2008). Some of these proteins are also multifunctional; for example IsdA, in addition to

haem utilisation, has been shown to bind the host factors fibrinogen and fibronectin and can aid in immune evasion, (Clarke *et al*., 2004; Clarke *et al*., 2007).

HrtAB is an additional haem *S. aureus* system but is most likely for efflux to resist high concentrations of haem rather than uptake as *hrtAB* negative strains have increased sensitivity to haem toxicity (Stauff *et al*., 2008). As previously described, *S. aureus* possesses several haemolysin proteases for the extracellular release of haem from host proteins. Therefore, *S. aureus* employs several methods for the acquisition of host iron during infection. However, many aspects of these systems still remain poorly defined.

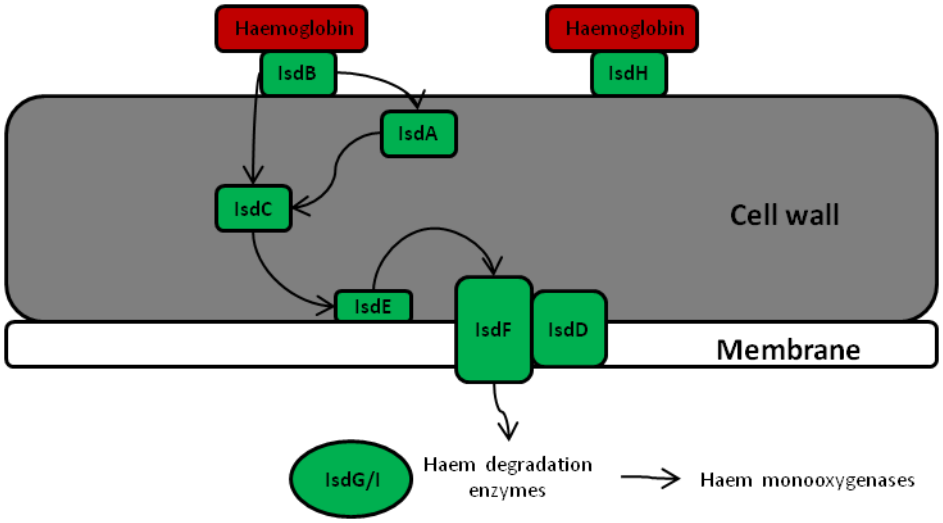


Figure 1.9. Model of *S. aureus* haem utilisation by the Isd proteins. Haemoglobin binds to IsdB and IsdH at the cell wall and haem is released and passed to IsdC directly or via IsdA. From here it moves to IsdE and enters the cytoplasm via the membrane IsdFD complex and the iron is released by haem degradation enzymes such as the cytoplasmic IsdG and IsdI proteins. Figure adapted from Figure 5 of Maresso & Schneewind (2006).

1.11. Bacterial adaptation to iron; storage

Iron acquired by pathogens needs to be stored to both reduce iron toxicity and to provide iron during periods of iron limitation. There are three bacterial iron storage proteins; ferritin, bacterioferritin and Dps proteins (ferritin like proteins) which all form spherical macromolecules of 24 (ferritin and bacterioferritin) or 12 (Dps) similar subunits (Andrews *et al.,* 2003). Ferritins are found in both prokaryotes and eukaryotes and *E. coli* ferritin A (FtnA) has been shown to be required for intracellular iron storage during stationary phase and subsequent growth on an iron limiting medium (Abdul-Tehrani *et al*., 1999). However, FtnA may have additional biological roles besides iron storage and it has been suggested that it is involved in restoring iron-sulphur centres which have been disrupted by hydrogen peroxide, possibly by releasing stored iron (Bitoun *et al*., 2008). However, the potential role of FtnA in oxidative stress resistance still needs to be established. FtnB is a putative additional ferritin of *E. coli* which has been better described in *Salmonella* species where it has been shown to be important for the repair of oxidative stress induced damage of Fe-sulphur clusters in addition to functioning as a secondary iron storage protein (Velayudhan *et al*., 2007).

Bacterioferritins (Bfr) are haem containing proteins, homologous to ferritins, which can store iron as phosphates or hydrites and are the most common of the iron storage proteins found in bacteria (Andrews *et al.,* 2003; Aitken-Rogers *et al*., 2004). The haem in Bfr and the ferridoxin Bfd, which reduces iron in the Bfr core, are both required for the utilisation of stored iron under iron restriction (Andrews *et al*., 1995; Weeratunga *et al*., 2009). However, there are few phenotypes described for bacterioferritin mutants but inactivation of either of the two bacterioferritins of *Synechocystis* sp. PCC 6803 results

in reduced growth under iron limiting conditions (Keren *et al*., 2004). The smaller *E. coli* Dps protein was found to bind DNA and protect from hydrogen peroxide oxidative stress (Almirón *et al*., 1992). This is likely its primary function as compared to the other iron storage proteins it is mainly expressed under conditions of oxidative stress in both the log and stationary growth phases (Zhao *et al*., 2002; Altuvia *et al*., 1994). However, recently *E. coli* Dps has also been implicated in copper tolerance as an *E. coli dps* mutant was more sensitive to low concentrations of copper (Thieme & Grass, 2009).

S. aureus iron storage

S. aureus has a ferritin (FtnA) and a Dps like protein, MrgA, but no identified bacterioferritin. FtnA contains the amino acids important for iron storage and ferroxidase activity, suggesting it functions as an iron storage protein (Morrissey *et al*., 2004). It is repressed in low metal ion media and induced by iron at the transcriptional level (Morrissey *et al*., 2004). A role for *S. aureus* FtnA in oxidative stress resistance is still undefined. MrgA is also repressed in low metal medium and iron induced (Morrissey *et al*., 2004). It has been shown that MrgA is involved in *S. aureus* nucleoid compaction and is induced by oxidative stress to reduce subsequent oxidative damage of DNA (Morikawa *et al*., 2006). However, there is no experimental evidence for the role of FtnA/MrgA in *S. aureus* iron storage.

1.12. Bacterial adaptation to iron; iron regulation

The ferric uptake regulator

For effective utilisation of host iron, the previously described systems must be expressed in response to the external iron availability. Also, due to iron toxicity, these systems must be repressed when external iron concentrations are high. The majority of iron regulated genes in bacteria are either directly or indirectly under the control of the ferric uptake regulator, Fur. The Fur protein homodimer consists of two homologous 17 kDa subunits with an N-terminal helix-turn-helix DNA binding domain and C-terminal dimerisation domain (Bai *et al*., 2006; Pecqueur *et al*., 2006; Stojiljkovic & Hantke, 1995). The binding of Fe^{2+} alters the quaternary structure of Fur and allows binding of the N-terminal domain to specific DNA sequences termed Fur boxes. These are located in target gene promoters and therefore the binding of iron-Fur to these sites prevents subsequent transcription by RNA polymerase (Andrews *et al.,* 2003). This is known as "classical" Fur regulation and allows for the repression of target genes, such as the previously described iron acquisition systems, in response to high iron conditions, summarised in Figure 1.10.

However, this is a simplified model and the Fur box sequence varies considerably between different Fur target promoters (Newman & Shapiro, 1999). In fact, Fur has been observed by DNase I footprinting experiments to bind multiple promoter sites of some Fur regulated genes and can even extend along the DNA sequence as numerous connected Fur units (Escolar *et al*., 2000; De Lorenzo *et al*., 1987). Nevertheless, Fur box consensus sequences have led to the subsequent discovery of numerous Fur regulated genes. The *E. coli* Fur is well described and classically regulates genes

involved in iron uptake including *fhuA, fepA, cir* and *feo* to ensure that the iron acquisition proteins are only expressed during times of iron limitation (Hantke, 1981; Kammler *et al*., 1993).

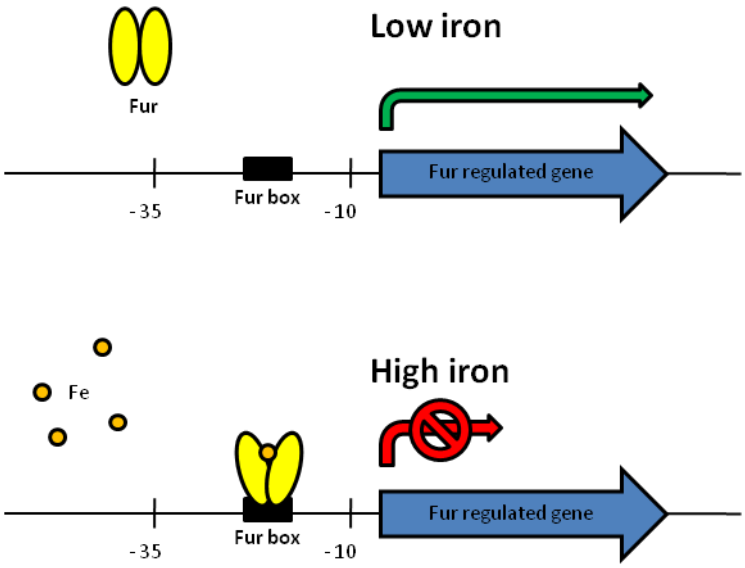


Figure 1.10. Iron regulation of genes through Fur. In low iron, the target gene is transcribed. However in high iron, iron-Fur binds promoter Fur box and prevents transcription.

E. coli Fur is also a global regulator as, in addition to the iron uptake systems, Fur controls the transcription of genes involved in glycolysis, DNA synthesis, respiration, virulence and redox stress resistance (Andrews *et al.,* 2003). Fur homologues have been identified in numerous other bacterial species that regulate genes in a similar fashion to the *E. coli* Fur. They are also global regulators, such as the Fur of *Neisseria meningitidis* which was found by transcriptional analysis to up-regulate a total of 153 and down-regulate 80 genes involved in iron acquisition and other cellular processes (Grifantini *et al*., 2003).

E. coli also uses external iron to sense that it has entered a host and can switch phenotypes to express virulence genes via Fur. For example, enterotoxigenic *E. coli* (ETEC) express fimbriae for intestinal colonisation and this phenotype is iron repressed by Fur and therefore only expressed when required in the iron restrictive host environment (Karjalainen *et al*., 1991). This link to virulence has been found in numerous other bacterial species. Examples include the requirement of Fur for expression of a type III secretion system of *Salmonella enterica* serovar Typhimurium, required for the invasion of intestinal epithelial cells (Ellermeier & Slauch, 2008) and the disruption of Fur in *Listeria monocytogenes* was found to reduce virulence in subsequent animal models (Rea *et al*., 2004).

Non-classical Fur regulation by small RNAs

In *E. coli*, it has been shown that Fur can also positively regulate gene expression. This is through post transcriptional regulation by the small RNA RhyB, which binds and leads to degradation of target mRNA, and is itself repressed by Fur in high iron (Massé & Gottesman, 2002). This leads to non-classical Fur regulation in *E. coli* by activating gene transcription in high iron which is summarised diagrammatically in Figure 1.11 and has been found for genes including *acnA* (aconitate hydratase), *sodB* (superoxide dismutase), *fumA* (fumarate hydratase) and *bfr* (bacterioferritin) (Massé & Gottesman, 2002).

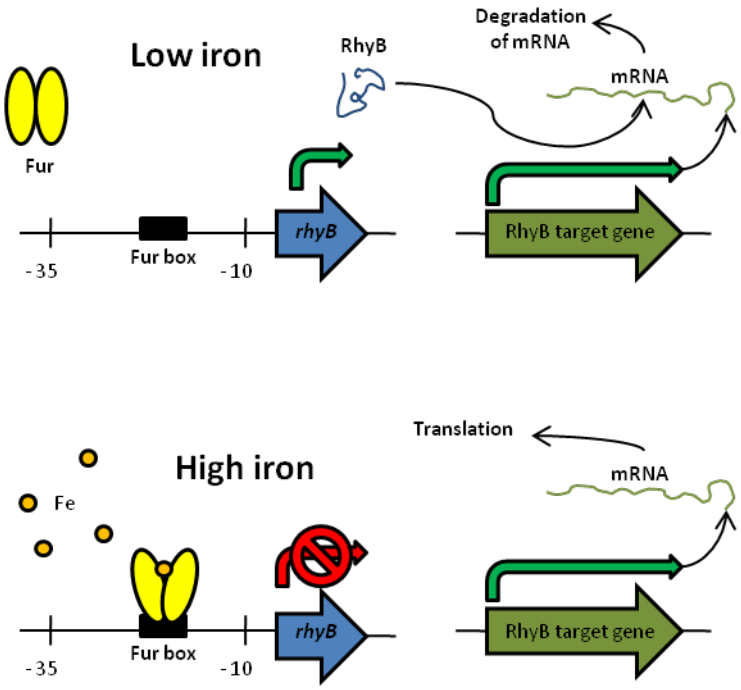


Figure 1.11. Positive activation of genes by Fur through the small RNA, RhyB. In low iron, RhyB is transcribed and this small RNA binds target gene mRNA leading to degradation. However in high iron, iron-Fur binds the *rhyB* promoter Fur box and prevents transcription of *rhyB* and therefore the RhyB target gene's mRNA can be translated. Figure created from information in Massé & Gottesman (2002).

A microarray study identified 56 genes potentially under control of RhyB that are involved in the TCA cycle, oxidative stress and both aerobic and anaerobic respiration but importantly are mostly genes which encode iron containing enzymes (Massé *et al.*, 2005). Additional studies have suggested that RhyB reduces non-essential iron enzymes under ion restriction to preserve iron for essential processes (Jacques *et al.*, 2006; Bollinger & Kallio, 2007). RhyB therefore represents a global regulator which, along with Fur, positively actives genes in high iron but is also essential in low iron for efficient utilisation of this important metal. The RNA binding protein Hfq is also required for some RhyB controlled regulation. For example, Hfq binds *sodB* RNA to induce a binding site for RhyB and prevent subsequent *sodB* translation by degrading *sodB* mRNA (Geissmann & Touati, 2004). Small RNAs are ubiquitously employed as

mechanisms for novel Fur regulation. Examples include NrrF of *N. meningitidis*, an Hfq-dependent sRNA which up-regulates the succinate dehydrogenase operon *sdhCDAB* in a Fur-dependent manner (Metruccio *et al*., 2009) and FsrA of *Bacillus subtilis*, which Fur acts through to limit non-essential iron enzymes during iron starvation (Gaballa *et al*., 2008).

Non-classical Fur regulation by alternative mechanisms

In addition to small RNAs, some important alternative mechanisms of Fur regulation have also been observed in several bacterial species. The *Helicobacter pylori* Fur repress the non-haem containing ferritin gene, *pfr*, in low iron by binding the promoter directly and at multiple sites (Delany *et al*., 2001). In *N. meningitidis* Fur and iron induce transcription of genes that code for metalloproteins/complexes with iron-active sites, which are involved in both anaerobic and aerobic respiration, by direct Fur binding to target gene promoters (Delany *et al.*, 2004). Also, although Fur functions to classically regulate iron uptake proteins in *B. subtilis*, Fur binds the *dhb* (dihydroxybenzoate siderophore biosynthesis) promoter independently of iron (Baichoo *et al*., 2002; Bsat & Helmann, 1999). This has also been observed for *L. monocytogenes*, where Fur binds the Fur box of *fhuDC* independently of Fe^{2+} and there is also no Fe^{2+} present in the active Fur protein (Ledala *et al*., 2007). Here, regulation is achieved by iron regulation of *fur* transcription (Ledala *et al*., 2007). Therefore while Fur homologues can share many similar characteristics, there can also be significant differences in how Fur functions as an iron responsive bacterial regulator.

The *S. aureus* Fur homologues

There are three Fur homologues in *S. aureus*, the ion limitation/oxidative stress dependant DNA repressor protein PerR, the zinc repressor Zur and the iron dependant repressor Fur (Horsburgh[2] *et al*., 2001; Lindsay & Foster, 2001; Xiong *et al.*, 2000). Zur represses the *S. aureus* zinc uptake operon *mreAB* in response to high Zn^{2+} but has no reported role in virulence (Lindsay & Foster, 2001). PerR is a metal ion limitation/oxidative stress dependant DNA repressor protein, which coordinates the oxidative stress response by regulating the catalase (*katA*) and alkyl hydroperoxide reductase (*ahpCF*) expression amongst others (Horsburgh[1] *et al*., 2001). This is through binding to specific DNA sequences termed PerR boxes in target gene promoters and *perR* is required for *S. aureus* virulence in a mouse model (Horsburgh[1] *et al*., 2001).

The *S. aureus* Fur is homologous in structure to the *E. coli* Fur protein and binds Fur boxes to repress target genes in high iron, including the iron uptake systems such as the *fhu* and *isd* operons (Xiong *et al.*, 2000; Friedman *et al*., 2006). However, currently there is no evidence that the active *S. aureus* Fur protein contains iron. Also like the *E. coli* Fur, the *S. aureus* Fur is a global regulator which regulates a diverse range of phenotypes in addition to iron acquisition, including; amino acid synthesis, oxidative stress resistance, virulence, metabolism and structural component synthesis (Friedman *et al*., 2006).

The *S. aureus* Fur has been shown to regulate genes non-classically. Catalase (*katA*) is an oxidative stress resistance enzyme which catalyses the conversion of hydrogen peroxide to water. Using a reporter assay, it has been found that *katA* is iron induced

with reduced expression in a *fur* mutant background (Horsburgh[2] *et al*., 2001). In addition, the previously described biofilm virulence factor has been found to be non-classically Fur regulated (Johnson *et al*., 2005). This is shown below in Figure 1.12.

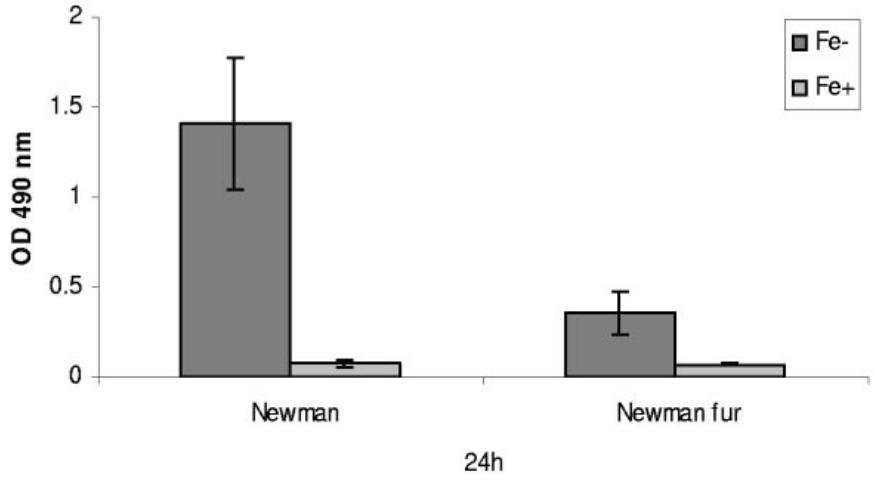


Figure 1.12. 24 hour *in vitro S. aureus* biofilm formation. In *S. aureus* Newman wild type, 24 hour biofilm is induced in low iron. Expression is significantly reduced in the Newman *fur* mutant however some Fur independent iron regulation is still seen. Figure taken from Johnson *et al*. (2005).

In the wild type Newman *S. aureus* strain, 24 hour biofilm formation is induced in low iron and expression is significantly reduced in a Newman *fur* mutant (Figure 1.12). In addition, there is still some Fur independent low iron biofilm induction in the Newman *fur* mutant (Figure 1.12). The protein factors involved in biofilm formation, Eap and Emp, have also been shown to be induced in low iron by Fur and have Fur independent iron regulation in a *fur* mutant (Johnson *et al*., 2008). Therefore, there appears to be another iron responsive regulator which is regulating independently of the *S. aureus* Fur. Unlike the non-classical Fur regulation observed in other bacterial species, the

mechanism(s) of this *S. aureus* novel Fur regulation is currently unknown but could be through small RNAs, novel Fur binding or some as yet undefined mechanism which may involve other iron responsive protein regulators.

Non-Fur iron regulators

Although most bacterial iron regulation is through the previously described Fur homologues, there are several other iron regulators that have been found. DxtR is the iron regulator of diphtheria toxin in *Corynebacterium diphtheriae* which also negatively regulates some iron homeostasis genes. Whilst showing no sequence homology to Fur, DtxR and its homologues consist of a dimerisation domain and an N-terminal DNA binding domain that also binds a specific sequence in target gene promoters in response to Fe^{2+} (Lee *et al*., 1997; Pohl *et al*., 1999). The *B. subtilis* DxtR homologue regulates the ferrichrome receptor FhuD homologue, IRP1 (Schmitt *et al*., 1997). Other DxtR homologues include the staphylococcal SirR, which regulates the iron response of the previously described *S. epidermidis sitABC* operon through binding a promoter located Sir box (Hill *et al*., 1998). MntR of *S. aureus* is a SirR homologue which represses the previously described *mntABC* operon. In *S. aureus* 8325-4, MntR was found to be a manganese responsive repressor rather than a novel iron repressor (Horsburgh[2] *et al*., 2002). Although a later study observed that the MntR protein could bind the *mntABC* promoter in a DNA gel shift binding assay, actual iron responsive iron regulation of this operon was only seen in a *S. aureus* strain which expressed the *C. diphtheriae* DtxR protein, not its native MntR (Ando *et al*., 2003). Therefore, the *S. aureus* MntR is unlikely to function as a novel iron regulator although this possibility is currently being investigated.

In addition to the DtxR homologues, other systems which involve non-Fur regulators have been described. These involve sigma factors, which are proteins that initiate transcription by enabling the binding of RNA polymerase to target gene promoters. For example, the *E. coli fec* operon is repressed by Fe^{2+} and Fur but activated by external ferric citrate, which is the substrate of outer membrane FecA, via the novel cytoplasmic membrane protein FecR and the sigma factor FecI (Angerer *et al*., 1995; Braun *et al*., 2003). This allows for *fec* expression only during iron limitation when the appropriate FecA substrate is available. Another example of a sigma factor that has been found to control iron responsive gene regulation is PvdS of *P. aeruginosa*. In low iron, PvdS functions to direct the transcription of numerous genes including siderophores, proteases and toxins (Tiburzi *et al*., 2008).

Therefore, bacterial environmental iron regulation can involve direct binding of the regulator Fur but the Fur mode of action can vary considerably between strains. Also, novel iron regulation can still involve Fur through a small RNA intermediary. Alternatively, iron responsive genes can be under the control of other iron regulators such as the DxtR homologues or sigma factors, which add a greater level of intricacy to iron regulation. These alternative systems may potentially be the regulators of the observed novel *S. aureus* iron regulation of *katA* and biofilm previously described. In addition, *S. aureus* may possess a novel Fur independent iron regulator which has yet to be identified (Figure 1.12).

1.13. Microorganism adaptation to copper

In this section, the mechanisms employed by bacteria for the acquisition and resistance of environmental copper will be discussed. There are numerous bacterial systems for the tolerance of copper and each of these will be detailed. Finally, our current understanding of the *S. aureus* response to copper challenge will be analysed to provide a basis for the subsequent work detailed in this thesis

Copper acquisition

Like iron**,** copper can also become a growth limiting factor when environmental concentrations are low and therefore uptake proteins are employed by some microorganisms to scavenge extracellular copper. The best studied copper influx mechanisms of a pathogen are those of the model eukaryotic organism *S. cerevisiae*. *S. cerevisiae* possesses numerous proteins for copper acquisition, summarised in Figure 1.13. The Fre proteins of *S. cerevisiae* are cell surface ferric and copper reductases which reduce extracellular iron and copper for transport by the membrane spanning Ctr1 and Ctr3 copper permeases. A *S. cerevisiae ctr1/3* double mutant has significantly reduced copper uptake when compared to the wild type or either single mutant, showing that both these proteins are needed for full *S. cerevisiae* copper influx (Knight *et al*., 1996).

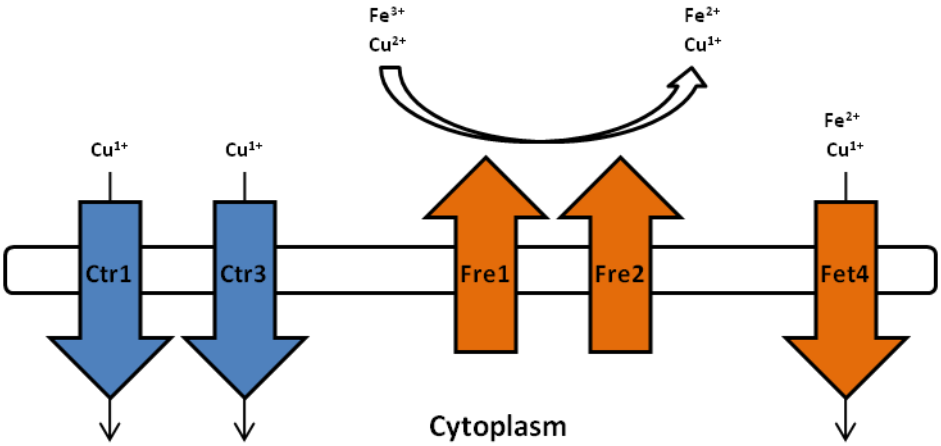


Figure 1.13. The copper influx systems of *S. cerevisiae*. Fre1 and Fre2 are membrane copper reductases that have been shown to be required for subsequent copper import and function to reduce extracellular membrane localised Cu^{2+} to Cu^{1+} for uptake. Ctr1 is a multi-spanning plasma membrane protein and Ctr3 is a copper uptake permease which, while functionally redundant to Ctr1, shows little amino acid homology. The iron permease Fet4 can also function as a low affinity copper permease. Figure created using information from Georgatsou *et al*. (1997), Dancis *et al*. (1994), Pena *et al*. (2000) and Hassett *et al*. (2000).

Fet4 is an iron permease which also functions as a low affinity copper permease, and a *fet4* mutant strain has reduced copper uptake (Hassett *et al*., 2000). Copper proteins are also required for *S. cerevisiae* iron uptake, such as the copper containing multi-copper oxadase (MCO) Fet3, a ferridoxase which oxidises Fe^{2+} to Fe^{3+} for influx into the cell via the iron permease Ftr1 (Wang *et al*., 2003). Fet5 of *S. cerevisiae* is a closely related copper containing MCO with a similar role in iron homeostasis but is localised to intracellular vesicles (Spizzo *et al*., 1997; Li *et al*., 2003). What is clear from *S. cerevisiae* Fet3 and Fet5 is the link between iron and copper metabolism in this organism. Copper is also required by some bacteria as a protein cofactor. Bacterial copper proteins include the Cu/Zn SODs of *Brucella abortus, Mycobacterium tuberculosis* and some *Salmonella* species, that convert superoxide to hydrogen peroxide and which are required for oxidative stress resistance (Piddington *et al*., 2001;

Gee *et al*., 2005; Sansone *et al*., 2002). Also, MCO proteins such as the *E. coli* CueO and proteins involved in respiration, such as *E. coli* cytochrome *Bo*, require copper for their function (Sakurai & Kataoka, 2007; Lindqvist *et al*., 2000).

Although copper can be required as a cofactor, not much is known about bacterial copper uptake systems. In the Gram negative bacterium *Pseudomonas fluorescens* SBW25, the periplasmic copper-binding protein, CopC, and the inner membrane protein, CopD, have been implicated in copper influx as their inactivation improves copper resistance whilst over-expression leads to sensitivity (Zhang & Rainey, 2008). The YcnJ protein of *B. subtilis*, which is homologous to the *Pseudomonas* CopC/D proteins, was recently shown to be required for copper uptake as an *ycnJ* mutant had a growth defective phenotype in copper limiting conditions (Chillappagari *et al*., 2009). However, the most common copper influx proteins are members of the P-type ATPase family. These integral membrane proteins utilise the energy from ATP hydrolysis to transport substrates, frequently against a concentration gradient. P-type ATPases transport metal ions by cycling through two structural conformations, named “P-type” after a conserved aspartic acid residue which forms a phosphorylated intermediate during the reaction (Palmgren & Axelsen, 1998). Based on sequence homology, there are five types of P-type ATPases and the copper ATPases are in a subset termed Type-1 P-type ATPases (Kühlbrandt, 2004). The structure of these proteins is shown in Figure 1.14 however, copper transfer/binding to these ATPases is poorly understood, particularly *in vivo*, but may involve direct transport of copper ions.

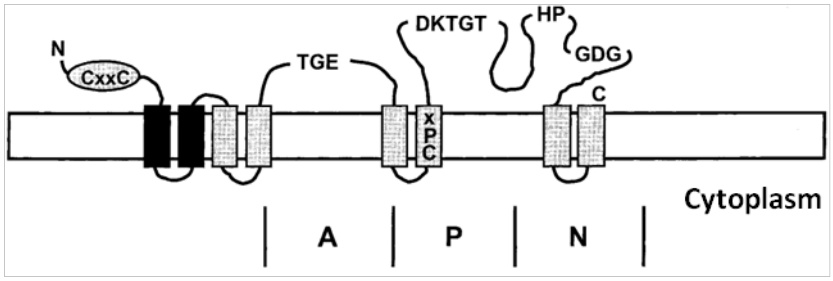


Figure 1.14. Type-1 P-type ATPases. These proteins consist of a single membrane-integrated protein with several conserved domains; a phosphorylation domain (P), a nucleotide-binding domain (N), an actuator domain (A) and a membrane domain. Copper ion binding occurs at the N-terminus, which features 1-6 metal binding sites with either CxxC motifs or consisting of histidine rich regions. The copper ATPases are distinctive and usually possess a total of eight transmembrane domains; four near the N-terminus, followed by two with a conserved intramembranous CPx motif and a final two at the C-terminus. The copper transporting ATPases are often named after the distinctive CPx intermembrane motif as "CPx-Type-1 ATPases". Figure adapted from Solioz & Stoyanov (2003).

Examples of Type-1 P-type ATPases which import copper have been found in *P. aeruginosa* (HmtA), *L. monocytogenes* (CtpA) and *Enterococcus hirae* (CopA) (Francis & Thomas, 1997; Lewinson *et al*., 2009; Odermatt *et al*., 1993). Whilst being the model organism for studies into copper toxicity and resistance, no copper import protein has yet been identified for *E. coli*. However, evidence suggests that copper influx may occur primarily through outer membrane pores and *E. coli* lacking certain outer membrane pore proteins have been found to be more copper tolerant (Lutkenhaus, 1977).

1.14. Resistance to copper toxicity

In addition to adaptations to acquire copper, microorganisms employ numerous methods for the resistance of copper toxicity. The variety and frequency of these systems suggests that environmental copper is a common and historically important challenge for prokaryotic organisms. The following sections will describe the various copper tolerance systems that have been identified in different bacterial species such as *E. coli*, *H. pylori*, *E. hirae* and *Salmonella* species. This systems include; chelators/chaperones, oxidation/reduction and active transport.

Active transport

The most commonly found copper resistance mechanism involves the active efflux of copper out of the cytoplasm. This is through members of the previously described Type-1 P-type ATPase family and examples have been identified in numerous Gram positive and negative bacterial species. These ATPases have been shown to be copper regulated and required for copper tolerance and oxidative stress resistance. Table 1.4 shows examples of identified cytoplasmic copper efflux proteins.

Organism	Type-1 P-type ATPases	Description	Reference
E. coli	CopA	Exports copper Cu^{1+} and silver ions and a *copA* mutant was copper sensitive in rich medium	Rensing *et al*. (2000)
S. typhimurium	CopA	Copper induced and is required for full copper tolerance	Espariz *et al*. (2007)
S. typhimurium	GolT	Confers resistance to gold salts. In a *copA* mutant GolT functions as an alternative copper efflux protein	Espariz *et al*. (2007)
C. jejuni	Cj1161	Cj1161 mutant showed copper sensitivity	Hall *et al*. (2008)
H. pylori	CopA	A *copA* mutant was copper sensitive	Bayle *et al*. (1998)
Pseudomonas putida	CueA	*Pseudomonas* copper resistance protein. *cueA* inactivation led to copper sensitivity and intracellular copper accumulation	Adaikkalam & Swarup (2002)
E. hirae	CopB	Copper and silver efflux pump	Solioz & Odermatt (1995)
B. subtilis	CopA	A *copA* mutant was copper sensitive	Gaballa & Helmann (2003)

Table 1.4. Examples of some Type-1 P-type ATPases which have been found to provide copper tolerance by exporting cytoplasmic copper.

However, Gram negative bacteria face the additional problem of toxic accumulation in the periplasm of cytoplasmic copper ions exported via the copper ATPases detailed above. Therefore additional periplasmic systems are required. For example, *E. coli* has several proteins for the detoxification of periplasmic copper which are show in Figure 1.15.

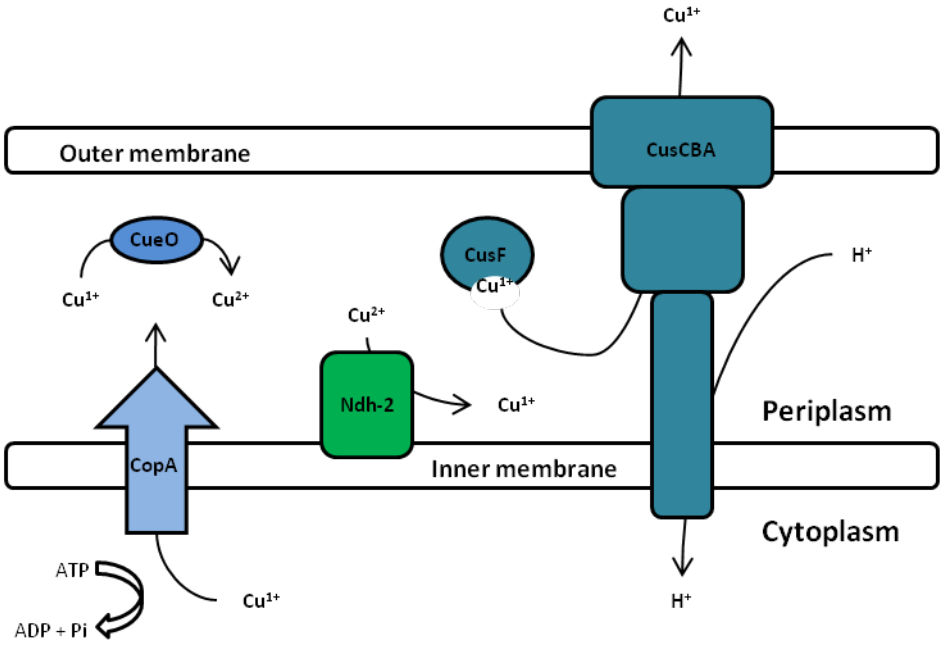


Figure 1.15. Detoxification of periplasmic copper ions in *E. coli*. Cytoplasmic Cu^{1+} is exported into the periplasm by CopA, where the MCO CueO (discussed later) can oxidise it to the less toxic Cu^{2+} state. The CusF chaperone protein binds and delivers periplasmic Cu^{1+} to the periplasmic spanning CusCBD protein which utilises proton motive force to efflux copper ions (and silver ions) into the external environment. Figure adapted from Fig. 1 of Rensing & Grass (2003).

The major periplasmic copper resistance protein is the CusCBA transporter. The *cus* operon encodes a copper chaperone CusF, which may aid copper tolerance by chelating periplasmic copper ions, and a CBA-type transport system consisting of an inner membrane pump (CusA), a periplasmic linker (CusB) and outer membrane protein (CusC), which transports copper ions into the extracellular environment (Rensing & Grass, 2003). A *copA/cusCFBA* mutant showed no difference in copper tolerance compared to a single *copA* mutant. CueO is the *E. coli* periplasmic MCO which catalyses the oxidation of the more toxic Cu^{1+} to Cu^{2+} and a *cueO/cusCFBA* mutant showed significantly more copper sensitivity then a single *cueO* mutant, therefore suggesting that CusCBA functions primarily as a periplasmic copper ion exporter

(Grass[2] & Rensing, 2001). There exist homologues of the *E. coli* Cus system, such as the *H. pylori* copper resistance complex encoded by *crdA*, *crdB*, *czcA* and *czcB* where *crdB*/*czcA*/*czcB* are orthologues of the *E. coli cusC*/*cusB*/*cusA* genes respectively (Waidner *et al*., 2002). However, currently it is unknown how the periplasmic copper is excreted from many of the Gram negative pathogens species such as *Salmonella*, which possess homologues to *E. coli* CopA and CueO but lack the periplasmic Cus system. However, this may involve the recently identified CueP, a periplasmic copper binding protein found in *Salmonella* species and of no known function but which is required for copper tolerance in anaerobic conditions (Pontel & Soncini, 2009). Homologues of this protein were identified in Gram negative species which lack a Cus system, including *Shewanella* and *Yersinia* species. Therefore, CueP may represent an alternative periplasmic copper detoxification system.

Copper oxidation by multi-copper oxidases

Bacteria can also resist copper toxicity through proteins which catalyse the oxidation of Cu^{1+} to Cu^{2+} to reduce subsequent copper induced oxidative damage through the production of the hydroxyl free radical. Multi-copper oxidases (MCOs) are a group of enzymes which usually contain several of the copper centres (types I, II and III) and as their name suggests, can act as substrate oxidisers to reduce oxygen or other molecules. MCOs are ubiquitous in both prokaryotic and eukaryotic organisms where they fulfil a wide range of roles from Fe^{2+} oxidation, such as the previously described Fet3 of *S. cerevisiae*, to the degradation of lignin, a complex molecule found in wood and plant cell walls, by laccase (Sakurai & Kataoka, 2007).

The Gram negative bacterial MCOs are also involved in copper tolerance. *E. coli* possesses two MCOs; CueO (YacK) and the plasmid encoded PcoA (Sakurai & Kataoka, 2007). CueO protects periplasmic proteins from copper damage by the oxidation of periplasmic cupric copper and has been shown experimentally to protect the periplasmic *E. coli* alkaline phosphatase protein from copper-induced damage (Grass[1] & Rensing, 2001; Singh *et al*., 2004). CueO can also oxidise Fe^{2+} and in an uropathogenic *E. coli cueO* mutant, iron uptake was increased which again shows the link between the homeostasis systems of iron and copper (Kim *et al*., 2001; Tree *et al*., 2008). The *pco* operon, encoded by the pRJ1004 plasmid, confers high copper tolerance in *E. coli* isolated from pigs that have been fed with dietary copper sulphate (Tetaz & Luke, 1983). PcoA interacts with periplasmic PcoC and similar to CueO, oxidises Cu^{1+} to the less toxic form in the periplasm (Djoko *et al*., 2008). Numerous MCOs have been found in Gram negative species which are homologues of either CueO or Fet3 but most show no ferroxidase activity. Some examples include the *C. jejuni* CueO homologue Cj1516, which is a ferroxidase and a mutant displays copper sensitivity (Hall *et al*., 2008). Also, the ferroxidase MCO of *P. aeruginosa* is required for growth on a minimal media with Fe^{2+} as the sole iron source but plays a lesser role in copper tolerance (Huston *et al*., 2002).

Plasmid encoded systems for high copper-tolerance

Copper tolerance proteins can be encoded on plasmids. This permits the expression of numerous copies of the resistance proteins and allows bacteria to survive in high levels of environmental copper. In addition, these plasmids can result in the horizontal transfer of these tolerance systems to copper sensitive strains. Some *Enterococcus* species

possess the plasmid encoded *tcrYAZB* operon. These four genes are homologous to the *E. hirae copYZAB* operon and provide hyper copper-tolerance that is transferable to copper sensitive *Enterococcus* (Hasman & Aarestrup, 2002; Hasman, 2005). The previously described *E. coli pco* system also confers high copper tolerance and consists of seven genes, *pcoABCDRSE*, whose functions are not fully understood, although several are periplasmic located and help detoxify copper ions in this location (Lee *et al*., 2002). PcoA is a CueO homologue that requires the outer membrane protein PcoB for efficient copper resistance and studies show that PcoC/PcoD may be involved in copper uptake (Rensing & Grass, 2003; Lee *et al*., 2002). Another example is the plasmid encoded *copABCD* operon found in *Pseudomonas syringae* pv. *Tomato*, which confers high copper tolerance in this strain (Bender & Cooksey, 1987; Lim & Cooksey, 1993). It is homologous to both the *pco* operon of *E. coli*, but without a *pcoE* homologue, and the chromosomally encoded *Pseudomonas cop* operon. However, these hyper copper-tolerant bacterial strains have only been isolated from plants and animals which have been fed high concentrations of copper. To date, no hyper copper-tolerant pathogens with plasmid encoded copper resistance proteins have been isolated from clinical sources.

Copper chelators and chaperone proteins

Bacteria can also passively reduce copper toxicity by keeping intracellular copper ions bound with high affinity to either large proteins as an enzyme cofactor or to smaller copper-binding proteins, in order to maintain a low free copper ion concentration in the cytoplasm. These smaller proteins can also be actively up-regulated in response to copper. The metallothioneins are cysteine rich proteins ubiquitously found intracellularly in eukaryotes. They are induced and bind metals such as cadmium, zinc

and copper (Andrews, 2000), such as the *Neurospora crassa* copper metallothionein which is up-regulated by copper (Kumar *et al*., 2005). Although most of the studied bacterial metallothioneins protect from zinc toxicity, recently a copper binding metallothionein was identified in *mycobacteria* which protected against copper toxicity, suggesting metallothioneins are also important for bacterial copper tolerance in some species (Gold *et al*., 2008). Copper chaperones are small soluble intracellular proteins which have several important functions; they chelate free copper ions to prevent intracellular toxicity, reduce oxidative stress and can deliver copper ions to other copper proteins (Field *et al*., 2002). They are found in many bacterial species usually as members of the previously described copper efflux systems such as *cusF* of the *E. coli cusCFBA* operon and *copZ* of the *E. hirae copYZAB* operon. They bind copper for transport in the cytoplasm which reduces copper toxicity by limiting the intracellular free copper ion concentration. In addition, the direct interaction between these copper-binding proteins and the copper ATPases may be required to pass the copper ions to the ATPase for transport (Argüello *et al*., 2007).

Copper reduction

Proteins that can reduce copper have been shown to protect against copper toxicity. The *E. coli* membrane bound NADH dehydrogenase-2 (Ndh-2) is a copper containing flavoprotein associated with the respiratory chain, which also has copper reductase activity (Rapisarda *et al*., 1999; Rapisarda *et al*., 2002). Although a *ndh* mutant is sensitive to both high and low concentrations of copper, the role of Ndh-2 in copper tolerance is poorly understood but may be due to Ndh-2 activity being required for subsequent export of Cu^{1+} copper by exporter proteins (Rodríguez-Montelongo *et al*.,

2006). However, this may be specific for this bacterial strain as no other copper reducers have been shown to have a protective effect against copper.

1.15. Copper regulation

To express the copper resistance proteins when required, bacteria sense and respond to elevated environmental copper concentrations. There are several families of copper regulators which include both activators and repressors. Interestingly, the copper responsive activators are usually found in the Gram negative species and the repressors in the Gram positive species. This may represent two opposing strategies that have evolved in bacteria for the control of the copper homeostasis response.

The Gram negative MerR copper activators

E. coli has two copper regulators; CueR, for activation of *copA/cueO* and CusRS, a two component regulator for the induction of the *cus* operon. Figure 1.16 summarises the known *E. coli* copper regulatory systems. The MerR like proteins are a protein family which consists of a group of environmental-responsive activators. MerR regulators are helix-turn-helix DNA binding proteins which sense external stimuli to control transcription (Brown *et al*., 2003). The model for MerR activation is that the MerR protein binds target gene promoters, recruits RNA polymerase and distorts DNA. Only the binding of the specific stimuli to the MerR protein results in the DNA straightening to allow RNA polymerase to begin transcription (Brown *et al*., 2003; Hobman, 2007). However, the structure of MerR has not been solved and therefore this model may be a simplistic view of the MerR mode of action.

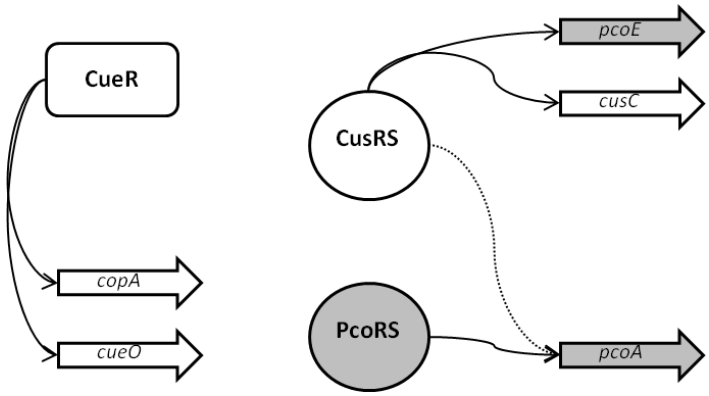


Figure 1.16. Regulation of copper homeostasis in *E. coli*. Arrows represent activation in response to elevated copper. Plasmid encoded genes/proteins are shown in grey. CueR activates *copA* and *cueO* in response to high copper. CusRS and PcoRS are two component regulators which function independently. CusRS induces plasmid *pcoE* and chromosomal *cusC* in response to copper. In addition, CusRS induces a low level of plasmid *pcoA*. However, full expression of *pcoA* requires the plasmid encode PcoRS. Figure created from information in Munson *et al*. (2000).

CueR is a MerR homologue which senses cytoplasmic copper and a *cueR* mutant is copper sensitive (Brown *et al*., 2003; Stoyanov *et al*., 2001). CueR binds the *copA* promoter encompassing the -10 and -35 upstream elements, is required for copper induction of *copA* and also regulates *cueO* in response to elevated copper (Figure 1.16) (Outten *et al*., 2000; Stoyanov *et al*., 2001). Homologues of the *E. coli* CueR have been identified in other bacterial species such as *Salmonella typhimurium*, where the *E. coli* CueR homologue, termed CueR, is required for *copA* and *cuiD* (MCO) copper induction (Espariz *et al*., 2007). The *Pseudomonas* copper ATPase, CueA, is also activated by the MerR type regulator, CueR (Zhang & Rainey, 2008).

The Gram negative two component copper activators

In addition to MerR-like regulators, Gram negative bacteria contain two component systems for the induction of copper genes. The organisation of TCR dual regulators has

been previously discussed and is shown in Figure 1.3. The *E. coli* chromosomal *cus* operon and plasmid encoded *pco* operon are regulated by the TCRs CusRS and PcoRS respectively (Rensing & Grass, 2003). CusRS is known to consist of a membrane located histidine kinase (CusS) and cytoplasmic response regulator (CusR). CusRS is required for copper dependent expression of chromosomal *cusC* and plasmid *pcoE*, whilst PcoRS controls the full copper regulation of plasmid *pcoA* (Figure 1.16). However, these regulatory systems act independently of each other and experimental evidence has shown that in a *cusRS* negative strain; *pcoRS* could not recover *cusC* or *pcoE* expression (Munson *et al*., 2000). Other copper responsive two component regulators include CopRS, which induces the *Pseudomonas* plasmid encoded and chromosomal encoded *cop* operons (Mills *et al*., 1993; Mills *et al*., 1994). Also in *H. pylori*, the copper resistance mechanism encoded by *crdA*/*crdB*/*czcA*/*czcB* is controlled by the two component regulator CrdRS and the response regulator part of this system, CrdR, has been shown to bind the *crdA* promoter for copper induction (Waidner *et al*., 2005).

Variable regulation of the *E. coli* copper homeostasis proteins

E. coli contains two regulators for the induction of copper tolerance genes in response to elevated copper, CueR and CusRS. However, it has been found that these regulators coordinate different responses to extracellular copper challenge (Outten *et al*., 2001). It is members of the *cue* regulon which function as the primary *E. coli* copper tolerance proteins, particularly periplasmic copper efflux by CopA (Outten *et al*., 2001). The *cus* regulon is only fully induced in very high copper and therefore represents an emergency system used by *E. coli* for high copper resistance (Outten *et al*., 2001). Both the *cue* and

cus systems are required for full aerobic copper tolerance. However, under anaerobic conditions the protective effect of periplasmic CueO is diminished. Under these conditions the *cus* systems become crucial for copper tolerance (Outten *et al*., 2001). For this reason, regulation of the two systems was found to be dependent on both copper and oxygen status. *copA* was up-regulated in moderate copper conditions and showed a greater basal expression rate in an anaerobic environment. *cueO* was induced by moderate copper in the presence of oxygen while *cusC* was mainly expressed under extreme copper stress (Outten *et al*., 2001). Therefore, both copper level and oxygen status affect the *E. coli* response to environmental copper.

The Gram positive CopY copper repressor proteins

Differing from the activator proteins of the Gram negative species, copper regulation is achieved in Gram positive bacteria by repressor proteins. The CopY family are a group of copper responsive repressors and the best studied is the CopY encoded by the *copYZAB* operon of *E. hirae*, which is shown in Figure 1.17. In addition to CopY, this operon encodes the chaperone CopZ and two copper ATPases, CopA and CopB (Figure 1.17). In an *E. hirae copY* mutant, operon expression is de-repressed (Odermatt & Solioz, 1995; Strausak & Solioz, 1997). CopY proteins repress *cop* operon transcription in low copper by binding to DNA regions upstream of target genes (Strausak & Solioz, 1997). In high copper, *E. hirae* CopZ functions to chaperone copper ions imported by CopA to CopY, which substitutes copper ions for CopY-bound zinc ions. This results in CopY being released from the *cop* promoter and allows for the transcription of the operon (Cobine *et al*., 1999). CopY regulators are found in several other Gram positive bacterial species including *Lactococcus lactis* and *Streptococcus mutans*, and these

CopY proteins have similar affinities for their own and homologous *cop* promoters including the upstream "Cop box" of the *E. hirae copYZAB* operon (Portmann *et al*., 2004).

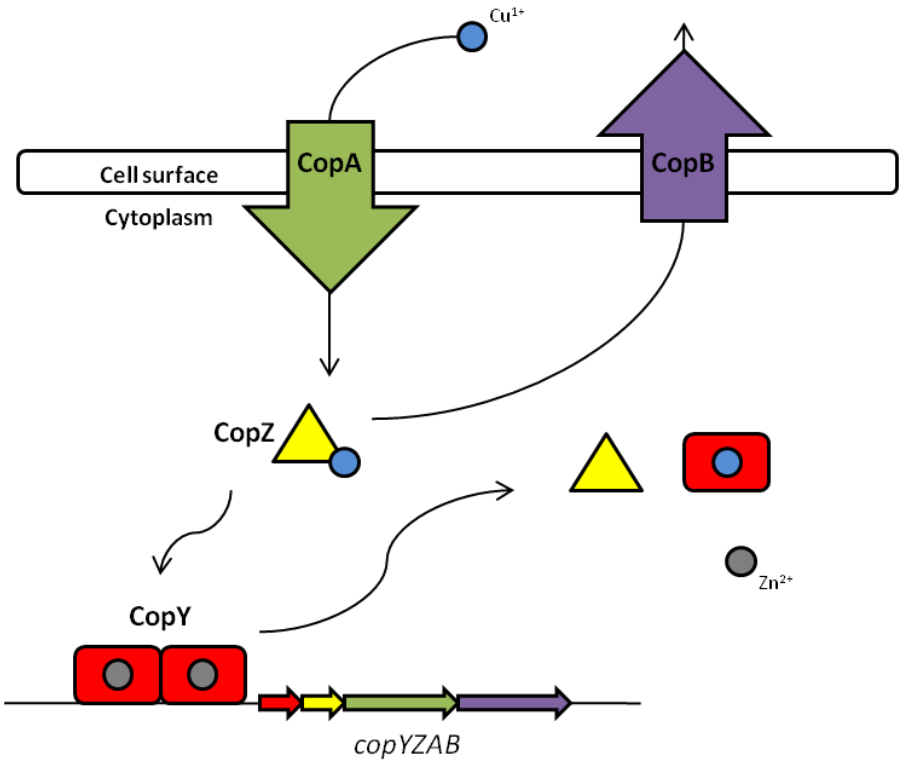


Figure 1.17. CopY regulation of the *E. hirae copYZAB* operon. CopA imports Cu^{1+} into the cytoplasm where it is bound to the cytoplasmic CopZ chaperone. CopY binds the promoter of *copYZAB* and represses transcription. CopZ can donate copper ions to CopY which replace CopY-bound zinc and results in the release of CopY from the *copYZAB* promoter. This allows for transcription of the *copYZAB* operon. Figure adapted from Figure 3 of Magnani & Solioz (2005).

The Gram positive CsoR copper repressor proteins

Recently a new type of copper repressor protein, termed CsoR, has been identified in Gram positive species. CsoR was first described in *M. tuberculosis* where it was shown that it bound copper to repress a downstream copper export ATPase through promoter

binding (Liu *et al*., 2007). Many other species possess CsoR homologues and the *B. subtilis* CsoR was shown to also repress a downstream copper ATPase system, *copZA*, in high copper by binding a promoter DNA site (Smaldone & Helmann, 2007). Furthermore, the *B. subtilis* CsoR also negatively regulates the previously described YcnJ copper uptake protein in conjunction with YcnK, a putative helix-turn-helix copper sensing regulator (Chillappagari *et al*., 2009).

The *S. aureus* copper systems

S. aureus contains no defined copper uptake systems and no homologue of the *B. subtilis* YcnJ protein. However, two CPx-Type-1 ATPases have been described and these reportedly are involved in copper efflux rather than influx (Sitthisak *et al*., 2005; Sitthisak *et al*., 2007). *S. aureus* strains have been found to contain either one or two of these *cop* operons which are displayed diagrammatically in Figure 1.18.

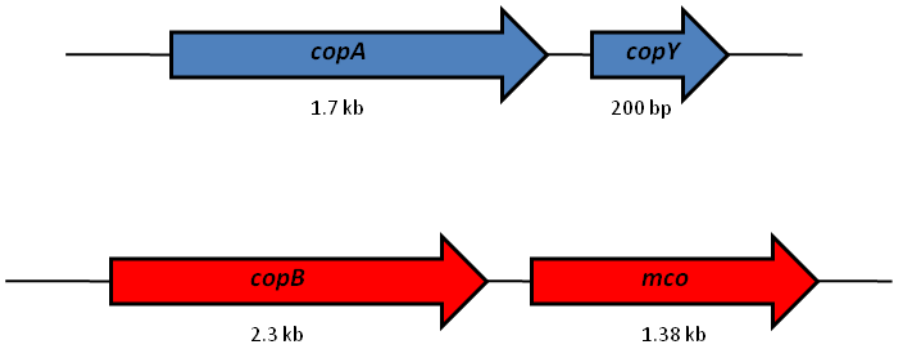


Figure 1.18. The two *S. aureus cop* operons. All sequenced strains contain the *copAZ* operon which encodes the copper efflux ATPase, CopA, and a copper chaperone, CopZ. A few strains have an additional *cop* operon which encodes the copper ATPase CopB and a MCO. Figure created using Sitthisak *et al*. (2005) and Sitthisak *et al*. (2007).

All sequenced *S. aureus* strains have been found to possess the Type-1 P-type ATPase gene *copA*, along with the downstream copper chaperone gene, *copZ* (Sitthisak *et al*., 2007). A *copA* mutant had increased intracellular copper levels, suggesting CopA functions as a copper exporter (Sitthisak *et al*., 2007). *copA* was found to be induced by copper in a concentration dependant manner and a *copA* mutant displayed no growth defect compared to wild type but was found to be copper sensitive, suggesting that the *S. aureus* CopA is critical for copper tolerance.

A second copper ATPase gene, *copB*, was only found in a few strains and is downstream of a gene encoding a *S. aureus* MCO termed *mco* (Sitthisak *et al*., 2007). The strains which contain this system are ATCC 12600, the EMRSA-16 MRSA252 and the more recently sequenced MSSA, MN8. The function of this additional ATPase is currently unknown, as is its role in copper tolerance (Sitthisak *et al*., 2007). However, the *S. aureus mco* is induced by copper and a *mco* mutant is hydrogen peroxide resistant but copper sensitive, suggesting the *S. aureus* MCO is somehow involved in *S. aureus* copper tolerance (Sitthisak *et al*., 2005). However, this *S. aureus mco* is clearly not essential, as it represents an accessory gene which is absent in the majority of the sequenced strains. In addition, the regulator(s) of these two *S. aureus* operons is currently undefined.

1.16. Project objectives

The pathogenic bacterium *S. aureus* is still of significant medical importance due to the emergence of new strains, such as the MRSAs, VISAs, VRSAs, EMRSAs and CA-MRSAs. These strains have often acquired additional resistances and virulence

factors from MGEs. The regulation of virulence factors is often under the control of regulators such as Fur and environmental signals such as iron concentration. In *S. aureus*, Fur is a global regulator that controls the expression of genes involved in iron uptake and other essential cellular processes. Similar to other bacterial species, the *S. aureus* Fur has been shown to regulate genes is a non-classical way. The catalase gene, *katA*, is iron activated but expression is lost in a *fur* mutant (Horsburgh[2] *et al*., 2001). Also, expression of the virulence factors *eap* and *emp* is induced by Fur in low iron (Johnson *et al*., 2005). In addition, there is evidence for a Fur independent iron regulator in *S. aureus* strain Newman (Figure 1.12), as *eap*, *emp* expression are still iron regulated in a *fur* mutant (Johnson *et al*., 2008). However, the mechanisms involved in this novel iron/Fur responsive gene regulation are currently unknown.

Copper is another important biological metal and numerous resistance mechanisms have been identified in bacterial species. In *S. aureus*, two copper resistance operons have been described; the *copAZ* operon which is ubiquitous in *S. aureus* strains and the *copB/mco* operon found in only a few strains (Sitthisak *et al*., 2005; Sitthisak *et al*., 2007). CopA is required for copper tolerance, as is MCO in the strains that contain it. However, the role of CopB in copper homeostasis is not defined and it is unknown how these copper operons are regulated. Furthermore, the response of *S. aureus* to copper challenge is undefined and in fact there is very little information about the cause of copper toxicity in Gram positive species.

Therefore the first part of this project will focus on novel Fur/iron regulation in *S. aureus*. It will attempt to find a mechanism for non-classical *S. aureus* gene

regulation. Potentially while exploring these systems, the Fur independent regulator, which the biofilm experiments suggest exists, will be identified. The second part of this project will investigate copper homeostasis in *S. aureus*. Initially, the copper response between different strains will be examined to determine if there is any strain variation and if found, the cause of this will be analysed. *S. aureus* possess either one or two *cop* operons and the regulator(s) of these systems will be investigated. Also currently unknown is the mechanism of copper toxicity in Gram positive species such as *S. aureus*. Therefore the final part of this project will attempt to define if oxidative stress induction is a major cause of copper induced toxicity in this species.

Project objectives

- Investigate *S. aureus* novel Fur regulation and define the mechanism(s) involved.

- Identify novel iron responsive regulators in *S. aureus* Newman.

- Examine the *S. aureus* strain response to copper and investigate any variation.

- Identify the copper responsive regulator(s) of *S. aureus copAZ* and *copB/mco*.

- Investigate the mechanism of copper induced toxicity in *S. aureus*.

Chapter 2. Materials and methods

2.1. Bacteria storage and growth

2.1.1. Storage and recovery of bacterial strains

Bacteria were stored at -80^{o}C in TSB containing 20% v/v sterile glycerol in a screw capped 1.5 ml eppendorf tube. To recover bacteria for experimental manipulation, the frozen glycerol stock was used to inoculate a Luria-Bertani agar plate containing the appropriate antibiotics. The plate was incubated overnight at 37^{o}C unless the strain contained a temperature sensitive plasmid, in which case incubation was at 30^{o}C. Fresh plates were used to inoculate broth cultures for further experimentation.

2.1.2. Growth conditions

Broth cultures were either grown statically at 37^{o}C and 5% CO_2 or with shaking at 37^{o}C in air. Strains which contained a temperature sensitive plasmid were grown with shaking at 30^{o}C.

2.2. Growth media and supplements

Where indicated, media were autoclaved at 120^{o}C for 15 minutes at 1 atmosphere pressure to produce sterility. To filter sterilise large volumes of liquid, 500 ml Stericup vacuum driven disposable filtration systems with a (Millipore – 0.22 µm pore size) were used. To filter sterilise smaller volumes of liquids, plastic syringes (BD Plastipak) and

0.2 μm Acrodisc® membranes (Pall) were employed. All supplements and media were made using dH_2O unless otherwise stated.

2.2.1. Luria Bertani medium (LB)

Luria Bertani medium consisted of 1% (w/v) tryptone (Oxoid), 0.5% (w/v) yeast extract (Oxoid) and 0.5% (w/v) sodium chloride. The medium was adjusted to pH 7.2 and solid medium (LA) was made by the addition of 1% Bioagar (Biogene.com). Medium was then autoclaved as previously described.

2.2.2. Trypticase soy broth (TSB)

Trypticase Soy broth consisted of 3% (w/v) BBL™ Trypticase™ Soy broth (BD Diagnostics) dissolved in 500 ml water. Medium was then autoclaved as previously described.

2.2.3. RPMI and CRPMI minimal medium (Morrissey *et al*., 2002)

RPMI-1640 medium (Sigma) which contained sodium bicarbonate but lacked L-glutamine (product reference R0833) was used as a minimal medium for bacterial growth. To create metal ion restrictive RPMI, 6% (w/v) chelex (Sigma) was added to a 500 ml bottle of RPMI-1640 medium. After 24 hours of continuous stirring at 4°C, this medium was filter sterilised to produce chelexed RPMI (CRPMI). This CRPMI medium was stored at 4°C for up to two weeks. To prepare the final metal ion restrictive CRPMI, 10% of RPMI was added to the CRPMI immediately before use, to provide bacterial cultures with the trace concentrations of metal ions required for growth.

2.2.4. LK broth and LK agar (LKA)

LK medium consisted of 1% (w/v) tryptone (Oxoid), 0.5% (w/v) yeast extract (Oxoid) and 0.7% (w/v) potassium chloride. 1.5% (w/v) Bioagar (Biogene.com) was added to produce LKA. Medium was then autoclaved as previously described. Immediately before use, molten LKA at 65^{o}C was supplemented with sterile sodium citrate to a final concentration of 0.05% (w/v).

2.2.5. Super optimal broth with catabolite repression (SOC) medium

To create SOC medium, 2% (w/v) tryptone (Oxoid) and 0.5% (w/v) yeast extract (Oxoid) were added to 950 ml of dH_2O which contained 8.5 mM sodium chloride and 2.5 mM potassium chloride. This solution was autoclaved as previously described. To this sterile solution, filter sterilised glucose was added to a concentration of 20 mM and filter sterilised magnesium chloride added to a concentration of 10 mM. The medium was stored in 20 ml aliquots at -20^{o}C until required. Before use, a 20 ml frozen aliquot was incubated at 37^{o}C until it was fully defrosted and warmed to 37^{o}C.

2.2.6. Staphylococcal siderophore detection (SSD) medium (Lindsay & Riley, 1994)

The following stocks were made and autoclaved: 200 ml of 20% (w/v) glucose, 200 ml of 1.5 M Tris-base adjusted to pH 8.8 with hydrochloric acid, 150 ml of 20% (w/v) casamino acids (Difco) and 500 ml of 25 × salts; which consisted of 0.68% (w/v) potassium dihydrogen-orthophosphate, 2.3% (w/v) ammonium chloride and 1.16% (w/v) sodium chloride. 50 × supplement was made through the addition of the following reagents to give the stated final concentrations: 0.04% (w/v) tryptophan, 0.02% (w/v) nicotinic acid and 0.01% (w/v) thiamine. This solution was subsequently filter sterilised.

500 ml of the final SSD medium consisted of:

- 20 ml 25 × salts
- 9 ml 20% (w/v) glucose
- 10 ml 1.5 M Tris pH 8.8
- 15 ml 20% (w/v) casamino acids
- 10 ml 50 × supplement

This solution was made up to 500 ml with dH_2O. After the addition of 1% (w/v) chelex (Sigma), it was continuously stirred at 4^{o}C for 24 hours and subsequently filter sterilised as previously described. The SSD medium was stored at 4^{o}C for no longer then 4 days as the medium produced inconsistent assay results if stored at this temperature for longer than 5 days. Before use, filter sterilised magnesium chloride was added to the SSD medium to a final concentration of 50 μM.

2.2.7. SMMP50 medium

Supplemented minimal medium (SMM) buffer was created made through the addition of the following reagents to give the stated final concentrations: 34.2% (w/v) sucrose, 0.4625% (w/v) maleic acid and 0.8% (w/v) magnesium chloride and the pH was adjusted to 6.5. This SMM buffer was autoclaved and stored at room temperature.

For 20 ml of SMMP50 medium, the following solutions were mixed and filter sterilised:

- 11 ml SMM buffer.
- 8 ml filter sterilised Penassay Broth (antibiotic solution 3 from Difco).
- 1 ml filter sterilised bovine serum albumen (10% w/v).

2.2.8. Antibiotics and metal supplements

10 mg/ml antibiotic stock solutions for *S. aureus* were made in dH_2O (kanomycin), 100% ethanol (erythromycin and chloramphenicol) or 1:1 dH_2O:ethanol (tetracycline). 100 mg/ml ampicillin stock solution for *E. coli* was made in dH_2O. Antibiotics were filter sterilised and stored at 4^oC for up to one month. Working solutions were as follows: 5-10 μg/ml tetracycline, 10 μg/ml erythromycin and chloramphenicol, 50 μg/ml kanomycin and 100 μg/ml ampicillin.

$CuCl_2$ and $Fe_2(SO_4)_3$ metal supplement stock solutions of 50 mg/ml were made up in 10 ml dH_2O and once the metals had fully dissolved, these solutions were filter sterilised. Fresh metal supplement stock solutions were made for each individual assay on the day it was performed.

2.2.9. X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside)

X-Gal (Melford) was made at 50 mg/ml in dimethylformamide and stored in an eppendorf at -20^oC for up to 1 month. For blue/white screening plates, 40 μl of this stock X-Gal was spread onto agar plates and allowed to dry before inoculation with bacteria.

2.3. Growth curves

2.3.1. Growth curves taking hourly time points

Fresh LA plates were used to inoculate 10 ml of medium and these cultures were grown overnight statically at 37^oC in 5% CO_2. The $OD_{595\ nm}$ was determined using a cuvette

spectrometer (Ultrospec® 2000 from Pharmacia Biotech) and by using un-inoculated medium as a blank. The 10 ml cultures were sub-cultured into pre-warmed 25 ml medium to an $OD_{595\ nm}$ of 0.05. These 25 ml cultures had the supplements to be assayed (metals and/or enzymes) added and after thorough mixing, the $OD_{595\ nm}$ was determined and recorded as the time point for zero hours. The 25 ml cultures were then incubated statically at 37°C in 5% CO_2. The $OD_{595\ nm}$ of the growing cultures was determined at desired time points with the growing cultures mixed thoroughly before each optical density reading. This mixing was essential, as bacterial cultures were found to settle after approximately 4 hours of static growth. This resulted in inaccurate readings of optical density after this period of incubation.

2.3.2. Growth curves using microtitre plates

An experiment for investigating *S. aureus* biofilm formation (Johnson *et al*., 2005) employed 96-well flat-bottomed tissue culture plates (Nunc) to which metal supplements such as $Fe_2(SO_4)_3$ could be added and which used CRPMI as the growth medium. This procedure was modified to create a high throughput growth experiment that was suitable for testing *S. aureus* copper tolerances. The method for setting up the microtitre growth assay is shown in Figure 2.1.

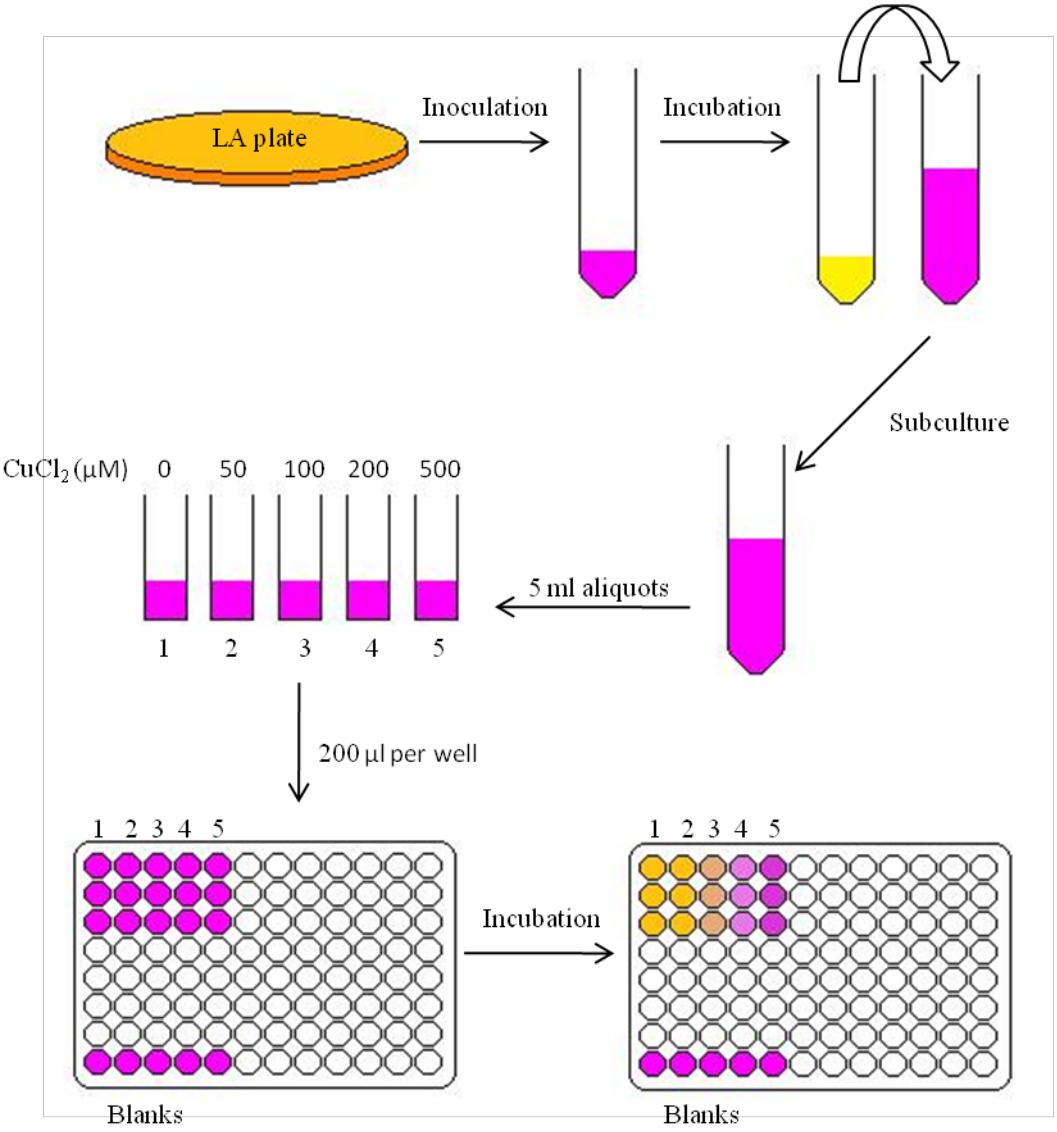


Figure 2.1. The 96 well microtitre plate growth assay. *S. aureus* were grown statically overnight in 10 ml CRPMI at 37°C in 5% CO_2 in air, diluted to an optical density at 600 nm of 0.05 into 25 ml fresh pre-warmed medium and aliquoted into 5 ml aliquots. After supplementation with cupric chloride, samples were inoculated into triplicate wells of 96-well flat-bottomed tissue culture plate and incubated statically at 37°C in 5% CO_2 and in air for 24 hours. After incubation, the plate was read at 595 nm in a Microplate reader (BioRad) to monitor total growth. Each copper concentration was blanked with medium containing the equivalent copper concentration.

A LA plate was used to inoculate 10 ml CRPMI supplemented with appropriate antibiotics. After static incubation overnight at 37^{o}C in 5% CO_2, in air, the $OD_{595\ nm}$ was determined and the culture used to inoculate 25 ml pre-warmed medium to an $OD_{595\ nm}$ of 0.05. After mixing, this culture was aliquoted into volumes of 5 ml. After supplementation with $CuCl_2$, 200 µl volumes were inoculated into three wells of a 96-well flat-bottomed tissue culture plate. The plate was incubated statically at 37^{o}C in 5% CO_2 and in air for 24 hours. After incubation, the plate was read at 595 nm in a Microplate reader (BioRad). The final growth reading was calculated by averaging the reading of the three replicate wells for each copper concentration and taking away the appropriate blank, which was $CuCl_2$ supplemented medium without bacterial inoculation. Each assay was repeated at least three times on different occasions using fresh LA plates, medium and $CuCl_2$.

Aliquoting into volumes of 5 ml was necessary to limit the effects of metal supplementation on the final culture volume. This reduced the subsequent dilution of the CRPMI and the nutrients it contained. Significant medium dilution could have affected the growth and copper tolerances of *S. aureus* strains. The 24 hour time point was chosen as initial cuvette copper growth curves (Figure 4.1 Chapter 4) showed that this reading was a satisfactory indicator of the total bacterial growth in response to copper challenge. Growth on the 96 well microtitre plate was also tested by taking hourly $OD_{595\ nm}$ readings and a normal pattern of growth was observed; a lag phase, an exponential phase ending in a stationary phase.

2.3.3. Oxidative stress growth curves

Fresh LA plates were used to inoculate 10 ml TSB and this was incubated overnight with shaking at 37°C. The $OD_{595\ nm}$ was determined using a cuvette spectrophotometer and using un-inoculated TSB as a blank. The 10 ml cultures were sub-cultured into 50 ml TSB to an $OD_{595\ nm}$ of 0.05. This suspension was split into two 25 ml cultures and one was supplemented with 0.003% H_2O_2 (Sigma (30% w/w)) and after thorough mixing, the $OD_{595\ nm}$ was determined and recorded as the time point for zero hours. The 25 ml cultures were then incubated with shaking at 37°C. The $OD_{595\ nm}$ of the growing cultures was determined at desired time points using un-inoculated TSB as the blank.

2.3.4. Biofilm assay (Deighton *et al*., 2001; Johnson *et al*., 2005)

10 ml CRPMI cultures were inoculated from a LA plate and incubated statically at 37°C overnight in 5% CO_2. These were centrifuged at 3200 × g for 10 minutes to pellet the cells and re-suspended in fresh CRPMI drop by drop until an OD_{595nm} of 0.01 was reached. This suspension was split into three for each condition being tested; no iron and 50 µM $Fe_2(SO_4)_3$. After addition of these supplements, four 200 µl volumes of each culture were inoculated into four replicate wells of a 96-well flat-bottomed tissue culture plate (Nunc). In addition, a well was inoculated with medium alone as a blank. After incubation for 24 hours at 37°C in 5% CO_2, this plate was read at 595 nm to measure bacterial growth. Excess medium was removed by flicking, followed by three sequential washes with phosphate buffered saline (PBS). After the final wash and removal of PBS, the plate was left to dry for approximately 15 minutes in a hybridisation oven set at 50°C. Each well was then strained with 200 µl 1% safranin and left at room temperature for 30 minutes. Next, all wells were gently rinsed with water until no more stain could be removed and then the plate dried and read at 490 nm. For

the final results, the readings of the four repeat wells for each condition were averaged. The $OD_{595\ nm}$ readings of total bacterial growth were used to equalise the final $OD_{490\ nm}$ biofilm readings to correct for growth variations.

2.4. Strains and plasmids used in this study

Strain	geneotype	Source/reference
E. coli TOPO10	F$^-$, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ* ΔM15 Δ*lac*X74 *deo*R *ara*D139 Δ(*ara-leu*)7697 *ga*/U *ga*/K *rps*L *end*A1 *nup*G	Invitrogen
Newman	Clinical isolate MSSA	Duthie and Lorenz (1952)
PM64	MRSA252 clone	Moore and Lindsay (2002)
MRSA252	EMRSA-16	Holden *et al*. (2004)
RN4220	Restriction negative 8325	Laboratory stock
ATCC 12600	Wild type	Sitthisak *et al*. (2005)
SH1000	NCTC 8325-4 with *rsbU* mutation repair	Horsburgh[1] *et al*. (2002)
8325-4	Wild-type strain cured of prophages	Horsburgh[1] *et al*. (2001)
ATCC 12600 *mco*	ATCC 12600 *mco::*Tn*917*	Sitthisak *et al*. (2005)
ATCC 12600 *mco* + pLI50$_{mco}$	ATCC *mco::Tn917* + pLI50$_{mco}$	Sitthisak *et al*. (2005)
SH1000 *sodA*	SH1000 *sodA::*Tn*917*	Clements *et al*. (1999)
SH1000 *sodM*	SH1000 *sodM::tet*	Karavolos *et al*. (2003)
SH1000 *ahpC*	SH1000 *ahpC::tet*	Cosgrove *et al*. (2007)
SH1000 *katA*	SH1000 *katA::Tn917*	Cosgrove *et al*. (2007)
8325-4 *perR*	8325-4 *perR::kan*	Horsburgh[1] *et al*. (2001)
Newman *fur*	Newman *Δfur::tet*	This study
PM64 *fur*	PM64 *Δfur::tet*	This study
Newman *lysR*	Newman *ΔlysR::tet*	This study
Newman *lysR* + pMK4$_{lysR}$	Newman *ΔlysR::tet* + pMK4$_{lysR}$	This study
Newman *csoR*	Newman *ΔcsoR::tet*	This study
PM64 *csoR*	PM64 *ΔcsoR::tet*	This study
ATCC 12600 *csoR*	ATCC 12600 *ΔcsoR::tet*	This study
Newman *csoR* + pMK4$_{csoR}$	Newman *ΔcsoR::tet* + pMK4$_{csoR}$	This study
PM64 *csoR* + pMK4$_{csoR}$	Newman *ΔcsoR::tet* + pMK4$_{csoR}$	This study
SH1000 *sodA/sodM*	SH1000 *sodM::tet/sodA::*Tn*917*	This study
SH1000 *ahpC/katA*	SH1000 *ahpC::tet/katA::*Tn*917*	This study

Table 2.1. Bacterial strains used in this study

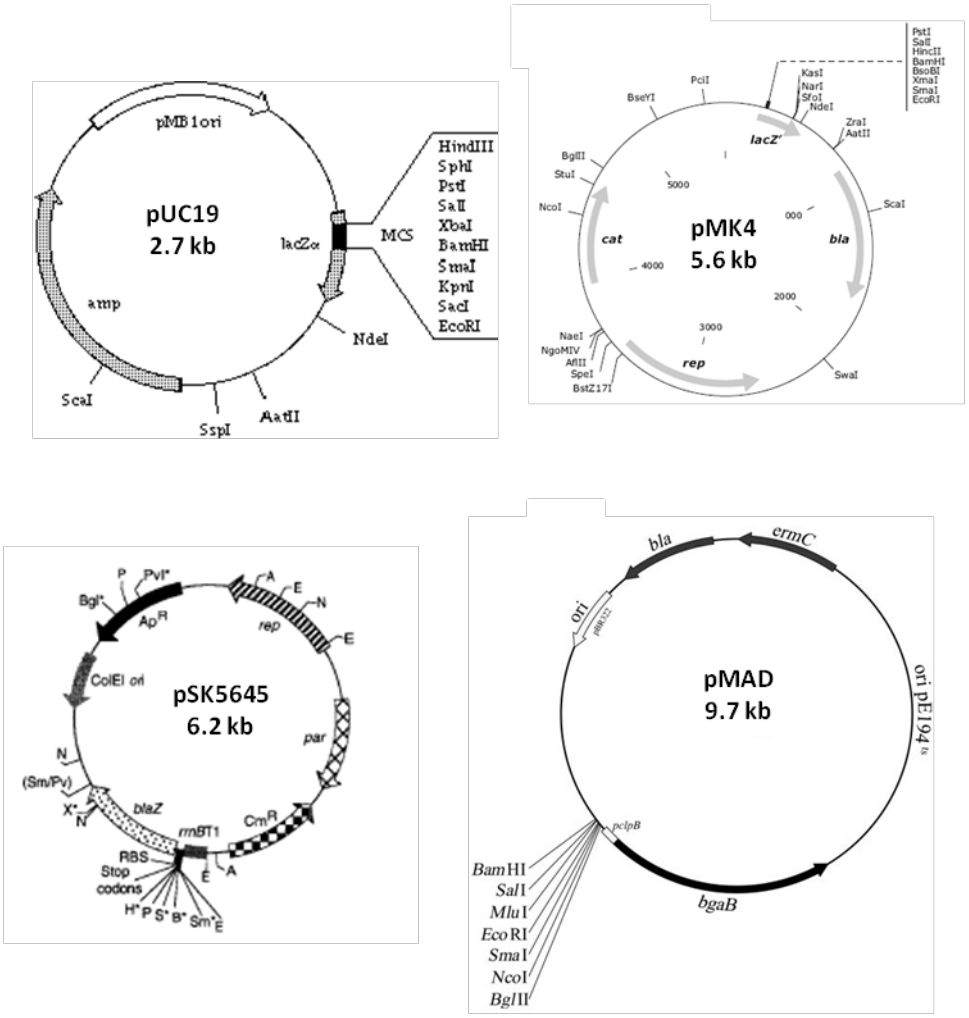


Figure 2.2. Plasmids used for this project. Shown are plasmid maps for pUC19, pMK4, pSK5645 and pMAD. Maps were modified from the following sources; pSK5645 from Grkovic *et al*. (2003), pMAD from Arnaud *et al*. (2004), pMK4 from http://www.bgsc.org/NewProducts/pMK3%20and%20pMK4%20revisited.pdf and pUC19 from http://www.bioron.net/pl/excellent-products-from-bioron/dna-marker-dna-plasmid/puc19-dna.html.

2.5. DNA preparations and manipulation

2.5.1. Plasmid preparations

Plasmid preparations were performed using a QIAGEN Qiaprep Spin Minprep Kit or an Omega E.Z.N.A Plasmid Mini Kit I. The standard manufacture's protocol for preparing bacterial plasmids using a micro-centrifuge was followed for both kits but with two alterations. When preparing plasmids from *S. aureus*, an additional 100 µg/ml lysostaphin (Sigma) was added to the lysis buffer (buffer P1 for QIAGEN or solution 1 for Omega) and this suspension incubated at 37^{o}C for 30 minutes to lyse the cells. Furthermore, the final elution was performed with dH_2O heated to 65^{o}C instead of the supplied elution buffer. Heating of the eluting water to 65^{o}C was necessary to obtain a good plasmid yield, particularly when isolating low copy number plasmids.

2.5.2. *S. aureus* total DNA preparations (Ausubel *et al*., 1995)

5 ml of LB broth with appropriate antibiotics was inoculated using a single *S. aureus* colony from a LA plate. This culture was incubated with shaking at 37^{o}C overnight. The following day, the culture was centrifuged at 12,000 × g for 10 minutes and the supernatant discarded. The remaining pellet was re-suspended in 250 µl P1 buffer (Qiagen) and this solution was decanted into an eppendorf tube. To this suspension, 100 µg/ml lysostaphin (Sigma) was added and this was incubated for 20-30 minutes at 37^{o}C until it became clear and viscous. After lysis had occurred, 0.125 mg of proteinase K (Sigma) was added followed by 27 µl 10% (w/v) SDS (sodium dodecyl sulphate). This solution was mixed well by inversion after each addition and incubated at 37^{o}C for 25 minutes. 97 µl of 5 M NaCl was then added and the suspension mixed again by inversion. This was followed by the addition of 81 µl of 65^{o}C CTAB (4.1%

(w/v) NaCl and 10% (w/v) cetyl trimethyl ammonium bromide) and this suspension again mixed by inversion. This was then incubated for 20 minutes at 65^{o}C. An equal volume of 24:1 chloroform:isoamylalcohol was added and after again mixing by inversion, was centrifuged at 12,000 × g for 10 minutes. The upper clear aqueous phase was transferred to an eppendorf tube whilst avoiding the removal of any precipitate. If separation was poor and precipitate removed along with the supernatant, an additional 200 µl of 24:1 chloroform:isoamylalcohol and 100 µl dH_2O was added to the solution which was again mixed and centrifuged at 12,000 × g for a further 10 minutes. This extra step was repeated until a completely clear supernatant could be removed without any precipitate. An equal volume of propan-2-ol was added to the clear supernatant and mixed gently to precipitate the DNA. This solution was then centrifuged for a further 10 minutes at 12,000 × g. Most of the alcohol was removed by aspiration and the remaining was allowed to evaporate overnight through aeration in a fume hood. To the dry pellet, 50 µl of sterile dH_2O was used to dissolve the DNA and this was kept on ice for all further experimentation and stored at -20^{o}C. To confirm the success of DNA isolation, 2-5 µl of the total preparation was run on a 1% (w/v) agarose gel and visualised with ethidium bromide (0.00005% (w/v)).

2.5.3. Gel electrophoresis

1% (w/v) agarose for electrophoresis gels was made by melting 4 g agarose (RESult ^{TM} LE general purpose agarose) in 400 ml TAE buffer (10 × TAE buffer is 48.4 g Tris-base, 10.9 g glacial acetic acid and 2.92 g EDTA made up to 1 L in dH_2O) with 20 µl of 10 mg/ml ethidium bromide added after cooling to 65^{o}C.

Agarose gels were run in 1 × TAE buffer. Samples were mixed with 6 × loading buffer (Thermo Scientific Type 2 gel buffer) and loaded into the wells of the gel. The gel was run at 80-100 V until the bands reached the required distance. The gel was then removed and visualised using UV light in an UV imaging system from Syngene. To analyse DNA in electrophoresis gels, samples were compared with ΦLambda DNA digested with *Hin*dIII and ΦX174 digested with *Hae*III (New England Biolabs Ltd). These were used as ladders containing digested fragments of known size, which with 5 µl loaded into a gel, contained a total of 500 ng DNA. Also employed was Hyperladder 1 (Bioline) which contained 14 fragments of known size, which when 5 µl of the ladder was loaded into a gel, contained a total of 720 ng DNA. These ladders were used to estimate DNA fragment size and concentration.

2.5.4. Restriction digests

10 µl DNA (100-200 ng) was mixed on ice in an eppendorf tube along with 3 µl 10 × Multi-core buffer (Promega) and 1 µl (20 U) of each restriction enzyme (New England Biolabs Ltd). Sterile water was added to produce a final reaction volume of 30 µl. This was incubated at 37°C for 60 minutes according to manufacturer's instructions.

2.5.5. Gel purification

A gel DNA recovery kit (Zymogen) was used to gel-purify digested plasmids or PCR products. After agarose gel electrophoresis, fragments were excised using a scalpel under UV visualisation. The manufacturer's instructions were followed to recover the DNA but in order to increase final yield, 15 µl of 65°C dH_2O was used to elute the DNA from the columns. 1 µl of this solution was run in a 1% (w/v) agarose gel to determine the concentration of DNA through visual comparison with DNA ladders as

previously described. This ultra-pure DNA was kept on ice at all times and stored at -20°C.

2.5.6. Ligations

Gel purified digested PCR product and plasmid were run on a 1% (w/v) agarose gel to determine DNA concentration as previously described. The insert and vector DNAs were mixed in an eppendorf to produce an insert:vector ratio of 3:1 (300:100 ng). 2 µl T4 DNA ligase buffer and 1 µl (3 U) T4 DNA ligase (New England Biolabs Ltd) was added and the solution made up to 20 µl with dH_2O. This ligation reaction was placed at 16°C overnight and stored at -20°C until required. As a control reaction, a ligation was performed which contained identical reaction reagents to the experimental ligation but with dH_2O replacing the insert DNA. This was to check the level of plasmid re-ligation after subsequent transformation into bacterial cells.

2.6. PCR reactions, primers and sequencing

2.6.1. Primers used in this project

Primer	Sequence	Enzyme	Description
M13-F	GTTGTAAAACGACGGCCAGTG		For plasmid construct screening
M13-R	GGAAACAGCTATGACCATGATTAC		For plasmid construct screening
SrrA-F	GTTGGGATCCGTTGGTCGTTTAGACTATGA	*Bam*HI	Amplification of *srrA* promoter
SrrA-R	TCCTAAGCTTGTGTGTACGAAAAGAAATGT	*Hin*dIII	Amplification of *srrA* promoter
CodY-F	ACATGGATCCGCTTCAAACAGAAGAAGTTA	*Bam*HI	Amplification of *codY* promoter
CodY-R	TACAAAGCTTGTATATTTTTATAGAATAAATG	*Hin*dIII	Amplification of *codY* promoter
LexA-F	GTTGGGATCCCGTACACTTGTTCCGAGTGA	*Bam*HI	Amplification of *lexA* promoter
LexA-R	TCCTAAGCTTCTAAATTAATTTAAATACAGTAT	*Hin*dIII	Amplification of *lexA* promoter
LysR-F	ACATGGATCCGCATTTCCAACTAACCCG	*Bam*HI	Amplification of *lysR* promoter
LysR-R	TTGCAAGCTTCTTTTATTTATAGATTCAAGC	*Hin*dIII	Amplification of *lysR* promoter
TetR-F	ACATGGATCCGTGCATGCCGATGTTGTTAT	*Bam*HI	Amplification of *tetR* promoter
TetR-R	TCCTAAGCTTGTTGTTTTAACACTTATTATCA	*Hin*dIII	Amplification of *tetR* promoter
pSK-R	TCATTTAACTCTTTGGCATG		For plasmid construct screening

Table 2.2. Primers used for reporter constructs

Primer	Sequence	Enzyme	Description
LysR-puc-F	GTCTTCTAGAGAATCGCTTGATGGAGGTC	*Sac*I	*lysR* mutant creation
LysR-puc-R	TCTAGAGCTCGCATTGCAGGGCAGAATGTG	*Xba*I	*lysR* mutant creation
LysR-inv-F	TCTAGGATCCGCGTAAACACTCCGAACAAG	*Bam*HI	*lysR* mutant creation
LysR-inv-R	TCTAGGTACCGTAGCAGTTAAAGATGGCTG	*Kpn*I	*lysR* mutant creation
LysR-flank-F	GCCATTGAAGCTTGTGACA		*lysR* mutant confirmation
LysR-flank-R	GTCTTCAGTTAGTATTGCTG		*lysR* mutant confirmation
CsoR-puc-F	GTCTGAGCTCGATGACGTTATATAGTCTAT	*Xba*I	*csoR* mutant creation
CsoR-puc-R	GTCTTCTAGAGATGGTTTCGATTGTTCATA	*Sac*I	*csoR* mutant creation
CsoR-inv-F	TCTAGGTACCGCATTCACTCCTTCATTTAC	*Kpn*I	*csoR* mutant creation
CsoR-inv-R	TCTAGGATCCGATTAAAGACTAAAGGAGTT	*Bam*HI	*csoR* mutant creation
CsoR-flank-F	GGAATAGGTATAGTCTTAT		*csoR* mutant confirmation
CsoR-flank-R	GCGTATCTCGCCAATAGC		*csoR* mutant confirmation
Tet-F	CGTCTGCCCTCATTATTGG		*tet* gene for mutants
Tet-R	TCTAGGATCCCGATTTAGAAATCCCTTTGAG	*Bam*HI	*tet* gene for mutants
CsoR-comp-F	TCTAGAATTCGACATACCTTTACTACATCA	*Eco*R1	*csoR* complementation
CsoR-comp-R	TCTAGGATCCGTATCATGAAGCAATTTAAA	*Bam*H I	*csoR* complementation
LysR-comp-F	ATACGAATTCGTTCTTTTAAGAAGTTAATTC	*Eco*RI	*lysR* complementation
LysR-comp-R	ATACGGATCCGATCTGCGCAACTGCATAAGA	*Bam*HI	*lysR* complementation
Fur-flank-F	GGCAGAATTCGGTACAGTTCACTTAGATG		*fur* mutant creation
Fur-flank-R	CTGAGGATCCCGCGCAATAGATTTAGCAG		*fur* mutant creation
SodA-F	GTAATCATACTTTATTTTGTTG		*sodA/M* mutant creation
SodA-R	GATTATTTATGGCTTTTGAA		*sodA/M* mutant creation
SodM-F	GAATATACTTATGGCATTTA		*sodA/M* mutant creation
SodM-R	GTTATATTATTTTGCTGCTTGG		*sodA/M* mutant creation
AhpC-F	GTAATGCCTCCTTAGATTTT		*ahpF/katA* mutant creation
AhpC-R	GAAGATATTTATGTCATTAA		*ahpF/katA* mutant creation
KatA-F	GACCTCTTTTAATGCAAGATA		*ahpF/katA* mutant creation
KatA-R	GAAACTACATATCAAATTTAT		*ahpF/katA* mutant creation

Table 2.3. Primers used for strain construction

Primer	Sequence	Description
16S-F	GATCCTGGGTCAGGATG	*16S* probe generation
16S-R	CTAGAGTTGTCAAAGGATG	*16S* probe generation
LysR-probe-F	GCTGCACAATTTTTACATA	*lysR* probe generation
LysR-probe-R	GAATGATAGCGTAACCTAA	*lysR* probe generation
Eap-probe-F	CTCGGATCCAAAAGGAGAGATAATTTATGA	*eap* probe generation
Eap-probe-R	CTCCTGCAGTTAAAATTTAATTTCAATGTCTACTTTTTTAATGTC	*eap* probe generation
HutU-probe-F	GCCTAACCAAGCAATACGT	*hutU* probe generation
HutU-probe-R	GAGAAAACGTTACGAGAATT	*hutU* probe generation
CopA-probe-F	GATAACAATAAAGATCAAAC	*copA* probe generation
CopA-probe-R	GCAATAACAGTATCTATGC	*copA* probe generation
CopB-probe-F	CGCCCATGATGGGAGTTAAAT	*copB* probe generation
CopB-probe-R	CTGCGCCAATTGCCATACC	*copB* probe generation
CsoR-probe-F	GTCTTTAATCAATTTTTGAAAA	*csoR* probe generation
CsoR-probe-R	GTATAGTAAGGAATGTAAATG	*csoR* probe generation
AhpF-probe-F	CGGTGCTCATGTCTTCGTAA	*ahpF* probe generation
AhpF-probe-R	GTGGTGGTCCTGCTAGTGGT	*ahpF* probe generation
SodM-probe-F	CAATGGAGTTTCATCACGACA	*sodM* probe generation
SodM-probe-R	GTAGGCATGCTCCCAAACAT	*sodM* probe generation

Table 2.4. Primers used for probe creation

2.6.2. PCR standard recipe

For a standard 10 µl PCR reaction the following recipe was used; 5 µl of FailSafe™ PCR 2 × PreMix D (Epicentre Biotechnologies), 2.5 µl of chromosomal or plasmid DNA (100-150 ng), 1 µM of each primer and 0.5 µl (2.5 U) of Kapa Taq polymerase (Kapa Biosystems). For larger concentrations of product, this recipe was increased to a total final volume of 50 µl with 25 µl of FailSafe™ PCR 2 × PreMix D, 2.5 µl of DNA, 5 µl of each primer and 2.5 µl of Kapa Taq polymerase. This mixture was made up to the final volume of 50 µl with dH_2O. For PCR products to be used in cloning, the Kapa Taq polymerase was replaced with an equal volume of the proof-reading enzyme BIO-X-ACT™ long DNA polymerase (Bioline). This enzyme possesses 5′-3′ polymerase activity and 3′-5′ proofreading activity and significantly reduces the error rate in PCR reactions.

2.6.3. PCR standard programme

A G-STORM GS1 thermal cycler was used for all PCRs. The following conditions were employed as the standard programme. To begin, a hot-lid start was used to prevent internal condensation of samples in the reaction tubes. An initial denaturing step was performed at 94^{o}C for 2 minutes. A thirty repeat sequence of three steps followed this initial denaturing step. These were; a 1 minute denaturing step at 94^{o}C, followed by a 1 minute annealing step at 50^{o}C and then a 3 minute elongation step at 72^{o}C. A final 4 minute elongation step at 72^{o}C was performed to ensure complete PCR product elongation.

2.6.4. Colony PCR

E. coli colonies were taken directly from a LA plate using sterile 200 µl pipette tips and dipped into 10 µl reaction mixes (5 µl of FailSafe™ PCR 2 × PreMix D, 2.5 µl of dH_2O, 1 µM of each primer and 0.5 µl (2.5 U) of Kapa Taq polymerase). PCR reactions were then run in a G-STORM GS1 thermal cycler using the standard programme but with an initial denaturing step of 10 minutes in order to lyse the cells.

2.6.5. Failsafe PCR

The TripleMaster PCR system (Eppendorf) was used for some PCRs. Two reaction tubes were set up. Tube 1 contained 16 µl dH_2O and 2 µM of each primer. Tube 2 contained 23 µl dH_2O, 5 µl buffer, 5 µl magnesium solution, 1 µl dNTP solution and 0.5 µl Failsafe Taq polymerase. These tubes were then mixed and 3 µl (250-350 ng) of template DNA was added. Using a thermal cycler, the PCR programme started with an initial denaturing step at 94^{o}C for 2 minutes. A thirty repeat sequence of three steps followed this initial denaturing step. These three steps were; a 20 second denaturing step at 94^{o}C, a 15 second annealing step at 50^{o}C then a 45 second elongation step at 72^{o}C. A final 10 minute elongation step at 72^{o}C was performed to ensure complete PCR product elongation.

2.6.6. TA-cloning with pCR®2.1 vector

All TA-cloning experiments were performed with The Original TA Cloning® Kit (Invitrogen) which uses the PCR® 2.1 vector. The standard protocol was followed and blue/white screening was employed using LB agar X-Gal plates as previously described.

2.6.7. DNA sequencing

Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The pre-mix terminator dye was diluted 1:8 with 5 × sequencing buffer (Applied Biosystems) before use. Sequencing reactions comprised of 4 µl diluted terminator pre-mix, 100-150 ng template DNA and 1 µM of primer in a final total volume of 10 µl in distilled water. Thermal cycler conditions were 29 repeated cycles at; 96 ºC for 10 seconds, 50 ºC for 10 seconds and 4 minutes at 60 ºC. After completion, 1 µl of 2.2% (w/v) SDS was added to the reaction which was then heated to 98^{o}C for 5 minutes in a thermal cycler and then cooled to room temperature. Unincorporated dye and excess primers were removed from the reaction using Performa® Gel Filtration Cartridges (Edge BioSystems) following the manufacturer's instructions. The purified reactions were analysed by the University of Leicester's Protein and Nucleic Acid Chemistry Laboratory (PNACL) using an Applied Biosystems 3730 sequencer.

2.7. Transformations and transductions

2.7.1. *E. coli* transformation with plasmid DNA

E. coli TOPO-10 cells were used to inoculate 100 ml pre-warmed LB broth in a 500 ml conical flask at a 1:100 dilution from an LB overnight of 10 ml. The 500 ml culture was grown with shaking until an $OD_{595\ nm}$ of between 0.5-0.6 was reached (about 3 hours). The flask was then plunged into ice and gently swirled to halt growth. Once the culture was chilled, the flask was split into two falcon tubes and centrifuged for 10 minutes at 3,184 × g. Four sequential washes were then performed whilst keeping cells and solutions on ice at all times. These four washes were as follows; two in 50 ml dH_2O, one in 25 ml 10% (v/v) glycerol and one in 5 ml 10% (v/v) glycerol. After each wash,

the cultures were centrifuged at 3,184 × g for 10 minutes. After the final wash, the cells in the two falcon tubes were combined and re-suspended in 500 µl 10% (v/v) glycerol and aliquoted in 50 µl volumes into eppendorf tubes. These competent cells were stored at -80^{o}C until required.

To transform competent TOP0-10 *E. coli*, the plasmid or plasmid construct to be transformed was first dialysed for 30 minutes by aspirating 30 µl onto a 0.025 µm 13 mm nitrocellulose disc (Millipore) floating on dH_2O in a Petri dish. For each transformation reaction, a 50 µl aliquot of competent cells was defrosted on ice. The DNA and the cells were mixed and allowed to rest for ten minutes on ice. This solution was aspirated into a 2 mm electroporation cuvette (Geneflow) and then pulsed briefly in a Biorad gene pulser^{TM} at 1.5 kV, 1000 Ω and 25 µF. The expected time constant was 24. Immediately following this, 1 ml of 37^{o}C SOC media was added and the solution transferred to an eppendorf and incubated at 37°C for 50 minutes. 100 µl of the transformation was then spread onto several replicate LA plates with the appropriate antibiotics. A control transformation was performed which lacked DNA and this was spread onto a LA plate which contained no antibiotics. This control established that the *E. coli* cells survived the electroporation process.

2.7.2. *S. aureus* transformation with plasmid DNA (Kraemer & Iandolo, 1990)

The procedure used to create electrocompetent *S. aureus* RN4220 cells was the same as the *E. coli* procedure previously described but here, the *S. aureus* RN4220 were initially grown in TSB and the washes performed in cold 0.5 M filter sterilised sucrose. To transform competent RN4220 cells, the DNA was dialysed as previously described, before being mixed with an aliquot of competent RN4220 cells and incubated for

30 minutes at room temperature. This solution was aspirated into an electropoation cuvette and then pulsed briefly in an electroporator at 2.5 kV, 200 Ω and 25 μF. The expected time constant was 4.8. Immediately following this, 900 μl of room temperature SMMP50 media was added and the solution transferred to a 15 ml Sterilin tube and incubated with shaking at 37°C for three hours. 100 μl of the transformation was then spread onto several replicate LB plates with the appropriate antibiotics. A control reaction was also performed on competent cells with no additional DNA.

2.7.3. *S. aureus* transduction: phage lysate production

The donor strain was plated onto LB agar containing appropriate antibiotics and grown at 37°C. This plate was used to inoculate 5 ml LB broth with appropriate antibiotics. 25 ml LB broth was sub-cultured with the overnight to an $OD_{595\ nm}$ of 0.05. To this, 0.25 ml 1 M $MgSO_4$ and 0.2 ml 1 M $CaCl_2$ were added and this solution incubated with shaking at 37°C until an $OD_{595\ nm}$ of 0.2 was reached (30 minutes to 2 hours). 10 ml of this culture was sub-cultured into fresh pre-warmed 25 ml LB broth, again with the previously added salts. Next 1 ml LB suspended phage phi11 (Cheung *et al*., 1992) was added and this culture incubated with shaking for 4^+ hours at 37°C or until full lysis had occurred. After centrifugation for 10 minutes at 3,184 × g, the culture was filter sterilised and stored at 4°C. Phage titre was checked by spotting 10 μl of serial 10 fold dilutions of the phage lysate onto a lawned LB plate of RN4220 and incubating at 37°C overnight.

2.7.4. *S. aureus* transduction

The recipient *S. aureus* strain was plated onto LB agar with the appropriate antibiotics and grown overnight at 37°C. This culture was used to inoculate 20 ml LK broth which

was grown with shaking at 37°C overnight. This culture was centrifuged at 3,184 × g for 5 minutes and re-suspended in 1 ml LK broth. Both a reaction tube and control tube were set up for each transduction as follows:

Sample	**Control**
500 µl cells	500 µl cells
1 ml LK + 10 mM $CaCl_2$	1.5 ml LK + 10 mM $CaCl_2$
500 µl donor phage lysate	

These two cultures were incubated statically at 37°C for 25 minutes and then with shaking at 37°C for 15 minutes. 1 ml ice cold 0.02 M sodium citrate was then added on ice and the reactions transferred to an eppendorf tube and centrifuged at 11,300 × g for 10 minutes. The supernatant was removed and the remaining pellet re-suspended in 1 ml ice cold 0.02 M sodium citrate and left for 2 hours on ice. 100 µl of the transduction was spread onto LK plates with appropriate antibiotics in triplicate (3 plates per reaction) and these were incubated for up to 72 hours at 37°C.

2.8. Strain construction

2.8.1. Mutant creation by inverse PCR

The same procedure was used to construct both the *lysR* and *csoR* mutant strains. Identical restriction enzymes were employed whilst the primers used are shown in (Table 2.3). First the gene and flanking regions were amplified with BIO-X-ACT™ long (Bioline) using Newman total DNA preparations as the template and the primers

CsoR-puc-F and CsoR-puc-R for *csoR* and LysR-puc-F and LysR-puc-R for *lysR*. The amplified PCR products were digested with *Sac*I and *Xba*I, ligated into pUC19 to create pUC19$_{csoR}$ and pUC19$_{lysR}$, and transformed into *E. coli* TOPO10 electrocompetent cells (Invitrogen). Recombinants were screened using X-Gal and selected with ampicillin and a successful cloned plasmid was sequenced. pUC19$_{csoR}$ and pUC19$_{lysR}$ were then used as the templates in two inverse PCR reactions with BIO-X-ACT™ long (Wren *et al*., 1994) using the primers CsoR-inv-F and CsoR-inv-R for *csoR* and LysR-inv-F and LysR-inv-R for *lysR*, which were subsequently digested with *Kpn*I and *Bam*HI. The primers Tet-F and Tet-R were used to amplify the *tet* cassette (minus the *tet* terminator) using pDG1515 as the template which was subsequently digested with *Kpn*I and *Bam*HI, ligated with the digested pUC19$_{csoR}$ and pUC19$_{lysR}$ to create pUC19$_{csoR::tet}$ and pUC19$_{lysR::tet}$ and transformed into *E. coli* TOPO10 electrocompetent cells. Recombinants were selected with ampicillin and tetracycline.

The *tet* cassette and *csoR* or *lysR* flanking regions were excised from pUC19$_{csoR::tet}$ and pUC19$_{lysR::tet}$ by digestion with *Eco*RI and *Sal*I, and ligated into pMAD to create pMAD$_{csoR::tet}$ and pMAD$_{lysR::tet}$ which were subsequently transformed into *E. coli* TOPO10 electrocompetent cells at 30^{o}C. Recombinants were screened using X-Gal and selected with ampicillin and tetracycline and a successful cloned plasmid was sequenced. pMAD$_{csoR::tet}$ and pMAD$_{lysR::tet}$ were then transformed into *S. aureus* RN4220 electrocompetent cells as previously described. Recombinants were screened using X-Gal and selected with erythromycin and tetracycline. The method described by Arnaud *et al*. (2004) was used to introduce the cloned *csoR::tet* or *lysR::tet* mutation into the RN4220 chromosome by homologous recombination. Colonies in which a double crossover event had occurred were selected through tetracycline resistance,

erythromycin sensitivity and screened by a white colour when grown on X-Gal plates. The resulting strains (RN4220 *csoR* and RN4220 *lysR*) were confirmed as containing the *csoR::tet* mutation using primers CsoR-flank-F and CsoR-flank-R or the *lysR::tet* mutation using primers LysR-flank-F and LysR-flank-R in PCR reactions using total DNA preparations of the potential mutants as the template. In addition, CsoR-inv-F, CsoR-inv-R, Tet-F and Tet-R were employed to confirm the correct construction of the *csoR::tet* in RN4220 *csoR*, while LysR-inv-F, LysR-inv-R, Tet-F and Tet-R were employed to confirm the correct construction of the *lysR::tet* in RN4220 *lysR*.

The *csoR::tet* mutation was transferred from RN4220 *csoR* to the *S. aureus* strains ATCC 12600, Newman and PM64 by transduction with phage phi11. Colonies containing the *csoR* mutation were again confirmed by PCR using primers CsoR-flank-F and CsoR-flank-R and total DNA preparations of the potential mutants as the template DNA. The *lysR::tet* mutation was similarly transferred from RN4220 *lysR* to the *S. aureus* strain Newman by transduction with phage phi11. Colonies containing the *lysR* mutation were again confirmed by PCR but using primers LysR-flank-F and LysR-flank-R and total DNA preparations of the potential Newman mutant as the template.

2.8.2. Re-introduction of wild type copies of the mutated genes

The same approach was used to re-introduce wild type copies of the mutated genes into both the *lysR* and *csoR* mutant strains. Primers CsoR-comp-F and CsoR-comp-R were used to amplify *csoR* and downstream gene (NWMN_1990) from a Newman total DNA preparation using BIO-X-ACT™ long (Bioline). Similarly, primers LysR-comp-F and LysR-comp-R were used to amplify *lysR* from a Newman DNA preparation also using

BIO-X-ACT™ long (Bioline). The PCR products were digested with both *Eco*RI and *Bam*HI, ligated into pMK4 to create pMK4$_{csoR}$ and pMK4$_{lsyR}$ and transformed into *E. coli* TOPO10 electrocompetent cells. Recombinants were selected with ampicillin and successfully cloned plasmids were sequenced. pMK4$_{csoR}$ and pMK4$_{lsyR}$ were next transformed into *S. aureus* RN4220 electrocompetent cells as previously described. Recombinants were selected with chloramphenicol and confirmed as containing the correct plasmid by plasmid isolation and subsequent *Eco*RI and *Bam*HI digestion.

The pMK4$_{csoR}$ plasmid was transferred from RN4220 to the *S. aureus* strains Newman *csoR* and PM64 *csoR* by transduction as previously described and recombinants selected with both chloramphenicol and tetracycline. Strains were confirmed as being *csoR* mutants that contained pMK4$_{csoR}$ through plasmid isolation and subsequent *Eco*RI and *Bam*HI digestion and total DNA PCR using the CsoR-flank-F and CsoR-flank-R primers. The pMK4$_{lysR}$ plasmid was transferred from RN4220 to Newman *lysR* by transduction as previously described and recombinants selected with both chloramphenicol and tetracycline resistance. Strains were confirmed as being *lysR* mutants that contained pMK4$_{lysR}$ through plasmid isolation and subsequent *Eco*RI and *Bam*HI digestion and total DNA PCR using the LysR-flank-F and LysR-flank-R primers.

2.8.3. Creation of β-lactamase reporter constructs using the pSK5645 reporter plasmid

The β-lactamase reporter primers listed in Table 2.2 were used in a PCR using the TripleMaster PCR system (Eppendorf) as previously described and total PM64 DNA preparations as the template DNA. These products were subsequently T-cloned into the

pCR2.1 vector and transformed into electrocompetent *E. coli*. Positive transformants were selected by ampicillin resistance and blue/white X-Gal selection and screened by colony PCR using the M13 primers. Plasmid preparations were performed and the insert sequenced with the M13 primers. Inserts were excised from the pCR2.1 vector with *Sma*I and *Bam*HI, subsequently ligated into pSK5645 and transformed into electrocompetent *E. coli*. Transformants were selected with chloramphenicol and screened by colony PCR using the specific forward primer of the insert and pSK-R, which anneals inside the plasmid located β-lactamase gene. The reporter plasmids were then transformed into *S. aureus* RN4220 electrocompetent cells as previously described. Recombinants were selected with chloramphenicol and confirmed as containing the plasmid by plasmid isolation and subsequent *Sma*I and *Bam*HI digestion. Next, the plasmids were transferred from RN4220 to the *S. aureus* strain Newman by transduction as previously described and recombinants selected for chloramphenicol resistance. Strains were confirmed as containing the reporter plasmids through plasmid isolation and subsequent *Sma*I and *Bam*HI digestion.

2.9. Protein isolation from *S. aureus*

2.9.1. *S. aureus* protein extraction

A 10 ml CRPMI 17 hour culture grown at 37°C in 5% CO_2 and was centrifuged at 3200 × g for 10 minutes to pellet the cells. All supernatant was removed and the pellet re-suspended in 200 µl residual supernatant and transferred to a sterile pre-weighed eppendorf. The cells were centrifuged at 11,300 × g for 10 minutes and all remaining

supernatant removed by aspiration and discarded. The eppendorf containing the pellet was then re-weighed and the pellet weight determined and recorded.

2.9.2. SDS (sodium dodecyl sulphate) extraction of non-covalently bound surface proteins

Depending on the pellet weight, cells were re-suspended in 2% (w/v) SDS based on 200 µl of SDS per 100 mg of bacterial cells. The preparations were then boiled for 3 minutes and the samples immediately centrifuged for 5 minutes at 11,300 × g and all supernatant removed as the SDS extract. 10 µl of 2 × Laemmli loading buffer (20% (v/v) glycerol, 4% (w/v) SDS, 200 mM Tris pH 6.8, 2% (w/v) bromophenol blue, 200 mM dithiothritol) was added to 10 µl of SDS extract which was then boiled for 5 minutes, briefly centrifuged and loaded onto a 10% (w/v) separating SDS PAGE (poly-acrylamide gel electrophoresis) mini-gel. Cell pellets treated with SDS in this way could not be used for further protein extractions.

2.9.3. Cell wall protein extraction

Cells were re-suspended in 200 µl/100 mg of cells in 1 mg/ml benzamidine, 30% (w/v) raffinose and 80 µg/ml lysostaphin in PBS. This suspension was incubated at 37°C for 15 minutes. The supernatant containing the cell wall extract was removed to a fresh tube after centrifugation at 11,300 × g for 10 minutes. 10 µl of 2 × Laemmli loading buffer was added to 10 µl of cell wall protein extract and was boiled for 5 minutes, briefly centrifuged and loaded onto a 10% (w/v) separating SDS PAGE gel. The remaining protoplast pellet was used to extract the membrane and cytoplasmic proteins.

2.9.3. Membrane and cytoplasmic protein extraction

Protoplasts obtained from the removal of the cell wall proteins were re-suspended in PBS depending on the previously determined cell pellet weight, based on 200 µl PBS per 100 mg cells. Cells were left on ice for 10 minutes and then sonicated for a total of 10 minutes in a Biorupter sonicating waterbath (Diagenode) containing ice and using the high setting for 30 seconds on and 30 seconds off. The supernatant containing the cytoplasmic proteins was then removed to a clean tube after centrifugation at 11,300 × g for 10 minutes. 10 µl of 2 × Laemmli loading buffer was added to 10 µl of the cytoplasmic protein extract which was boiled for 5 minutes, briefly centrifuged and loaded onto a 10% (w/v) separating SDS PAGE gel.

The remaining protoplast pellet was re-suspended in 1 × Laemmli sample buffer depending on the previously determined cell pellet weight, based on 200 µl sample buffer per 100 mg of cells. This was boiled for 5 minutes, briefly centrifuged and loaded onto a 10% (w/v) separating SDS PAGE gel.

2.10. SDS PAGE protein gels

2.10.1. 10 × SDS PAGE running buffer

30.2 g Tris, 144 g glycine and 10 g SDS were dissolved in 1 l of dH_2O and stored at room temperature. The pH was checked but not adjusted if found to be between 8 and 8.5. The buffer was diluted to 1 × just before use in electrophoresis using dH_2O.

2.10.2. SDS buffer A

45.2 g Tris and 1 g SDS was dissolved in 500 ml water. The pH was adjusted to 8.8 with HCl and the buffer stored at room temperature.

2.10.3. SDS buffer B

15.1 g Tris and 1 g SDS was dissolved in 500 ml water. The pH was adjusted to 6.8 with HCl and the buffer stored at room temperature.

2.10.4. Coomassie brilliant blue stain (100 ml)

0.25 g Coomassie R250 (Sigma) was dissolved in 90 ml 50% (v/v) methanol and 10 ml glacial acetic acid. This was stored at room temperature.

2.10.5. SDS de-stain

SDS de-stain was 7.5% (v/v) glacial acetic acid and 20% (v/v) methanol in water.

2.10.6. SDS PAGE mini-gels

Proteins were separated on a SDS mini-gel by electrophoresis (SDS-PAGE). 5.5 ml of 10% (w/v) separating gel was prepared by mixing 2.7 ml of buffer A, 1.83 ml of acrylamide mix (37.5: 1 acrylamide:bisacrylamide), 190 µl 1% (w/v) APS (ammonium persulphate) and 765 µl dH_2O. 15µl TEMED (N,N,N',N'-tetramethylethylenediamine) was added directly before pouring the gel. The surface of the gel was then overlaid with dH_2O until polymerisation occurred, after which the water was decanted and the excess removed carefully with blotting paper. A 5% (w/v) stacking gel was poured on top of the separating gel layer into which the comb was placed to create the wells. 2 ml 5%

(w/v) stacking gel consisted of 330 µl acrylamide mix (as above), 1 ml Buffer B, 50 µl 1% (w/v) APS and 616 µl dH2O. 10 µl TEMED was added just before pouring the solution. After polymerisation of the stacking layer, the wells were washed out with dH2O and the protein samples loaded after boiling with Laemmli sample buffer as previously described. To estimate band sizes two ladders were employed (FAVORGEN); either a pre-strained single colour protein ladder or a pre-strained dual colour protein ladder.

The gel was run in 1 × SDS-PAGE running buffer at constant 20 mA for approximately 30-40 minutes. Protein bands were visualised by overnight staining in 200 ml Coomassie stain followed by sequential washes in SDS-PAGE de-staining solution to remove non-specific staining. Bands of interest were sequenced using the MALDI-TOF (Matrix Assisted Laser Desorption Ionisation - time-of-flight) mass-spec system by the Protein and Nucleic Acid Chemistry Laboratories (PNACL) of Leicester University.

2.11. Nitrocefin reporter assay (Grkovic *et al*., 2003)

5 ml SSD medium was inoculated with reporter strains and this suspension vortex mixed until the cells were fully in suspension. The $OD_{595\ nm}$ was determined and recorded and this suspension was used to inoculate several 10 ml cultures of SSD to an $OD_{595\ nm}$ of 0.05. These contained the appropriate antibiotics and the supplements to be assayed. The cultures were grown with shaking for 17 hours at 37°C. After this incubation, the $OD_{595\ nm}$ was determined and recorded, the cultures centrifuged at 3200 × g for 8 minutes and all the supernatant removed by decanting. The remaining cell pellets were washed in 50 mM sodium phosphate buffer (pH 6.5) and again

centrifuged at 3200 × g for 8 minutes. Next, all supernatant was removed by decanting and the pellets re-suspended in 700 µl of 50 mM sodium phosphate buffer (pH 6.5).

In an eppendorf tube, 300 µl of the cell suspension (neat or diluted depending on promoter strength of the reporter promoter) was mixed with 200 µl 50 mM sodium phosphate buffer (pH 6.5) and 200 µl of 37°C working stock nitrocefin solution. Nitrocefin (Oxoid) solution was made through mixing the supplied nitrocefin powder (1 mg) with the supplied rehydration fluid (1.9 ml) and this 500 µg/ml stock solution was stored at -20°C for up to two weeks. The 125 ug/ml nitrocefin working solution was made on the same day as the assay in 50 mM sodium phosphate buffer (pH 6.5). A control reaction was made in which 300 µl of 50 mM sodium phosphate buffer (pH 6.5) replaced the cell suspension. Immediately after the addition of the nitrocefin solution, the time was recorded and the tubes incubated at 37°C for 10 minutes to one hour depending on the promoter activity of the gene being assayed. The samples were then centrifuged at 11,300 × g for 10 minutes and two 200 µl volumes of the supernatant removed and loaded into a round bottomed 96 microwell plate (Nunclon) and the $OD_{490\,nm}$ determined using a Bio-Rad 3550 Microplate reader. The control reaction containing no cell suspension was used as a blank to calculate the final reading of OD_{490nm} for each experimental reaction. This reading was equalised to account for minor growth differences by using the initial $OD_{595\,nm}$ reading of the 10 ml overnight culture. For the calculation of enzyme units the following equation was used:

$$\frac{\left(\frac{\Delta A}{\Delta T}\right)}{\varepsilon} \times Vf \times 1000 \times \frac{Vt}{Vs} = \text{Enzyme activity } \mu\text{mol min}^{-1}$$

ΔA = Change in absorbance $OD_{490\ nm}$
ΔT = Time taken for change in absorbance
ε = Molar extinction coefficient variation
Vf = Final volume of sample in which $OD_{490\ nm}$ taken
Vt = Total volume of reaction sample
Vs = Volume of nitrocefin 125μg/ml used

This equation converts the $OD_{490\ nm}$ results of the nitrocefin assay into enzyme units, measured as μ/mol of nitrocefin used per minute. The ΔA values were corrected for growth differences of the bacterial culture. Molar extinction coefficient variation of nitrocefin is 15000 M/cm.

2.12. Northern blot transcriptional analysis

2.12.1. DEPC treated water (RNase free)

In a fume hood, 0.1% (v/v) DEPC (di-ethyl-pyrocarbinate) (Sigma) was added to dH_2O in a Duran bottle and was shaken rigorously until the DEPC was fully in suspension. This bottle was left overnight in a fume hood and with the lid loose to allow the DEPC to evaporate. The next day, the DEPC treated water was autoclaved at 120^{o}C for 15 minutes at 1 atmosphere pressure to destroy any residual DEPC. This DEPC treated water was used to make all solutions which were required to be RNase free.

2.12.2. AE buffer (RNase free)

50 mM sodium acetate pH 5.3 was mixed with 10 mM EDTA in 500 ml DEPC treated water.

2.12.3. 10 × 3-(N-Morpholino)propanesulfonic acid (MOPS) (RNase free)

MOPS was created through the addition of the following reagents to give the stated final concentrations: 0.2 M MOPS pH 7.0 (Sambrook), 50 mM sodium acetate and 1 mM EDTA in DEPC treated water.

2.12.4. Sephadex column elution buffer (50 ml)

Sephadex column elution buffer was made through the addition of the following reagents to give the stated final concentrations: 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1% (w/v) SDS.

2.12.5. 20 × Sodium chloride sodium citrate (SSC) solution

20 × SSC was made through the addition of the following reagents to give the stated final concentrations: 0.3 M tri-sodium citrate and 3 M sodium chloride in high purity dH_2O, pH 7.0.

2.12.6. Church and Gilbert's buffer (1 litre)

51.11 g Na_2HPO_4, 21.84 g NaH_2PO_4 and 70 g SDS were dissolved in 998 ml dH_2O plus 2 ml 0.5 M EDTA. Buffer was heated to 65^{o}C before use.

2.12.7. 5 × RNA loading buffer (RNase free)

5 × RNA sample loading buffer contained 200 µl of saturated bromophenol blue solution, 720 µl of 37% formaldehyde, 3.084 ml of formamide, 80 µl of 500 mM EDTA pH 8.0, 2 ml of glycerol and 10 ml of 10 × MOPS per 10 ml of RNase-free water.

2.12.8. Isolating RNA (Schmitt *et al*., 1990)

30 ml pre-warmed CRPMI or 20 ml pre-warmed LB was inoculated to an $OD_{595\ nm}$ at 0.1 from 10 ml cultures which had been incubated statically at 37°C in 5% CO_2 overnight. Metal supplements were added as required from fresh filter-sterilised stocks. CRPMI cultures were incubated statically for 6 hours while LB cultures were incubated for only 4 hours, both at 37°C in 5% CO_2. The $OD_{595\ nm}$ was then determined and recorded and the cultures centrifuged at 3200 × g for 10 minutes to pellet the cells. The supernatants were discarded and the pellets re-suspended in 1 ml RNAlater (Ambion) and these suspensions aspirated into 1.5 ml screw cap RNase free tubes (Sarstedt) and left at 4°C overnight. The following day, the tubes were centrifuged at 11,300 × g for 10 minutes and the supernatants removed and discarded. These cell pellets were stored at -80°C for at least 2 hours or until needed.

The pellets were re-suspended in 400 µl RNase free 10 mM Tris pH 8.5 which contained 30 µl 10 mg/ml RECOMB lysostaphin (AMBI) and after brief vortex mixing, were incubated at 37°C for 10-20 minutes until lysis occurred. 80 µl of 10% (w/v) SDS was added then and the samples vortexed for exactly 30 seconds followed by a brief centrifuging to prevent contamination when removing lids. To these, an equal volume (480 µl) of phenol/AE pH 4 (Fisher) was added and the tubes vortexed for 30 seconds and then centrifuged at 11, 300 × g for 10 minutes. The upper aqueous phase was

removed to a clean tube and the remaining solution discarded. This phenol/AE pH 4 step was repeated. To the clean aqueous phase, an equal volume of phenol:chloroform: IAA (25:24:1) was added and the samples vortexed for 30 seconds and then centrifuged at 11, 300 × g for 10 minutes. The upper aqueous phase was removed to a clean tube and mixed with twice the volume of 100% (v/v) ethanol and precipitated overnight at -20°C.

The next day, the RNA was pelleted by a 15 minute centrifugation at 11,300 × g and then washed once in 70% ethanol. After a 10 minute centrifugation at 11,300 × g, most of the supernatant was removed by aspiration and the remaining supernatant allowed to evaporate at room temperature for 1 hour. The pellet was re-suspended in 50 µl DEPC treated water and the concentration determined using a Thermo Scientific NanoDrop™ 1000 spectrophotometer. The purity of the RNA was then checked by gel electrophoresis and the RNA preparations stored at -80°C until required.

2.12.9. Formaldehyde denaturing gel electrophoresis for RNA

1.8 g agarose was dissolved in 130.5 ml DEPC treated H_20. 3 µl of 10 mg/ml ethidium bromide, 15 ml 10 × MOPS solution and 4.5 ml 37% (v/v) formaldehyde solution (Sigma) were mixed. Once the agarose cooled, the solution was added and mixed before being poured into a gel cassette free from RNases. The RNA preparations were mixed with DEPC water to give final concentrations of 20 µg RNA in 20 µl. 5 µl of 5 × RNA loading buffer was also added and these samples then incubated at 65°C for 10 minutes. The samples were loaded into the formaldehyde denaturing gel and run in 1 × MOPS buffer. The gel was run at 120 volts until the bands had migrated the required distance down the gel. The gel was removed and photographed using UV light in an UV imaging

system from Syngene to check RNA separation had occurred and loading was even. Sizes were estimated using 4 µl RNA ladder (Lonza).

2.12.10. Northern blotting

A plastic tray was filled with 20 × SSC to a depth of 2 cm. In the centre of this tray a plastic gel tray of the same size as the one used for the formaldehyde gel was placed upside down to act as a base. Onto this, a wick made from 3 mm chromatography paper (Whatman) was placed, which covered the length of the base and also dipped into the 20 × SSC at either end. The formaldehyde gel was placed onto this wick-covered base and the air bubbles removed. On top of the gel was placed a piece of nitrocellulose membrane (Osmonics Inc.) with the same dimensions as the gel which was pre-soaked in 2 × SSC. A piece of 3 mm paper was also soaked in 2 × SSC and placed onto the membrane and all air bubbles again removed. The surrounding wick and lower tray were then covered with Clingfilm to prevent direct contact between blotting paper and wick. Once only the 3mm paper covering the membrane was exposed, 20-30 paper blotting towels were stacked on top of this in order to start the blotting process. These towels were covered by a glass plate onto which a weight was added to aid blotting. After overnight blotting, the towels and 3 mm paper were removed and the gel and attached membrane transferred to a dry piece of 3 mm paper. The locations of the wells were marked onto the membrane and the gel removed. The membrane was cross-linked at 70,000 µJ/cm^{-2} using an Ultraviolet Cross Linker (Amersham Life Science).

2.12.11. Pre-hybridisation

The cross-linked membrane was placed in a glass hybridisation tube which was 1/3rd full of 65°C Church Gilberts buffer. The side of the membrane containing the RNA was

the side that was exposed to the buffer. The tube was placed in a dual hybridisation oven (Hybaid), turning gently at 65°C for a minimum of twelve hours.

2.12.12. Probe creation and probing procedure

A standard PCR was performed with specific probe primers and a Cycle Pure PCR clean up kit (E.Z.N.A) was used to purify the PCR product. This PCR product was diluted in dH_2O to give a concentration of 30 ng in 18 µl. The sample was denatured by boiling for 10 minutes followed by incubation on ice for 5 minutes and 1 µl (10 ng) of BSA (New England Biolabs), 1 µl Klenow fragment of DNA Pol I (USb), 5 µl of Oligolabelling buffer and 2.5 µl of α-^{32}P dCTP (specific activity of 0.925 Mbq) were added and the reaction incubated at 37°C for two hours. Oligolabelling buffer was created from 3 solutions; A, B and C in a ratio of 2:5:3. Solution A contained 1 ml Solution O (1.25 M Tris-Cl, pH 8.0 and 0.125 M magnesium chloride), 18 µl β-mercaptoethanol and 5 µl of dATP, dTTP and dGTP (100 µM each). Solution B contained 2 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 6.6 and Solution C contained random hexamers (GE Healthcare) re-suspended in TE pH 8.0 at a concentration of 90 $OD_{595\ nm}$ units/ml. The random hexamers acted as primers in the labelling reaction (Feinberg & Vogelstein, 1983).

Unincorporated nucleotides were then removed using Illustra NICK™ columns (GE Healthcare) as directed by the manufacture and using elution buffer to equilibrate the column and to recover the probe. The solution recovered from the column which gave the highest count of radioactivity was used as the probe. This probe was heated for 10 minutes to 95°C in a heating block followed by incubation on ice for 10 minutes.

Next, the probe was added to the glass hybridisation tubes containing the membranes in Church Gilberts buffer and this was hybridised overnight with gentle turning at 65°C.

2.12.13. Washing blots

The blot was washed with 65°C 3 × SSC/0.1% (w/v) SDS, repeated three to five times depending on the efficiency of the wash. Once removed wash buffer contained low radioactivity, the blot was ready for autoradiography.

2.12.14. Autoradiography

The blot was removed from the tube using tweezers and placed onto a similar sized piece of 3 mm paper soaked in 3 × SSC/0.1% (w/v) SDS and wrapped in a layer of Clingfilm with the edges sealed with tape. The membrane was transferred into a cassette containing intensifier screens and a piece of X-ray film (Super RX Fuji medical X-ray film) and exposed at -80°C for 3-18 days depending on the strength of the bound probe and the blots were then developed.

2.12.15. Stripping

The blots were stripped of probes by washing with two/three changes of boiling 0.1% (w/v) SDS for approximately 15 minutes followed by a final rinsing with 65^{o}C 2 × SSC. The blots were then pre-hybridized as previously described and again probed.

Chapter 3. Novel iron regulation in *S. aureus*

3.1. Introduction

Most cases of *S. aureus* Fur regulation have been observed to act in the classical way, through Fur binding to specific DNA promoter sites in high iron to repress target gene transcription. Similar to other bacterial species, *S. aureus* Fur has also been shown to non-classically regulate gene transcription. In total there have been four distinct types of Fur regulation observed in *S. aureus* which are only fully distinguishable from one another through comparing iron dependant regulation in both a wild type and a *fur* mutant. These four different mechanisms are shown in Figure 3.1, along with an identified example. Classical Fur regulation is highlighted and is found for numerous *S. aureus* genes, such as the siderophore uptake *sstA* gene (Morrissey *et al*., 2000). The *S. aureus* Fur was also found to repress the expression of glyceraldehyde-3-phosphate dehydrogenase (*gapA*) in low iron (Figure 3.1D) as *gapA* expression is induced in high iron and constitutive in a *fur* mutant (unpublished data). The *S. aureus* Fur has also been shown to activate gene transcription. Reporter assays using a *lacZ* reporter fusion found that the catalase gene (*katA*) had reduced expression in a *fur* mutant background but was iron induced in a wild type background (Figure 3.1B) (Horsburgh[2] *et al*., 2001). Conversely, Fur activates *eap* and *emp* expression in low iron where expression is lost in a *fur* mutant (Figure 3.1C) (Johnson *et al*., 2008). However, the mechanisms involved in these examples of novel *S. aureus* Fur regulation are currently unknown. In addition, there is evidence from studies into *in vitro* biofilm formation for a Fur independent iron responsive regulator in *S. aureus* (Johnson *et al*., 2005)

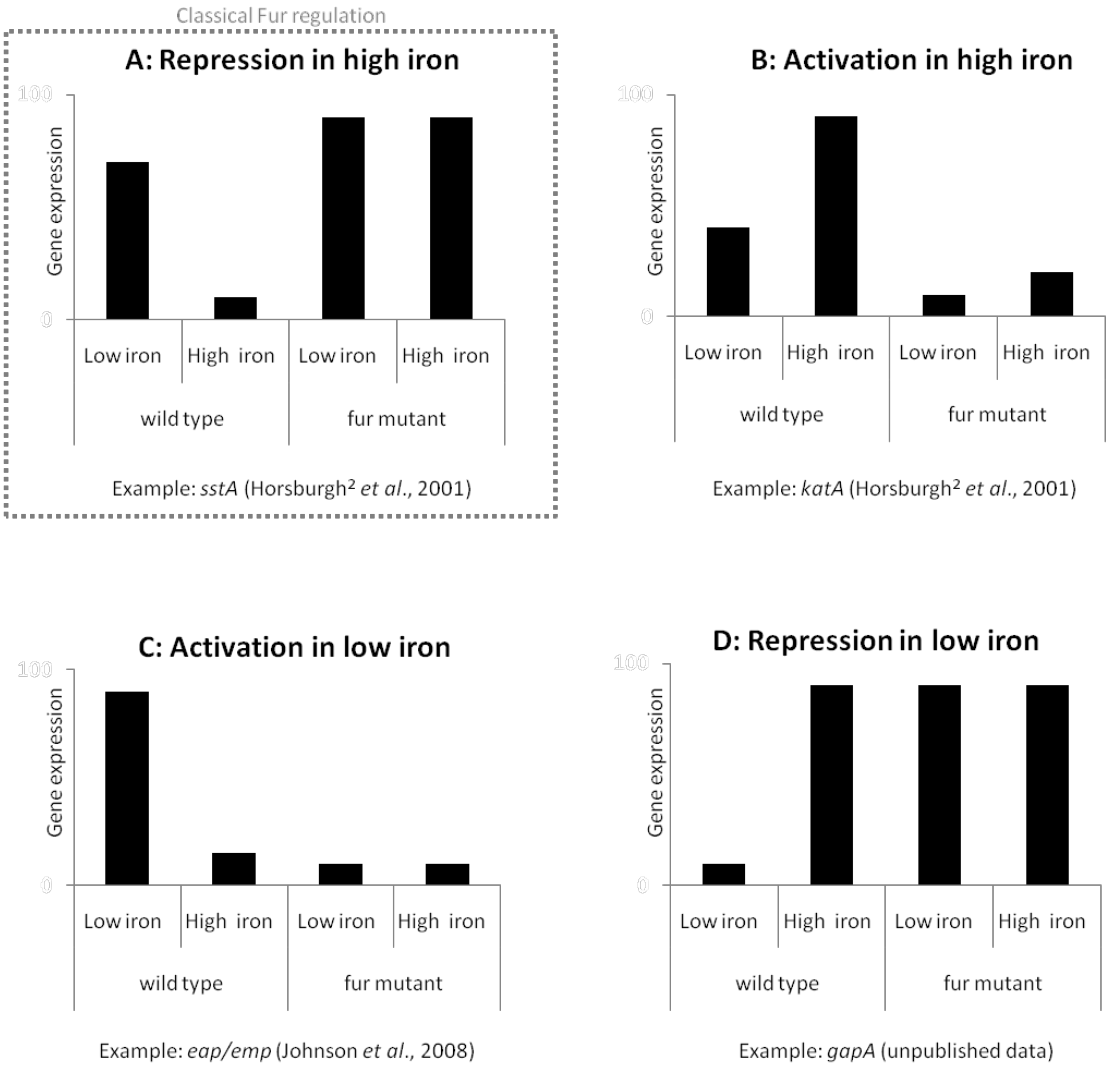


Figure 3.1. Representative graphs showing the four types of Fur regulation observed in *S. aureus*. Classical Fur regulation is highlighted and an example is given for each of the four types of Fur/iron regulation that have been observed. The relative gene expression low and high iron is shown for both a wild type and a *fur* mutant *S. aureus* strain. *y*-axis represents relative gene expression.

Potentially, novel *S. aureus* Fur regulation could be through direct Fur-promoter binding to either activate or repress target genes in high or low iron. However, the non-classically Fur regulated *S. aureus* genes have been reported to not contain promoter located Fur boxes. Alternatively, this novel regulation of Fur target genes may be through another factor such as a small RNA or protein regulator. Although *S. aureus* has been found to express several small RNAs (Pichon & Felden, 2005), none has been implicated in iron/Fur regulation. The role of sRNAs in *S. aureus* iron/Fur regulation is currently being investigated by a colleague. Therefore, the aim of this chapter was to identify any protein regulator whose expression is under the control of Fur and/or iron and determine this second regulator's role in novel Fur regulation.

3.2. Results

3.2.1. Identification of targets for the reporter assay

A hypothesis for novel *S. aureus* iron regulation is that Fur acts through another regulatory protein. Consequently, regulators under the control of Fur needed to be identified and two different methods were chosen to provide targets for further investigation: a bioinformatic search for genes with upstream Fur boxes and published reports showing known regulators potentially under the control of iron/Fur. The regulation of putative iron regulators was investigated using reporter assays, as they allowed for quantitative data of gene expression to be collected in a variety of conditions and strain backgrounds. Initially to identify potential Fur regulated regulatory proteins for the reporter assay, a bioinformatic search was performed to find regulator genes with an upstream Fur box. Horsburgh[2] *et al*. (2001) identified the 19 bp *S. aureus* Fur box consensus sequence by comparing the putative Fur boxes from

several known classically Fur regulated *S. aureus* operons, including *fhuCBD* and *sstABCD*. Therefore, a genome-scale DNA-pattern search using this 19 bp sequence was performed on the RSAT database. The RSAT data base allows for the detection of regulatory signals in non-coding sequences of published genomes. Three searches were performed using the published sequences of two *S. aureus* strains and partial sections of the Fur box consensus sequence. This was because initial searches identified no matches using the entire Fur box sequence, even allowing for one base pair substitution.

Searches 1 and 2 used the MRSA252 sequence whilst search 3 used the N315 sequence. The sections of the Fur box consensus sequence chosen for the three searches are shown in Figure 3.2A. These searches identified genes which contained these partial Fur box sequences upstream of their transcriptional start sites. Genes detected more than once from the same upstream Fur box were only included once. Also, genes which contained the putative Fur box more than 100 bp from their +1 site were removed, as these putative Fur boxes will be located outside of the region that is bound by RNA polymerase and so their transcription will be unlikely to be blocked by Fur-DNA binding. For search 1 this left 18 genes and for hypothetical genes, the NCBI and BLAST databases were used to find putative functions using their annotated sequence (Figure 3.2B). After similar editing, search 2 produced a list of 21 genes (Figure 3.3). Both these searches detected several well characterised Fur regulated genes including; *isdB*, *isdC*, and *fhuA*, which justified using partial sequences of the Fur box. Of the total 34 genes detected only one was a putative regulator of unknown function (Figure 3.3 SAR2652, highlighted in blue). This gene encodes a putative TetR family regulatory protein and therefore was subsequently designated *tetR*.

A

Search 1 - GATAATGATTATCATTATC

Search 2 - GATAATGATTATCATTATC

Search 3 - GATAATGATTATCATTATC

B

	Strand	Start	End	Gene	Known/putative function
1	D	-47	-36	*sirA*	lipoprotein
2	R	-64	-53	SAR0119	pyridoxal-phosphate dependent enzyme
3	R	-81	-70	SAR0177	hypothetical protein - cytoplasmic protein
4	D	-33	-22	SAR0265	hypothetical protein - methyltransferase
5	D	-64	-53	SAR0344	putative Sec-independent protein translocase protein
6	R	-98	-87	*fhuA*	ferrichrome transport ATP-binding protein
7	D	-40	-29	*sstA*	FecCD transport family protein
8	D	-53	-42	SAR0825	hypothetical protein - NAD dependent epimerase
9	D	-53	-42	SAR0907	hypothetical protein - FAD-dependent oxidoreductase
10	D	-49	-38	*isdB*	iron-regulated heme-iron binding protein
11	R	-75	-64	*isdA*	iron-regulated heme-iron binding protein
12	D	-66	-55	*isdC*	putative surface anchored protein
13	D	-62	-51	*uppS*	undecaprenyl pyrophosphate synthetase
14	D	-42	-31	SAR2271	hypothetical protein - membrane transporter
15	R	-71	-60	SAR2272	hypothetical protein - aerobactin biosynthesis protein
16	R	-26	-15	SAR2368	putative ferrichrome-binding lipoprotein precursor
17	D	-67	-56	SAR2461	pyridine nucleotide-disulphide oxidoreductase protein
18	D	-38	-27	SAR2631	hypothetical protein - FeoA family protein

Figure 3.2. Bioinformatic search for putative *S. aureus* Fur boxes.

(A) The regions of the *S. aureus* Fur box consensus sequence used in the three genome-scale DNA-pattern searches on the RSAT database.

(B) Fur box search 1 using the MRSA252 sequence. Table shows the 18 identified genes, including; gene direction on the chromosome (D = Forward R = Reverse), distance from transcriptional start site, gene name and known function. Highlighted in red are putative functions of hypothetical genes found through performing a BLASTP search using amino acid sequences. Highlighted in yellow are genes identified in both searches 1 and 2.

	Strand	Start	End	Gene	Known/putative function
1	D	-40	-29	SAR0057	hypothetical protein
2	R	-104	-93	SAR0103	hypothetical protein - possible DNA helicase
3	D	-63	-52	SAR0344	putative Sec-independent protein translocase protein
4	R	-67	-56	SAR0467	putative acetyltransferase
5	R	-85	-74	SAR0503	peptidyl-tRNA hydrolase
6	D	-98	-87	*fhuA*	ferrichrome transport ATP-binding protein
7	R	-80	-69	SAR0766	glutamine amidotransferase class-I protein
8	R	-45	-34	*trpS*	tryptophanyl-tRNA synthetase
9	R	-92	-81	*purF*	amidophosphoribosyltransferase
10	R	-49	-38	*isdB*	iron-regulated heme-iron binding protein
11	R	-72	-61	*isdC*	putative surface anchored protein
12	R	-39	-28	SAR1113	ribonuclease HIII
13	R	-40	-29	SAR1303	hypothetical protein
14	D	-89	-78	SAR1875	hypothetical protein - possible alpha-hemolysin
15	R	-104	-93	SAR1917	enterotoxin
16	R	-88	-77	SAR2009	putative sodium:sulfate symporter
17	R	-73	-62	*int*	integrase
18	R	-62	-51	SAR2268	transport binding lipoprotein - ferrichrome transporter
19	D	-83	-72	SAR2513	6-carboxyhexanoate-CoA ligase
20	R	-38	-27	SAR2631	hypothetical protein - FeoA family protein
21	R	-47	-36	SAR2652	TetR family regulatory protein - transcriptional regulator

Figure 3.3. Fur box search 2 using the MRSA252 sequence. Table shows the final 21 identified genes, including; gene direction on the chromosome (D = Forward R = Reverse), distance from transcriptional start site, gene name and known function. Highlighted in red are putative functions of hypothetical genes found through performing a BLASTP search using the amino acid sequences. Highlighted in yellow are genes identified in both searches 1 and 2. Highlighted in blue are potential regulator proteins.

As only one potential target was found an additional search (search 3) was performed which used the *S. aureus* N315 published sequence and a less stringent sequence, the first 12 base pairs of the Fur box consensus sequence minus the first guanine base (Figure 3.2A). This identified 83 genes, which again included several genes known to be classically Fur regulated, such as; *isdA*, *isdB*, *isdC* and *fhuA* (Table A1, Appendix). Two potentially Fur-repressed regulator genes were also detected; SA0908 and SA2123 (Table A1, Appendix). A BLASTP search using their deduced amino acid sequences found that the SA0908 protein had no obvious homologues or putative functions. However, the SA2123 protein represented a LysR type transcriptional regulator which was highly conserved between *S. aureus* strains but which also had no putative function. This gene was subsequently designated *lysR*.

In addition to the three RSAT searches, published reports were examined for potential targets. Friedman *et al*. (2006) used two-dimensional difference gel electrophoresis to examine the changes in *S. aureus* global protein levels in response to changes in the bacterial iron status. They identified several regulators that appeared to be iron/Fur regulated including the two component regulator SrrA and the global regulator CodY, which were both positively regulated by iron and Fur. Also identified was the SOS response regulator LexA, which showed Fur regulation that was independent of iron. Therefore, these three regulators plus two from the Fur box searches were chosen for further investigation and are listed in Table 3.1.

Gene	Function	Source
srrA	2 component global regulator	Friedman *et al*. (2006)
codY	Global regulator - virulence and stationary phase genes	Friedman *et al*. (2006)
lexA	SOS response regulator	Friedman *et al*. (2006)
tetR	Putative hypothetical TetR-like regulator	Fur box search 3 (N315)
lysR	Putative hypothetical LysR type regulator	Fur box search 2 (MRSA252)

Table 3.1. Genes chosen for further investigation

3.2.2. pSK5645, reporter strain construction and reporter assay results

The reporter assay chosen to initially investigate the potential iron regulation of the five genes shown in Table 3.1 used the pSK5645 reporter plasmid created by Grkovic *et al*. (2003). This contains a promoterless *blaZ* gene upstream of a multi-cloning site which allows for the transcriptional activity of promoters cloned into this site to be measured in a β-lactamase assay. The β-lactamase reporter assay chosen employed nitrocefin, a chromogenic substrate which undergoes a colour change from yellow to red as the amide bond in its β-lactam ring is hydrolyzed by β-lactamase activity. This is detectable by reading the optical density at 490 nm.

A pSK5645 construct containing the promoter of the ABC transporter *opp1A* had been shown to be iron repressed and this was used as a control (personal communication from Dr Cockayne, Nottingham University). This gene was also detected in the Fur box search 3 (Table A1, Appendix). Primers were designed to amplify between 250 bp - 850 bp of the promoters and upstream regions of the genes listed in Table 3.1 (Figure 3.4).

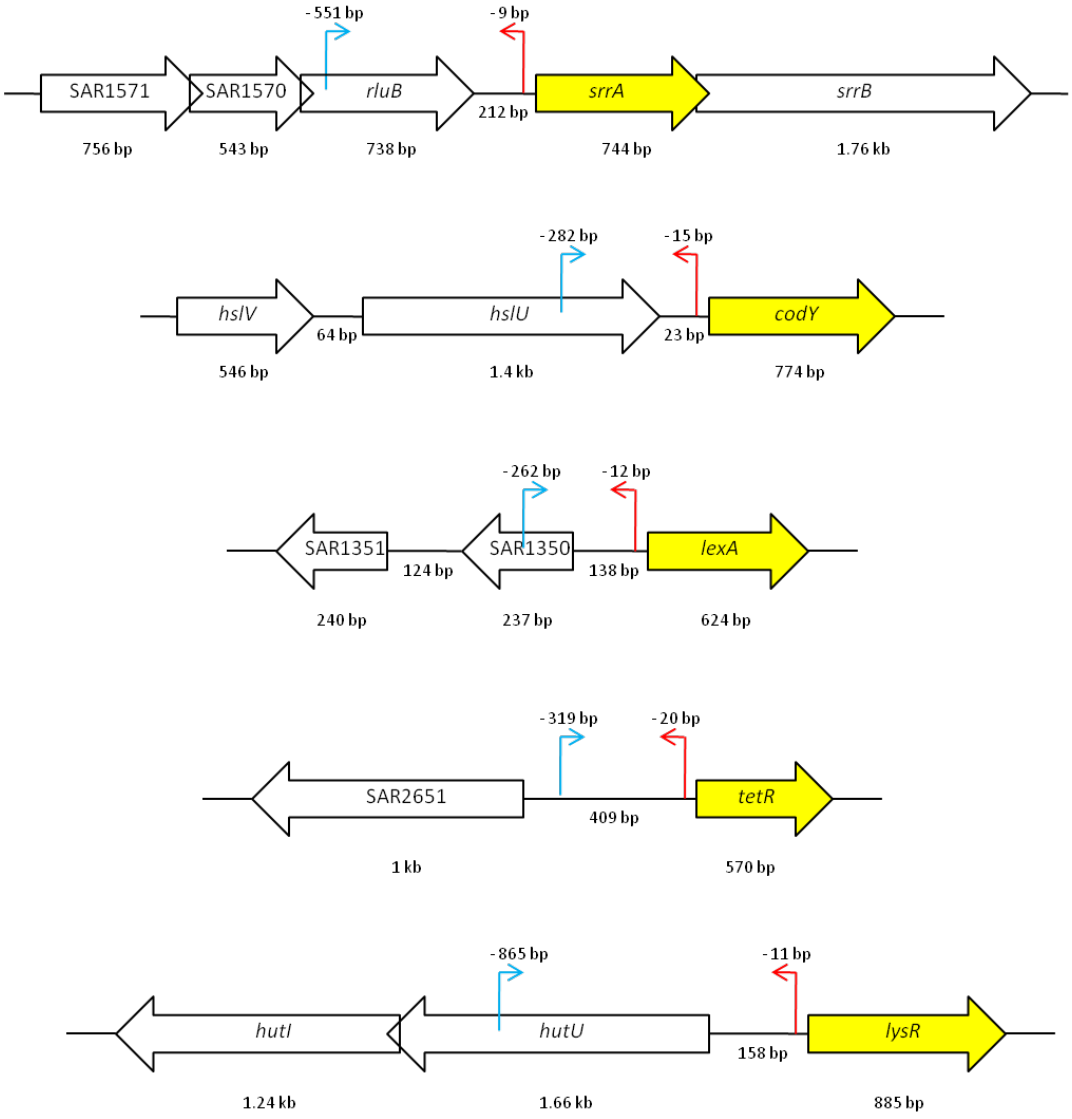


Figure 3.4. Region of target gene promoters cloned into the reporter plasmid pSK5645. Highlighted in yellow are the genes chosen for the transcriptional analysis. Upstream genes are shown with sizes. Putative ribosomal binding sites were identified and not included in the upstream region which was amplified by PCR. Coloured arrows represent the reverse (red) and forward (blue) primers used for the reporter construction, along with distances from the transcriptional start sites.

The target gene's ribosomal binding site (RBS) was not included in this fragment to avoid complications from posttranscriptional regulation as the *blaZ* gene in pSK5645 includes its own RBS (Grkovic *et al*., 2003). Construction of the reporter plasmids is fully described in section 2.8.3. The reporter constructs pSK5645$_{srrA}$, pSK5645$_{codY}$, pSK5645$_{lexA}$, pSK5645$_{lysR}$ and pSK5645$_{tetR}$ were transduced into *S. aureus* strain Newman. Also constructed was a control Newman strain which contained the reporter plasmid without a cloned gene promoter; Newman + pSK5645.

The Newman + pSK5645$_{opp1A}$ construct was used to test and refine the nitrocefin reporter assay. Initially, CRPMI was used to provide iron restrictive growth conditions as previous reports have used this medium to characterise *S. aureus* Fur/iron protein regulation (Morrissey *et al*., 2002). However, the assay was found to require substantial cell numbers and preliminary experiments failed to generate reproducible results using this minimal medium (data not shown). Therefore, CRPMI was replaced with the iron restrictive SSD medium, which permits almost 3-fold greater *S. aureus* growth than CRPMI (Lindsay & Riley, 1994). The Newman reporter strains were grown to stationary phase as the nitrocefin reporter assays by Grkovic *et al*. (2003) used a similar growth stage for their experiments. The optical densities at 595 nm were recorded and used to normalise different samples after the final assay measurement at 490 nm in order to account for minor growth differences. Enzyme activity was calculated using the equation shown in section 2.11.

The control Newman strain which contained only the pSK5645 plasmid showed a low basal level of enzyme activity and no significant iron regulation (Figure 3.5). Initial nitrocefin reporter assays using the pSK5645$_{opp1A}$ construct observed repression in

50 μM $Fe_2(SO_4)_3$, which subsequently will be termed Fe(50). The P-value for *opp1A* iron repression was 0.04 and this iron regulation correlated with previous experiments using this *opp1A* reporter construct (personal communication from Dr Cockayne, Nottingham University). Therefore, nitrocefin assays were performed using the Newman reporter constructs which were repeated on different occasions with different stocks of media, metals and fresh bacterial cultures. Due to variations in promoter strength, the results are shown as two separate graphs for easier visualisation. Copper responsive transcription was also analysed as a control transition metal, in order to confirm that any iron repression was iron specific and not a general response to extracellular metal.

In contrast to the previous publication which showed LexA and CodY protein iron regulation (Friedman *et al*., 2006), the *lexA* and *codY* reporter constructs showed no iron or copper regulation (Figure 3.6). However, the *srrA*, *tetR* and *lysR* Newman reporter constructs all showed statistically significant transcriptional iron repression. The *srrA* construct had a high level of transcription and was repressed by both Fe(50) (P = 0.028) and 50 μM $CuCl_2$, which subsequently will be termed Cu(50) (P = 0.003) (Figure 3.6A). While the *tetR* construct showed Fe(50) repression, no consistent results could be obtained when examining transcription in copper and therefore it was unknown if this repression was iron specific (Figure 3.6A). However, the *lysR* construct showed 2.66-fold repression in Fe(50) (P = 0.004) but no significant copper regulation (P = 0.276) (Figure 3.6B). Therefore, only *lysR* showed iron specific regulation and this putative LysR type transcriptional regulator (LTTR) was selected as the best candidate for a novel Fur controlled regulator for further investigation.

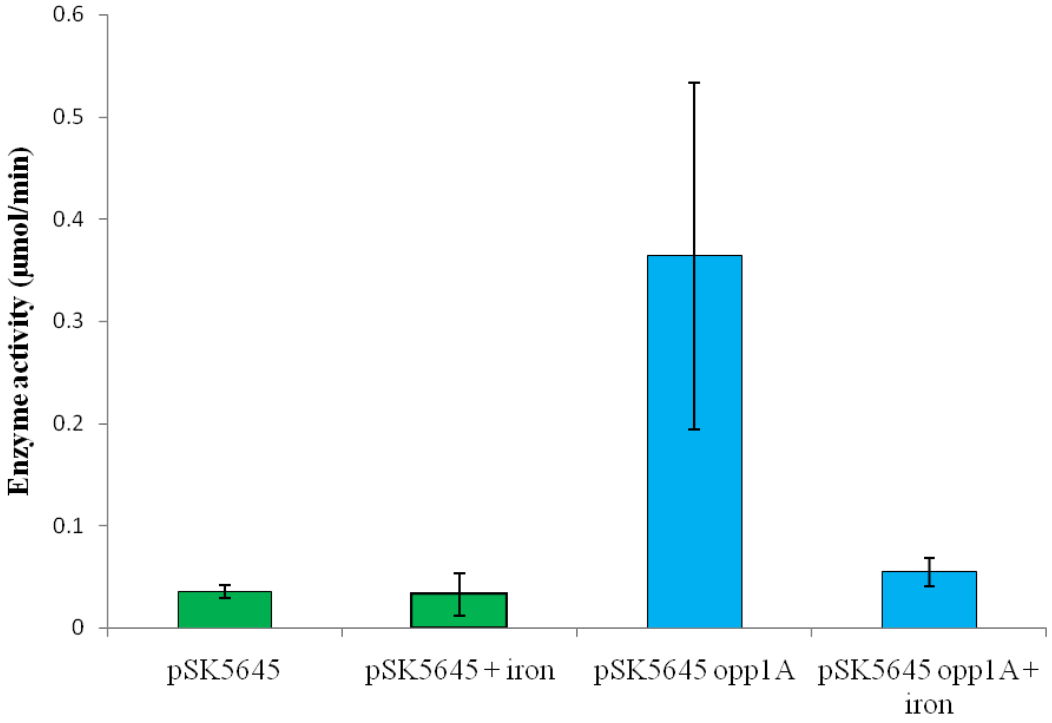
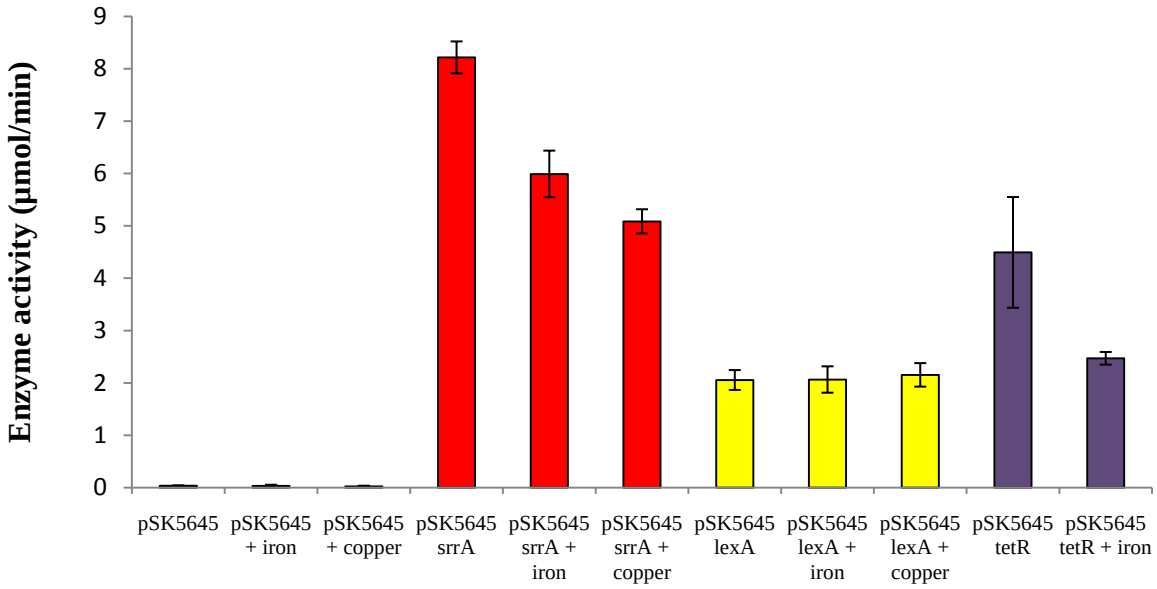


Figure 3.5. Reporter assay optimisation. Reporter Newman strains carrying the reporter plasmid were grown to stationary phase in SSD with or without 50 µM $Fe_2(SO_4)_3$. Shown are the pSK5645 control strain (green) and the pSK5645 *opp1A* construct (blue). *y*-axis represents the enzyme activity. Results shown represent the means and standard deviations of three independent experiments.

A



B

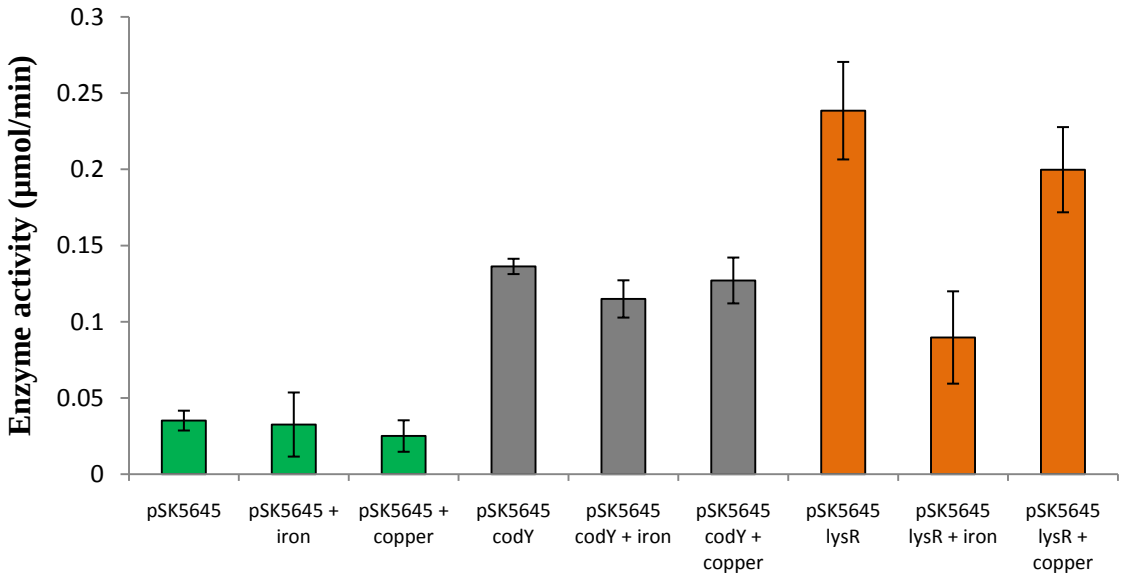


Figure 3.6. Nitrocefin reporter assays. Reporter Newman strains carrying the reporter plasmid were grown to stationary phase in SSD with no supplements, 50 µM $Fe_2(SO_4)_3$ or 50 µM $CuCl_2$. **(A)** pSK5645 control (green), pSK5645$_{srrA}$ (red), pSK5645$_{lexA}$ (yellow) and pSK5645$_{tetR}$ (purple). **(B)** pSK5645 control (green), pSK5645$_{codY}$ (grey) and pSK5645$_{lysR}$ (orange). *y*-axis represents the enzyme activity. Results shown represent the means and standard deviations of three independent experiments.

3.2.3. The LTTR family and the *S. aureus* LysR regulator

The LysR type transcriptional regulator (LTTR) family was first described by Henikoff *et al*. (1988) and now represents one of the largest groups of bacterial transcriptional regulators (Schell, 1993). There are examples of LTTRs which activate and repress their target genes and some have been identified that globally regulate diverse and unrelated sets of genes/operons. For example, LeuO of *Salmonella enterica* serovar Typhi is a well characterised LTTR which positively or negatively regulates seven proteins with different physiological functions; including some virulence factors (Hernández-Lucas *et al*., 2006). LTTRs often require a co-inducer for regulation, which is usually a product, substrate or intermediate of the biochemical pathway under their control.

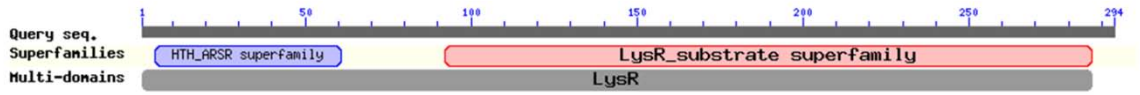
DNA-footprinting and DNase I-protection assays have shown that LTTRs typically bind to two separate upstream DNA regions of their targets which interact further to achieve the regulation (Belitsky *et al*., 1995; Porrúa *et al*., 2007). Also, the quaternary protein structure of an active LTTR protein is frequently a tetramer (Muraoka *et al*., 2003). Auto-regulation and DNA bending are common features of positive LTTR gene regulation. Typically, the regulator protein is always attached to target DNA. Upon LTTR and co-inducer interaction, the LTTR protein undergoes a conformational change leading to an alteration in the degree of DNA bending which subsequently allows the LTTR protein tetramer to interact with RNA polymerase to begin transcription (Maddocks & Oyston, 2008). Structurally, LTTRs possess a highly conserved N-terminal helix-turn-helix DNA-binding domain and a variable C-terminal co-inducer binding domain.

The 294 residue *S. aureus* LysR protein is highly conserved, with 99% homology between the sequenced strains. The variation is due to one, or sometimes two, amino acid substitutions. Using the NCBI database, *S. aureus* LysR was found to contain three protein domains, shown in Figure 3.7A. The multi-domain LysR identifies this putative protein as belonging to the LTTR regulatory family. The N-terminal HTH_ARSR domain suggests LysR regulates through direct DNA binding. The *S. aureus lysR* gene is located near genes encoding enzymes of the histidine utilisation pathway (Figure 3.7B). However, *lysR* is separated from *hutG* by the phosphomycin resistance gene *fofB.*

3.2.4. Newman *lysR* is Fur activated in low iron

To further investigate *lysR* regulation, the reporter construct was transduced into a Newman *fur* background and the nitrocefin assay repeated (Figure 3.8A). In this background, *lysR* expression was significantly reduced; suggesting that Fur activates *lysR* transcription in Fe(0). Although there appeared to be a low level of iron induction in the Newman *fur* mutant ($P = 0.026$), there was no statistical significance between Fe(50) *lysR* expression in Newman and Newman *fur* ($P = 0.064$).

A



B

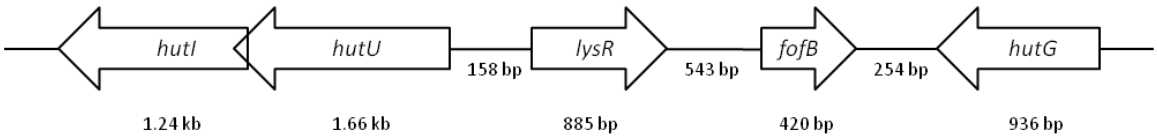
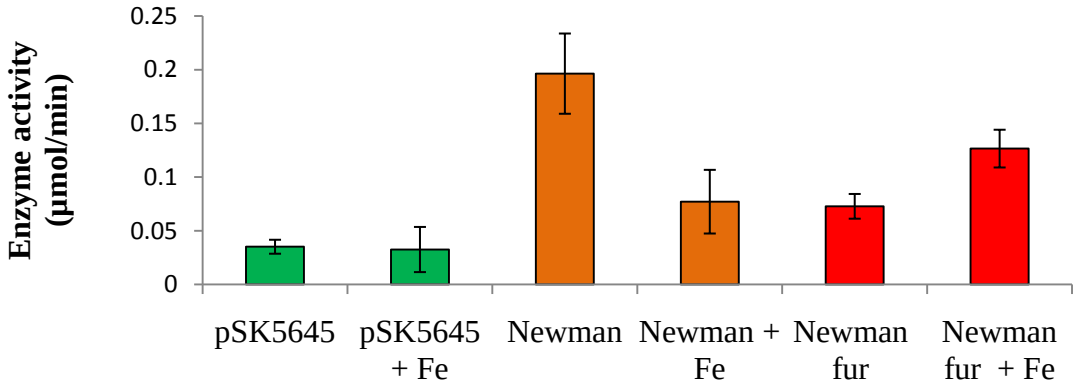


Figure 3.7. The Newman LysR.

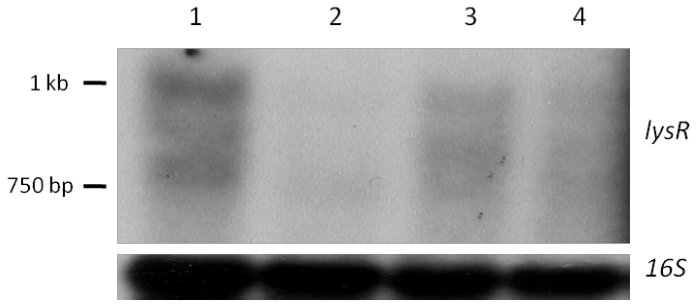
(A) Using the NCBI database, the Newman LysR (NWMN_2233) was found to be 294 residues in length and contained 3 putative protein domains; the multi-domain LysR, the N-terminal HTH_ARSR domain and the C-terminal LysR-substrate domain. Image is taken from the NWMN_2233 (LysR) page in the NCBI "Conserved Domain" database.

(B) Newman *lysR* and surrounding region. The Newman *lysR* gene is located downstream of *hutUI* and upstream of *hutG*, separated by the phosphomycin resistance gene *fofB*.

A



B



C

Lane	**1**	**2**	**3**	**4**
Expression level	1.02	0.12	0.22	0.25

Figure 3.8. Fur regulation of *lysR*. **(A)** Nitrocefin assay of the *lysR* pSK5645 reporter in a Newman and Newman *fur* background. Newman strains carrying the reporter plasmid were grown to stationary phase in SSD with or without 50 µM $Fe_2(SO_4)_3$. Shown is the pSK5645 control (green), the pSK5645$_{lysR}$ construct in a Newman background (orange) and the pSK5645$_{lysR}$ construct in a Newman *fur* background (red). *y*-axis represents enzyme activity. Results shown represent the means and standard deviations of four independent experiments.

(B) Northern blot analysis of Newman *lysR*. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *lysR* DNA probe. (1) Newman, (2) Newman plus 50 µM $Fe_2(SO_4)_3$, (4) Newman *fur*, (5) Newman *fur* plus 50 µM $Fe_2(SO_4)_3$. The blot was then stripped and re-hybridised with a *16S* rRNA control probe. Blot shown is representative of two repeat experiments. **(C)** Expression levels of the genes in **(B)** are shown as a proportion of the expression level of the *16S* control.

Northern blot analysis was also used to examine Newman wild type and *fur* mutant *lysR* transcription in both Fe(0) and Fe(50). RNA proved to be difficult to extract from SSD medium cultures and so CRPMI was used in this experiment. The *lysR* probe was created using primers LysR-probe-F and LysR-probe-R and detected 2 transcripts of 1 kb and 750 bp (Figure 3.8B). These sizes are estimates but the 750 bp transcript may represent the *lysR* gene. Densitometry analysis showed that Newman *lysR* transcription was reduced 8.5-fold in Fe(50). However, in the Newman *fur* mutant this regulation was lost (Figure 3.8B). This was similar to the *lysR* Fur transcription observed in the nitrocefin assays (Figure 3.8A). Therefore, this is the first evidence of novel Fur regulation of a gene with a putative upstream Fur box in *S. aureus*. Also, this gene encodes a putative regulator of the LTTR family and therefore may be involved in additional novel Fur/iron regulation.

3.2.5. Newman *lysR* may be auto-regulated

For further investigations, inverse PCR was used to construct a Newman *lysR* mutant by replacing 721 bp of *lysR* with the 1.5 kb tetracycline cassette from Newman *fur*, which is fully described in section 2.8.1. The pSK5645$_{lysR}$ construct was transduced into Newman *lysR* and was subsequently used in a nitrocefin reporter assay as previously described (Figure 3.9). *lysR* remained iron regulated in the Newman *lysR* background but transcription was increased 1.4-fold in Fe(0), with a P-value of 0.027 (Figure 3.9). This suggests that, like many LTTRs, *lysR* is auto-regulated. In addition, LysR was shown to not be required for the low iron induction of *lysR* (Figure 3.9).

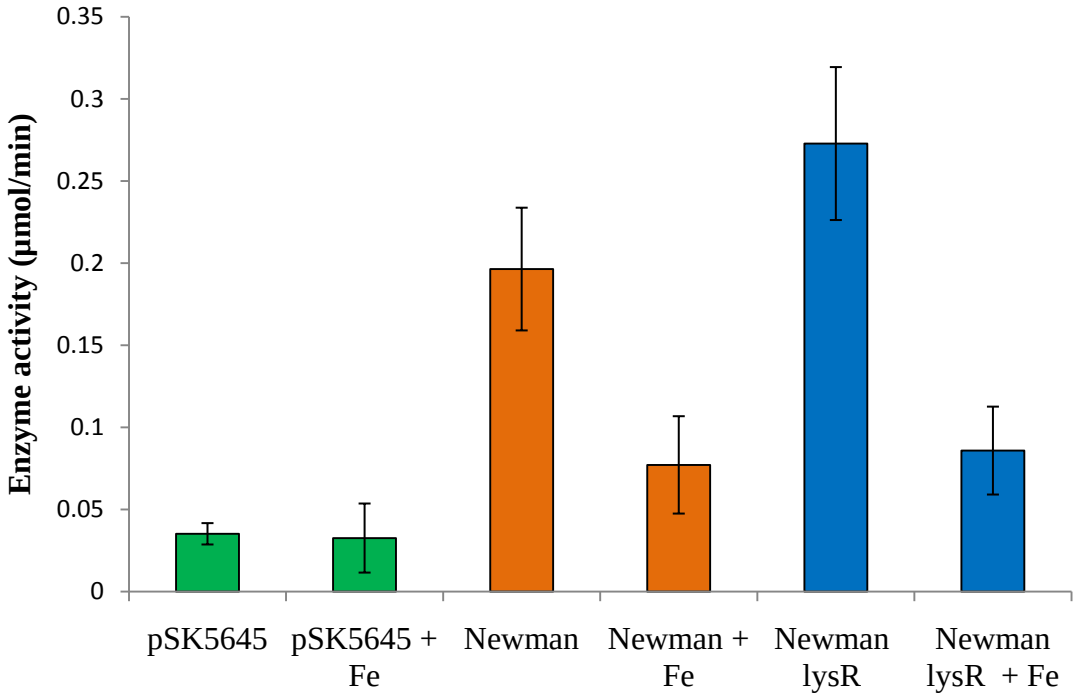


Figure 3.9. Nitrocefin assay of the *lysR* pSK5645 reporter in a Newman and Newman *lysR* background. Newman strains carrying the reporter plasmid were grown to stationary phase in SSD with or without 50 µM $Fe_2(SO_4)_3$. Shown is the pSK5645 control (green), the pSK5645$_{lysR}$ construct in a Newman background (orange) and the pSK5645$_{lysR}$ construct in a Newman *lysR* background (blue). *y*-axis represents the enzyme activity. Results shown represent the means and standard deviations of four independent experiments.

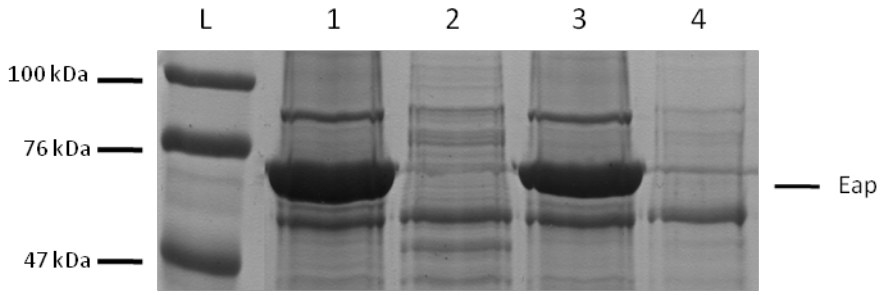
3.2.6. The expression of Eap was slightly reduced in Newman *lysR*

To further investigate the role of LysR in Newman, protein profiles were examined to compare the protein expression of the Newman wild type with the *lysR* mutant. Protein extraction was found to be variable when using the SSD medium and could not provide reproducible results. Therefore, proteins were harvested from CRPMI cultures. The membrane and cytoplasmic extracts of the Newman wild type and *lysR* mutant were identical (data not shown). However in both the cell wall proteins and non-covalently attached proteins removed by SDS treatment, a 70 kDa protein showed an overall reduced level of expression in Newman *lysR* (Figure 3.10). This protein band was sequenced as described in section 2.10.6., and found to be the anchorless protein Eap. Eap has been shown to be activated in low iron (Figure 3.1) and these protein profiles observed the same pattern of regulation. This low iron activation was maintained in the Newman *lysR* mutant but overall expression was slightly reduced (Figure 3.10). Therefore, the absence of LysR led to a reduced expression of Eap at the cell surface but did not affect the low iron dependant Eap activation.

3.2.7. The inactivation of *lsyR* reduced Newman *eap* transcription

Northern blot analysis was used to determine if the reduction of Eap cell surface protein levels in Newman *lysR* was due to a reduction in *eap* transcription. Total RNA was prepared from mid-log cultures in the minimal medium CRPMI. An *eap* probe created using the primers Eap-probe-F and Eap-probe-R detected two transcripts of 2 kb and 1.3 kb, which in the Newman wild type were reduced 5-fold in Fe(50) (Figure 3.11A lanes 1 and 2). However, the precise identity of these two transcripts is unknown (Johnson *et al*., 2008).

A



B

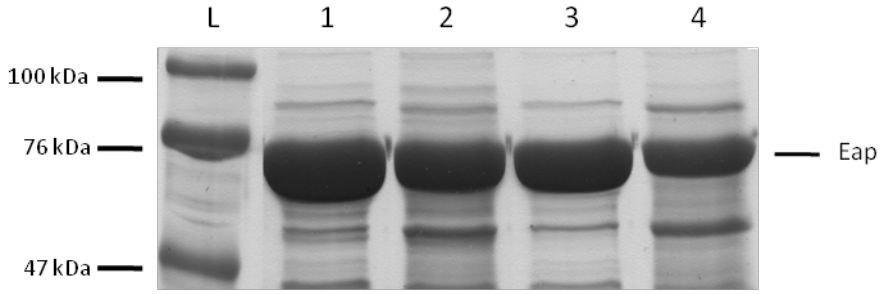
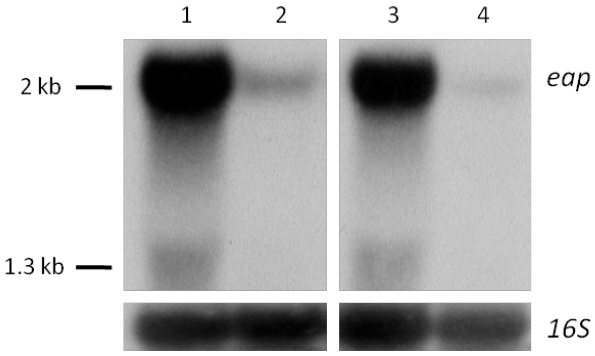


Figure 3.10. SDS-PAGE gels of Newman and Newman *lysR* CRPMI protein extracts. Protein was harvested from stationary cultures after 17 hours. **(A)** Cell wall extracts; (1) Newman, (2) Newman plus 50 µM $Fe_2(SO_4)_3$, (3) Newman *lysR* and (4) Newman *lysR* plus 50 µM $Fe_2(SO_4)_3$. **(B)** SDS extracts; (1) Newman, (2) Newman plus 50 µM $Fe_2(SO_4)_3$, (3) Newman *lysR* and (4) Newman *lysR* plus 50 µM $Fe_2(SO_4)_3$. Highlighted is the Eap protein which was sequenced from lane 3 of both gels. Gels shown are representative of two repeat experiments. Product size was estimated using 5 µl of pre-strained single colour protein ladder (L).

A



B

Lane	**1**	**2**	**3**	**4**
Expression level	1.21	0.25	0.74	0.07

C

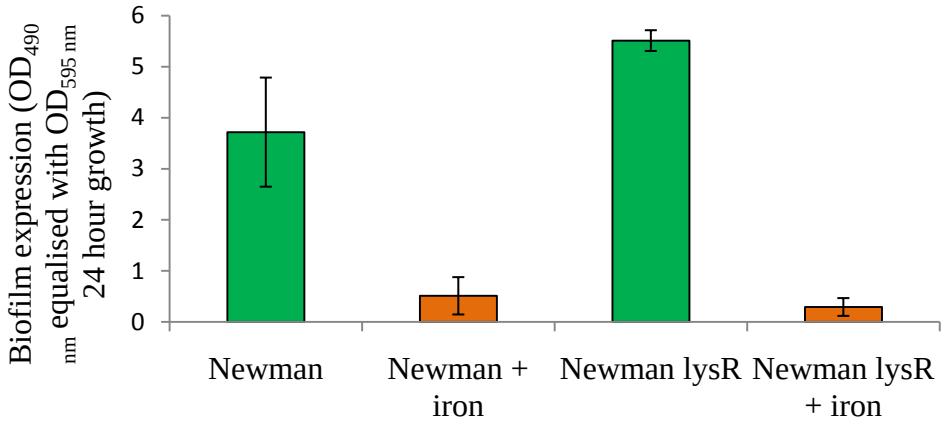


Figure 3.11. *eap* transcription and *in vitro* biofilm formation of Newman *lysR*.

(A) Northern blot analysis of *eap* transcription. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with an *eap* DNA probe. (1) Newman, (2) Newman plus 50 µM $Fe_2(SO_4)_3$, (3) Newman *lysR* and (4) Newman *lysR* plus 50 µM $Fe_2(SO_4)_3$. The blot was then stripped and re-hybridised with a *16S* rRNA control probe. Blot shown is representative of two repeat experiments. **(B)** Expression levels of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

(C) 24 hour biofilm formation in the Newman wild type and *lysR* mutant. CRPMI cultures were incubated for 24 hours in microtitre plates and biofilm formation was detected by safranin staining. Cultures were grown with or without 50 µM $Fe_2(SO_4)_3$. Results shown represent the means and standard deviations of three independent experiments.

In Newman *lysR*, the iron regulation of *eap* was similar to wild type (Figure 3.11A lanes 3 and 4). However, the Newman *lysR* mutant displayed an overall reduction in *eap* transcription, comparable to the protein data (Figure 3.10) and which showed that *lysR* inactivation was either directly or indirectly affecting *eap* transcription.

3.2.8. Biofilm formation in Newman *lysR* was identical to wild type

Newman *lysR* displayed a minor reduction in *eap* transcription and a lower cell surface expression of the Eap protein (Figures 3.10 and 3.11A). Eap has numerous biological functions but has been implicated in biofilm production (Rohde *et al*., 2007; Johnson *et al*., 2008). Therefore, the level of 24 hour CRPMI biofilm expression was examined in response to iron using the Newman wild type and *lysR* mutant. Similar to a previous report by Johnson *et al*. (2005), Newman 24 hour biofilm was highly expressed in Fe(0) but reduced in Fe(50) (Figure 3.11C). This was also observed for Newman *lysR*, where there was no significant alteration to the biofilm response in either Fe(0) ($P = 0.1$) or Fe(50) ($P = 0.21$). Therefore, the observed Eap reduction in Newman *lysR* had no measurable effect on its *in vitro* ability to form a 24 hour biofilm.

3.2.9. Newman *lysR* showed reduced growth in rich but not minimal media

For further investigations, a Newman *lysR* mutant with a restored copy of the *lysR* gene was constructed. This was created using the shuttle vector pMK4 which contained a 1.5 kb cloned DNA fragment of the Newman *lysR* gene and its promoter, which is fully described in section 2.8.2. Growth curves were used to examine the effect of inactivating *lysR* in the Newman strain on growth in different media (Figure 3.12). There was no growth variation when the two strains were cultured in the minimal

medium CRPMI (Figure 3.12A). However in richer media, the *lysR* mutant showed a significant growth defect. After 8 hours incubation, Newman *lysR* growth was reduced by 1.1-fold in LB and 1.34-fold in TSB when compared to wild type Newman (Figures 3.12B and 3.12C).

As the *lysR* mutant showed a growth defect in two rich media, the minimal medium CRPMI was supplemented with casamino acids and the growth was again compared. In this medium, 8 hour growth for the mutant was reduced by 1.7-fold compared to the wild type (Figure 3.12D). Growth of Newman *lysR* + pMK4$_{lysR}$ was assayed in CRPMI, LB and TSB and was found to be reduced in all three media when compared to Newman wild type and Newman *lysR*. Also, a Newman *lysR* strain which possessed the pMK4 plasmid without the *lysR* construct displayed an identical growth defect to Newman *lysR* + pMK4$_{lysR}$ (Figure 3.13). Consequently, it is likely that the increased growth reduction observed for Newman *lysR* + pMK4$_{lysR}$ was due to the additional metabolic burden having to replicate the pMK4 plasmid. Therefore, inactivating *lysR* negatively affects Newman growth in nutrient rich media but not in the CRPMI minimal medium (Figure 3.12).

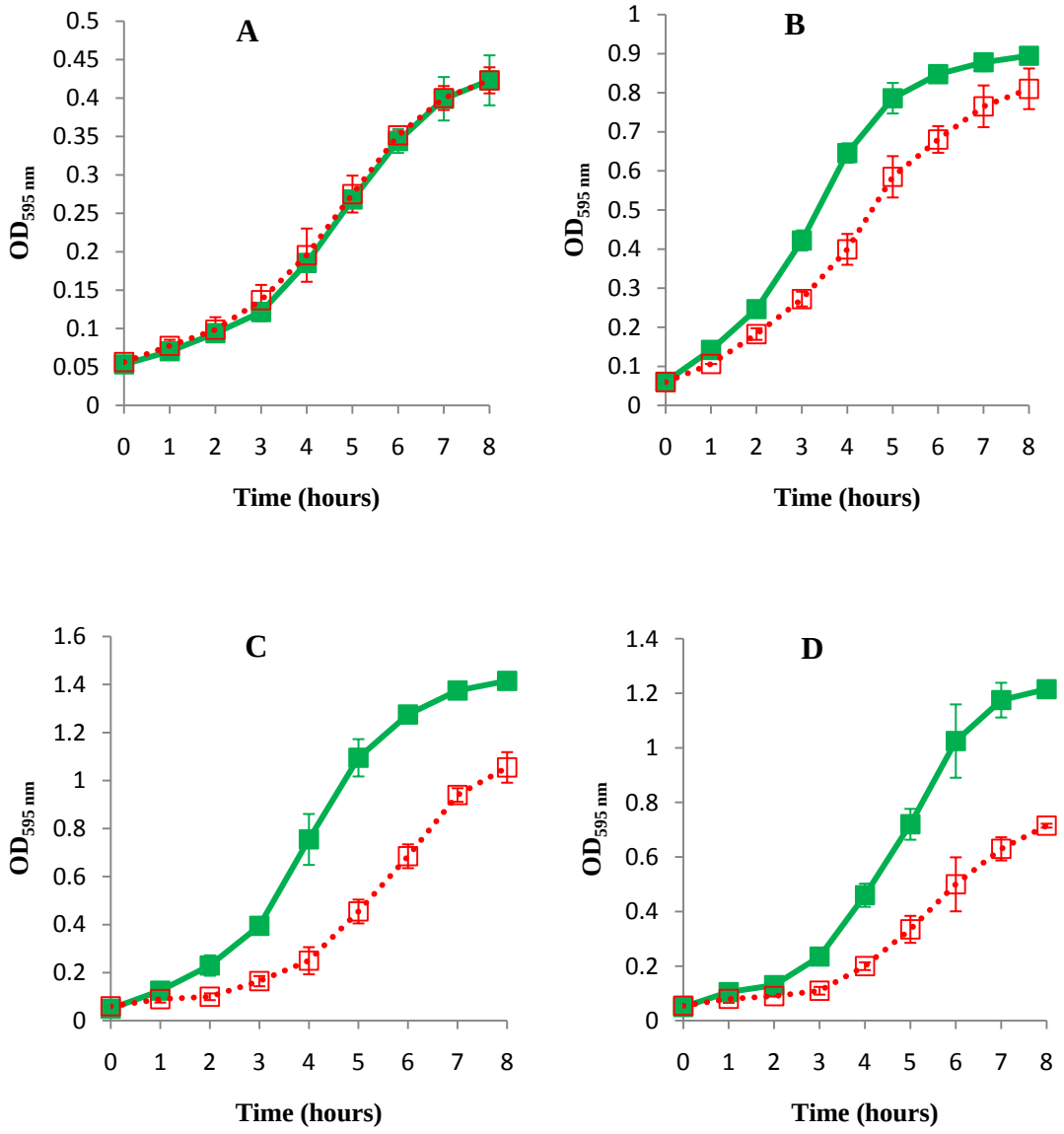


Figure 3.12. Growth curves of Newman wild type and *lysR* mutant. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 and growth was measured at $OD_{595\ nm}$ each hour for 8 hours. The strains Newman (■) and Newman *lysR* (□) were grown in four different media; **(A)** CRPMI, **(B)** LB broth, **(C)** TSB and **(D)** CRPMI with an additional 3% (w/v) casamino acids. Results shown represent the means and standard deviations of two independent experiments.

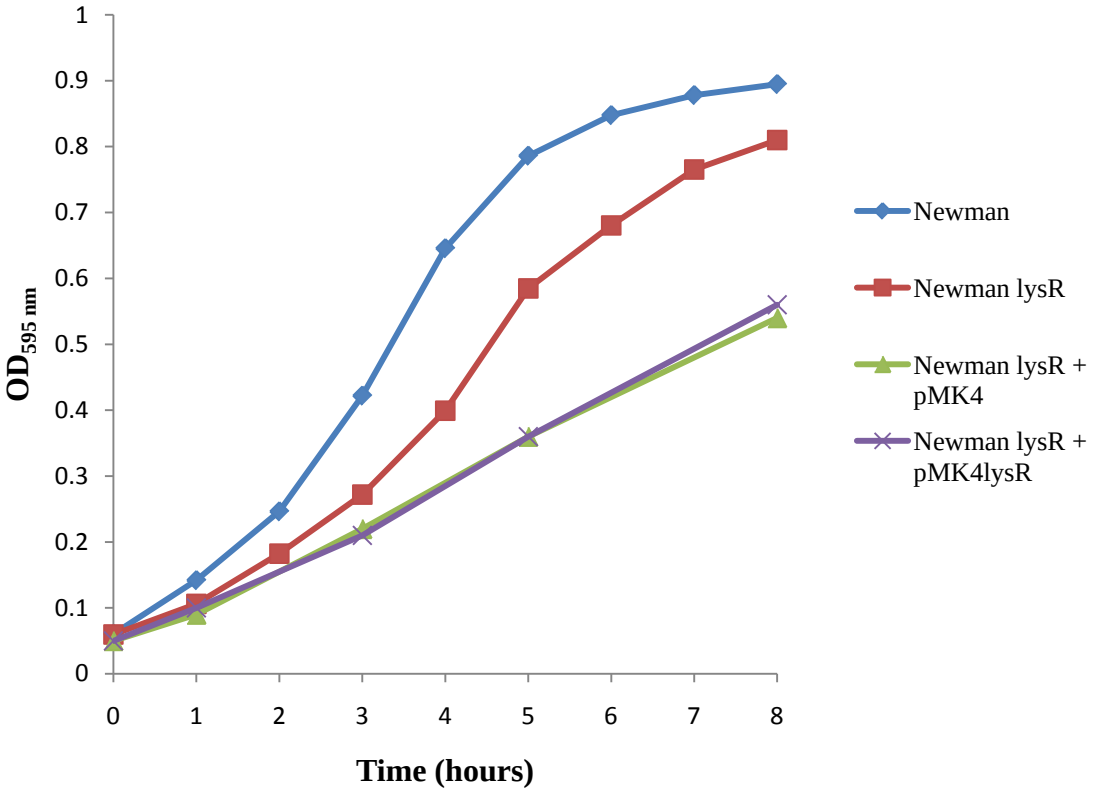


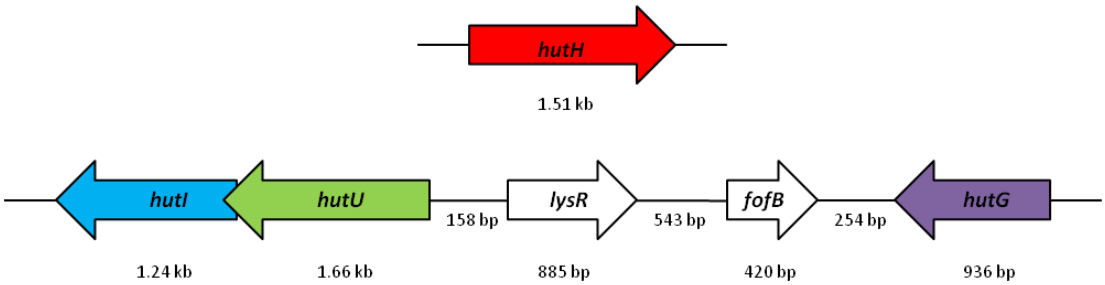
Figure 3.13. Growth curve of Newman wild type, *lysR* mutant and *lysR* mutant containing pMK4$_{lysR}$ or pMK4 alone. Cultures were grown statically in LB from a starting $OD_{595\ nm}$ of 0.05 and growth was measured at $OD_{595\ nm}$ each hour for 8 hours. For Newman *lysR* + pMK4 and Newman *lysR* + pMK4$_{lysR}$ enough time points were taken to confirm that the growth defect was due to pMK4 alone and not the cloned *lysR* gene.

3.2.10. Newman *lysR* is located next to several genes of the *hut* operon

As the *lysR* mutant displayed reduced growth in nutrient rich media (Figure 3.12), an explanation for this effect was investigated. When examining the regions directly upstream and downstream of Newman *lysR*, it was found that this putative LTTR is near to several genes of the *hut* system (Figure 3.14A). The four *hut* genes of Newman are *hutH*, *hutU*, *hutI* and *hutG*, three of which are located close to *lysR*, while *hutH* is separated from the rest of the *S. aureus hut* system and *lysR* by approximately 400 genes. Newman *lysR* is divergently located 158 bp upstream of *hutUI* and downstream of *hutG*, separated by the phosphomycin resistance gene *fofB* (also termed *fosB*) (Figure 3.14A).

These *hut* genes encode for enzymes that catalyse the pathway to degrade L-histidine to L-glutamate. This pathway utilises water to acquire nitrogen when an alternative nitrogen source is unavailable (Figure 3.14B). If these genes are under the control of LysR it may explain why *lysR* disruption leads to a growth defect in a nutrient rich media, as available histidine in the rich media may potentially not be able to be utilised by the mutant. The putative Newman L-histidine degradation pathway is shown in Figure 3.14B. The L-histidine to L-glutamate degradation pathway involves three intermediates, urocanate, imidazolonylpropionate and *N*-formimino-L-glutamate (Figure 3.14B) (Coote & Hassall, 1973).

A



B

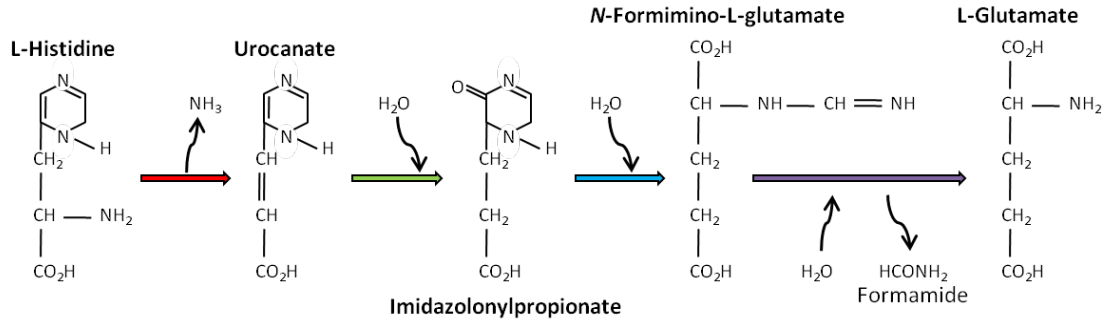


Figure 3.14. The Newman *hut* genes and the putative *S. aureus* histidine utilisation pathway based on the known protein functions of homologues of the four *S. aureus* Hut proteins. **(A)** The Newman *lysR* gene is located upstream of *hutUI* and downstream of *hutG*, separated by the phosphomycin resistance gene *fofB*. The first gene of the histidine degradation pathway *hutH* is separated from the rest of the *hut* system by approximately 400 genes. **(B)** The degradation pathway catalysed by the four known *S. aureus* Hut enzymes. Colour of the arrows represents the Hut enzyme that catalyses the pathway step and correlates to the gene colours illustrated in **(A)**. Figure is adapted from Coote & Hassall (1973).

The presence of the phosphomycin resistance gene *fofB* in this region may represent a gene transfer event where Newman, or a descendant strain, acquired this antibiotic resistance gene through a chromosomal DNA insertion. Analysis of the region separating Newman *lysR* and *fofB* identified a series of 60 bases repeated a total of 7 times, suggesting that an insertion by foreign DNA has indeed occurred. In *S. aureus* strain RF122, located between *hutG* and *lysR* is a 1.3 kb transposase (SAB2210c) not *fofB*, which signifies that this is a potential site of recombination.

Using the NCBI database, the majority of *S. aureus* strains were found to be like Newman and have a *lysR* adjacent to a phosphomycin resistance gene and *hut* genes (Figure 3.13B). However, the MSSA476 *lysR* (SAS2225) and surrounding *hut* genes show a similar arrangement to Newman *lysR* (Figure 3.14A) but without the phosphomycin resistance gene. Similar LTTRs with approximately 50% protein identity to *S. aureus* Newman *lysR* are found in several other bacterial species and are also located near to histidine degradation pathway genes. This includes; *Staphylococcus saprophyticus* subspecies *saprophyticus* ATCC 15305 (SSP0571), *Staphylococcus carnosus* subspecies *carnosus* TM300 (Sca_1830) and *Macrococcus caseolyticus* JCSC5402 (MCCL_1458). Therefore, this particular LTTR and *hut* association may represent a conserved gene arrangement while the *fofB* insertion is an accessory gene which varies between sequenced *S. aureus* strains.

In other bacterial species several regulators of the *hut* system have been identified. The *B. subtilis hut* system is arranged as a single operon (*hutPHUIGM*) which is induced by histidine (Chasin & Magasanik, 1968; Wray & Fisher, 1994). The first *hut* gene (*hutP*)

encodes the positive regulator of this system, a mRNA binding protein which requires L-histidine and magnesium for binding (Oda *et al*., 1988; Wray & Fisher, 1994; Kumarevel *et al*., 2005). This *B. subtilis hut* system is also regulated by carbon catabolite availability and amino acids (Chasin & Magasanik, 1968; Atkinson *et al*., 1990). The *hutC* gene of *P. putida* encodes a repressor which regulates the five *hut* genes although *hutG* can be transcribed independently (Allison & Phillips, 1990; Hu & Phillips, 1988). The HutC repressor is also found in *S. typhimurium* which similarly represses its *hut* operon (Smith & Magasanik, 1971). In *Klebsiella aerogenes*, the *hut* operon is activated by the LTTR NAC (nitrogen assimilation control), which is up-regulated during nitrogen starvation, and this *hut* system is also repressed by a HutC protein (Schwacha & Bender, 1990; Bender, 1991).

3.2.11. Regulation of the *hut* system by LysR

S. aureus Newman does not possess homologues of the known *B. subtilis* and *S. typhimurium hut* regulators; *hutC* and *hutP*. Also, Newman has no *K. aerogenes* NAC homologue. Therefore, the possibility of LysR being the *S. aureus hut* regulator was investigated. It is possible that the growth reduction in rich media observed for Newman *lysR* (Figure 3.12) was due to unregulated L-histidine conversion to L-glutamate which results in histidine deficiency leading to lower mutant growth. Therefore, a growth comparison was performed in LB medium with histidine excess. However, exogenous histidine did not prevent the inhibited LB growth for the *lysR* mutant (Figure 3.15), suggesting that another factor was responsible for the growth defect observed in rich media (Figure 3.12).

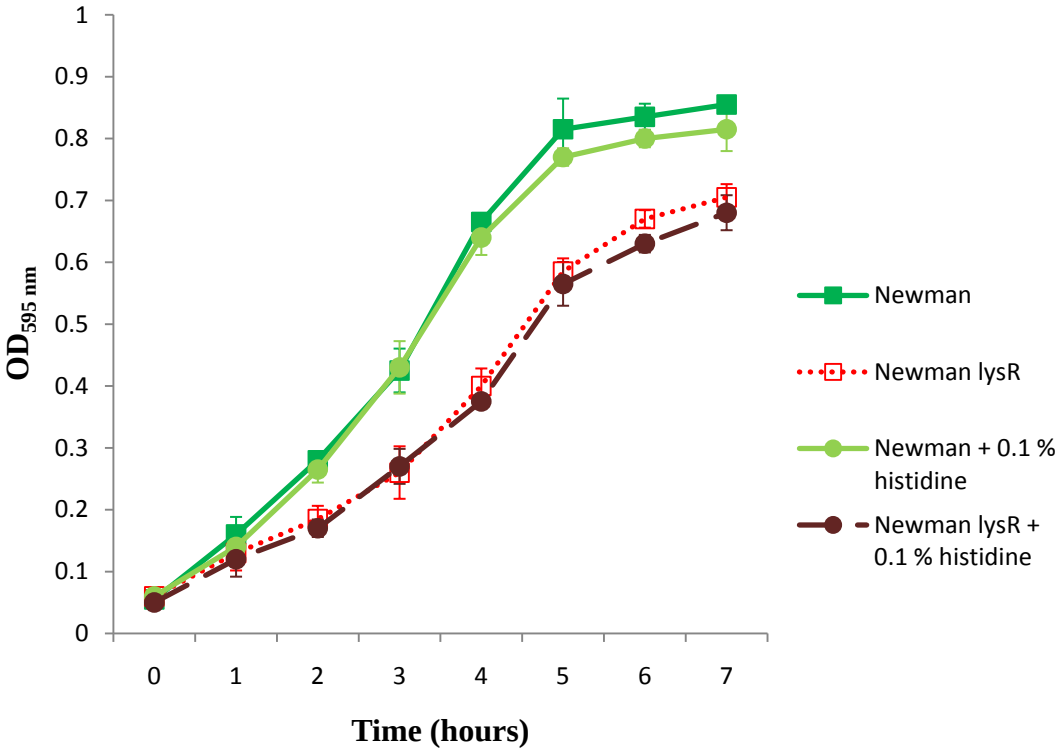
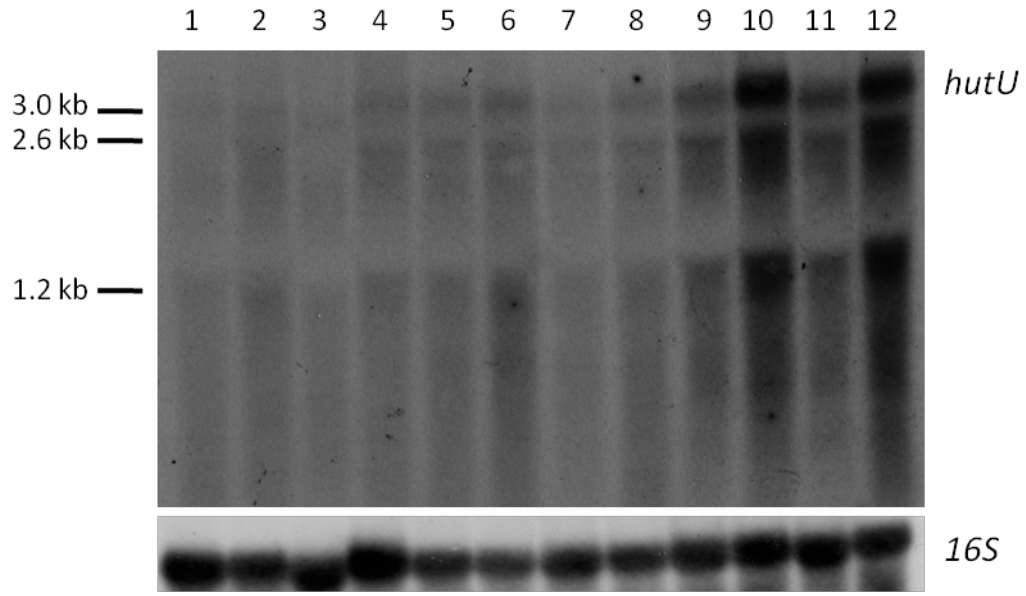


Figure 3.15. LB growth comparison of Newman wild type and *lysR* mutant with exogenous L-histidine. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 and growth was measured at $OD_{595\ nm}$ each hour for 7 hours. Strains were incubated with or without 0.1% (w/v) L-histidine. Results shown represent the means and standard deviations of two independent experiments.

When analysing the *hut* pathway (Figure 3.14B), it can be seen that one intermediate and the final product of the putative Newman *hut* pathway are both acids (urocanate and L-glutamate). Therefore to test if disruption of this pathway was causing medium pH alterations which were subsequently affecting growth in the *lysR* mutant, the pH of growing Newman and Newman *lysR* LB cultures was measured over the course of the growth phases. However, no variation in culture pH was detected (data not shown).

Northern blot analysis was also used to investigate the potential regulation of the *hut* system by LysR. A growth medium was required which was low in carbon catabolites and amino acids, as these have both been reported to repress the *B. subtilis hut* system (Chasin & Magasanik, 1968; Atkinson *et al*., 1990). RPMI was the medium chosen, as its concentrations of amino acids are significantly lower (between 4-fold and 40-fold) than the levels previously found by Atkinson *et al*. (1990) to repress *B. subtilis* histidase (HutH) expression. Therefore, RNA was extracted from mid-exponential phase RPMI cultures and used to investigate transcription. A *hutU* probe created using primers HutU-probe-F and HutU-probe-R detected 3 transcripts of 3 kb, 2.6 kb and 1.2 kb, with the 3 kb product most likely representing a RNA transcript of the entire *hutUI* operon. The expression level of these transcripts for both the wild type and *lysR* mutant was extremely low and no accurate regulation could be detected (Figure 3.16A lanes 1-8). However, densitometry analysis of the Newman *lysR* + pMK4$_{lysR}$ transcripts showed an overall increased expression when compared to the Newman wild type and *lysR* mutant. Also, a 2.8-fold iron induction could be observed in this strain. (Figure 3.16A lanes 9-12). Surprisingly, the addition of histidine had no clear effect on *hutU* expression, possibly as there was a stronger repressing substrate present in the RPMI medium.

A



B

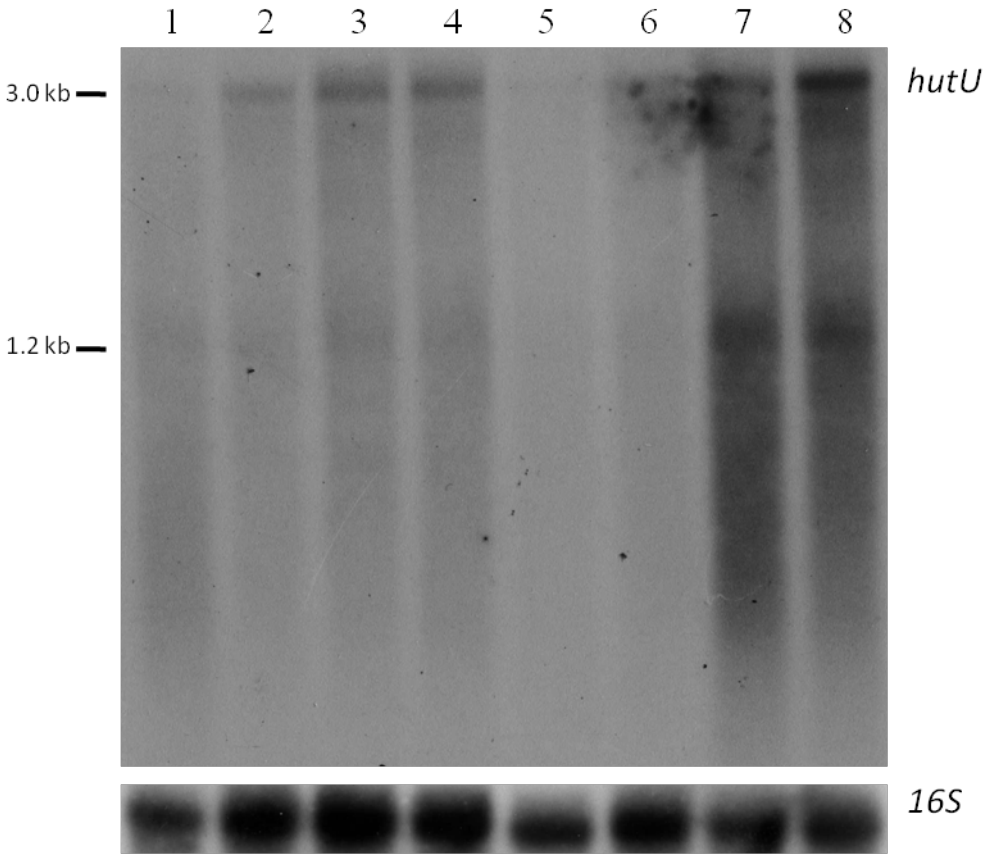
Lane	**9**	**10**	**11**	**12**
Expression level	0.17	0.47	0.22	0.51

Figure 3.16. Northern blot analysis of *hutU*. RNA was prepared from mid-exponentially RPMI growing cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *hutU* DNA probe. **(A)**; (1) Newman, (2) Newman plus 50 µM $Fe_2(SO_4)_3$, (3) Newman plus 0.01% (w/v) histidine, (4) Newman plus 50 µM $Fe_2(SO_4)_3$/0.01% (w/v) histidine, (5) Newman *lysR*, (6) Newman *lysR* plus 50 µM $Fe_2(SO_4)_3$, (7) Newman *lysR* plus 0.01% (w/v) histidine, (8) Newman *lysR* plus 50 µM $Fe_2(SO_4)_3$/0.01% (w/v) histidine, (9) Newman *lysR* + pMK4$_{lysR}$, (10) Newman *lysR* + pMK4$_{lysR}$ plus 50 µM $Fe_2(SO_4)_3$, (11) Newman *lysR* + pMK4$_{lysR}$ plus 0.01% (w/v) histidine and (12) Newman *lysR* + pMK4$_{lysR}$ plus 50 µM $Fe_2(SO_4)$3/0.01% (w/v) histidine. The blot was then stripped and re-hybridised with a *16S* rRNA control probe. Blot shown is representative of two repeat experiments. **(B)** Expression levels that could be detected of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

While the growth of Newman wild type and *lysR* mutant in CRPMI was identical, the two strains did display different growth phenotypes in LB (Figure 3.12). Therefore, the northern blot analysis was repeated using RNA extracted from 3 hour cultures grown in LB broth. Due to time constraints only a single northern blot experiment could be performed under these conditions (Figure 3.17A). As with RPMI, *hutU* expression was exceptionally low in LB medium but in this experiment only the 3 kb and 1.2 kb transcripts were detected (Figure 3.17A). From this preliminary result, *hutU* appears to be iron induced and this regulation is lost in Newman *lysR* and restored in Newman *lysR* + pMK4$_{lysR}$ (Figure 3.17A). However, iron regulation appears to be maintained in the *fur* mutant. Similar to the *hutU* transcription in RPMI, expression of *hutU* in Newman *lysR* + pMK4$_{lysR}$ was greatly increased compared to wild type (Figure 3.17 lanes 7 and 8).

From these preliminary results LysR appears to be involved in the regulation of *hutU*, which is potentially iron induced. In both RPMI and LB, over expression of LysR from the pMK4$_{lysR}$ plasmid increases the overall transcription of *hutU* (Figures 3.16A and 3.17A). This implies that LysR is an iron dependant activator of *hutU*. However, *hutU* transcription was found to be very low in these two media, suggesting that the optimal conditions for *hutU* expression have not been used.

A



B

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Expression level	0.06	0.11	0.18	0.18	0.07	0.09	0.41	0.55

Figure 3.17. Northern blot analysis of *hutU*. RNA was prepared from mid-exponentially LB growing cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *hutU* DNA probe. **(A)** (1) Newman, (2) Newman plus 50 µM $Fe_2(SO_4)_3$, (3) Newman *lysR*, (4) Newman *lysR* plus 50 µM $Fe_2(SO_4)_3$, (5) Newman *fur*, (6) Newman *fur* plus 50 µM $Fe_2(SO_4)_3$, (7) Newman *lysR* + pMK4$_{lysR}$ and (8) Newman *lysR* + pMK4$_{lysR}$ plus 50 µM $Fe_2(SO_4)_3$. The blot was then stripped and re-hybridised with a *16S* rRNA control probe. **(B)** Expression levels of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

3.2.12. The effect of *lysR* inactivation on peroxide stress resistance

A microarray analysis using *S. aureus* strain SACOL-2325 found 2.5-fold increased *lysR* mRNA levels after 20 minutes exposure to hydrogen peroxide in a rich medium (Chang *et al*., 2006). Therefore, the hydrogen peroxide stress resistances of actively growing Newman wild type, Newman *lysR* and Newman *lysR* + pMK4$_{lysR}$ were compared in the rich medium TSB. As these three strains all exhibited different growth rates in rich media (Figure 3.12), two TSB cultures for each strain were inoculated from a single overnight culture and simultaneously incubated with shaking but with one culture containing 0.003% (v/v) hydrogen peroxide. Growth at hourly time points was recorded and used to calculate the relative growth of strains in response to peroxide, which removed the variation caused by the differing growth rates. Significant differences were seen between the wild type and *lysR* mutant during the exponential stage. This was statistically significant at 3 hours and at every additional hourly time point examined. The peroxide tolerance was not restored by the addition of the wild type *lysR* gene to the mutant strain but was instead decreased further and this was also statistically significant (Figure 3.18). Therefore, the inactivation of *lysR* increases hydrogen peroxide induced stress but the reintroduction of the wild type *lysR* gene leads to an even greater increase.

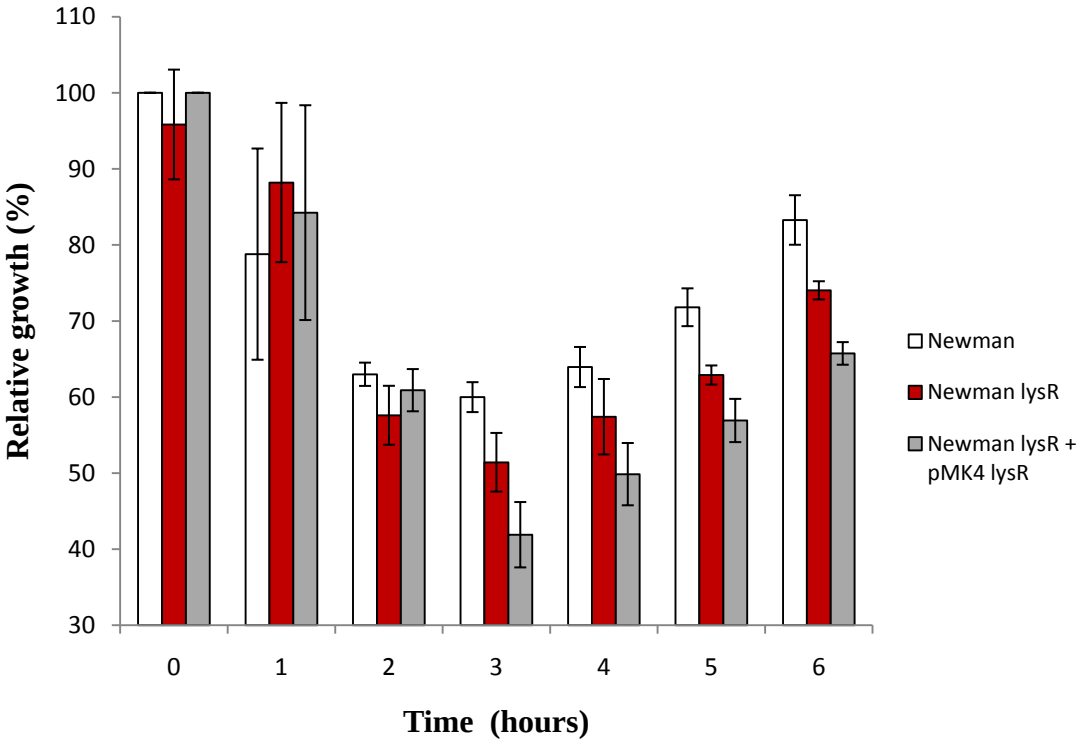


Figure 3.18. A comparison of peroxide stress resistance. Two cultures, one of which contained 0.003% (v/v) H_2O_2, were grown with shaking from a starting $OD_{595\ nm}$ of 0.05 and growth was measured at $OD_{595\ nm}$ each hour for 6 hours. The $OD_{595\ nm}$ of the two cultures was used to calculate relative growth in peroxide, expressed as a percentage. Three strains are shown; Newman, Newman *lysR* and Newman *lysR* + pMK4$_{lysR}$. Results represent the means and standard deviations of three independent experiments.

3.3. Discussion

The original aim of this work was to identify a regulator under the control of Fur/iron which could be involved in a regulatory pathway for non-classical Fur gene regulation. The regulator identified by this research, LysR, was shown to be iron regulated and require Fur for this transcription and therefore may play a role in *S. aureus* non-classical Fur regulation. Interestingly, *lysR* itself was found to be non-classically Fur regulated even though it has a putative upstream Fur box. However without further investigation, this Fur induction in low iron cannot be known to be direct or indirect. LysR is a member of the LTTR family which represents one of the largest groups of bacterial transcriptional regulators, with a diverse range of functions (Schell, 1993). Therefore finding the role of LysR in *S. aureus* presented a challenge. Some minor phenotypic effects were observed including; reduced rich media growth, *eap* expression and oxidative stress resistance. However, the most likely function of LysR is the induction of the *S. aureus* histidine utilisation gene, *hutU* (Figures 3.16A and 3.17A). However, due to the low expression of this operon in the chosen media and time constraints, this effect could not be fully characterised, although there appears to be a link.

The Fur box search and published regulatory study identified five potential regulators under the control of Fur/iron which were investigated further using reporter assays. These showed that transcription of *srrA*, *lysR* and *tetR* was repressed by Fe(50) but only *lysR* showed repression that was specific for iron (Figure 3.6). *lysR* is a member of the LTTR family and subsequent investigations identified that this putative regulator was non-classically Fur regulated. Similar to the regulation of *S. aureus eap* and *emp* (Figure

3.1), *lysR* is induced by Fur in Fe(0) (Figure 3.8). This result was unexpected but *lysR* now represents an additional gene regulated by Fur in a novel way. It is also the first *S. aureus* non-classically regulated gene with a putative upstream Fur box. A Fur DNA binding assay is required to show if this novel regulation is through direct Fur-DNA binding. Also, in addition to representing a new non-classically Fur regulated gene, *lysR* encodes a putative regulator protein and therefore may be part of a hierarchical Fur regulation pathway.

The Newman *lysR* mutant displayed a growth defect in rich media (Figure 3.12). *lysR* inactivation may be disrupting a detoxifying pathway that is leading to a build up of a toxic product in rich media, which subsequently affects growth. Also, the *lysR* mutant is more susceptible to peroxide stress (Figure 3.18) and induced by peroxide (Chang *et al*., 2006). Therefore, this may be the contributing factor for the observed rich media growth defect. However, during this project the peroxide induction of *lysR* was not confirmed and this would be essential before any further study into this putative function of LysR. Also for future work, the effect of inactivating *lysR* on the transcription of the *S. aureus* oxidative stress resistance genes should be examined to investigate if this is the cause of the observed decrease in peroxide tolerance in the *lysR* mutant. In addition to the rich media growth defect, both the surface expression of Eap and *eap* transcription was reduced in Newman *lysR* (Figures 3.10 and 3.11A). However this effect was minor and did not alter the Eap iron regulation and had no subsequent effect on Newman *in vitro* biofilm formation (Figure 3.11C).

The results obtained suggest that LysR is involved in the regulation of the *S. aureus hut* system. *lysR* is adjacent to *hutUI* but divergently transcribed (Figure 3.14A), which is a common genetic arrangement of many LTTRs with their target gene(s) (Henikoff *et al*., 1988). *lysR* was also observed to be auto-regulated (Figure 3.9) and LTTRs bind to sites upstream of target genes to regulate transcription (Maddocks & Oyston, 2008). It is therefore likely that if *lysR* is indeed auto-regulated and LysR binds the *lysR* promoter, then transcription of the adjacent *hutUI* operon is similarly affected by the LysR protein. The subsequent transcriptional analysis proved difficult due to the low expression of *hutU* in the two selected media (Figures 3.16A and 3.17A). However, in LB, *hutU* displayed iron regulation which was lost in Newman *lysR* but restored in Newman *lysR* + pMK4$_{lysR}$ (Figure 3.17A). In addition, in both RPMI and LB, *hutU* transcription was increased in Newman *lysR* + pMK4$_{lysR}$, which suggests that LysR is an activator of *hutU* (Figures 3.16A and 3.17A). However, more analysis is required to establish the regulatory relationships between *hutU*, *lysR*, LysR and Fur in response to iron. This would include DNA binding assays to investigate if Fur and LysR directly or indirectly regulate *lysR* and *hutU* through binding their promoters. In addition, the effect of inactivating *lysR* on the other *hut* genes of *S. aureus* (Figure 3.14A) should be examined but this would require a medium better suited to high expression of this system. Finally, the co-inducer of LysR also requires identification. If LysR was confirmed to indeed regulate the *hut* genes, then histidine or glutamate as the first and final compounds of the Hut enzyme pathway would be the two most likely candidates for the LysR co-inducer (Figure 3.14B).

The replacement of the wild type copy of *lysR* in the Newman *lysR* mutant led to an increased growth defect due to the pMK4 plasmid (Figure 3.13). Therefore, for any

future work an alternative complement would be constructed which would not use the pMK4 plasmid. Instead an integrated copy of *lysR* would be placed into the Newman *lysR* chromosome. The Newman LysR described in this work represents a new *S. aureus* regulator which is non-classically Fur regulated. It is also the first non-classically Fur regulated gene to contain a putative upstream Fur box. *lysR* is required for wild type growth in rich media and full peroxide resistance. In addition, LysR is likely to be the regulator of the *S. aureus hutU* gene but additional study is required to fully define this relationship. A microarray analysis using the Newman *lysR* mutant would be of interest to identify additional LysR targets.

Chapter 4. Copper homeostasis in *S. aureus*

4.1. Introduction

Copper is an essential biometal which is used as a cofactor in some important enzymes and is required for several essential biochemical reactions. However, free copper ions can be toxic if the intracellular concentration is not tightly regulated. Two copper operons have been identified in *S. aureus*. The first is conserved in all sequenced *S. aureus* strains and encodes the copper ATPase CopA and its downstream copper chaperone CopZ (Sitthisak *et al*., 2007). *copA* is induced by copper in a concentration dependant manner and also showed some induction by the metals iron and lead. Sitthisak *et al*. (2007) found that a *copA* mutant displayed no growth defect compared to wild type but was found to be copper, iron and lead sensitive. Also, intracellular copper was increased 7-fold in the *copA* mutant, which suggested CopA functions as a copper ion exporter. These phenotypes were all successfully complemented with a 2.7 kb fragment which contained *copA* and its promoter cloned into the shuttle vector pLI50. The metal binding domain of *copA* was also investigated and this CXXC motif was found to bind copper, cadmium and cobalt ions *in vitro* (Sitthisak *et al*., 2007).

The second copper *S. aureus* operon was found via a Tn*917* induced mutant library in strain ATCC 12600 (Sitthisak *et al*., 2005). Streptonigrin is a metal-dependent quinine-containing antibiotic which requires copper ions to function and a streptonigrin resistant mutant from the library was analysed and a multi-copper oxidase (MCO) was identified. Upstream of this *mco* gene is a putative copper ATPase gene, *copB*. This

operon is present in only a few strains, including EMRSA-16. The transcription of *mco* was copper induced and a *mco* mutant was hydrogen peroxide resistant but copper sensitive. Introduction of a wild type *mco* gene restored peroxide sensitivity but not copper tolerance, possibly due to upstream disruption of *copB*. These two copper operons represent the only identified *S. aureus* copper homeostasis systems to date.

However, there are many aspects of *S. aureus* copper homeostasis which are still undefined, such as the regulator(s) of the two *S. aureus cop* operons. In the copper homeostasis systems of other bacteria, numerous proteins have been identified which regulate the copper ATPases. These include the *E. coli* MerR-like activator CueR, the *E. hirae* CopY repressor and the recently identified *B. subtilis* CsoR repressor (Stoyanov *et al*., 2001; Outten *et al*., 2000; Solioz & Stoyanov, 2003; Smaldone & Helmann, 2007). Therefore, *S. aureus* copper tolerance and the regulation of the two identified copper operons were investigated further, in order to increase our understanding of copper homeostasis in this important pathogen.

4.2. Results

4.2.1. Copper was toxic to *S. aureus* in a growth limiting environment

Uğur & Ceylan (2003) identified several metal resistances in staphylococcal species which include different levels of copper tolerance between clinically isolated *S. aureus* strains. The minimal medium CRPMI was used to further investigate *S. aureus* copper toxicity by testing the tolerance of the clinically isolated strain Newman to a selection of environmental copper concentrations in a nutrient poor environment, reflective of *in vivo* growth. After 24 hours, Newman growth was significantly reduced by the

addition of copper ($CuCl_2$) to the medium, confirming that copper is inhibitory to *S. aureus* growth. This effect was amplified with increasing concentrations of $CuCl_2$ (Figure 4.1).

4.2.2. Environmental copper tolerance varied between *S. aureus* strains

Since tolerance to copper has been found to vary between clinically isolated staphylococcal species (Uğur & Ceylan, 2003), a number of different *S. aureus* strains were grown with several concentrations of copper in the minimal medium CRPMI and the effect of copper on total growth was compared. The strains chosen to investigate *S. aureus* copper tolerance included two well studied strains; Newman and 8325-4. However, 8325-4 is deficient in a functional sigma factor σ^B and so the σ^B positive isogenic strain SH1000 was also included (Horsburgh[1] *et al*., 2002). The final two strains examined were the EMRSA-16 MRSA252 clone PM64, and ATCC 12600. These both contain the additional *copB/mco* operon described by Sitthisak *et al*. (2005), which has been implicated in copper tolerance. The effects of four concentrations of cupric chloride on *S. aureus* CRPMI growth were tested; 50, 100, 200 and 500 µM.

There were significant variations in the copper tolerances of these five *S. aureus* strains (Figure 4.2A). Cu(100) was toxic to all strains, except ATCC 12600 which only showed growth inhibition in Cu(500). In Cu(50), different levels of growth in response to copper were observed. However in Cu(0), strains grew to different optical densities after the 24 hour incubation which distorted the subsequent inhibitory effect of Cu(50). For example, ATCC 12600 only reached an $OD_{595\ nm}$ of 0.18 while SH1000 grew to an $OD_{595\ nm}$ of 0.26, a 24 hour growth difference of 1.7-fold (Figure 4.2A). These variations in Cu(0) growth had to be taken into account before comparisons of *S. aureus* strain Cu(50) tolerance could be made.

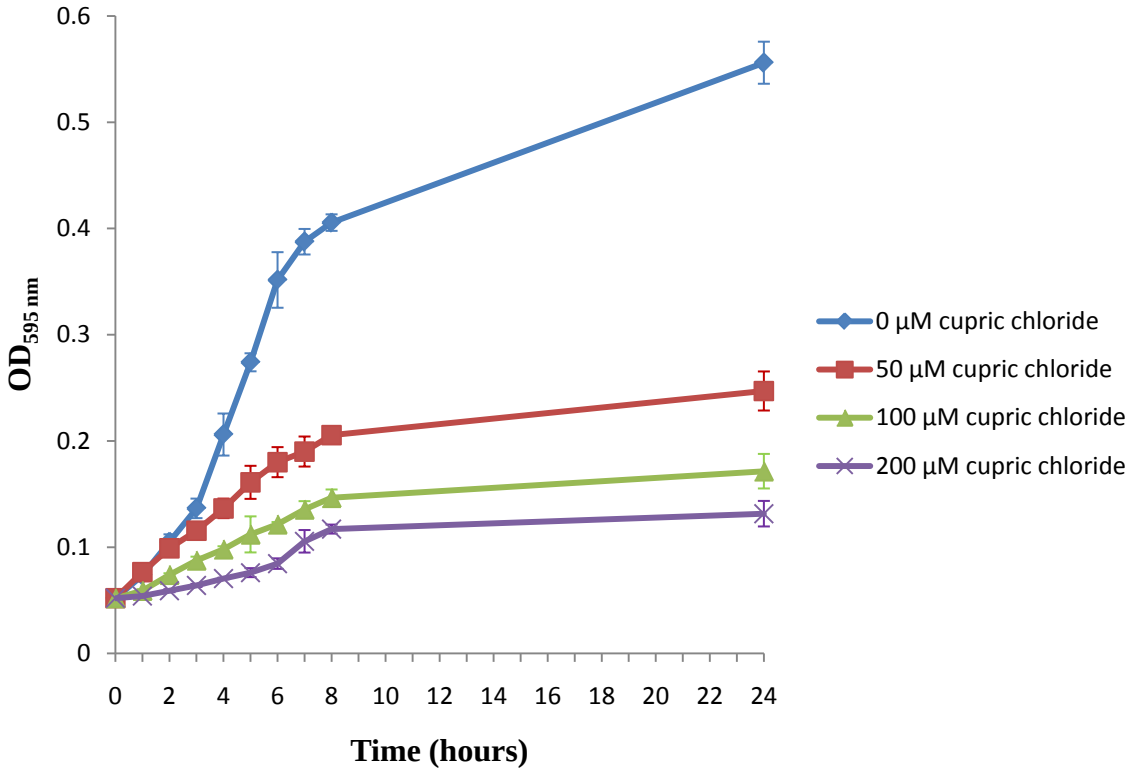
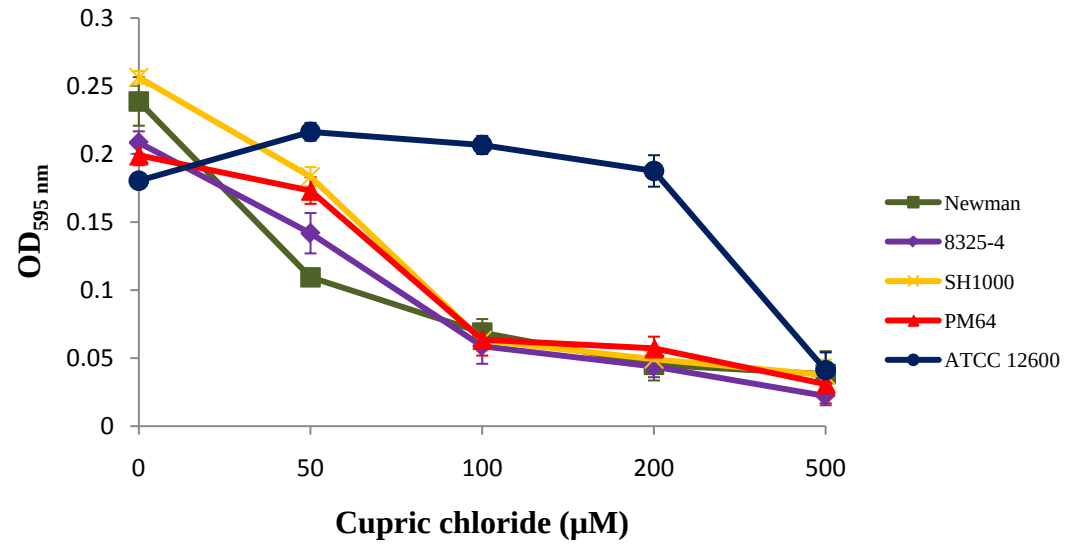


Figure 4.1. The effect of copper on the growth of *S. aureus* Newman in the minimal medium CRPMI. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 and growth was measured at $OD_{595\ nm}$ each hour for 8 hours and also once at 24 hours. Newman was grown in 0, 50, 100 or 200 µM $CuCl_2$. Results shown represent the means and standard deviations of three independent experiments.

A



B

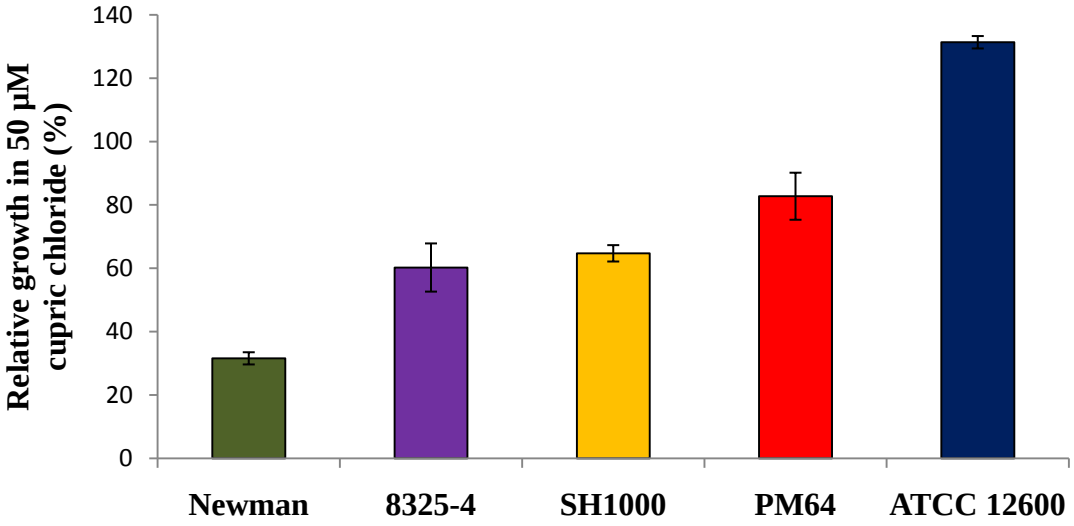


Figure 4.2. The effect of copper on the growth of *S. aureus* strains in the minimal medium CRPMI. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. The strains shown are; Newman, 8325-4, SH1000, PM64 and ATCC 12600. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Results shown represent the means and standard deviations of three independent experiments.

(A) *S. aureus* strains total growth when incubated in 0, 50, 100, 200 or 500 µM $CuCl_2$.

(B) Relative growth of the *S. aureus* strains in Cu(50) compared to growth in Cu(0).

Therefore, the relative growth in Cu(50) for each strain was calculated from the $OD_{595\,nm}$ change observed in Cu(50) when compared to Cu(0) and expressed as a percentage (Figure 4.2B). Newman was found to be the least copper tolerant strain with the lowest growth in CRPMI with Cu(50). Despite SH1000 displaying a better 24 hour $OD_{595\,nm}$ in Cu(50) than 8325-4 (Figure 4.2A), both SH1000 and 8325-4 had similar relative growth in this copper concentration when compared to growth in Cu(0) ($P = 0.46$) (Figure 4.2B). PM64 was the second most copper tolerant strain, with almost 3-fold better relative growth in Cu(50) compared to Newman ($P = 0.0009$). Interestingly, ATCC 12600 actually showed improved 24 hour $OD_{595\,nm}$ in Cu(50) compared to Cu(0) ($P = 0.00002$) and no inhibition in Cu(100) or Cu(200), whilst all other tested strains could only survive in Cu(50) (Figure 4.2A). Therefore, ATCC 12600 was found to be a hyper copper-tolerant *S. aureus* which exhibited a copper tolerance significantly greater than all other tested strains.

Previous studies have observed an improvement in copper tolerance when *S. aureus* are grown in a rich medium (Sitthisak *et al.*, 2007). Therefore, TSB was used to investigate the copper tolerance of three different *S. aureus* strains in a nutrient rich environment. Newman was included as a representative of the copper sensitive strains, while PM64 and ATCC 12600 were included as the two most copper tolerant strains which also possessed the additional *S. aureus* copper operon, *copB*/*mco*. Growth in rich medium resulted in a significant increase in copper tolerance compared to incubation in minimal medium (Figure 4.3).

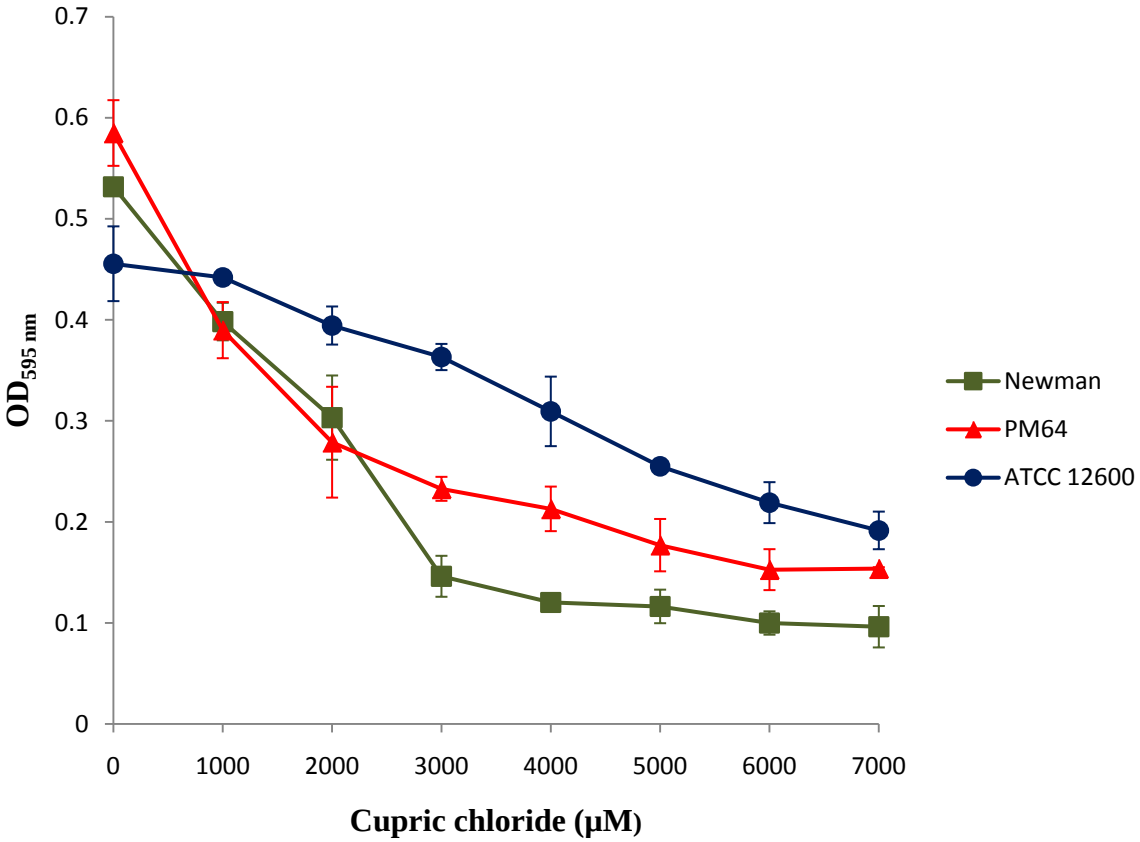


Figure 4.3. The effect of copper on the growth of *S. aureus* in TSB. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. The strains shown are; Newman, PM64 and ATCC 12600. Strains were incubated in 0, 1000, 2000, 3000, 4000, 5000, 6000 or 7000 µM cupric chloride. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Results shown represent the means and standard deviations of three independent experiments.

In CRPMI Newman and PM64 could only tolerate Cu(50) (Figure 4.2), whereas in TSB Newman grew in copper concentrations of up to 2000 µM whilst PM64 and ATCC 12600 still showed some growth in 7000 µM $CuCl_2$ (Figure 4.3). Furthermore, 7000 µM $CuCl_2$ was found not to alter the pH of the TSB medium. Therefore, this inhibition was due to the added copper. However, the pattern of strain copper tolerance in TSB was similar to that previously seen in CRPMI; with Newman the least tolerant and ATCC 12600 the most tolerant to copper. These assays confirmed previous reports of higher *S. aureus* copper tolerance in a richer medium (Sitthisak *et al*., 2005) but also demonstrated that strain variation still occurred.

4.2.3. Increased copper tolerance in nutrient rich media was independent of improved *S. aureus* growth

E. coli copper tolerance has been found to be dependent on the availability of nutrients and certain metals in the growth medium (Tree *et al*., 2005) which may act to reduce the toxic effects of copper. To investigate the effect of additional nutrients on copper tolerance, Newman was grown in CRPMI supplemented with casamino acids (Difco). In Cu(0), the growth of Newman was not significantly improved by supplementing CRPMI with either 0.01% (w/v) or 0.05% (w/v) casamino acids, P = 0.223 and P = 0.08 respectively (Figure 4.4 green bars). However, in Cu(50), Newman growth was improved in CRPMI supplemented with either 0.01% (w/v) or 0.05% (w/v) casamino acids, P = 0.0008 and P = 0.004 respectively (Figure 4.4 blue bars). This indicated that increased copper tolerance in a nutrient rich medium is not entirely due to improved bacterial growth, as in these experiments additional nutrients in the CRPMI medium allowed Newman to improve copper tolerance independently of growth.

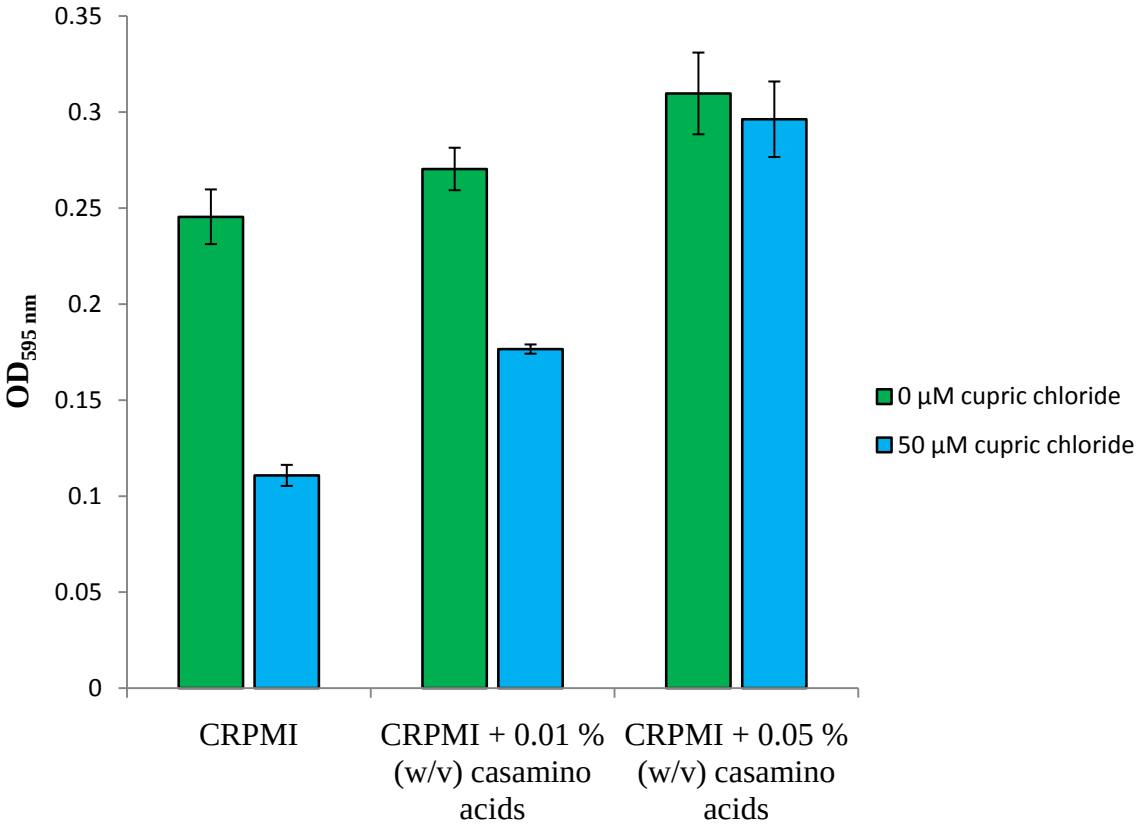


Figure 4.4. The effect on Newman copper tolerance by the supplementation of CRPMI with casamino acids. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. Two levels of casamino acids supplementation are shown; 0.01% and 0.05% (w/v). Newman was incubated with (blue bars) or without (green bars) 50 µM cupric chloride. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Results shown represent the means and standard deviations of three independent experiments.

4.2.4. The hyper copper-tolerance of ATCC 12600 was found to be linked to the *copB/mco* operon

As the copper tolerance of ATCC 12600 was quite distinctive (Figures 4.2 and 4.3), further investigations were undertaken to identify additional strains with an equally high tolerance to copper. However, even though a variety of *S. aureus* strains were examined none displayed a similar copper hyper-tolerant phenotype. The strains tested were; the clinical strains Mu50 and WCUH29, the bovine strain RF122 and another of the dominant EMRSA strains E15 (data not shown). Therefore, the ATCC 12600 hyper copper-tolerance was investigated further. Both ATCC 12600 and PM64 contain the *copB/mco* operon (Sitthisak *et al*., 2005) and the *mco* gene has been implicated in copper tolerance. Therefore, both the ATCC 12600 *mco* mutant and *mco* mutant containing a wild type *mco* gene on the shuttle vector pLI50 (Sitthisak *et al*., 2005), were assayed in CRPMI and their copper tolerances compared to wild type ATCC 12600.

Interestingly, growth in Cu(0) CRPMI was improved for ATCC 12600 *mco* when compared to wild type ATCC 12600 and was now similar to the copper sensitive strains such as Newman (Figure 4.5B). This wild type Cu(0) growth was not restored in ATCC 12600 *mco* + $pLI50_{mco}$. Hyper copper-tolerance was lost in the *mco* mutant, showing that it was linked to the *copB/mco* operon but this phenotype was not complemented in ATCC 12600 *mco* + $pLI50_{mco}$. However, Sitthisak *et al*. (2005) suggest that the *mco* transposon insertion may also be affecting the upstream *copB* and the hyper copper-tolerance of ATCC 12600 may therefore be entirely due to CopB. However, this strain may also contain other mutations and therefore this conclusion cannot be confirmed from the results shown in Figure 4.5 alone.

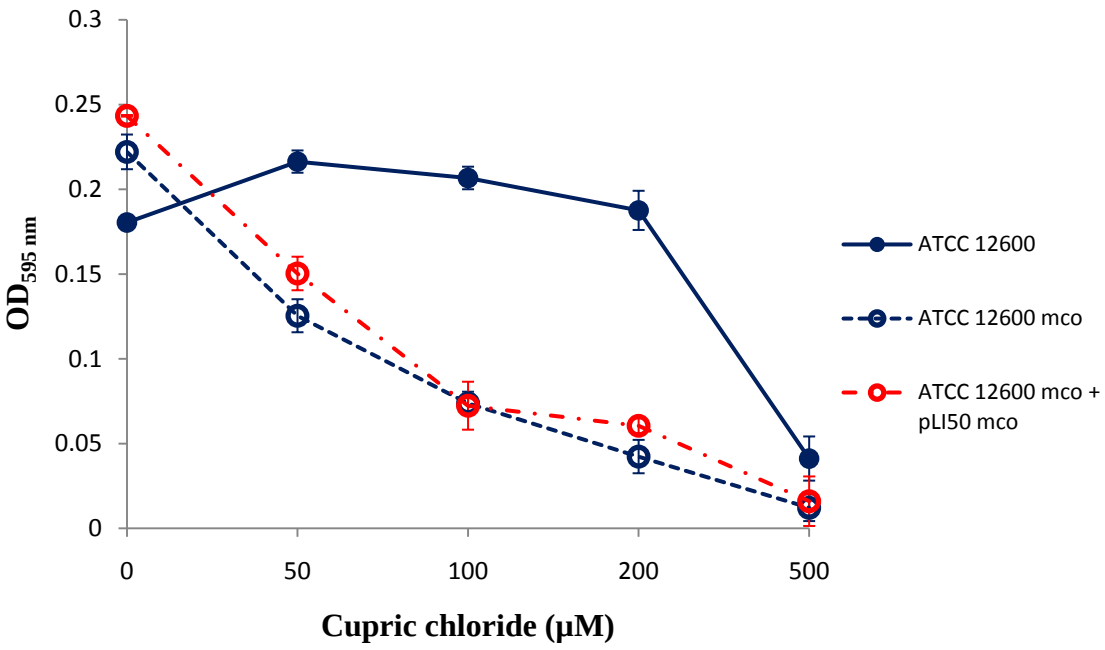


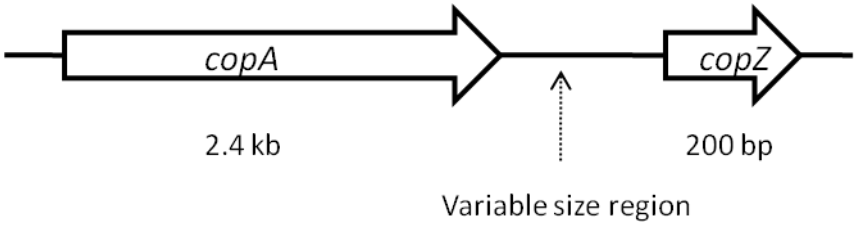
Figure 4.5. The effect of copper on the CRPMI growth of *S. aureus* strain ATCC 12600, its *mco::*Tn*917 lacZ* mutant, and the *mco* mutant containing a wild type *mco* gene. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. Strains were incubated in 0, 50, 100, 200 or 500 µM $CuCl_2$. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Results shown represent the means and standard deviations of three independent experiments.

4.2.5. The *copA* and *copB* expression varied between *S. aureus* strains

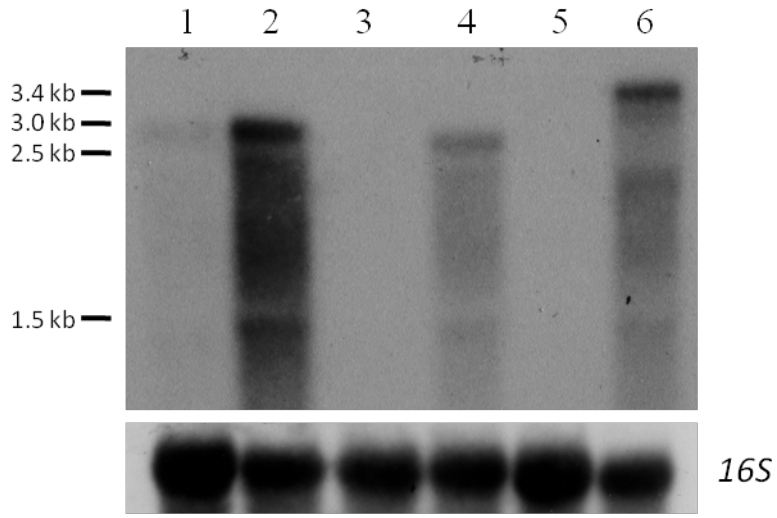
The *copB* ATPase is found in both ATCC 12600 and PM64. However, although the copper tolerances of these two strains were significantly different (Figure 4.2) the ATCC 12600 hyper copper-tolerance was observed to be linked to *copB/mco* (Figure 4.5B). The transcription levels of *copA* or *copB* have not been compared between strains. Therefore, northern blot analysis was used to examine *copA* and *copB* expression in *S. aureus* Newman, ATCC 12600 and PM64 in CRPMI with either Cu(0) and Cu(10). For RNA extraction, cultures were only challenged with 10 µM $CuCl_2$ as higher concentrations resulted in growth inhibition which affected the subsequent efficiency of the extraction process.

A *copA* probe was created using primers CopA-probe-F and CopA-probe-R. Transcription of *copA* was induced by copper for Newman, ATCC 12600 and PM64, producing three transcripts (Figure 4.6B). Transcripts of 2.5 kb and 1.5 kb were found in all three strains with the 2.5 kb product probably representing the 2.4 kb *copA* gene alone. However, the largest transcript varied in size of: 3.4 kb in PM64, 3 kb in Newman and 2.9 kb in ATCC 12600. This was due to strain variation in the intergenic distance between *copA* and *copZ* (Figure 4.6A). There was some strain variation in *copA* expression in Cu(10) as densitometry analysis showed that ATCC 12600 *copA* transcription was 3.7-fold lower than Newman *copA* and 2.8-fold lower than PM64 *copA* (Figure 4.6B).

A



B



C

Lane	**1**	**2**	**3**	**4**	**5**	**6**
Expression level	0.07	0.73	0.02	0.20	0.02	0.55

Figure 4.6. Operon genomic arrangement and northern blot analysis of *copA*.

(A) The 2.4 kb *copA* gene is separated from the 200 bp *copZ* gene by 359 bp in Newman and 820 bp in MRSA252.

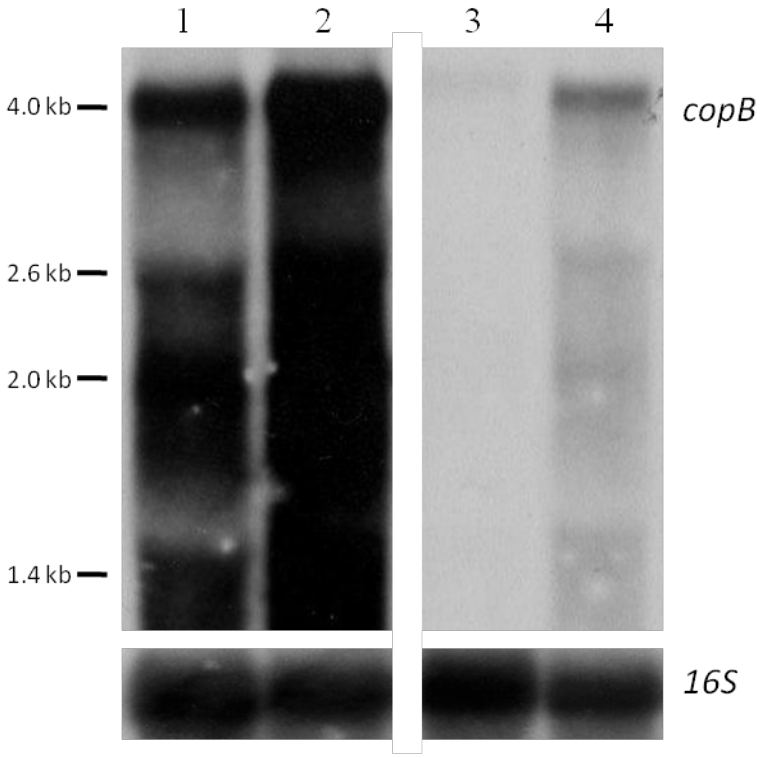
(B) RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *copA* DNA probe. (1) Newman, (2) Newman plus 10 µM $CuCl_2$, (3) ATCC 12600, (4) ATCC 12600 plus 10 µM $CuCl_2$, (5) PM64 and (6) PM64 plus 10 µM $CuCl_2$. The blot was then stripped and re-hybridised with a *16S* rRNA control probe. Blot shown is representative of two repeat experiments. **(C)** Expression levels of the genes in **(B)** are shown as a proportion of the expression level of the *16S* control.

A *copB* probe was created using primers CopB-probe-F and CopB-probe-R. *copB* transcript levels differed significantly between PM64 and ATCC 12600 (Figure 4.7). Copper induced *copB* expression in these strains and produced four transcripts of 1.4 kb, 2 kb, 2.6 kb and 4 kb. The largest 4 kb transcript probably represents the entire *copB/mco* operon and the 2.0 kb transcript is likely to be the *copB* gene alone. In Cu(0), ATCC 12600 exhibited a 15.8-fold higher level of *copB* transcription than PM64 (Figure 4.7A lanes 1 and 3). Also, ATCC 12600 *copB* expression was significantly higher (3.4-fold) in Cu(10) compared to PM64 (Figure 4.7A lanes 2 and 4). Therefore, overall expression of ATCC 12600 *copB* was significantly more elevated than that of PM64 *copB*, regardless of the environmental copper concentration and this could explain the hyper copper tolerance of ATCC 12600. This also suggested that ATCC 12600 *copB* is different from PM64 *copB*, either in regulation or copy number.

4.2.6. The *copB* of ATCC 12600 is encoded on a multi-copy plasmid which was transferred to another *S. aureus* strain and provided hyper copper-tolerance

Bioinformatic analysis revealed that the *copB/mco* operon in MRSA252, of which PM64 is a clonal variant, is encoded by an integrated plasmid (Holden *et al*., 2004). The increased ATCC 12600 *copB* expression and the hyper copper-tolerance could therefore be explained if ATCC 12600 *copB* is encoded on a multi-copy plasmid. Therefore, plasmid DNA was prepared from ATCC 12600 and PM64, and ATCC 12600 but not PM64 was found to contain a plasmid (Figure 4.8A). Serial 10-fold dilutions of both these plasmid preparations were used as templates for a PCR amplification of the *copB* gene and the known chromosomally located *fur* gene, which was employed to identify chromosomal contamination of the plasmid preparations. A *fur* product was seen at a 1:10000 dilution for PM64 and ATCC 12600 (Figure 4.8C lanes 6 and 15).

A



B

Lane	**1**	**2**	**3**	**4**
Expression level	0.95	1.18	0.06	0.35

Figure 4.7. (A) Northern blot analysis of *copB*. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *copB* DNA probe. The four lanes shown are from the same blot. (1) ATCC 12600, (2) ATCC 12600 plus 10 µM $CuCl_2$, (3) PM64 and (4) PM64 plus 10 µM $CuCl_2$. The blot was then stripped and re-hybridised with a *16S* rRNA control probe. Blot shown is representative of three repeat experiments. **(B)** Expression levels of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

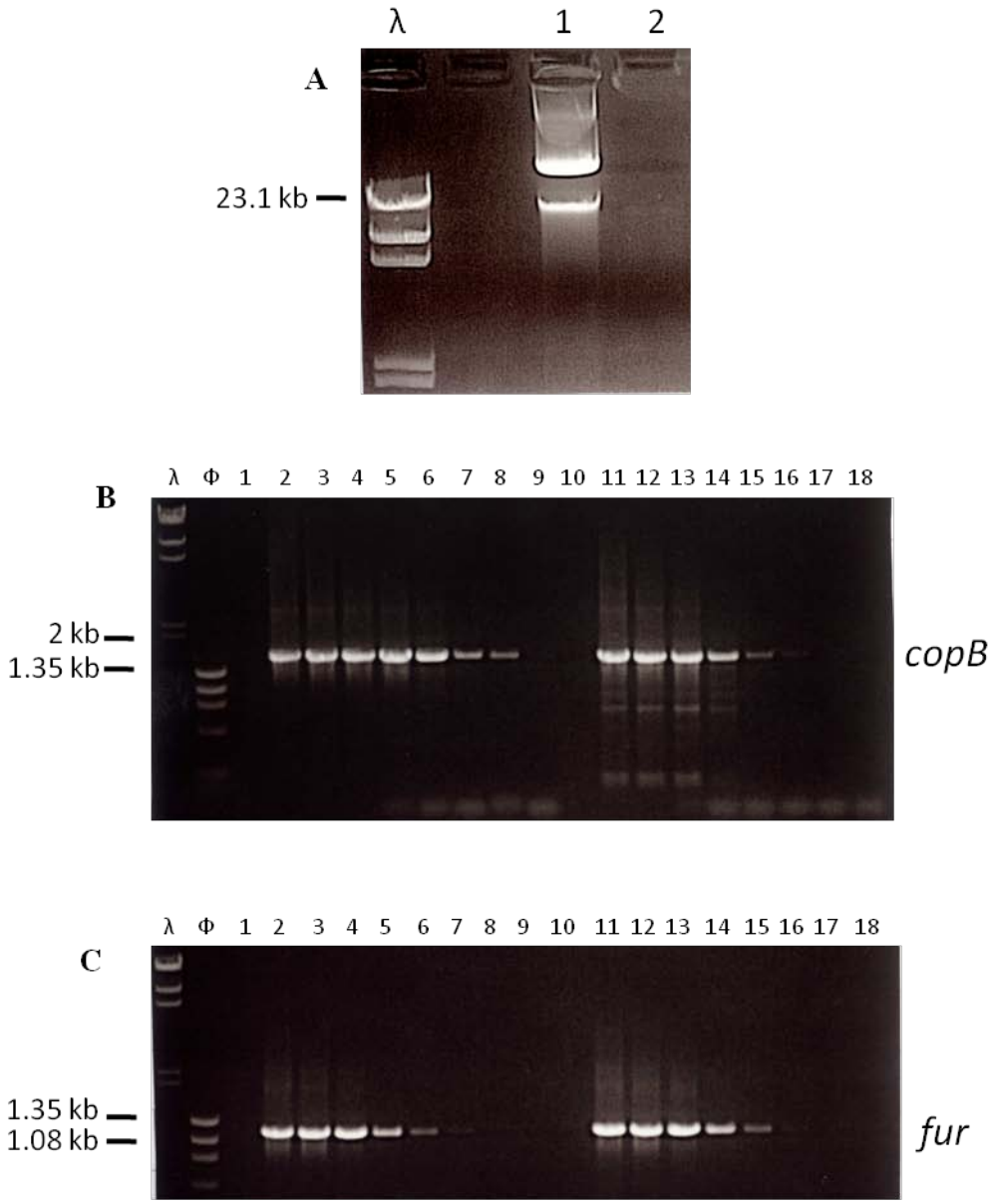


Figure 4.8. Identification of the ATCC 12600 plasmid and confirmation that the ATCC 12600 *copB* gene is plasmid located.

(A) Agarose gel of 5 µl plasmid preparations from (1) ATCC 12600 and (2) PM64.

(B) *copB* and **(C)** *fur* PCR amplification using the plasmid preparations as the DNA template. (1) Blank, (2 – 10) serial tenfold dilutions of the ATCC 12600 plasmid DNA preparation and (11 – 18) serial tenfold dilutions of the PM64 plasmid DNA preparation. Product size was estimated using 5 ul of lambda (λ) or ΦX174 (Φ) ladder.

In PM64, a *copB* product was only amplified from the same template dilution which produced a *fur* product (Figure 4.8B lane 15) whereas in ATCC 12600, a *copB* product was seen at the much higher dilution of 1:1000000 (Figure 4.8B lane 8). This showed that *copB* in ATCC 12600 was being amplified from a more concentrated source than chromosomal DNA contamination and therefore the ATCC 12600 *copB* is encoded by the ATCC 12600 plasmid (Figure 4.8A).

To determine if this plasmid could be transferred to another *S. aureus* strain to confer hyper copper-tolerance, phage lysate using phage phi11 was produced from ATCC 12600. This lysate was used in a transduction of *S. aureus* Newman and the transductants selected by growth on LK agar plates containing 3 mM $CuCl_2$, which inhibited all Newman wild type growth but crucially did not affect ATCC 12600 growth. A plasmid was found to be present in several of the transductants through plasmid preparations and subsequent electrophoresis on an agarose gel, one of which was cultured and this strain was named NAP1 (Newman with the ATCC 12600 Plasmid). Plasmid preparations identified a plasmid in both ATCC 12600 and NAP1 but not Newman; although Newman was observed to possibly contain a low copy plasmid (Figure 4.9A). Furthermore, a *copB* PCR product of 1.6 kb was only produced using the ATCC 12600 and NAP1 plasmid preparations as the template DNA (Figure 4.9B). Restriction digests with either *Eco*RI or *Hin*dIII, showed that the plasmid had been transferred successfully as the two digests produced equivalent sized products from the two plasmid DNA preparations (Figure 4.9C). However, the ATCC 12600 plasmid digests produced one extra product with each of the restriction enzymes (Figure 4.9C). This could have been due to incomplete digestion of the NAP1 plasmid or the presence of two plasmids in the ATCC 12600 strain.

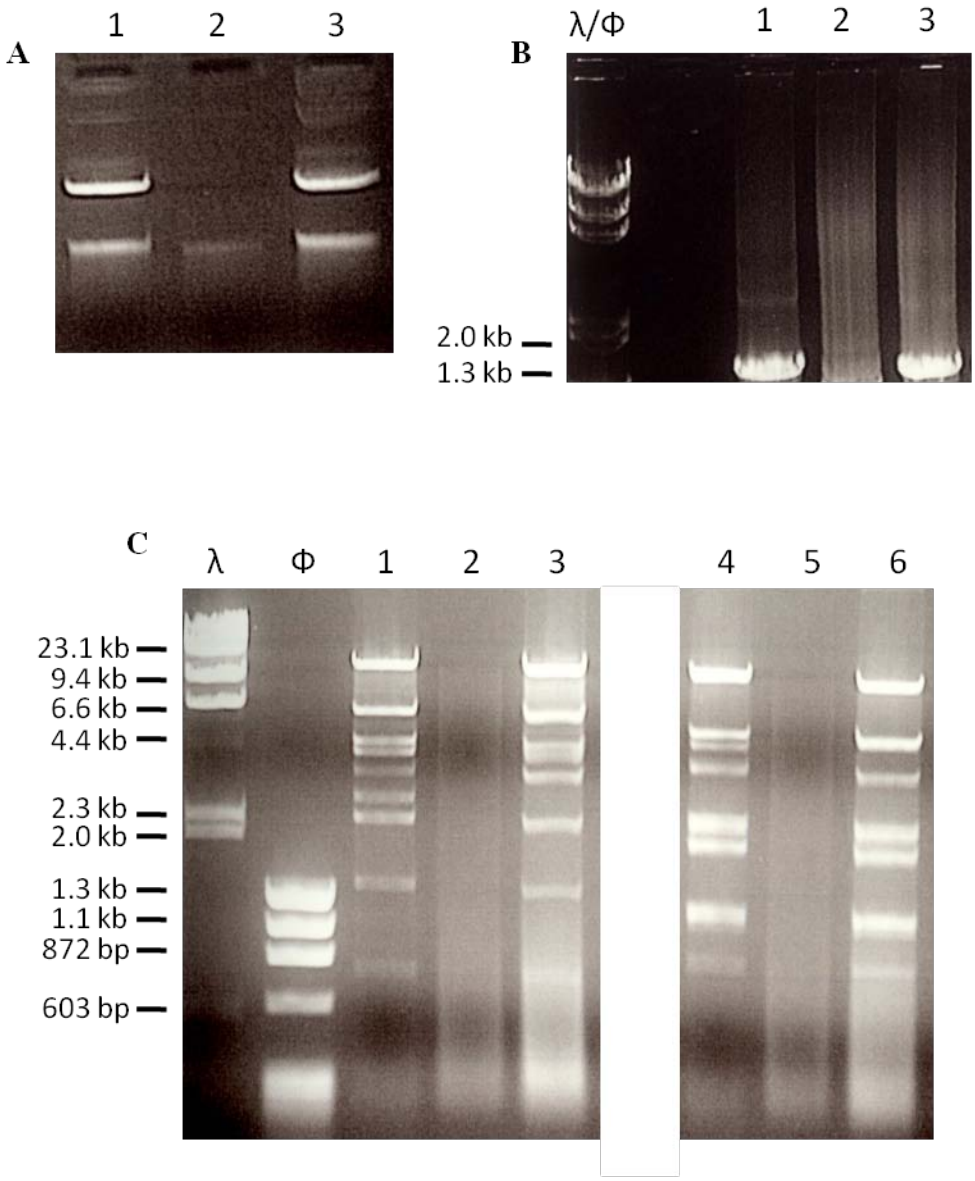


Figure 4.9. Conformation of ATCC 12600 plasmid transfer into Newman to create strain NAP1.

(A) Agarose gel of 5 µl plasmid preparation of (1) ATCC 12600, (2) Newman and (3) NAP1.

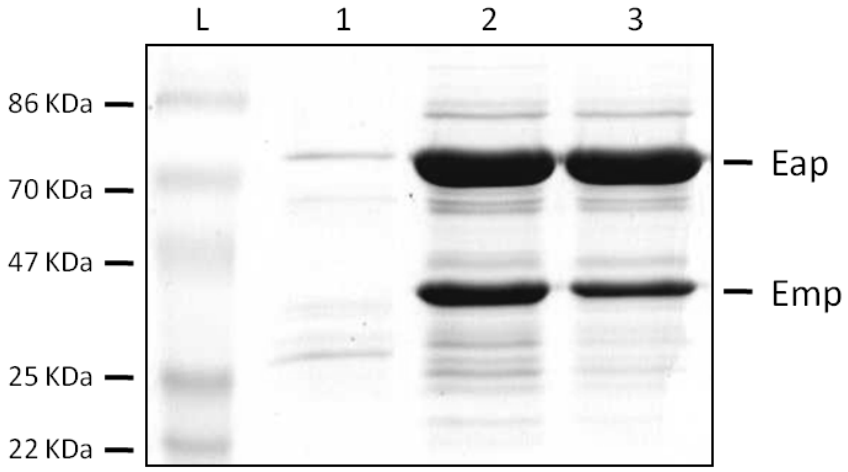
(B) PCR amplification with the *copB* primers using the plasmid preparations from (1) ATCC 12600, (2) Newman and (3) NAP1 as the template DNA. Product size was estimated using a 10 µl 1:1 (v/v) mix of lambda (λ) and ΦX174 (Φ) ladders.

(C) Restriction digests with *Eco*RI on the plasmid preparations of (1) ATCC 12600, (2) Newman and (3) NAP1 and with *Hin*dIII on the plasmid preparations of (4) ATCC 12600, (5) Newman and (6) NAP1. Product size was estimated using 5 ul of lambda (λ) or ΦX174 (Φ) ladder.

Compared to other commonly studied strains, Newman has a high expression level of the cell surface proteins Eap (70 kDa) and Emp (30 kDa), which is distinctive of this strain (Johnson *et al*., 2008). Therefore to establish that NAP1 was Newman which contained the ATCC 12600 plasmid and not ATCC 12600 itself, SDS protein profiles were compared after 17 hours growth in CRPMI using SDS PAGE mini gels (Figure 4.10A). The NAP1 SDS protein profile was identical to that of Newman and showed high levels of both Eap and Emp (Figure 4.10A lanes 2 and 3), whilst ATCC 12600 displayed few non-covalently attached surface proteins (Figure 4.10A lane 1), which confirmed that NAP1 was indeed Newman and not ATCC 12600.

To investigate if the plasmid acquisition increased copper tolerance, the growth in Cu(100) of NAP1, Newman and ATCC 12600 was analysed in CRPMI (Figure 4.10B). The relative growth in Cu(100) for each strain was calculated from the growth change observed in Cu(100) when compared to Cu(0) and expressed as a percentage (Figure 4.2B). The Cu(100) relative growth of NAP1 was 87%, which was a substantial increase when compared to the relative growth of Newman and which was now equivalent to ATCC 12600 ($P = 0.0085$). Therefore, the transfer of the ATCC 12600 plasmid to Newman had significantly increased copper-tolerance, showing that this *copB* containing plasmid could be moved into other *S. aureus* strains to confer a hyper copper-tolerance phenotype.

A



B

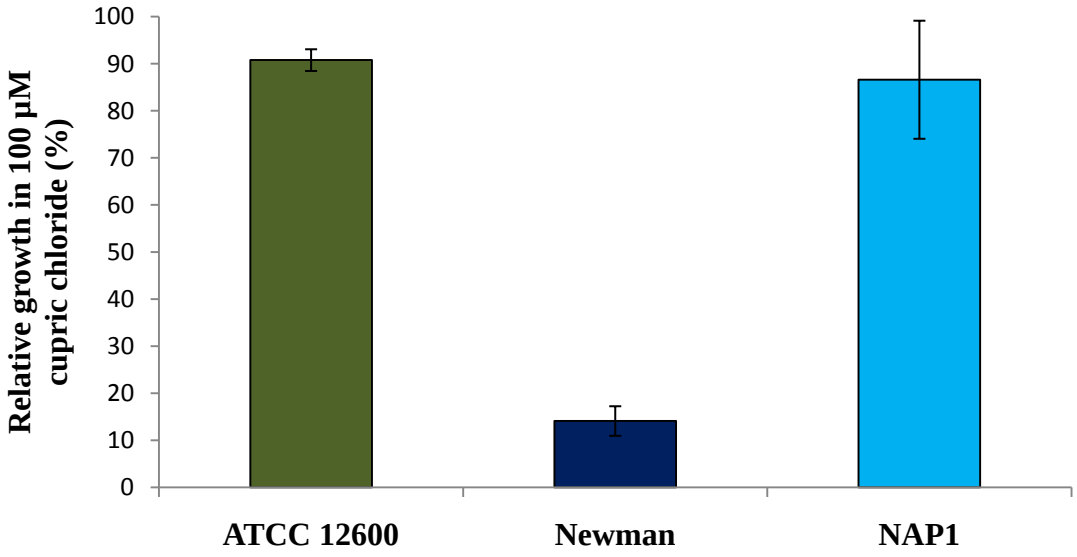


Figure 4.10. Confirmation that NAP1 was Newman and analysis of the NAP1 copper tolerance to 100 µM $CuCl_2$.

(A) SDS PAGE mini-gel of the CRPMI SDS extracts. Protein was harvested from stationary cultures after 17 hours incubation. (1) ATCC 12600, (2) Newman and (3) NAP1. Product size was estimated using 5 µl of pre-strained dual colour protein ladder (L).

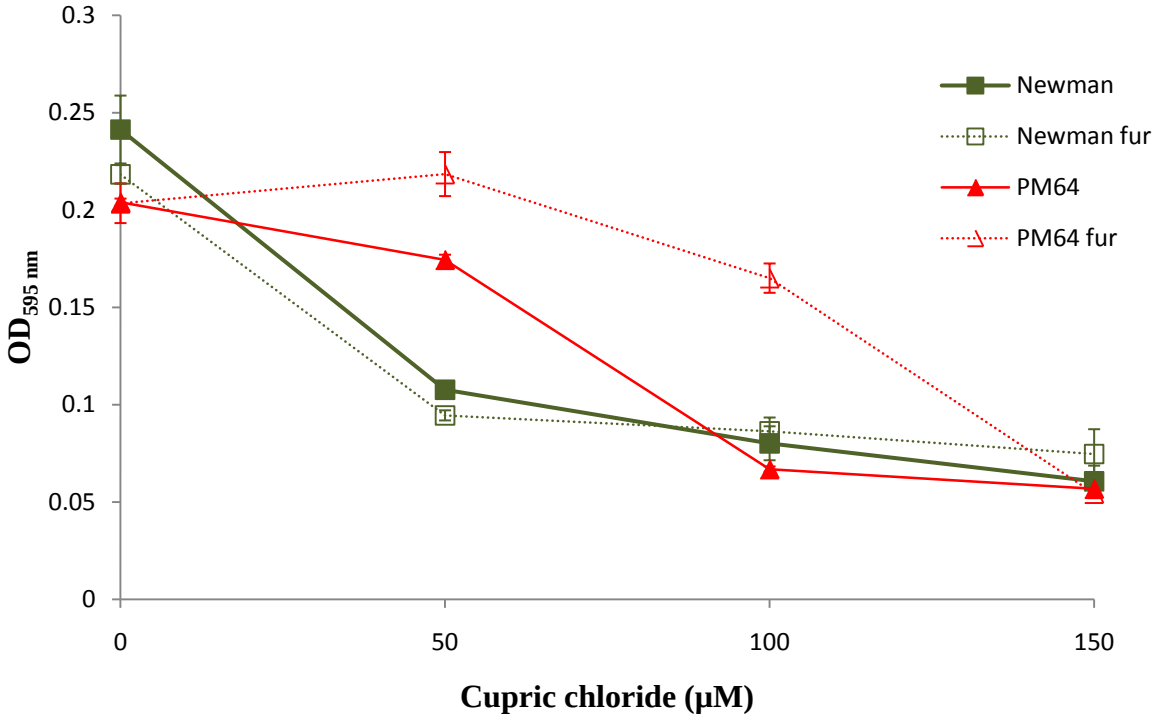
(B) CRPMI cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. The strains shown are; Newman, ATCC 12600 and NAP1. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Displayed is the relative growth of the *S. aureus* strains in Cu(100) compared to growth in Cu(0). Results shown represent the means and standard deviations of three independent experiments.

4.2.7. The Ferric-Uptake-Regulator (Fur) is involved in *S. aureus* copper tolerance

The previous experiments identified *copB/mco* as a major contributor to *S. aureus* copper tolerance but the copper-responsive regulator of either this or the *copA* operon was unknown. Previous studies have implicated iron as a regulator of *copA*. Allard *et al*. (2006) observed *in vivo copA* iron regulation but did not analyse a *fur* mutant, and a microarray study showed de-repression of *copA* in a *S. aureus* SH1000 *fur* mutant (Personal communication from Dr Jayaswal, Illinois State University). Therefore to further investigate the role of Fur in the copper-dependant regulation of *copA* and *copB*, the CRPMI copper tolerances of Newman, PM64 and ATCC 12600 were compared to those of their isogenic *fur* mutants. Newman *fur* was used as the donor strain to transduce the *fur* mutation into ATCC 12600 using phage phi11 which were selected by resistance to tetracycline. To confirm successful transductants, a *fur* flanking PCR was performed using transductant colony total DNA preparations as the template DNA which showed the successful replacement of 402 bp of ATCC 12600 *fur* with the 1.5 kb of *tet* cassette to create strain ATCC 12600 *fur* (data not shown).

There was no difference in the copper tolerance of Newman and Newman *fur*, as both strains displayed significantly reduced growth in Cu(50) and no growth in Cu(100) (Figure 4.11A). However, both the ATCC 12600 and PM64 *fur* mutants had an increased copper tolerance compared to their wild types. PM64 *fur* was able to survive in Cu(100) whereas the wild type could not (Figure 4.11A). In addition, ATCC 12600 *fur* could survive in Cu(600) but the wild type could only grow in Cu(200) (Figure 4.11B). Therefore, Fur was potentially involved in the copper homeostasis/tolerance of both ATCC 12600 and PM64 but not Newman, suggesting a potential link between copper and iron in *S. aureus*.

A



B

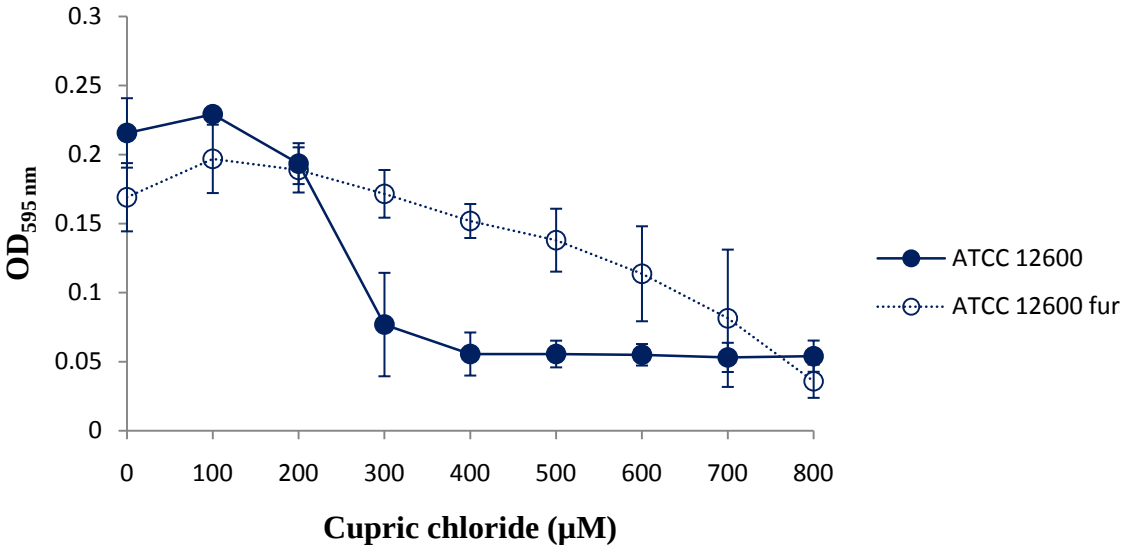


Figure 4.11. The effect of copper on the CRPMI growth of the three *S. aureus* strains and their isogenic *fur* mutants. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. Results shown represent the means and standard deviations of three independent experiments.

(A) Newman, Newman *fur*, PM64 and PM64 *fur*.

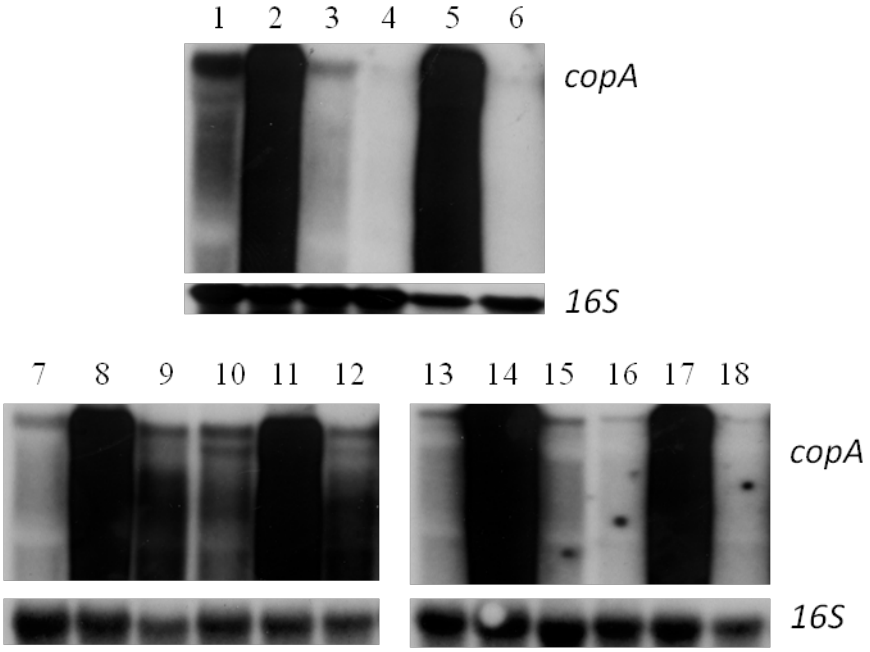
(B) ATCC 12600 and ATCC 12600 *fur*.

If Fur is a regulator of *copA* or *copB* then the increased copper tolerances of ATCC 12600 *fur* and PM64 *fur* may have been due to full de-repression of these resistance genes in the mutants. Therefore to investigate this possibility, northern blot analysis was used to examine CRPMI *copA* and *copB* transcription in Cu/Fe(0), Cu(10) and Fe(50) using the three strains and their *fur* mutants. Due to the low levels of transcription, blots required significant exposure and therefore densitometry readings for induction by Cu(10) although shown, were above the detectable range.

copA expression was repressed by iron in Newman but this effect was minor when compared to the massive induction caused by copper (Figure 4.12A). However, there was no significant iron regulation of *copA* in either ATCC 12600 or PM64 (Figure 4.12A). In the Newman *fur* mutant, *copA* expression in Cu(0) was reduced (Figure 4.12A) but *copA* expression was unchanged in ATCC 12600 *fur* and PM64 *fur*. Copper induction of *copA* was not affected by the *fur* mutation in any of the three strains. Therefore, although the minor Newman *copA* iron repression was disrupted in Newman *fur*, iron and Fur were not involved in *copA* regulation of ATCC 12600 or PM64.

Similar to *copA*, the iron status did not affect ATCC 12600 *copB* transcription (Figure 4.13A). There was also no change in *copB* expression in ATCC 12600 *fur* or PM64 *fur* compared to expression in the wild types. Therefore, the increase in copper tolerance previously observed for the *fur* mutants of PM64 and ATCC 12600 (Figure 4.11) could not be simply explained by Fur de-repression of the *copA* or *copB* operons, as Fur and iron played a minor role in their regulation.

A



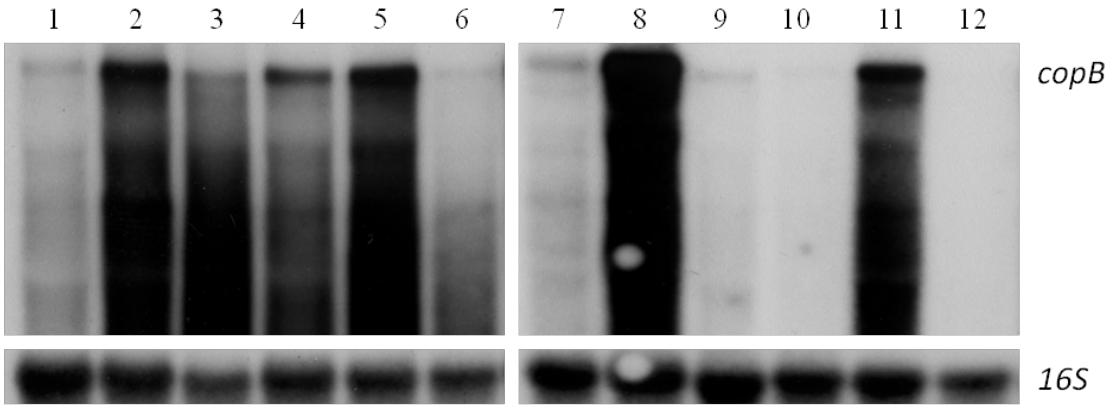
B

Lane	1	2	3	4	5	6
Expression level	0.81	0.96	0.37	0.13	1.28	0.22

Lane	7	8	9	10	11	12	13	14	15	16	17	18
Expression level	0.94	1.34	1.06	1.04	1.60	1.19	0.67	1.57	0.61	0.55	1.48	0.65

Figure 4.12. Northern blot analysis of *copA*. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *copA* DNA probe. The blots were then stripped and re-hybridised with a *16S* rRNA control probe. Blots shown are representative of at two repeat experiments. **(A)** (1) Newman, (2) Newman plus 10 µM $CuCl_2$, (3) Newman plus 50 µM $Fe_2(SO_4)_3$, (4) Newman *fur*, (5) Newman *fur* plus 10 µM $CuCl_2$, (6) Newman *fur* plus 50 µM $Fe_2(SO_4)_3$, (7) ATCC 12600, (8) ATCC 12600 plus 10 µM $CuCl_2$, (9) ATCC 12600 plus 50 µM $Fe_2(SO_4)_3$, (10) ATCC 12600 *fur*, (11) ATCC 12600 *fur* plus 10 µM $CuCl_2$, (12) ATCC 12600 *fur* plus 50 µM $Fe_2(SO_4)_3$, (13) PM64, (8) PM64 plus 10 µM $CuCl_2$, (14) PM64 plus 50 µM $Fe_2(SO_4)_3$, (15) PM64 *fur*, (16) PM64 *fur* plus 10 µM $CuCl_2$ and (17) PM64 *fur* plus 50 µM $Fe_2(SO_4)_3$. **(B)** Expression levels of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

A



B

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Expression level	0.43	1.32	0.64	0.76	1.40	0.56	0.34	1.61	0.27	0.24	1.46	0.23

Figure 4.13. Northern blot analysis of *copB*. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *copB* DNA probe. The blots were then stripped and re-hybridised with a *16S* rRNA control probe. Blots shown are representative of two repeat experiments. **(A)** (1) ATCC 12600, (2) ATCC 12600 plus 10 µM $CuCl_2$, (3) ATCC 12600 plus 50 µM $Fe_2(SO_4)_3$, (4) ATCC 12600 *fur*, (5) ATCC 12600 *fur* plus 10 µM $CuCl_2$, (6) ATCC 12600 *fur* plus 50 µM $Fe_2(SO_4)_3$, (7) PM64, (8) PM64 plus 10 µM $CuCl_2$, (9) PM64 plus 50 µM $Fe_2(SO_4)_3$, (10) PM64 *fur*, (11) PM64 *fur* plus 10 µM $CuCl_2$, (12) PM64 *fur* plus 50 µM $Fe_2(SO_4)_3$. **(B)** Expression levels of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

4.2.8. Identification of the *S. aureus* CsoR repressor

As Fur was found not to be the copper regulator of *copAZ* or *copB/mco*, the Newman genome was used to search for a homologue of known bacterial ATPase copper regulators such as *copY* or *cueR*, but none was found. However, a BLASTP (NCBI) search using the *M. tuberculosis* CsoR protein sequence and selecting for the subset of *S. aureus*, identified a Newman CsoR orthologue (NWMN_1991). This *S. aureus* CsoR (112 residues) was 100% conserved in most sequenced strains and 99% in a few, and varied in size from the CsoR proteins of *M. tuberculosis* (119 residues) and *B. subtilis* (101 residues). Newman CsoR contained DUF156, a conserved domain of unknown function and also the three amino acids that have previously been recognized as being important for *M. tuberculosis* CsoR copper ion binding; a histidine at position 61 and two cysteines at positions 36 and 65 (Liu *et al*., 2007). These were used as a template for an alignment which was created utilising the ClustalW2 programme (EBI). The Newman CsoR was found to be 24% identical to the *M. tuberculosis* CsoR and 36% identical to the *B. subtilis* CsoR at the amino acid level (Figure 4.14A).

Figure 4.14B shows the chromosomal arrangement of the *csoR* from *M. tuberculosis*, *B. subtilis* and *S. aureus* Newman. Analysis of the region flanking Newman *csoR* identified a small downstream 210 bp gene termed *csoZ*. The predicted CsoZ protein contains a conserved heavy-metal associated protein domain (HMA) which is approximately 30 residues in length and typically found in proteins that transport or detoxify heavy metals, such as heavy metal ATPases and copper chaperones.

A

```
                                                                    *
M. tuberculosis    1 MSKELTAKKRAALNRLKTVRGHLDGIVRMLESDAYCVDVMKQISAVQSSL
B. subtilis        9 LNHKSSKEKDQITNRLKRIEGQVRGIQNMVENDRYCVDILVQISAVQAAM
S. aureus Newman  21 NAHHSEQIKTNLKSRLNRIEGQVRAINRMIEEDVYCDDVLTQIRATRSAL

                                    *   *
M. tuberculosis   51 ERANRVMLHNHLETCFSTAVLDGHGQAAIEELIDAVKFTPALTGPHARLG
B. subtilis       59 KNVALHLLEDHAHHCVADAIKSGDGEQAISELLDVFKKFTKS
S. aureus Newman  71 NSVAIKLLEQHMKSCIMNKVNQGAQEEAMEELLVTFQKLIKD

M. tuberculosis  102 GAAVGESATEEPMPDASNM
```

B

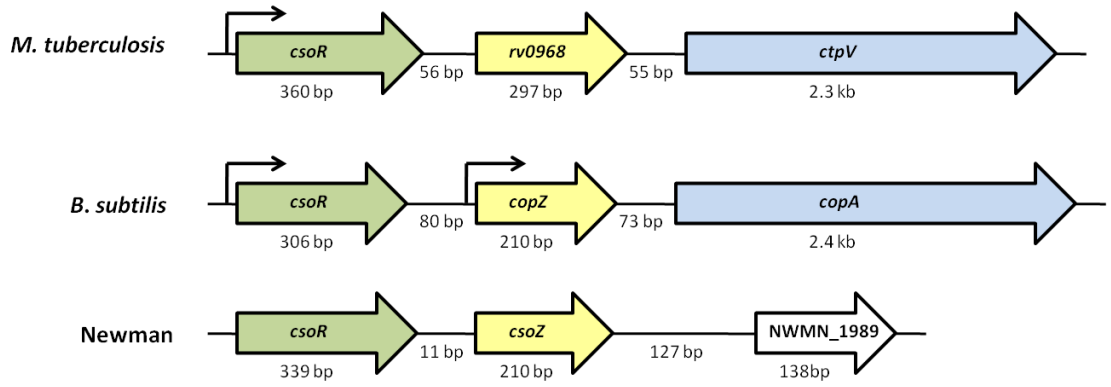


Figure 4.14. The Newman CsoR regulator.

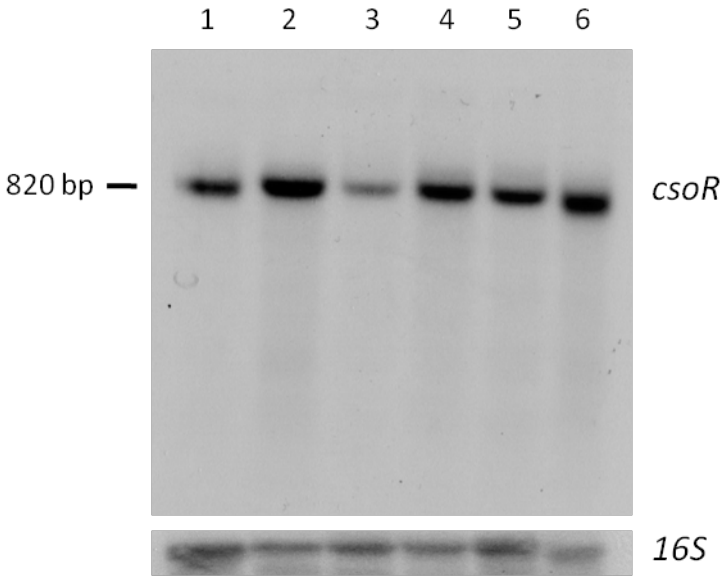
(A) BLASTP amino acid sequence alignment of CsoR from *M. tuberculosis*, *B. subtilis* and *S. aureus* strain Newman. The highlighted residues (*) Cys36, His61 and Cys65 have been identified as being important for copper ion binding and were used as a template for the alignment utilising the ClustalW2 programme (EBI). Black residues represent exact matches. The *S. aureus* CsoR has 41% of the residues for the DUF156 domain.

(B) Genetic arrangement of *M. tuberculosis*, *B. subtilis* and *S. aureus* Newman *csoR* genes with sizes and known transcriptional start sites shown as black arrows. The *csoR* regulators are shown in green and the downstream copper chaperones in yellow. The downstream ATPases regulated by CsoR are shown in blue.

The small size of CsoZ (69 residues) suggests it is a copper chaperone rather than a copper transporter, which are typically over 600 residues in length. The close proximity and similar putative function of these two genes suggests that they form an operon. There is also a putative transcriptional terminator approximately 40 bp downstream of the *csoZ* gene. There were significant differences in the genetic arrangement of Newman *csoR* compared to the *csoR* of *M. tuberculosis* and *B. subtilis*. Like that of *M. tuberculosis* and *B. subtilis*, Newman *csoR* was located upstream of a putative copper chaperone (*csoZ*) and an unknown gene (NWMN_1989) (Liu *et al*., 2007; Smaldone & Helmann, 2007). However, even though *csoZ* and the *B. subtilis copZ* both contained the HMA domain and were 210 bp in size, they were not homologous at the amino acid level. Also, unlike the *csoR* of the other two bacteria the Newman *csoRZ* operon (NWMN_1991/1990) was not located near to the *copAZ* operon (NWMN_2457/2458).

Northern blot analysis was used to analyse *csoR* transcription in the *S. aureus* strains Newman, ATCC 12600 and PM64. A *csoR* probe was created using primers CsoR-probe-F and CsoR-probe-R. A single transcript of 820 bp was detected in all three strains and its expression was increased 2-fold in Cu(10) (Figure 4.15A). The size of this transcript was larger than the predicted 600 bp for *csoRZ* alone. Therefore, this transcript may have began further upstream than the start of *csoR*. However, it is also possible that this larger transcript includes *csoRZ* and the 138 bp gene located 127 bp downstream of *csoZ* (Figure 4.14B). This additional gene showed no homology with any known proteins and contained no recognised protein domains.

A



B

Lane	**1**	**2**	**3**	**4**	**5**	**6**
Expression level	1.95	3.96	1.22	3.37	2.20	4.40

Figure 4.15. *S. aureus csoR* transcription.

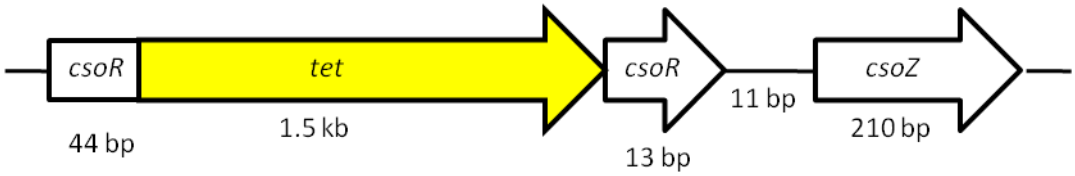
(A) Northern blot analysis of Newman, ATCC 12600 and PM64 *csoR* transcription. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *csoR* DNA probe. (1) Newman, (2) Newman plus 10 µM $CuCl_2$, (3) ATCC 12600, (4) ATCC 12600 plus 10 µM $CuCl_2$, (5) PM64 and (6) PM64 plus 10 µM $CuCl_2$. The blot was then stripped and re-hybridised with a *16S* rRNA control probe. Blot shown is representative of two repeat experiments. **(B)** Expression levels of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

This northern blot analysis showed that the *S. aureus csoR* regulation was similar to *csoR* of *M. tuberculosis*, in that it was copper induced and transcribed along with at least one of the downstream genes. Copper regulation of *S. aureus csoR* suggested either that another unidentified copper regulator was involved in *csoR* transcription or, if CsoR represent the only *S. aureus* copper regulator, that it was auto-regulated similar to the auto-regulation of the *M. tuberculosis csoR.*

4.2.9. Construction of the *csoR* mutants and *csoR* mutants with a wild type copy of the *csoR* gene encoded on the pMK4 plasmid

To investigate the role of CsoR in *S. aureus*, *csoR* mutants and *csoR* mutants with a wild type copy of the *csoR* gene on the pMK4 plasmid were created. The construction of these strains is fully described in section 2.8.1. Figure 4.16 shows the disruption of *csoR* by a tetracycline cassette and the region of Newman *csoR* cloned into pMK4. PCR amplification using DNA preparations confirmed construction of the three *csoR* mutants. PCR product size was increased from 2.1 kb in the wild types to 3.3 kb in the mutants by the replacement of 282 bp of *csoR* by the 1.5 kb *tet* cassette (Figure 4.17A). The *csoR* flanking PCR using PM64 DNA as the template produced a larger product of 3.1 kb which was due to a longer (1 kb) region separating the third PM64 gene of the *csoR* operon from the flanking primer. A similar PCR was used to verify that Newman + pMK4$_{csoR}$ and PM64 + pMK4$_{csoR}$ were *csoR* mutants (Figure 4.17B lanes 1 and 3). Plasmid preparations and digestion with *Eco*RI and *Bam*HI excised the 711 bp *csoRZ* fragment from the 5.5 kb pMK4 plasmid, showing that the plasmid and cloned *csoR* region were both present (Figure 4.17B lanes 2 and 4). ATCC 12600 was found to be naturally chloramphenicol resistant, which prevented selection for pMK4$_{csoR}$.

A



B

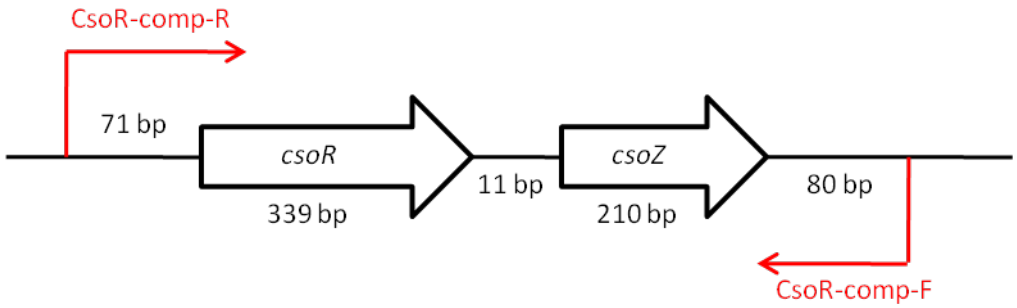
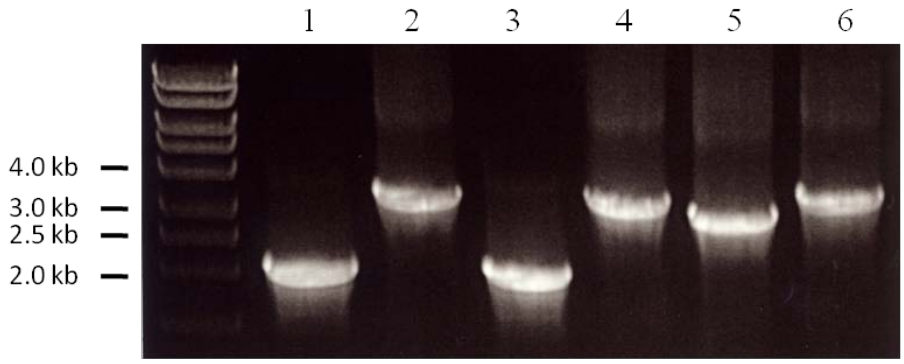


Figure 4.16. The *csoR* mutant and *csoR* mutant with a wild type copy of the *csoR* gene.

(A) Chromosomal gene arrangement of the *csoR* mutants. 282 bp of *csoR* was replaced by the 1.5 kb *tet* cassette which lacked its own terminator to maintain downstream operon transcription.

(B) Region of Newman *csoR* cloned into the expression plasmid pMK4 and introduced into the Newman and PM64 mutant strains by phage transduction as described in Chapter 2. Shown are the locations of the two complementing primers

A



B

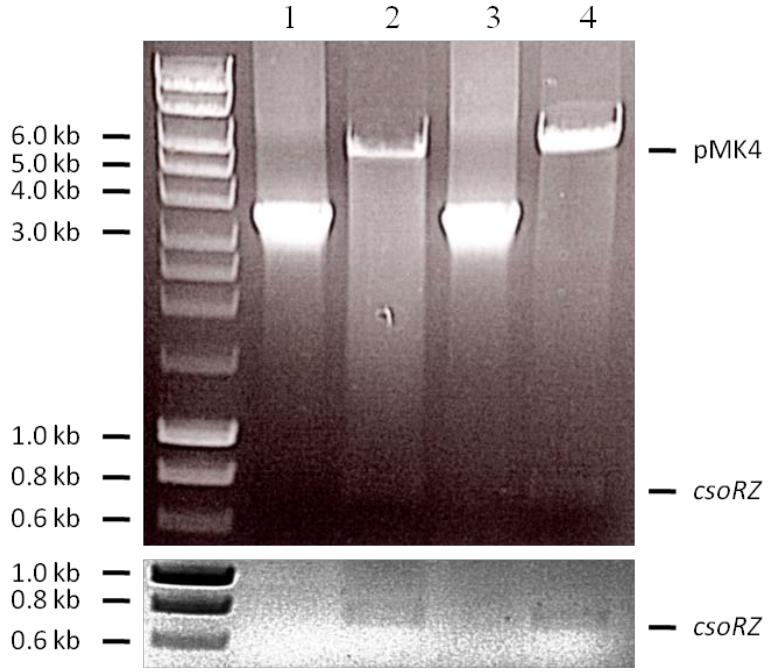


Figure 4.17. Confirmation of the *csoR* mutagenesis and pMK4$_{csoR}$ transduction.

(A) Agarose gel of 5 µl PCR product when using primers CsoR-flank-F and CsoR-flank-R and total DNA preparations of the *csoR* mutants and wild types as the template. (1) Newman, (2) Newman *csoR*, (3) ATCC 12600, (4) ATCC 12600 *csoR*, (5) PM64 and (6) PM64 *csoR*.

(B) CsoR-flank-F and CsoR-flank-R PCR using whole DNA preparations as the DNA template. (1) Newman *csoR* + pMK4$_{csoR}$ and (3) PM64 *csoR* + pMK4$_{csoR}$. *Eco*RI and *Bam*HI digestion of plasmid preparations from (2) Newman *csoR* + pMK4$_{csoR}$ and (4) PM64 *csoR* + pMK4$_{csoR}$.

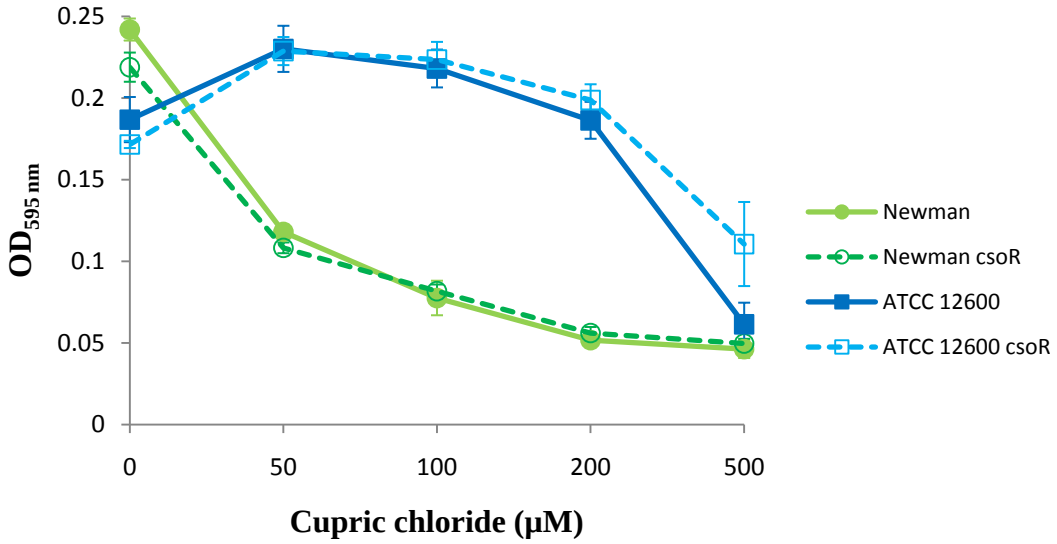
4.2.10. Copper tolerances of the three *csoR* mutants

The CRPMI copper tolerances of Newman, ATCC 12600 and PM64 wild types and *csoR* mutants were investigated (Figure 4.18). Newman and the Newman *csoR* mutant showed no significant differences in CRPMI copper tolerance (Figure 4.18A). However, ATCC 12600 *csoR* showed increased tolerance to Cu(500) (Figure 4.18A). This increase was variable but statistically significant (P = 0.042). The PM64 *csoR* mutant did not grow to the same $OD_{595\ nm}$ as the wild type in either Cu(0) or Cu(50) (Figure 4.18B). However, their copper tolerance was found to be identical when the relative growth in Cu(50) was calculated. Conversely in Cu(100), PM64 *csoR* + $pMK4_{csoR}$ displayed improved copper tolerance compared to both the wild type and *csoR* mutant strain (Figure 4.18B). This was not seen for the Newman *csoR* + $pMK4_{csoR}$ (data not shown). Therefore, disruption of the Newman and ATCC 12600 *csoR* had no effect on copper tolerance, except in Cu(500) for ATCC 12600. However, although disruption of the PM64 *csoR* had no effect on copper tolerance, the over expression of *csoR* in PM64 *csoR* + $pMK4_{csoR}$ improved tolerance in Cu(100).

4.2.11. CsoR regulates both *copA* and *copB* in response to environmental copper

Northern blot analysis was used to analyse the copper-dependant transcriptional regulation of *copA* and *copB* in the three *csoR* mutant strains. Disruption of *csoR* led to de-repression of *copA* in Cu(0) for all three *S. aureus* strains, whilst the wild type CsoR-dependant copper regulation was restored in both Newman + $pMK4_{csoR}$ and PM64 + $pMK4_{csoR}$ (Figure 4.19). This correlated with the known function of the CsoR repressor in *B. subtilis* which prevents transcription of *copAZ* in Cu(0) through direct operon promoter binding.

A



B

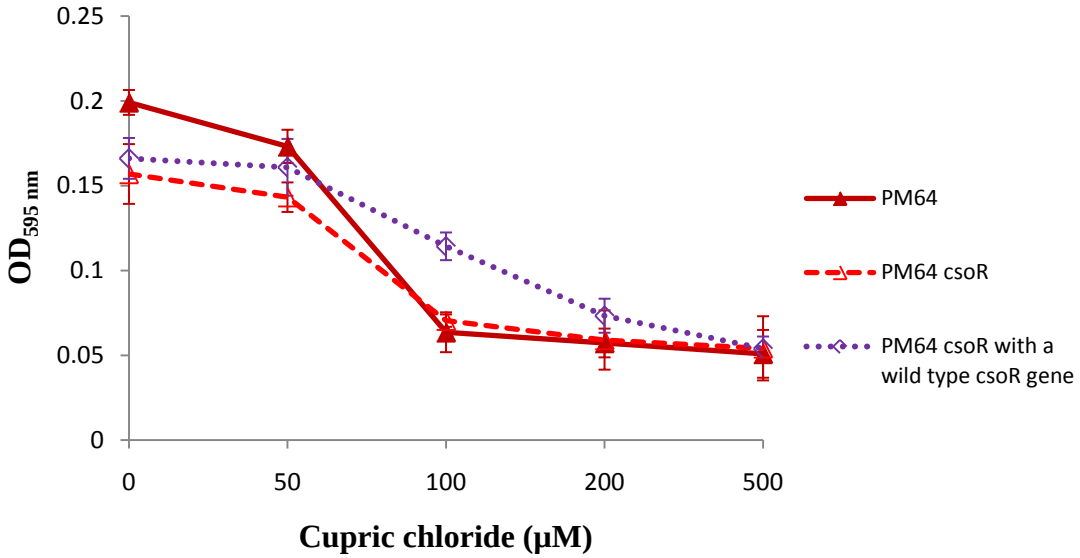
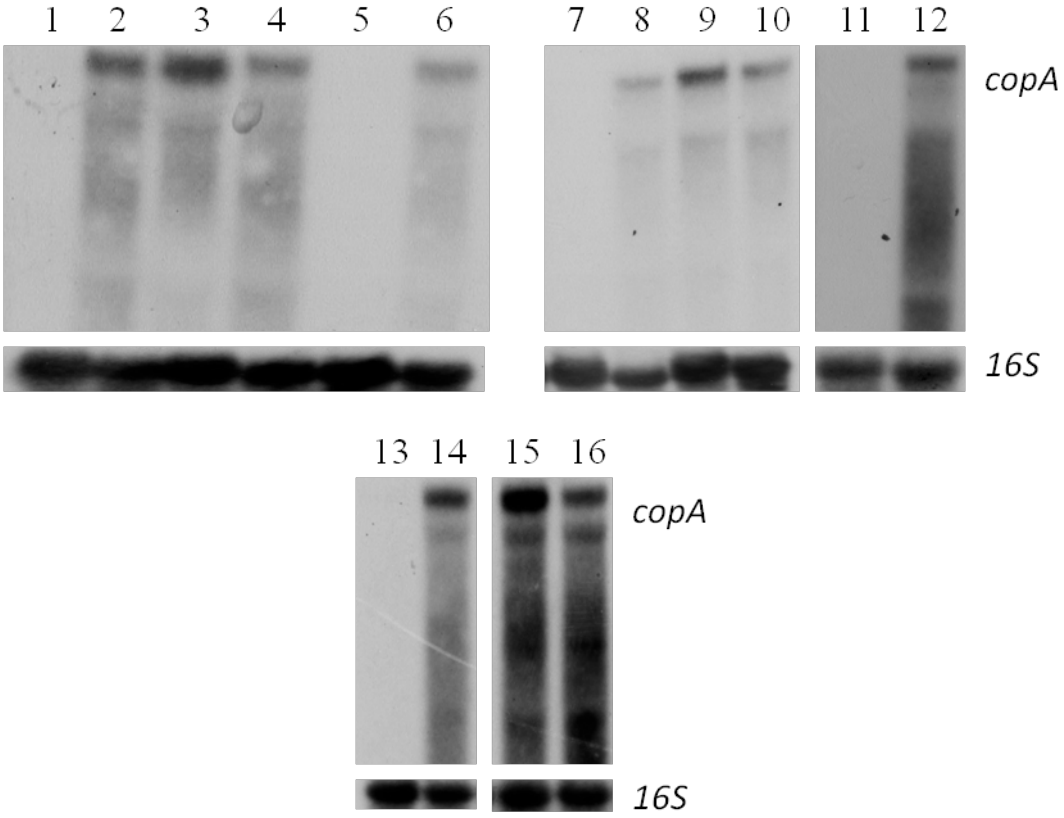


Figure 4.18. The effect of copper on the CRPMI growth of the *S. aureus* wild type and *csoR* mutant strains. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Results shown represent the means and standard deviations of three independent experiments.

(A) Newman and ATCC 12600 wild types and *csoR* mutants.

(B) PM64, PM64 *csoR* and PM64 *csoR* + $pMK4_{csoR}$.

A



B

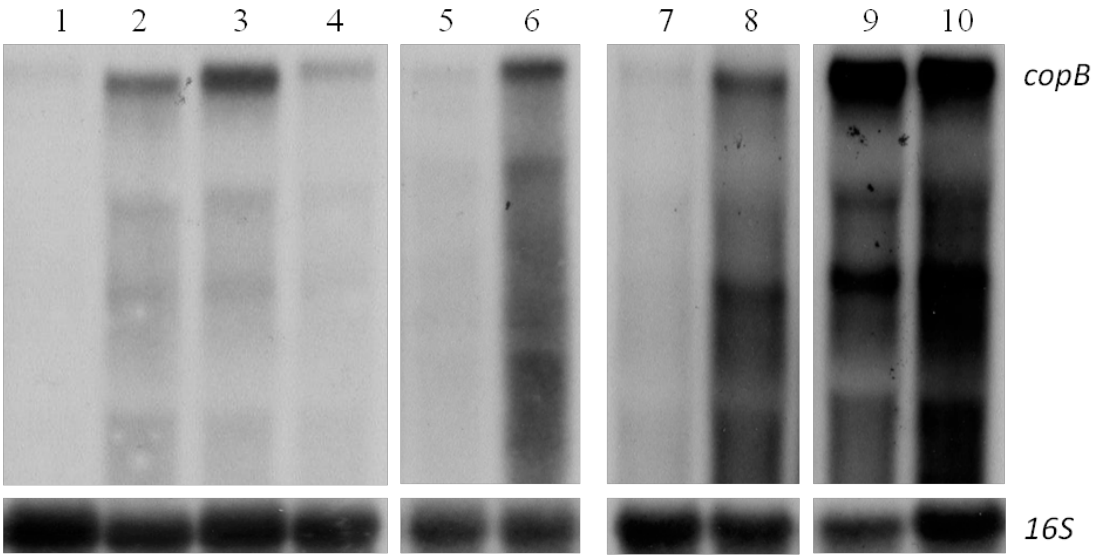
Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Expression level	0.05	0.45	0.55	0.27	0.01	0.24	0.01	0.15	0.42	0.21	0.01	0.45

Lane	**13**	**14**	**15**	**16**
Expression level	0.02	0.45	0.78	0.48

Figure 4.19. Northern blot analysis of *copA* transcription in the *csoR* mutants. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *copA* DNA probe. **(A)** (1) Newman, (2) Newman plus 10 µM $CuCl_2$, (3) Newman *csoR*, (4) Newman *csoR* plus 10 µM $CuCl_2$, (5) Newman *csoR* + $pMK4_{csoR}$, (6) Newman *csoR* + $pMK4_{csoR}$ plus 10 µM $CuCl_2$, (7) PM64, (8), PM64 plus 10 µM $CuCl_2$, (9) PM64 *csoR* and (10) PM64 *csoR* plus 10 µM $CuCl_2$, (11) PM64 *csoR* + $pMK4_{csoR}$, (12) PM64 *csoR* + $pMK4_{csoR}$ plus 10 µM $CuCl_2$, (13) ATCC 12600, (14) ATCC 12600 plus 10 µM $CuCl_2$, (15) ATCC 12600 *csoR* and (16) ATCC 12600 *csoR* plus 10 µM $CuCl_2$. The blots were then stripped and re-hybridised with a *16S* rRNA control probe. Blots shown are representative of two repeat experiments. **(B)** Expression levels of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

The pattern of the CsoR-dependant copper regulation of *copB* transcription in PM64 and ATCC 12600 was identical to that found for *copA* (Figure 4.20). In Cu(0), *copB* was de-repressed in the ATCC 12600 and PM64 *csoR* mutants and restored in PM64 *csoR* + pMK4$_{csoR}$ strain. This showed that in Cu(0), *S. aureus* CsoR functions as a repressor of both chromosomally encoded *copA* and *copB* in addition to the ATCC 12600 plasmid encoded *copB*. However, during this transcriptional analysis, the de-repression of both *copA* and *copB* in the *csoR* mutants varied between Cu(0) and Cu(10). In Cu(10), the expression of both *copA* and *copB* was reduced in the *csoR* mutants when compared to the Cu(0) de-repression (Figures 4.19 and 4.20). Densitometry analysis showed that this difference varied between 1.25-fold and 1.75-fold but was always observed for both of the *cop* operons and in all the three strains. This suggested the possible involvement of another regulator, which was still repressing some *copA/B* transcription in Cu(10) but allowed for full *copA/B* Cu(0) de-repression in the *csoR* mutants. Alternatively, a functional CsoR may be required for full expression of both *copA* and *copB* in Cu(10).

A



B

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Expression level	0.06	0.33	0.64	0.18	0.10	0.66	0.13	0.77	1.25	1.10

Figure 4.20. Northern blot analysis of *copB* transcription in the *csoR* mutants. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *copB* DNA probe. **(A)** (1) PM64, (2) PM64 plus 10 µM $CuCl_2$, (3) PM64 *csoR*, (4) PM64 *csoR* plus 10 µM $CuCl_2$, (5) PM64 *csoR* + pMK4$_{csoR}$, (6) PM64 *csoR* + pMK4$_{csoR}$ plus 10 µM $CuCl_2$, (7) ATCC 12600, (8), ATCC 12600 plus 10 µM $CuCl_2$, (9) ATCC 12600 *csoR* and (10) ATCC 12600 *csoR* plus 10 µM $CuCl_2$. The blots were then stripped and re-hybridised with a *16S* rRNA control probe. Blots shown are representative of two repeat experiments. **(B)** Expression levels of the genes in **(A)** are shown as a proportion of the expression level of the *16S* control.

4.3. Discussion

Copper tolerance varies between *S. aureus* strains and in different growth environments. The majority of strains tested were sensitive to copper but two strains, the EMRSA-16 clone PM64 and ATCC 12600, showed an increased tolerance to copper. This agrees with previous studies which have shown that EMRSA-16 clones have a higher resistance to copper surfaces than other tested staphylococci (Noyce *et al*., 2006). This study showed that increased copper tolerance is due to the presence of an additional copper homeostasis locus encoding an ATPase and a multi-copper oxidase (*copB/mco*). ATCC 12600 was found to be hyper copper-tolerant and it was discovered that this was due to the increased copy number of the *copB/mco* locus, as it is encoded on a multi-copy plasmid in ATCC 12600. A copper responsive regulator CsoR, was identified which represses both chromosomally and plasmid encoded copper homeostasis genes in response to low copper.

Environmental copper was found to be toxic in a dose dependant manner to *S. aureus* growing in a nutrient poor medium (Figure 4.1). However, copper tolerance varied between the tested strains in both rich and minimal media. *S. aureus* growth was doubled in the rich medium TSB when compared to growth in the minimal medium CRPMI, whilst copper tolerances were significantly increased (Figure 4.3). Through supplementing CRPMI with casamino acids, the copper tolerance increase for *S. aureus* strain Newman was found to be independent of the improved growth (Figure 4.4). There are two possible explanations for higher *S. aureus* copper tolerance in rich media. The extra availability of nutrients in TSB may reduce the rate of copper-displacement of protein metal-cofactors through removing the necessity for some biosynthetic pathways

(Macomber & Imlay, 2008) or alternatively, constituents of the casamino acids are able to chelate copper ions and reduce their toxic effects.

The majority of the *S. aureus* analysed were copper sensitive, with Newman representative of these strains. 8325-4 displayed a greater copper tolerance than Newman and although the σ^B positive isogenic strain SH1000 showed improved Cu(50) growth, its copper tolerance was identical to that of 8325-4 when relative growth was calculated, suggesting σ^B does not have a role in copper tolerance (Figure 4.2B). Two strains were found have a high copper tolerance, PM64 and ATCC 12600, with the latter discovered as being hyper copper-tolerant. These two strains both possess the additional copper operon *copB/mco*, and analysis of the ATCC 12600 *mco* mutant suggested that this operon was important for the hyper copper-tolerance. The *mco* mutant lost the hyper copper-tolerance phenotype, which was not restored when the *mco* gene alone was reintroduced into the mutant (Figure 4.5). This data, combined with the likelihood that the ATCC 12600 *mco* mutant is actually a *copB/mco* double mutant (Sitthisak *et al*., 2005), indicates that *copB* is the major copper tolerance gene of this operon.

The *copB* gene is found in a few other *S. aureus*, such as the community acquired strain USA300 and MN8. Interestingly, the USA300 *copB* (USA300HOU_0084) lacks a downstream *mco* gene. The *copB/mco* operon is also found in *Staphylococcus epidermidis* strains (Sitthisak *et al.,* 2005), such as ATCC 12228. This suggests that the coagulase negative staphylococci may possess high copper tolerances and may also be the source of horizontal transfer of the *copB/mco* operon into *S. aureus* strains such as PM64. *copB* and *mco* single mutants are required to fully define the role

of both these genes in *S. aureus* copper homeostasis. In addition, there may be other genes involved in copper tolerance yet to be defined.

MCOs can oxidise cytoplasmic Cu^{1+} to Cu^{2+}, which has been shown to be protective against copper induced toxicity in other bacterial species (Grass[1] & Rensing, 2001; Singh *et al*., 2004). The *S. aureus* MCO may also provide the same protection as other MCOs, and therefore the hyper copper-tolerance conferred by the plasmid encoded *copB/mco* operon may potentially be due to increased MCO copper oxidation rather than improved copper efflux by CopB. If the pLI50 plasmid used to expresses the wild type *mco* gene in ATCC 12600 *mco* is at a significantly lower copy number than the identified ATCC 12600 *copB/mco* plasmid and if the hyper copper-tolerance is due to the high copy number of the *mco* gene, the replacement of *mco* in ATCC 12600 *mco* + $pLI50_{mco}$ would not complement copper tolerance, which was indeed observed in Figure 4.5B. Therefore, as previously mentioned, single *copB* and *mco* ATCC 12600 mutants are required before the hyper tolerance phenotype can be definitively linked to either of these single genes alone. Alternatively, hyper copper-tolerance may require the entire operon and the MCO protein may oxidise Cu^{2+} for high affinity transport by CopB.

PM64 is a clonal variant of one of the major UK nosocomial MRSA strains, MRSA252 (Moore and Lindsay, 2002). High copper tolerance may therefore contribute to the prevalence of MRSA252 as a hospital pathogen. The hyper copper-tolerant *S. aureus* strain ATCC 12600 was originally isolated from lung pleural fluid. Lung tissue contains high concentrations of copper and zinc due to their involvement as cofactors in Cu/Zn superoxide dismutase (Catalani *et al*., 2008). This is an essential protective enzyme for this oxygen exposed region of the body. *S. aureus* can cause several respiratory tract

infections including; tracheitis, bronchitis and pneumonia (Crossley & Archer, 1997). Therefore hyper copper-tolerance, like that observed for strain ATCC 12600, may be an important virulence factor for a *S. aureus* strain attempting to survive and establish an infection in this copper rich tissue. When growing in copper rich environments such as the lungs, high copper tolerance may also provide an advantage over other, less copper tolerant *S. aureus* species.

The ATCC 12600 hyper copper-tolerance was investigated further and northern blot transcriptional analysis found that the expression of ATCC 12600 *copB* was significantly greater than that seen for PM64 regardless of the environmental copper concentration, although copper induction was still observed (Figure 4.7A). This increased expression was due to the ATCC 12600 *copB* being encoded by an unidentified multi-copy plasmid. Transference of this plasmid into the copper sensitive strain Newman (to create strain NAP1) and the resulting increase in NAP1 copper tolerance showed that it was this plasmid that caused the ATCC 12600 hyper copper-tolerance phenotype (Figures 4.8, 4.9 and 4.10). This also suggested a worrying potential for the *S. aureus* hyper copper-tolerance to spread, as if copper continues to be used as a hospital antibacterial agent, selective pressure will encourage the dissemination of this ATCC 12600 hyper copper-tolerance plasmid to other *S. aureus* strains. This could represent a serious health risk as hyper copper-tolerant *S. aureus* would not only be better equipped to resist nosocomial copper antibacterial treatments but might also more easily infect and persist in the copper rich tissues of the body.

Interestingly, digestion of the ATCC 12600 plasmid with *Eco*RI produced eight products of sizes: 10, 6.9, 4.8, 4.2, 3.4, 2.7, 2.3 and 1.6 kb (Figure 4.9C) and all of these

products (except the largest and smallest) were also observed when the *S. aureus* plasmid pXU5, which encodes high level cadmium resistance, was similarly digested by Udo *et al*. (2000). The integrated *copB/mco* of MRSA252 is flanked by cadmium and arsenic resistance operons which all may have integrated from the same plasmid (Holden *et al*., 2004). This evidence suggests that the ATCC 12600 plasmid may be a derivate of pXU5 and may contain further heavy-metal resistance operons in addition to *copB/mco*. This would increase the likelihood of this plasmid being transferred horizontally from strain to strain, as if it could indeed confer several advantageous phenotypes rather than just hyper copper-tolerance it would more likely be retained even in low copper environments. Subsequent plasmid isolations of NAP1 found that the plasmid was lost if the copper selection was absent (data not shown). This could have been due to incompatibility with the putative Newman plasmid. However, the multi-copy plasmid was stable in ATCC 12600 and remained in the absence of copper challenge (data not shown).

ATCC 12600 *copA* expression was lower than the *copA* expression of both Newman and PM64 (Figure 4.6B), which could have been due to ATCC 12600 possessing a plasmid encoded *copB*. For instance; if both *copA* and *copB* require the same activator for transcription then the two genes may be in competition for this regulator. ATCC 12600 *copA* transcription would be reduced if this activator preferentially bound the multiple copies of *copB* promoter rather than the single chromosomally encoded *copA* promoter. Alternatively, this may simply be a result of the different copper tolerances of the three strains, with the more copper tolerant ATCC 12600 requiring less CopA if the highly expressed CopB was fulfilling a similar role of copper ion export. A *copB* mutant and intracellular copper ion analysis is needed to characterise the role of

copB, which is potentially involved in copper tolerance but as yet, has not been proved to be a copper ion exporter. However, Sitthisak *et al*. (2005) observed significantly increased intracellular copper in the *mco* mutant and higher than wild type intracellular copper levels in ATCC 12600 *mco* + pLI50$_{mco}$, suggesting that *copB* had also been affected by the *mco* mutagenesis and that CopB is a copper exporter.

Investigations into *copA* or *copB* operon regulation first examined Fur as the candidate regulator since there was evidence from several microarray studies for Fur involvement in *copA* transcription. However, no significant iron/Fur regulation was observed for the two *cop* operons compared to their high induction by copper (Figures 4.12 and 4.13). Interestingly, both the ATCC 12600 and PM64 *fur* mutants were more resistant to copper than wild types, which was not observed for Newman *fur* (Figure 4.11). The previous transcriptional experiments showed that this was not due to de-repression of the *cop* operons in the *fur* mutants and the exact cause of this increased tolerance remains unknown. Fur is a global regulator and its disruption affects numerous diverse cellular systems, such as; iron homeostasis, oxidative stress resistance, virulence, metabolism and structural component synthesis (Allard *et al.*, 2006). Therefore, there are many diverse *S. aureus* biological systems affected by the inactivation of *fur* that, either individually or in combination, may be contributing to increase the ATCC 12600 and PM64 copper tolerances. Links between iron and copper tolerance were investigated as numerous interactions between the two metals have been observed. However, no clear connection could be established between the two systems using the project protocols and iron in the medium did not obviously affect copper tolerance (data not shown). However, the *fur* mutant results (Figure 4.11) suggest that there is linkage between the two homeostasis systems and *copA* has been shown to be de-repressed in a

S. aureus SH1000 *fur* mutant (Personal communication from Dr Jayaswal, Illinois State University).

The *S. aureus* CsoR represents the first copper regulator found in *S. aureus*. Although it showed some homology to the CsoRs of other bacterial species, it was unique in many respects. The transcription of *csoR* is different in *B. subtilis* and *M. tuberculosis*. The *M. tuberculosis csoR* is co-transcribed with the copper chaperone *rv0968* gene and the copper ATPase *ctpV* gene as a polycistronic operon and both *csoR* and *ctpV* are copper induced (Liu *et al*., 2007). The *M. tuberculosis* CsoR is also auto-regulated. The *B. subtilis csoR* is not co-transcribed with the downstream *copZA* operon and is not induced by copper (Smaldone & Helmann, 2007). Like the *M. tuberculosis csoR*, *S. aureus csoR* was copper induced (Figure 4.15A). However, the genetic arrangement of the Newman *S. aureus csoR* differed significantly from that of *M. tuberculosis* and *B. subtilis*. While it similarly sat upstream of a putative copper chaperone gene (*csoZ*), it was not located anywhere near its regulatory target operon, *copAZ*. Also, there is potentially an additional gene included in the *S. aureus csoRZ* operon, located 127 bp downstream of *csoZ*. This putative protein showed no homology to any known proteins and contained no conserved protein domains. Northern blot analysis is required to confirm the copper regulation of this additional gene and its potential inclusion in the *csoRZ* operon. Also, the inactivation of this gene followed by a *copA* and *copB* transcriptional analysis would identify its role in the copper responsive regulation of the two *cop* operons.

Like the Newman *csoR*, the MRSA252 *csoR* (SAR2175) is not located near either the *copAZ* (SAR2637/2639) or *copB/mco* (SAR0720/0721) operons. Examination of the

published MRSA252 genome showed that *csoR* was a pseudo gene which terminated at amino acid 62 due to a frame shift, making it 50 amino acids smaller than Newman CsoR. The putative copper chaperone gene *csoZ* (SAR2174) was still present, as was the third unidentified gene which is potentially part of this operon. From the transcription experiments, the CsoR protein of PM64 (a variant of MRSA252) appeared to function normally as a transcriptional repressor. Therefore, both the PM64 and MRSA252 *csoR* genes were sequenced but no frame shift mutation was found in either of their *csoR* genes, suggesting an error in the published MRSA252 genome. The ATCC 12600 *csoR* was also sequenced and found to be 100% identical to Newman *csoR*.

Mutation of the *csoR* gene had no effect on subsequent copper tolerances when compared to wild type, which was expected as the copper efflux systems de-repressed in the mutants would be analogous to the induction by copper in the wild type strains (Figure 4.18A). However, growth of the Newman and PM64 *csoR* mutants was decreased in Cu(0) (Figure 4.18). This could be due to additional stress caused by the de-repressed expression of the copper efflux transporters, which may have limited internal copper that may be required for optimal growth. No growth defect was observed for the ATCC 12600 *csoR* mutant, possibly as the wild type ATCC 12600 growth is already decreased in Cu(0) when compared to other *S. aureus* strains, due to the high plasmid encoded expression of *copB* (Figure 4.18). However, the PM64 *csoR* mutant containing a wild type *csoR* gene did show increased copper tolerance. This was not found with Newman *csoR* + pMK4$_{csoR}$ which may be due to PM64 but not Newman encoding the additional copper operon *copB/mco* (Figure 4.18B).

The *S. aureus* CsoR was found to represses the *copAZ* and *copB/mco* operons in Cu(0), similar to the function of the CsoR homologues in *M. tuberculosis* and *B. subtilis*, but whether this is through direct DNA binding or via another regulator is at this time unknown (Figures 4.19 and 4.20). *S. aureus* CsoR was also found to regulate chromosomally encoded *copA* and *copB* as well as plasmid encoded *copB*. This suggests that the *S. aureus copB/mco* originated from a bacterial strain with an analogous CsoR protein, possibly one of the other staphylococci species. In *B. subtilis*, CsoR binds a GC-rich inverted repeat between the -35 and -10 boxes, upstream of *copZ* (Smaldone & Helmann, 2007). However, no such repeat could be located in the promoter of Newman *copA/Z*. DNA-CsoR binding assays are required to test if the CsoR protein binds target gene promoters directly. There was also evidence of its requirement for full operon expression in Cu(10). Alternatively, there may be other copper regulators involved in regulating these genes, illustrated by the recently identified *B. subtilis* protein YcnK, which, along with CsoR regulates the copper responsive transcription of *ycnJ* (Chillappagari *et al*., 2009). There is no *ycnK* homologue in *S. aureus* but the YcnK-CsoR linked regulation demonstrates that CsoR proteins can be involved in copper regulatory networks along with other regulators. Therefore, *S. aureus* CsoR may represent only the first of several copper regulators to be identified.

Chapter 5. Copper toxicity in *S. aureus* through oxidative stress

5.1. Introduction

One of the known causes of copper-induced cellular toxicity is increased oxidative stress (Kershaw *et al*., 2005; Teitzel *et al*., 2006), primarily due to the creation of harmful reactive oxygen species (ROS) such as the hydroxyl radical ($OH^{\bullet}$). This radical is one of the most reactive biological compounds produced in cells and can lead to the damage of macromolecules such as DNA and lipids (Zastawny *et al*., 1995; Avery *et al*., 1996). The creation of ROS by copper ions occurs by the Haber-Weiss reaction (below), where free Cu^{1+} reacts with hydrogen peroxide to create hydroxyl radicals. Cellular hydrogen peroxide is produced by NADPH oxidation and subsequent superoxide dismutase (SOD) activity (Teitzel *et al*., 2006). The more toxic Cu^{1+} can be created from Cu^{2+} by reductants such as superoxide (Bremner *et al*., 1998).

$$O_2^- + Cu^{2+} \rightarrow O_2 + Cu^+$$

$$Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + OH^- + OH^{\bullet}$$

Therefore in many bacteria, excess copper induces the expression of oxidative stress resistance genes which function as an important copper tolerance system. In *P. aeruginosa*, copper shock leads to the up-regulation of several genes involved in oxidative stress resistance including; *ahpF, sodM* and *katA* (Teitzel *et al*., 2006). These *P. aeruginosa* genes were not induced when copper adapted rather than copper shock cultures were analysed. However in *E. coli*, it has been shown that copper neither

induces the oxidative stress response nor leads to DNA damage (Macomber *et al*., 2007).

S. aureus possesses several oxidative resistance proteins, including two SODs. These are metal containing proteins that detoxify superoxide (O_2^-) to hydrogen peroxide, which is then further reduced to water by catalase activity. The *S. aureus* SodA is a manganese SOD which is induced when superoxide levels become elevated (Clements *et al*., 1999). SodM is also proposed to be a manganese SOD, which rescued a SOD deficient *E. coli* (Valderas *et al*., 2001). While both SodA and SodM are oxidative resistance proteins, they deal with oxidative stress at different stages of the bacterial growth cycle. *sodM* is induced by external oxidative stress and expressed mainly in stationary phase while *sodA* is induced by internal oxidative stress and expressed primarily in exponential phase (Karavolos *et al*., 2003). However, Karavolos *et al*. (2003) found that SodA alone was sufficient to resist external superoxide stress and that both SOD proteins contributed to virulence. The *S. aureus* proteins KatA (catalase) and AhpC (alkyl hydroperoxide reductase) have compensatory roles in resisting peroxide stress by converting hydrogen peroxide to water and oxygen, but in *S. aureus* KatA acts as the primary H_2O_2 scavenger protein (Cosgrove *et al*., 2007). These two proteins are regulated by the *S. aureus* Fur homologue PerR, a peroxide-responsive regulator which induces *ahpC* and *katA* in response to hydrogen peroxide (Horsburgh[1] *et al*., 2001).

Transcriptional profiling of *S. aureus* strain SH1000, grown to mid-log phase in defined medium, identified a number of genes that are regulated in response to copper stress (Personal communication from Dr Jayaswal, Illinois State University). cDNA was made from 3 repeat RNA extracts of mid-log *S. aureus* cultures via reverse transcription and

hybridised to DNA chips which were subsequently scanned and normalised. Copper excess led to a global gene response which included the up-regulation of the *S. aureus* oxidative stress proteins *ahpF* and *sodM*. SodM is one of the two known *S. aureus* SODs and AhpF (alkyl hydroperoxide reductase subunit F) functions to catalyse the NADH-dependent reduction of AhpC in order to re-generate AhpC every catalytic cycle. This microarray data suggested that environmental copper excess leads to increased *S. aureus* oxidative stress and induces an appropriate phenotypic response. It also implied that the oxidative stress resistance proteins SodM and AhpF play an important role in *S. aureus* copper tolerance and are copper regulated, similar to the observed copper induction of oxidative stress resistance proteins in other well studied pathogens, such as *P. aeruginosa* (Teitzel *et al*., 2006).

5.2. Results

5.2.1. Newman *csoR* was more susceptible to peroxide stress than wild type

The CsoR protein of Newman (described in Chapter 4) represents the first copper homeostasis regulator identified in *S. aureus*. This regulator was found to control copper ion efflux by repressing *copA* transcription in Cu(0) (Figure 4.19 Chapter 4). In addition, a *copA* mutant was more sensitive to hydrogen peroxide stress (Sitthisak *et al*., 2007). Therefore, to examine if CsoR was also involved in copper induced *S. aureus* oxidative stress resistance, the hydrogen peroxide resistances of Newman, its *csoR* mutant and the Newman *csoR* mutant with a wild type copy of the *csoR* gene were compared.

Several methods to examine peroxide-induced oxidative stress can be used, including viable counts and growth curves (Horsburgh[1] *et al*., 2001; Sitthisak *et al*., 2007). Growth curves were chosen for this investigation as they allowed for the effects of oxidative stress to be examined throughout the *S. aureus* growth cycle, which provided a better representation of the peroxide growth response. Initial experiments could not provide reproducible results using CRPMI medium. Therefore, the rich medium TSB was used for this comparison but a preliminary growth curve with Newman, its *csoR* mutant and the Newman *csoR* mutant with a wild type copy of the *csoR* gene, observed variable growth rates for these three strains (data not shown). This had to be taken into account before a direct comparison of their growth responses to hydrogen peroxide could be made. Therefore, relative growth was determined for each strain from two simultaneously grown 25 ml cultures inoculated from the same 10 ml overnight culture, with one containing 0.003% (v/v) hydrogen peroxide. This eliminated variation caused by the differing growth rates of the strains.

Growth readings for the time points at 1 and 2 hours were extremely variable due to low initial $OD_{595\ nm}$ readings, which caused massive differences in the relative growth from only minor deviations in optical density (Figure 5.1). However, once exponential growth had begun (3 hours), the *csoR* mutant was found to be more sensitive to peroxide stress then the wild type. This was statistically significant at 3 hours and at every additional hourly time point examined. Peroxide tolerance was restored in Newman *csoR* with a wild type copy of the *csoR* gene and this was also statistically significant. Therefore, disruption of *csoR* increased Newman hydrogen peroxide induced stress in TSB.

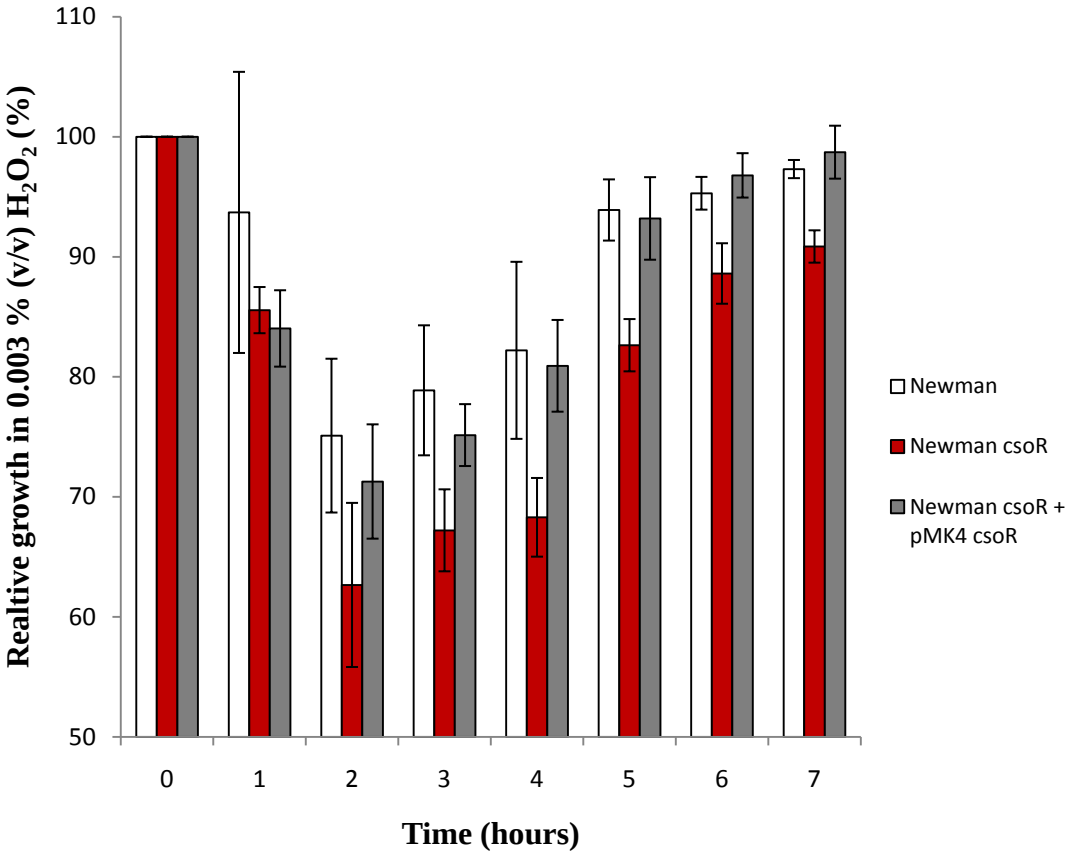


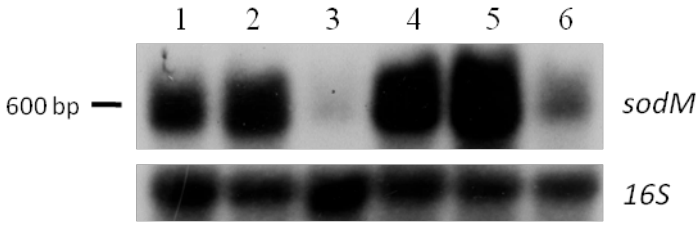
Figure 5.1. The hydrogen peroxide stress resistance of the Newman *csoR* mutant. TSB cultures were grown with shaking from a starting $OD_{595\ nm}$ of 0.05. Two 25 ml cultures, one of which contained 0.003% (v/v) H_2O_2, were incubated for seven hours and growth was measured at $OD_{595\ nm}$ at hourly time points. The $OD_{595\ nm}$ of the two cultures was used to calculate relative growth in peroxide, expressed as a percentage. Three strains are shown; Newman, Newman *csoR* and Newman *csoR* with a wild type copy of the *csoR* gene. Results represent the means and standard deviations of three independent experiments.

5.2.2. Newman CsoR does not directly regulate *ahpF* or *sodM*

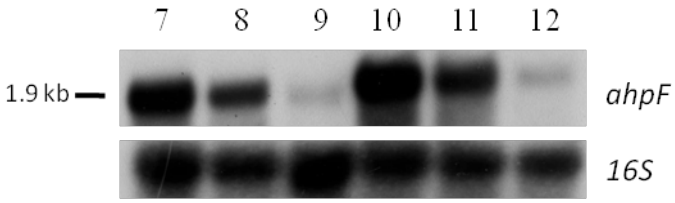
The microarray study observed copper induction of the oxidative stress genes *ahpF* and *sodM* in defined medium (Personal communication from Dr Jayaswal, Illinois State University). As Newman CsoR was found to regulate the copper response of the *copA* operon (Figure 4.19 Chapter 4), the transcription of both *ahpF* and *sodM* were examined in the Newman *csoR* mutant to investigate if CsoR also controlled the copper regulation of these two genes. Total RNA was prepared from mid-log cultures in the minimal medium CRPMI which is reflective of *in vivo* growth and transcription was examined using northern blots.

The *sodM* probe created using primers SodM-probe-F and SodM-probe-R, detected a single transcript of 600 bp and densitometry analysis showed it was induced 1.28-fold in Cu(10) but was reduced 4.5-fold in Fe(50) (Figure 5.2A). The same pattern of regulation was seen in the Newman *csoR* mutant but overall *sodM* expression was increased 1.4-fold in both Cu/Fe(0) and Cu(10), and 3.3-fold in Fe(50). The *ahpF* probe created using primers AphF-probe-F and AphF-probe-R, detected a single product of 1.9 kb which represented the entire 2 kb *ahpCF* operon (Figure 5.2B). Transcription was decreased 1.5-fold in Cu(10) and 6-fold in Fe(50) when compared to Cu/Fe(0) CRPMI. This pattern was retained in the *csoR* mutant but overall expression was increased 1.1-fold in Cu/Fe(0), 1.4-fold in Cu(10) and 1.1-fold in Fe(50). Therefore, although CsoR does not appear to control the copper regulation of these genes, disruption of *csoR* increased their overall transcription level.

A



B



C

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Expression level	0.68	0.87	0.15	0.96	1.20	0.50	0.84	0.55	0.14	0.96	0.78	0.16

Figure 5.2. Northern blot analysis of *sodM* and *ahpF* copper and iron regulation. RNA was prepared from mid-exponentially growing CRPMI cultures and 20 µg of total RNA were resolved by agarose gel electrophoresis and hybridised with a *sodM* or *ahpF* DNA probe. The blots were then stripped and re-hybridised with a *16S* rRNA control probe. Blots shown are representative of two repeat experiments.

(A) *sodM* probe; (1) Newman, (2) Newman plus 10 µM $CuCl_2$, (3) Newman plus 50 µM $Fe_2(SO_4)_3$, (4) Newman *csoR*, (5) Newman *csoR* plus 10 µM $CuCl_2$ and (6) Newman *csoR* plus 50 µM $Fe_2(SO_4)_3$.

(B) *ahpF* probe; (7) Newman, (8) Newman plus 10 µM $CuCl_2$, (9) Newman plus 50 µM $Fe_2(SO_4)_3$, (10) Newman *csoR*, (11) Newman *csoR* plus 10 µM $CuCl_2$ and (12) Newman *csoR* plus 50 µM $Fe_2(SO_4)_3$.

(C) Expression levels of the genes in **(A)** and **(B)** are shown as a proportion of the expression level of the *16S* control.

5.2.3. The *sodA*, *sodM*, *ahpC* and *katA* SH1000 single mutations did not affect the copper tolerance in a minimal medium

The microarray study which identified the induction of the oxidative stress genes *sodM* and *ahpF* used the *S. aureus* strain SH1000 (Personal communication from Dr Jayaswal, Illinois State University).Therefore, to investigate the role of oxidative stress in *S. aureus* copper tolerance, the CRPMI growth of the *sodA*, *sodM*, *ahpC* and *katA* SH1000 mutants was examined in two copper concentrations. Compared to wild type, in Cu(0) both SH1000 *sodA* and SH1000 *katA* showed a minor reduction in total growth while SH1000 *sodM* and SH1000 *ahpC* did not show any significant growth defect (Figure 5.3). There was also no statistically significant change in the copper tolerances for the four mutants in both Cu(50) and Cu(100) compared to wild type SH1000.

5.2.4. Construction of the *sodA/sodM* and *ahpC/katA* double SH1000 mutants

The single *sodA*, *sodM*, *ahpC* and *katA* SH1000 mutants did not show any alteration in copper tolerance. However, this may have been due to *S. aureus* SodA/SodM and AhpC/KatA having analogous functions which may have been compensating for the single mutations. Therefore, double mutants were required to fully investigate the role of these oxidative stress proteins in copper tolerance. Phage lysates were created from strains SH1000 *sodM* and SH1000 *ahpC* and used to transduce the mutations into SH1000 *sodA* and SH1000 *katA* respectively. Colonies were selected with tetracycline and erythromycin and screened by PCR amplification using the four pairs of flanking primers and DNA preparations from transductants as the template (Figure 5.4A). For the *sodM* and *ahpC* mutants created by tetracycline cassette insertion, PCR amplification produced a larger product (Figure 5.4B lanes 4 and 7).

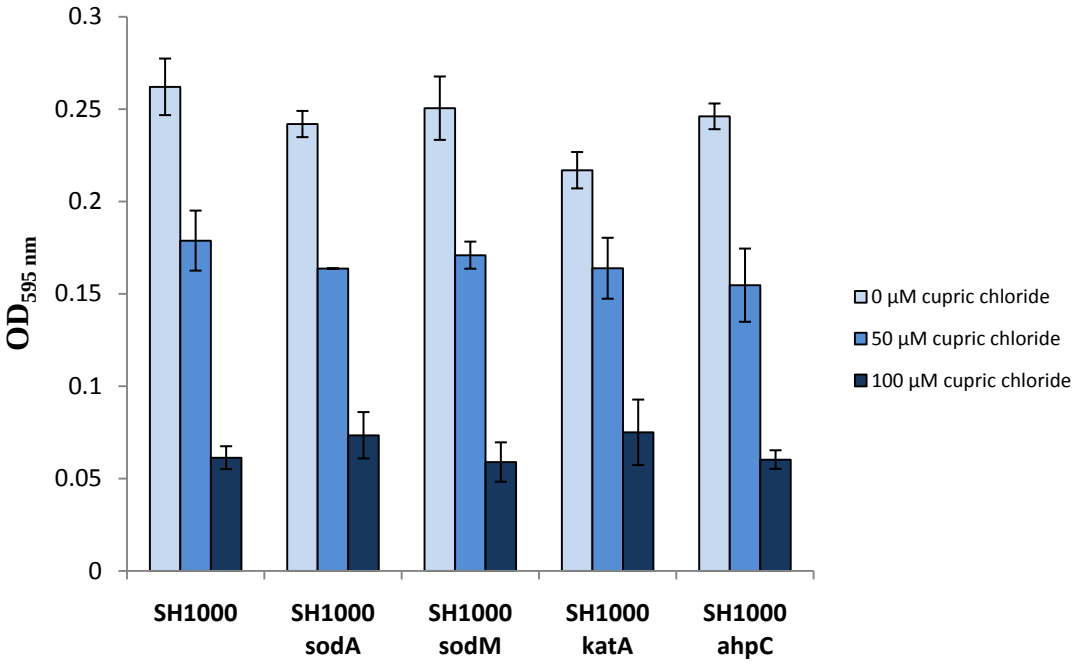
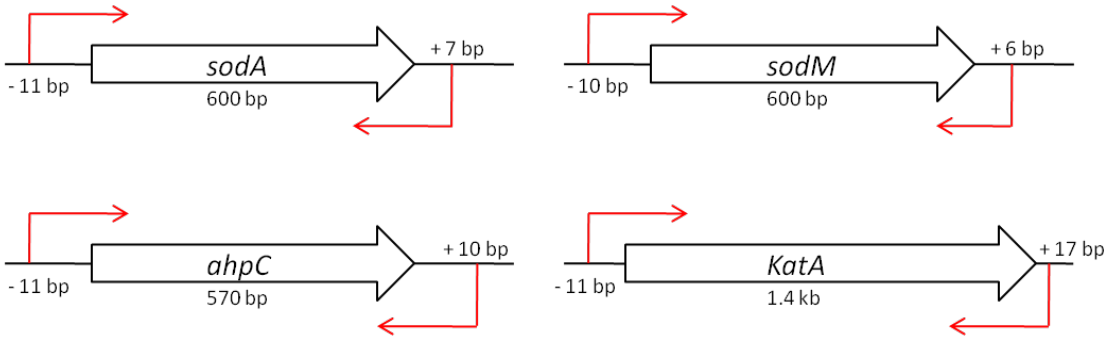


Figure 5.3. The effect of copper on the growth of the four SH1000 single mutants. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. The strains shown are; SH1000, SH1000 *sodA*, SH1000 *sodM*, SH1000 *katA* and SH1000 *ahpC*. Three concentrations of $CuCl_2$ are shown; 0, 50 and 100 µM. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Results shown represent the means and standard deviations of three independent experiments.

A



B

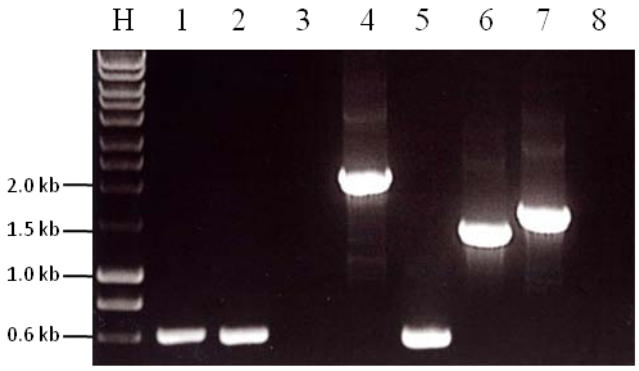


Figure 5.4. Construction of the *sodA/sodM* and *ahpC/katA* double mutants.

(A) The sizes of the oxidative stress resistance genes and the locations of the four primer pairs used to analyse the SH1000 mutants, which are represented by red arrows.

(B) Confirmation of the *sodA/sodM* and *ahpC/katA* double mutants by PCR using total DNA preparations as the template. SH1000 with (1) SodA-F and SodA-R or (2) SodM-F and SodM-R primers. SH1000 *sodA/sodM* with (3) SodA-F and SodA-R or (4) SodM-F and SodM-R primers. SH1000 with (5) AhpC-F and AhpC-R or (6) KatA-F and KatA-R primers. SH1000 *ahpC/katA* with (7) AhpC-F and AhpC-R or (8) KatA-F and KatA-R primers. Product size was estimated using 5 µl of Hyperladder I (H).

Conversely, for the *sodA* and *katA* mutants created by a Tn*917* insertion, PCR amplification produced no product as the greatly increased primer distances prevented the primers from amplifying (Figure 5.4B lanes 3 and 8). The transduction was successful and created the two double mutants; SH1000 *sodA/sodM* and SH1000 *ahpC/katA*.

5.2.5. Copper tolerance was altered for the *sodM*/*sodA* and *ahpC*/*katA* SH1000 double mutants

The SH1000 double mutants were assayed to investigate if the loss of all catalase/AhpC or SOD activity affected CRPMI copper tolerance. In Cu(0), there was a small 1.3-fold growth reduction for the *sodM*/*sodA* ($P = 0.0027$) and *ahpC*/*katA* ($P = 0.0008$) double mutants compared to wild type (Figure 5.5). These were statistically significant, unlike the small reductions observed in Cu(0) growth for the single *sodA* and *katA* mutants (Figure 5.3). In Cu(50), there was a significant decrease in the copper tolerance of SH1000 *ahpC/katA* compared to SH1000 ($P = 0.0004$) (Figure 5.5). This suggested that hydrogen peroxide scavenging by catalase and AhpC is an important *S. aureus* copper tolerance mechanism. In Cu(100), the SH1000 *sodA/sodM* copper tolerance was significantly increased when compared to SH1000 wild type ($P = 0.0043$), which suggested that SOD activity was actually contributing to copper toxicity.

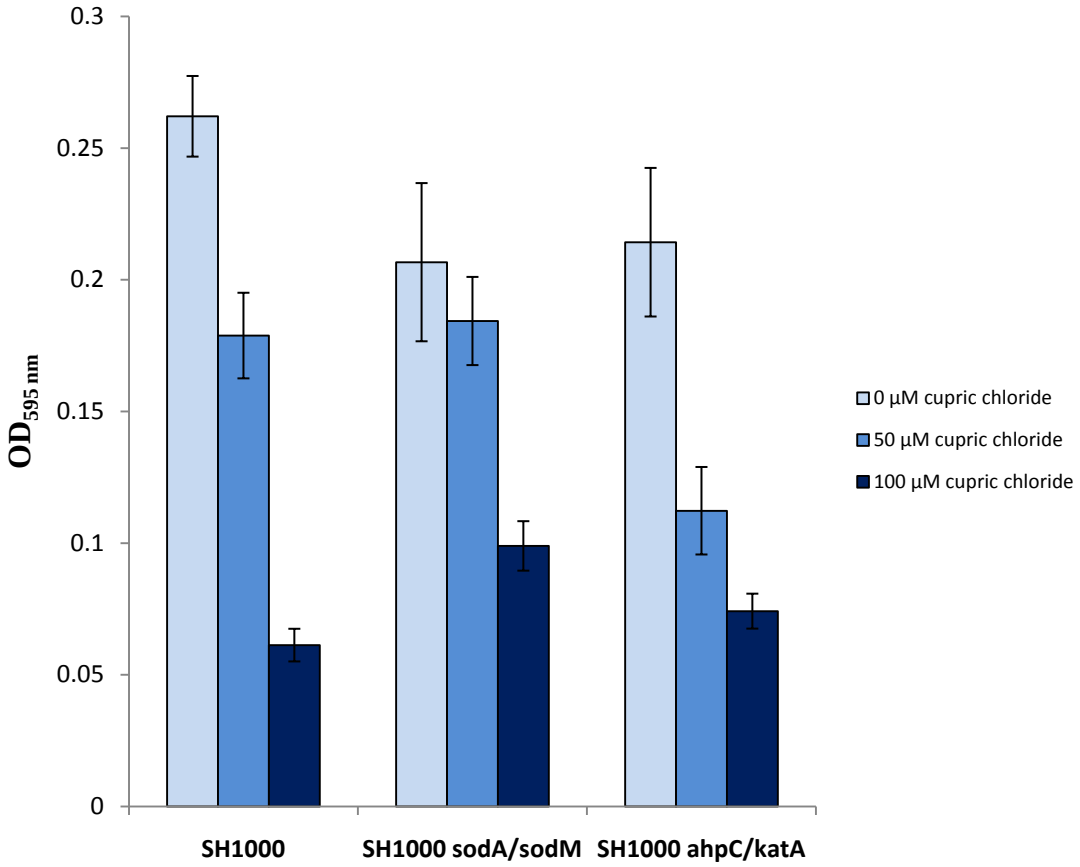


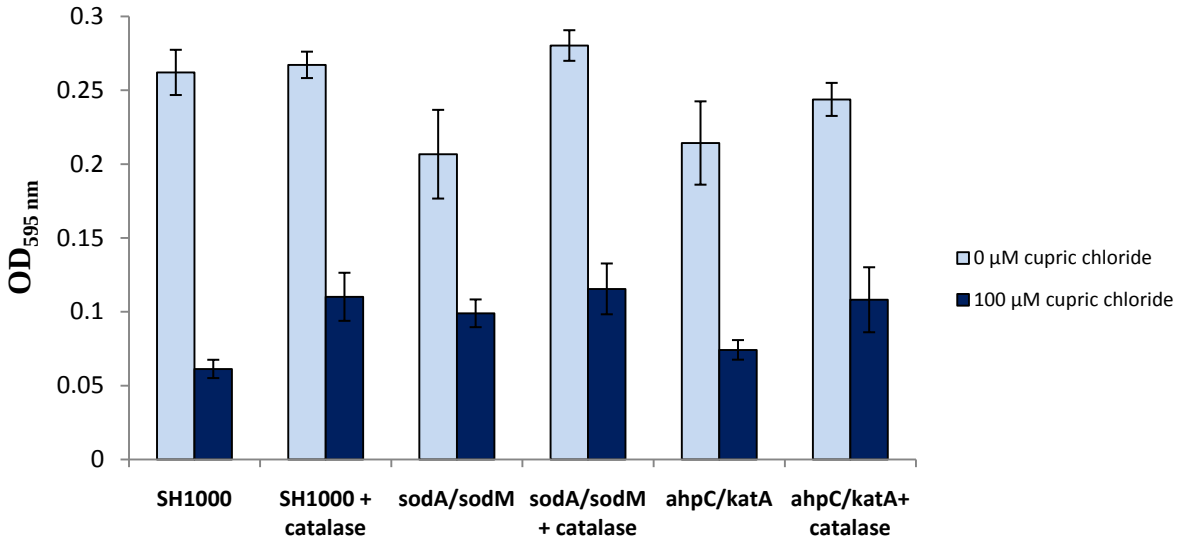
Figure 5.5. CRPMI copper tolerance of the SH1000 double mutants. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. The strains shown are; SH1000, SH1000 *sodA/sodM* and SH1000 *ahpC*/*katA*. Three concentrations of $CuCl_2$ were compared; 0, 50 and 100 µM. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Results shown represent the means and standard deviations of three independent experiments.

5.2.6. The copper tolerances of the SH1000 wild type and double mutants were either increased or decreased by exogenous catalase or SOD

Espírito Santo *et al*. (2008) observed that *E. coli* copper-surface toxicity was reduced by the addition of exogenous oxidative stress enzymes, particularly catalase and SOD. Therefore, to determine if surface oxidative stress is important for copper toxicity in *S. aureus*, the wild type and double SH1000 mutants were challenged with Cu(0) or Cu(100) in the presence of either exogenous catalase or SOD and the differences in copper tolerance compared. In Cu(0), the addition of exogenous catalase did not affect the growth of SH1000 wild type or SH1000 *ahpC/katA*, whilst the growth of SH1000 *sodA/sodM* was increased 1.5-fold (P = 0.0015) (Figure 5.6A). However in Cu(100), the copper tolerance of wild type was increased 5-fold by exogenous catalase (P = 0.0015) but copper tolerance was not affected in the two double mutants. Exogenous SOD did not affect the Cu(0) growth of the SH1000 wild type and mutant strains (Figure 5.6B). However for the wild type, the addition of exogenous SOD in Cu(50) had a similar effect to catalase and increased copper tolerance 5-fold (P = 0.0043). Conversely, SOD had the opposite effect on the copper tolerances of the double mutants and decreased copper tolerance 2.4-fold in SH1000 *sodM*/*sodA* (P = 0.042) and 3.2-fold in SH1000 *ahpC/katA* (P = 0.001).

Therefore, SH1000 copper tolerance was significantly increased with both SOD and catalase in the growth medium. However in Cu(100), exogenous SOD caused the SH1000 double mutants to become more sensitive to copper than either the wild type with exogenous SOD or the double mutants without SOD.

A



B

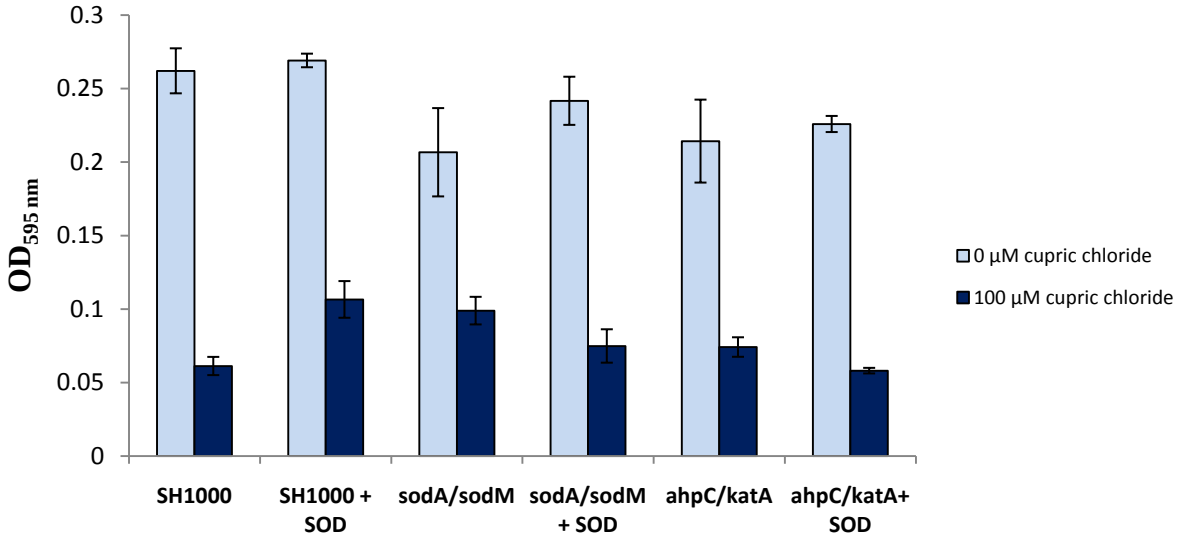


Figure 5.6. The effect of exogenous catalase or SOD on the CRPMI copper tolerances of SH1000 wild type and double mutants. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Cultures were challenged with either 0 or 100 µM $CuCl_2$. Results shown represent the means and standard deviations of three independent experiments.

(A) Wild type and double mutants with or without 500 U/ml catalase.

(B) Wild type and double mutants with or without 10 U/ml SOD.

5.2.7. The copper tolerance of the 8325-4 *perR* mutant was equivalent to wild type

The Fur homologue PerR regulates the oxidative stress genes *katA* and *ahpCF* (Horsburgh[1] *et al*., 2001) in addition to *sodA* and *sodM*. Catalase is involved in copper tolerance and *ahpF* was found to be copper regulated independently of CsoR (Figure 5.2). SH1000 is the σ^B positive isogenic strain of 8325-4 and the 8325-4 *perR* mutant was already available (Horsburgh[1] *et al*., 2001). Therefore, to investigate the possibility that PerR is involved in copper tolerance, the CRPMI 8325-4 *perR* copper tolerance was compared to wild type. There were no statistically significant differences in the copper tolerances of these two strains in either Cu(50) or Cu(100) (Figure 5.7). Therefore, the inactivation of *perR* had no subsequent affect on the 8325-4 copper tolerance.

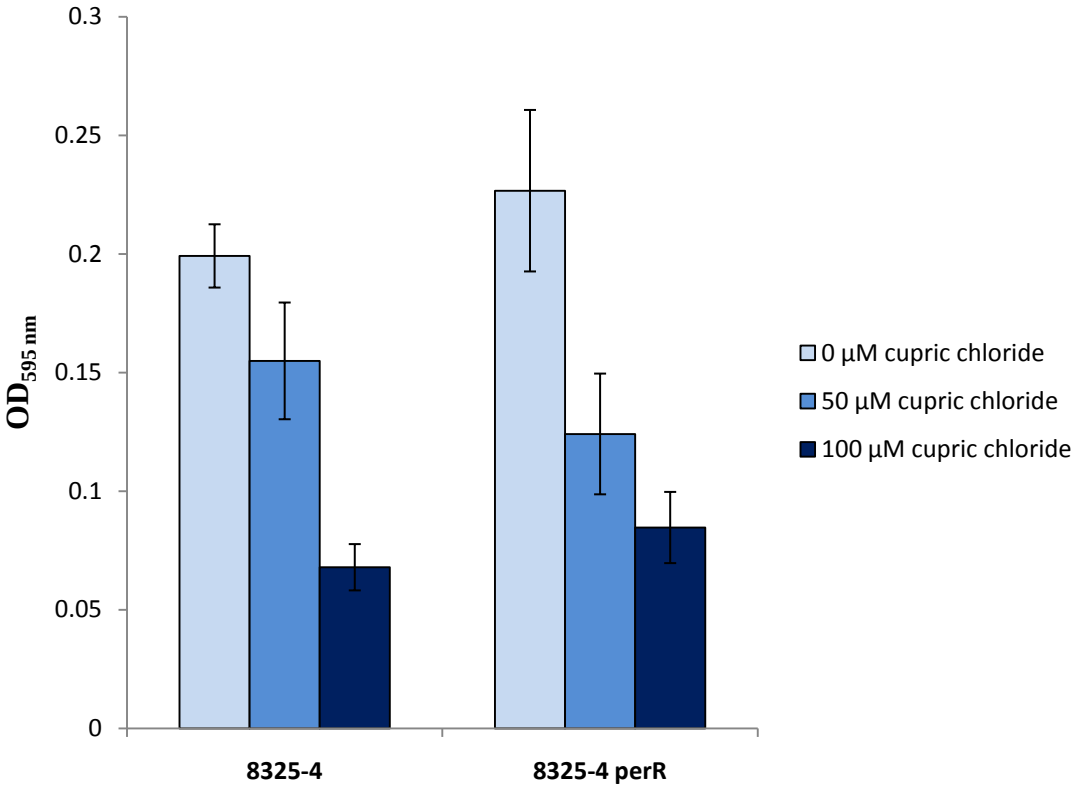


Figure 5.7. The CRPMI copper tolerances of 8325-4 wild type and *perR* mutant. Cultures were grown statically from a starting $OD_{595\ nm}$ of 0.05 in triplicate in a microtitre plate. Three concentrations of $CuCl_2$ are shown; 0, 50 and 100 µM. Total growth was measured at $OD_{595\ nm}$ after 24 hours. Results shown represent the means and standard deviations of three independent experiments.

5.3. Discussion

In most bacterial species, an increase in oxidative stress is a major cause of copper toxicity in the cell, where copper elicits an oxidative resistance gene response (Teitzel *et al*., 2006). This was shown to also be the case for *S. aureus*, as the oxidative stress resistance genes *sodM* and *ahpF* were both induced by copper excess in a microarray study (Personal communication from Dr Jayaswal, Illinois State University). The regulator of this response is currently unknown but transcriptional analysis showed that it was not the previously identified copper repressor, CsoR (Figure 5.2). A candidate for this unknown regulator is PerR, which had already been identified as an important regulator of *katA* and *ahpCF* expression (Horsburgh[1] *et al*., 2001). Northern blot analysis of the *sodM* and *ahpF* transcriptional copper response would confirm if PerR was responsible for their copper regulation if this regulation was found to be lost in a *perR* mutant. Although, a 8325-4 *perR* mutant showed no change in copper tolerance compared to wild type (Figure 5.7), this does not exclude PerR as being a potential copper responsive regulator as the Newman *csoR* mutant also had an identical copper tolerance compared to the wild type (Figure 4.18A Chapter 4) but was subsequently shown to repress the copper efflux operon, *copAZ*.

Here, the copper induction of *sodM* agreed with the copper induction observed in the microarray study (Figure 5.2A). However, *ahpF* transcription was repressed by copper in this analysis whilst in the microarray it was induced 2-fold by copper. This may have been due to strain variation, as the microarray examined SH1000 rather than Newman expression. Alternatively, this may have been a result of using copper adapted cultures (northern blot) rather than copper shock (microarray) as previous studies have observed

significant differences in the bacterial gene response to these two types of copper challenge (Teitzel *et al*., 2006).

S. aureus Newman peroxide resistance was found to be decreased in the Newman *csoR* mutant compared to the wild type (Figure 5.1). Furthermore, even though CsoR was found to not regulate the copper expression of *sodM* or *ahpF* directly, overall expression for both these genes was increased in the Newman *csoR* mutant (Figure 5.2). This suggested that disrupting the Newman *csoR* gene was somehow affecting Newman's ability to deal with oxidative stress. The *copAZ* operon was previously observed to be de-repressed in the *csoR* mutant and the resulting uncontrolled extracellular export of copper ions may be detrimental to peroxide stress resistance. This may be more apparent when, like in the TSB growth assay, peroxide is added exogenously and can react with exported copper ions located near to the *S. aureus* cell surface to create ROS. Alternatively, CsoR could be regulating one or both of the other SOD or catalase genes; *sodA* and *katA*. Transcriptional analysis would identify if this is the case. Indeed, if one of these genes is regulated by the Newman CsoR, *csoR* inactivation may be disrupting one of the key oxidative stress resistance pathways, leading to an intracellular accumulation or inability to detoxify one of the harmful products of these systems such as H_2O_2, which would increase overall internal oxidative stress. A copper microarray analysis using the Newman wild type and *csoR* mutant would identify which genes and pathways are controlled by CsoR and may help explain why *csoR* disruption increases oxidative stress and peroxide sensitivity.

As oxidative stress in *S. aureus* has an important role in copper homeostasis, copper tolerance was examined using four SH1000 single mutants which encompassed the

major *S. aureus* oxidative stress resistance genes; *sodM*, *sodA*, *ahpC* and *katA*. Despite both SH1000 *sodA* and SH1000 *katA* showing an apparent minor reduction in Cu(0) total growth, it was not significant and the copper tolerances were found to be identical to wild type (Figure 5.3). The compensatory function of the two SODs and catalase/AhpC proteins was a possible reason for this lack of copper phenotype and therefore double mutants were constructed and their copper tolerances examined. The copper tolerances of the *sodM*/*sodA* and *ahpC*/*katA* SH1000 mutants differed significantly from the wild type (Figure 5.5). The loss of all SOD activity in SH1000 *sodA*/*sodM* led to increased tolerance to Cu(100) (Figure 5.5). Conversely, the loss of all catalase activity in SH1000 *ahpC/katA* led to reduced tolerance to Cu(50) (Figure 5.5). This can be explained by examining the reactions below. (1) SOD catalyses the reaction of superoxide with hydrogen ions to produce water and oxygen. (2/3) Copper ions can react with hydrogen peroxide to produce harmful radicals.

$$(1)\ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$(2)\ Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + O_2 + OH^- + OH^\bullet$$

$$(3)\ Cu^{2+} + H_2O_2 \rightarrow OOH^\bullet + H^+ + Cu^{1+}$$

Therefore, no SOD activity in SH1000 *sodA*/*sodM* would prevent reaction 1 from occurring. This would increase copper tolerance by reducing the levels of hydrogen peroxide, so preventing free copper ions producing ROS via reactions 2 and 3. Alternatively, no catalase activity in SH1000 *ahpC/katA* would eliminate all scavenging of cellular hydrogen peroxide and, in the presence of copper ions, would increase the production of ROS via reactions 2 and 3, resulting in reduced copper tolerance.

To further investigate copper and oxidative stress; the strains SH1000, SH1000 *ahpC/katA* and SH1000 *sodA/sodM* were grown in Cu(0) and Cu(100), with or without the addition of exogenous catalase and SOD (Figure 5.6). In Cu(0), the enzymes had no effect on SH1000 growth. However, SH1000 copper tolerance was significantly increased with both SOD and catalase (P = 0.03 and 0.01 respectively). Both these enzymes are likely to remain extracellular and therefore would not protect the cells from internal copper induced toxicity, suggesting that exogenous copper is toxic to *S. aureus*, potentially at the cell surface.

In Cu(100), exogenous SOD caused the SH1000 double mutants to become more sensitive to copper than either the wild type with exogenous SOD or the double mutants without SOD (Figure 5.6A). This suggests that extracellular H_2O_2 produced by the action of exogenous SOD was not dealt with correctly by either the SH1000 *sodA/sodM* or *ahpC/katA* mutant and is contributing to copper toxicity. Therefore in addition to intracellular copper, extracellular copper may also toxic to *S. aureus* and potentially there are different mechanisms for dealing with copper induced oxidative stress at these two cellular locations.

In a preliminary intracellular metal analysis using *S. aureus* PM64, copper was found to cause a massive decrease in cellular potassium levels (data not shown). This corresponds with previous reports which have shown copper-induced membrane disruption in *S. cerevisiae* (Ohsumi *et al*., 1988; Avery *et al*., 1996; Howlett & Avery, 1997). Possibly, exogenous copper disrupts the *S. aureus* membrane in a similar fashion leading to cytoplasmic leakage, perhaps by the creation of ROS at this site by copper-hydrogen peroxide interaction. This would explain why in Cu(0), the Newman

csoR mutant was more sensitive to hydrogen peroxide stress (Figure 5.1) as exported copper ions would be localised at the cell surface to react with the exogenously added peroxide. Also, this would explain why exogenous SOD decreased copper tolerance for SH1000 *ahpC/katA* (Figure 5.6B) as in this strain, SOD activity could create cell surface hydrogen peroxide which would not be reduced by catalase activity and which would react with copper to cause toxicity. However, copper toxicity in *S. aureus* may involve many pathways/interactions yet to be identified, linking the oxidative stress enzymes with different cellular locations and toxic effects of copper.

Chapter 6. Final discussion

S. aureus is an important pathogenic microorganism which is still a major cause of nosocomial infections. This thesis explored two aspects of environmental gene regulation in *S. aureus*; novel iron/Fur regulation and copper homeostasis. This work identified the new regulator LysR, which was found to be non-classically regulated by Fur and iron. LysR represents the first *S. aureus* regulator under the control of Fur which potentially could be part of a regulatory system for non-classical Fur regulation. Furthermore, *lysR* is an additional non-classically Fur regulated *S. aureus* gene and the first to contain an upstream Fur box. Several putative functions of LysR were identified. LysR was found to be required for wild type growth in rich media, which might be linked to oxidative stress as *lysR* is induced by peroxide and a mutant showed increased peroxide sensitivity. There was also evidence that LysR may be the *S. aureus* regulator of the enzymes involved in the histidine utilisation pathway.

Investigations into copper tolerance found variation between different *S. aureus* strains. This led to the identification of the hyper copper-tolerant ATCC 12600 strain and the discovery of its copper resistance plasmid. This is the first copper tolerance plasmid found in a clinical isolate and has important implications for the dissemination of copper resistance in the *S. aureus* population. In fact, this plasmid was successfully transferred to a copper sensitive strain to provide the copper tolerance phenotype. *S. aureus* strains can contain one or two of the described *S. aureus cop* operons (Sitthisak *et al*., 2005; Sitthisak *et al*., 2005). Here it was shown that both these operons

are under the control of the CsoR repressor, a homologue of a recently identified regulatory family. The final part of this work examined the mechanism of copper toxicity in *S. aureus*. It was found that like many bacterial species oxidative stress induction, particularly hydrogen peroxide scavenging, is an important aspect of *S. aureus* copper tolerance. Also, surface toxicity appears to play a role in copper induced *S. aureus* toxicity and a preliminary study suggested that copper may be disrupting bacterial cell membranes leading to cytoplasmic leakage.

The two aims for the first section of this thesis were; to find a regulator under the control of Fur which could be part of non-classical *S. aureus* Fur regulation and to identify the Fur independent iron regulator that is controlling *eap* and *emp* transcription in the Newman *fur* mutant (Johnson *et al*., 2008). From a sequence search and published reports, five potential targets were chosen for a reporter assay (Table 3.1). These assays demonstrated that three of the chosen target genes are repressed by iron (Figure 3.6). Of these three, only *lysR* showed repression that was specific for iron and so this regulator was chosen for further study. Therefore, although the novel *S. aureus* iron regulator was not found through this work there are potential candidates for future studies. In particular *tetR*, a regulator of unknown function that showed iron regulation (Figure 3.6A). The transcription of this gene in response to iron in a *fur* mutant background would demonstrate if this regulation is Fur dependant. If it was found to act independently of Fur, then transcriptional analysis of *eap* and *emp* expression in a *tetR/fur* double mutant would identify if *tetR* is responsible for their previously observed Fur independent iron regulation. A role for LysR in non-classical Fur regulation could not be established. An investigation using a *S. aureus* microarray and the *lysR* mutant would be of interest to examine which genes are under *lysR* control and

if any of these show novel regulation in response to Fur. Therefore, although not fulfilling the original aim of this work, it still remains possible that Fur acts through LysR for novel iron regulation but the target gene remains undefined.

Initial examinations into *lysR* transcription unexpectedly found that this gene is non-classically Fur regulated (Figure 3.8). Therefore, *lysR* represents the first *S. aureus* gene with a putative upstream Fur box that is regulated by Fur in a novel manner. It would be of great interest to perform DNA binding assays on the *lysR* promoter to confirm if Fur is binding directly to initiate *lysR* transcription in low iron. This mechanism of Fur regulation has been found in other bacterial species such as *N. meningitidis* (Delany *et al.*, 2004). However, if this was found to be the mechanism of *lysR* induction in low iron by Fur, it would still not explain the non-classical regulation of the other *S. aureus* genes which do not contain upstream Fur boxes. In addition, *lysR* showed auto-regulation (Figure 3.9) and again DNA binding assays would confirm this result. If both Fur and LysR bind the *lysR* promoter, then *lysR* transcription may be more complicated than first thought.

Attempts were then made into finding a phenotype for a *lysR* mutant and identify the function of LysR in *S. aureus*. *lysR* inactivation had a minor negative effect on *eap* transcription (Figure 3.11A) but no subsequent effect on *in vitro* biofilm formation. Therefore LysR is not responsible for the Fur independent iron regulation of biofilm expression that has previously been described (Johnson *et al*., 2008). However, the Newman *lysR* mutant showed significant growth defects in rich media when compare to the Newman wild type (Figure 3.12). Although a *lysR* mutant with a replaced copy of

lysR on a pMK4 plasmid showed further growth inhibition, this was shown to be due to pMK4 and not the cloned *lysR* gene (Figure 3.13). The reason for the mutant growth defect is unknown but potentially may be due to a decreased ability to resist oxidative stress. A published report has shown that *lysR* is induced by hydrogen peroxide (Chang *et al*., 2006) and in this thesis it was found that both a *lysR* mutant and a Newman strain which was overproducing LysR had increased peroxide sensitivity (Figure 3.18). However, additional research into this possible phenotype is required before a definitive function of LysR as an oxidative stress regulator can be concluded. However, both iron and Fur are involved in the regulation of *S. aureus* oxidative stress genes including the catalase gene *katA*, which is non-classically Fur regulated (Horsburgh[2] *et al*., 2001). Therefore, as a regulator that is both iron and peroxide controlled, LysR may be involved in Fur non-classical *katA* regulation. Transcriptional analysis of *katA* expression in Newman *lysR* would confirm this hypothesis. In addition, a microarray analysis using the *lysR* mutant in response to peroxide may identify regulatory targets of LysR involved in oxidative stress.

The second most likely role of LysR is as the regulator of the *S. aureus hut* operon. *S. aureus* contains no homologues of the known bacterial *hut* regulators *hutC, hutP* or NAC (Allison & Phillips, 1990; Hu & Phillips, 1988; Oda *et al*., 1988; Wray & Fisher, 1994; Kumarevel *et al*., 2005; Schwacha & Bender, 1990; Bender, 1991). Transcriptional experiments suggested that LysR is an activator of *hutU* (Figures 3.16 and 3.17). Additional work is required before this phenotype of LysR can be confirmed and repeats of the *hutU* transcriptional experiments in a medium more appropriate for high *hutU* expression are required. However, no obvious reason for iron regulation of the *S. aureus hut* genes could be identified. These enzymes (Figure 3.14B) do not

contain iron or have any obvious connection to iron homeostasis. However, the pathway for the conversion of histidine to glutamate requires three molecules of H_2O_2 (Figure 3.14B). Therefore it is possible that Hut enzymes in high iron protect the bacterial cell by scavenging cellular peroxide which would normally lead to free radical production and subsequent iron toxicity. However, this function should be performed by catalase and requires further investigation, such as inactivating both *katA* and *lysR* and testing iron toxicity resistance. Therefore, *lysR* is a new non-classically Fur regulated gene in *S. aureus* and the first with an upstream Fur box. Potentially, LysR may be involved in oxidative stress resistance and regulation of the *hut* genes but more work is required to fully define LysR function.

The second part of this thesis examined the copper response of *S. aureus*. Initial investigations into *S. aureus* copper homeostasis identified that instead of a uniform growth response to copper challenge, there is significant variation between *S. aureus* strains (Figure 4.1). This was shown to most likely be due to the *copB/mco* operon, which is only present in a few of the sequenced strains, but found in the two most tolerant *S. aureus* tested; PM64 and ATCC 12600. However, PM64 possesses a chromosomally encoded *copB/mco* and the hyper copper tolerant ATCC 12600 was found to contain a plasmid encoded *copB/mco* (Figures 4.8, 4.9 and 4.10). Hyper copper-tolerance plasmids encoding copper homeostasis operons have been previously identified, but these have either been isolates from animals and plants fed high concentrations of copper or non-clinical human isolates. These plasmids have been shown to encode; the *tcrYAZB* operon of *E. hirae*, the *pcoABCDRSE* operon of *E. coli* and the *copABCD* operon found in *Pseudomonas syringae* pv. *Tomato* (Hasman & Aarestrup, 2002; Lee *et al*., 2002; Bender & Cooksey, 1987; Lim & Cooksey, 1993).

Therefore, this ATCC 12600 plasmid represents the first which confers high copper tolerance in a clinical strain. This is significant for *S. aureus* as copper is beginning to be employed as an antibacterial agent and from this study at least one hyper copper tolerant clinical strain has been found to exist. In addition, this *S. aureus* hyper copper tolerance is plasmid encoded so the likelihood of this phenotype being spread throughout the *S. aureus* population is increased by it being encoded on a MGE. Indeed, this plasmid was shown to be easily transferable to copper sensitive strains, such as Newman, which subsequently lead to the copper tolerance phenotype (Figure 4.10). To continue this investigation, the effect of *S. aureus* hyper copper tolerance on virulence should be examined. ATCC 12600 was isolated from the lungs and potentially this may be a tissue where copper tolerance is an important factor *in vivo*. Therefore, lung animal infection models would be of great interest to determine if the copper tolerance provided by the *S. aureus* plasmid is a virulence factor.

However, although the hyper tolerance was shown to be linked to the ATCC 12600 plasmid, it could not be confirmed if it was directly due to either *copB* or *mco*, and further investigations are therefore required. The ATCC 12600 *mco* mutant was created using a transposon (Sitthisak *et al*., 2005) which may have affected downstream genes. In this work, the ATCC 12600 *mco* mutant with a replaced *mco* gene encoded on pLI50 was found not to complement the loss of hyper copper tolerance in ATCC 12600 *mco* (Figure 4.5). However, this work also found that the ATCC 12600 *mco* gene is plasmid encoded and therefore the pLI50 *mco* may be at a lower copy number then the wild type *mco* gene which is a possible explanation for the failure to complement this mutant. The creation of a PM64 *mco* mutant would eliminate the problem of *mco* copy number and allow for easier investigation of *mco* function.

Restriction digests of the ATCC 12600 plasmid suggest that it is large and also that it may be related to *S. aureus* plasmid pXU5, which encodes high level cadmium resistance (Udo *et al*., 2000). Full characterisation of the ATCC 12600 would be of great interest, as it may encode additional and unknown copper tolerance systems that may be contributing to the hyper tolerance phenotype it provides. This could be achieved by creating mutants of individual genes on this plasmid and testing the affect on ATCC 12600 copper tolerance, providing these mutations did not affect plasmid copy number. Also as previously discussed, this plasmid likely encodes other metal resistance genes and therefore may provide additional useful phenotypes as well as copper tolerance. ATCC 12600 was also found to be chloramphenicol resistant which, due to time constraints, was not investigated further. The ATCC 12600 plasmid may potentially be the source of this antibiotic resistance and therefore may contain one or more virulence factors, which may provide further useful resistances to recipient *S. aureus* strains, thus increasing their pathogenicity. Full sequencing of the ATCC 12600 plasmid would allow for all the potentially provided phenotypes to be investigated further through experimentation.

One use of copper as a nosocomial antibacterial agent is through replacing steel surfaces with those made from copper. It has been shown that copper is superior to steel in killing surface *S. aureus* (Noyce *et al*., 2006). However, the discovery of the ATCC 12600 hyper copper-tolerance means that further investigations into the effectiveness of copper surfaces is required. EMRSA-16 was found to be more resistant to copper surfaces than other strains (Noyce *et al*., 2006) but in this study, ATCC 12600 was observed to possess a far superior copper tolerance when compared to the EMRSA-16, PM64 (Figure 4.2). Investigations into ATCC 12600 copper surface

survival are therefore required to establish if the hyper tolerance to copper in liquid media also provides protection against copper surfaces. As previously stated, any advantage bestowed by the ATCC 12600 plasmid against these surfaces has the potential of moving to other *S. aureus* strains and so reduce their effectiveness as a new *S. aureus* preventative measure.

The *S. aureus* CsoR characterised in this thesis represents the first *S. aureus* copper responsive regulator identified. Through transcriptional analysis it was shown that CsoR controls the copper regulation of chromosomal *S. aureus copA* and *copB* plus the plasmid encoded *copB* of ATCC 12600 (Figures 4.19 and 4.20). From the data obtained, CsoR represses these target genes transcription in Cu(0). However, two important aspects of this regulation remain unanswered. Firstly, the northern blot experiments suggested that in Cu(10) CsoR may be required for full expression of target genes. This has not been observed for the CsoR homologues of either *M. tuberculosis* or *B. subtilis*. Alternatively there may be another copper *S. aureus* regulator, similar to *ycnJ* copper regulation in *B. subtilis* which involves both CsoR and the recently identified YcnK regulator (Chillappagari *et al*., 2009). If CsoR is acting through another regulator, such as a sRNA or protein regulator, this may explain why no obvious CsoR binding site motif could be located in the promoters or either *copA* or *copB*. However, a reason for hierarchical copper regulation in *S. aureus* is unclear and this has not been observed in the regulation of any other bacterial copper ATPase. Most likely, the *S. aureus* CsoR binding site is not highly conserved between targets and is impossible to designate with only the two CsoR target genes that have been found in this study. As previously described, DNA protection assays would confirm CsoR direct regulation by DNA

promoter binding and a microarray analysis using the *csoR* mutants would be of great interest in finding the CsoR controlled genes which respond to environmental copper.

The final part of this thesis examined the nature of copper toxicity in *S. aureus* as very little is known about Gram positive copper toxicity. Similar to most other bacterial species, copper stress induces the up-regulation of oxidative stress genes in *S. aureus* strain SH1000 (Personal communication from Dr Jayaswal, Illinois State University). This study showed that the removal of the protective effect of catalase decreased copper tolerance (Figure 5.5). Also, inactivation of both *S. aureus* SODs increased copper tolerance and both these results taken together strongly suggest that it is hydrogen peroxide that is leading to *S. aureus* copper induced toxicity, most likely through the formation of harmful hydroxyl radicals by copper ions.

There was also evidence that surface toxicity is import for copper tolerance. Exogenous SOD and catalase, which are likely to remain extracellular, improved copper tolerance for SH1000 (Figure 5.6). However, this protection was not evident for the SH1000 double mutants with added exogenous SOD where copper tolerance was actually decreased (Figure 5.6B). Therefore, there is a relationship between the oxidative stress enzymes and surface *S. aureus* copper toxicity. This is potentially through the formation of peroxide radicals at the cell surface which leads to membrane disruption. Preliminary results showed some evidence for this in PM64, where copper led to a decrease in intracellular potassium which is suggestive of lipid disruption and cytoplasmic leakage. However, this requires further study for conformation. For future work, investigations into the formation of free radicals at the *S. aureus* cell surface for SH1000 wild type and

oxidative stress gene mutants in response to copper would help define the mechanisms that are occurring to disrupt the *S. aureus* membrane.

In summary, *S. aureus* has two separate systems for the resistance of environmental copper ions. The CopA efflux pump has been shown to aid copper tolerance and is found in all the sequenced *S. aureus* strains (Sitthisak *et al*., 2007). Some strains also encode an additional copper ATPase, CopB. This work found that the ATCC 12600 plasmid, which encodes *copB/mco*, can provide hyper copper tolerance. However, the exact function of CopB and the plasmid gene(s) responsible for copper tolerance still require to be fully defined in *S. aureus*. The other consequence of environmental copper investigated in this thesis was the *S. aureus* oxidative stress response. This system is both up-regulated in response to copper and catalase/AhpC activity was found to be required for copper tolerance. In addition, surface oxidative stress induction appears to be an important aspect of copper toxicity and would be the subject of further investigations.

Appendix

	Strand	Start	End	Gene	Known/putative function
1	D	-51	-41	SA0022	hypothetical protein - 5'-nucleotidase precursor
2	R	-40	-30	SA0055	hypothetical protein
3	R	-71	-61	SA0094	hypothetical protein - putative lipoprotein
4	D	-43	-33	SA0098	hypothetical protein - aminoacylase
5	D	-46	-36	*sirA*	lipoprotein
6	R	-64	-54	SA0112	hypothetical protein
7	D	-72	-62	*sodM*	superoxide dismutase
8	D	-87	-77	SA0160	hypothetical protein - heme-degrading monooxygenase IsdI
9	R	-90	-80	SA0170	hypothetical protein
10	D	-48	-38	*acpD*	azoreductase
11	D	-58	-48	SA0212	hypothetical protein - sugar phosphate isomerase/epimerase
12	D	-32	-22	SA0257	hypothetical protein - methyltransferase
13	D	-63	-53	SA0335	hypothetical protein
14	R	-69	-59	*lpl9*	Uncharacterized lipoprotein
15	D	-84	-74	*pth*	peptidyl-tRNA hydrolase
16	R	-31	-21	SA0507	hypothetical protein - amidohydrolase
17	D	-90	-80	SA0509	chaperone protein HchA
18	D	-78	-68	SA0511	hypothetical protein - putative epimerase/reductase
19	D	-60	-50	SA0574	hypothetical protein
20	D	-98	-88	*fhuA*	ferrichrome transport ATP-binding protein
21	D	-64	-54	SA0636	hypothetical protein
22	D	-56	-46	SA0679	hypothetical protein - histidinol-phosphate aminotransferase
23	D	-39	-29	SA0688	hypothetical protein - ferrichrome ABC transporter permease
24	D	-32	-22	*trxB*	thioredoxine reductase
25	D	-52	-42	SA0721	hypothetical protein
26	D	-52	-42	SA0724	hypothetical protein
27	D	-40	-30	*eno*	enolase
28	D	-22	-12	SA0754	hypothetical protein - acetyltransferase
29	D	-30	-20	SA0756	3-dehydroquinate dehydratase
30	R	-70	-60	SA0786	hypothetical protein
31	D	-42	-32	SA0797	hypothetical protein - nitrogen fixation protein NifU
32	R	-84	-74	SA0798	hypothetical protein
33	D	-105	-95	SA0804	hypothetical protein - Na+/H+ antiporter family protein

	Strand	Start	End	Gene	Known/putative function
34	D	-44	-34	*trpS*	tryptophanyl-tRNA synthetase
35	D	-75	-65	SA0864	GTP pyrophosphokinase
36	D	-107	-97	SA0887	hypothetical protein
37	D	-45	-35	SA0908	hypothetical protein - putative transcriptional regulator
38	D	-23	-13	SA0931	hypothetical protein
39	D	-48	-38	*isdB*	iron-regulated heme-iron binding protein
40	R	-75	-65	*isdA*	iron-regulated heme-iron binding protein
41	D	-65	-55	*isdC*	putative surface anchored protein
42	D	-52	-42	SA1007	alpha-hemolysin
43	D	-36	-26	*lytN*	LytN protein
44	D	-61	-51	*uppS*	undecaprenyl pyrophosphate synthetase
45	R	-71	-61	*glpP*	glycerol uptake operon antiterminator regulatory protein
46	D	-24	-14	*katA*	catalase
47	D	-20	-10	SAS044	4-oxalocrotonate tautomerase
48	D	-48	-38	SA1208	hypothetical protein
49	R	-74	-64	SA1209	hypothetical protein - hydrolase-related protein
50	R	-97	-87	*dfrA*	dihydrofolate reductase
51	D	-68	-58	SA1278	hypothetical protein
52	R	-66	-56	*xseA*	exodeoxyribonuclease VII large subunit
53	R	-47	-37	*coaE*	dephospho-CoA kinase
54	R	-46	-36	SA1552	hypothetical protein
55	R	-27	-17	SA1565	hypothetical protein - iron-regulated cell wall-anchored protein
56	D	-62	-52	*ribH*	6,7-dimethyl-8-ribityllumazine synthase
57	D	-51	-41	SA1636	hypothetical protein
58	D	-82	-72	*sen*	enterotoxin
59	D	-61	-51	SA1671	hypothetical protein - glucosamine-6-phosphate isomerase
60	R	-35	-25	SA1678	hypothetical protein - FUR family transcriptional regulator - PerR
61	D	-54	-44	SA1706	hypothetical protein
62	R	-90	-80	SA1709	hypothetical protein - ferritin
63	R	-24	-14	SA1732	hypothetical protein - sodium-dependent transporter
64	D	-73	-63	SAS056	hypothetical protein
65	R	-45	-35	SA1857	hypothetical protein - ATP/GTP hydrolase
66	D	-66	-56	SA1925	hypothetical protein

	Strand	Start	End	Gene	Known/putative function
67	R	-69	-59	*dps*	general stress protein 20U
68	R	-28	-18	SAS073	hypothetical protein
69	D	-41	-31	SA1982	hypothetical protein - membrane transporter
70	R	-71	-61	SA1983	hypothetical protein - aerobactin biosynthesis protein
71	D	-78	-68	SA2000	hypothetical protein
72	R	-26	-16	SA2079	hypothetical protein - ferric hydroxamate receptor 1
73	D	-79	-69	*hutU*	urocanate hydratase
74	R	-91	-81	SA2123	hypothetical protein - lysR type transcriptional regulator
75	R	-28	-18	*tcaB*	hypothetical protein - bicyclomycin resistance protein
76	R	-106	-96	SA2162	hypothetical protein - thioredoxin reductase
77	D	-73	-63	SA2180	hypothetical protein - two component sensor histidine kinase
78	R	-59	-49	*opp-1A*	hypothetical protein - oligopeptide transporter substrate protein
79	R	-66	-56	*fbp*	fructose-bisphosphatase
80	D	-37	-27	SA2338	hypothetical protein - ferrous iron transport protein A
81	D	-80	-70	SA2396	hypothetical protein - amino acid transporter
82	R	-83	-73	*isaB*	iron-regulated heme-iron binding protein
83	D	-79	-69	SA2432	hypothetical protein
84	D	-96	-86	SA2443	hypothetical protein
85	D	-59	-49	SA2454	hypothetical protein - putative acetyltransferase

Table A1: Fur box search 3. The first 12 base pairs (minus the initial G) of the *S. aureus* Fur box consensus sequence was used in a genome-scale DNA-pattern search using the RSAT database, selecting for the sequence of N315 and allowing for 1 base substation. Table shows the final 85 identified genes, including; gene direction on the chromosome (D = Forward R = Reverse), distance from transcriptional start site, gene name and known function. Highlighted in red are putative functions of hypothetical genes found through performing a BLASTP search using amino acid sequence. Highlighted in blue are potential regulator proteins.

Bibliography

Abdul-Tehrani, H., Hudson, A. J., Chang, Y. S., Timms, A. R., Hawkins, C., Williams, J. M., Harrison, P. M., Guest, J. R. and Andrews, S. C. (1999). Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and *fur* mutants are iron deficient. *J. Bacteriol.* **181**(5):1415-1428.

Adaikkalam, V. and Swarup, S. (2002). Molecular characterization of an operon, *cueAR*, encoding a putative P1-type ATPase and a MerR-type regulatory protein involved in copper homeostasis in *Pseudomonas putida*. *Microbiology* **148**(pt. 9):2857-2867.

Ahmer, B. M., Thomas, M. G., Larsen, R. A. and Postle, K. (1995). Characterization of the *exbBD* operon of *Escherichia coli* and the role of ExbB and ExbD in TonB function and stability. *J. Bacteriol.* **177**(16):4742-4747.

Airey, P. and Verran, J. (2007). Potential use of copper as a hygienic surface; problems associated with cumulative soiling and cleaning. *J. Hosp. Infect.* **67**(3):271-277.

Aitken-Rogers, H., Singleton, C., Lewin, A., Taylor-Gee, A., Moore, G. R., Le Brun, N. E. (2004). Effect of phosphate on bacterioferritin-catalysed iron(II) oxidation. *J. Biol. Inorg. Chem.* **9**(2):161-170.

Allison, S. L. and Phillips, A. T. (1990). Nucleotide sequence of the gene encoding the repressor for the histidine utilization genes of *Pseudomonas putida*. *J. Bacteriol.* **172**(9):5470-5476.

Allard, M., Moisan, H., Brouillette, E., Gervais, A. L., Jacques, M., Lacasse, P., Diarra, M. S. and Malouin, F. (2006). Transcriptional modulation of some *Staphylococcus aureus* iron-regulated genes during growth *in vitro* and in a tissue cage model *in vivo*. *Microbes. Infect.* **8**(7):1679-1690.

Almirón, M., Link, A. J., Furlong, D. and Kolter, R. (1992). A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. *Genes Dev.* **6**(12B):2646-2654.

Altuvia, S., Almirón, M., Huisman, G., Kolter, R. and Storz, G. (1994). The *dps* promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. *Mol. Microbiol.* **13**(2):265-272.

Ando, M., Manabe, Y. C., Converse, P. J., Miyazaki, E., Harrison, R., Murphy, J. R. and Bishai, W. R. (2003). Characterization of the role of the divalent metal ion-dependent transcriptional repressor MntR in the virulence of *Staphylococcus aureus*. *Infect. Immun.* **71**(5):2584-90.

Andrews, G. K. (2000). Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem. Pharmacol.* **59**(1):95-104.

Andrews, S. C., Le Brun, N. E., Barynin, V., Thomson, A. J., Moore, G. R., Guest, J. R. and Harrison, P. M. (1995). Site-directed replacement of the coaxial heme ligands of bacterioferritin generates heme-free variants. *J. Biol. Chem.* **270**(40):23268-23274.

Andrews, S. C., Robinson, A. K., Rodríguez-Quiñones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol. Rev.* **27**(2-3):215-237.

Angerer, A., Enz, S., Ochs, M. and Braun, V. (1995). Transcriptional regulation of ferric citrate transport in *Escherichia coli* K-12. Fecl belongs to a new subfamily of sigma 70-type factors that respond to extracytoplasmic stimuli. *Mol. Microbiol.* **18**(1):163-174.

Arciola, C. R., Campoccia, D., Gamberini, S., Baldassarri, L. and Montanaro, L. (2005). Prevalence of *cna*, *fnbA* and *fnbB* adhesin genes among *Staphylococcus aureus* isolates from orthopedic infections associated to different types of implant. *FEMS Microbiol. Lett.* **246**(1):81-86.

Ardic, N., Ozyurt, M., Sareyyupoglu, B. and Haznedaroglu, T. (2005). Investigation of erythromycin and tetracycline resistance genes in methicillin-resistant staphylococci. *Int. J. Antimicrob. Agents* **26**(3):213-318.

Argüello, J. M., Eren, E. and González-Guerrero, M. (2007). The structure and function of heavy metal transport P1B-ATPases. *Biometals* **20**(3-4):233-248.

Arnaud, M., Chastanet, A. and Débarbouillé, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *App. Environ. Microbiol.* **70**(11):6887-6891.

Arrecubieta, C., Matsunaga, I., Asai, T., Naka, Y., Deng, M. C. and Lowy, F. D. (2008). Vaccination with clumping factor A and fibronectin binding protein A to prevent *Staphylococcus aureus* infection of an aortic patch in mice. *J. Infect. Dis.* **198**(4):571-575.

Arredondo, M. and Núñez, M. T. (2005). Iron and copper metabolism. *Mol. Aspects Med.* **26**(4-5):313-327.

Aruoma, O. I., Halliwell, B., Gajewski, E. and Dizdaroglu, M. (1991). Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. *Biochem. J.* **273**(Pt. 3):601-604.

Asmuss, M., Mullenders, L.H., Eker, A. and Hartwig, A. (2000). Differential effects of toxic metal compounds on the activities of Fpg and XPA, two zinc finger proteins involved in DNA repair. *Carcinogenesis* **21**(11):2097-2104.

Atkinson, M. R., Wray, L. V. Jr. and Fisher, S. H. (1990). Regulation of histidine and proline degradation enzymes by amino acid availability in *Bacillus subtilis*. *J. Bacteriol.* **172**(9):4758-4765.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seldman, J. G., Smith, J. A. and Struhl, K., Ed. (1995). Current Protocols in Molecular Biology, John Wiley and Sons.

Avery, S. V., Howlett, N. G. and Radice, S. (1996). Copper toxicity towards *Saccharomyces cerevisiae*: dependence on plasma membrane fatty acid composition. *Appl. Environ. Microbiol.* **62**(11):3960-3966.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K. and Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* **359**(9320):1819-1827.

Bai, E., Rosell, F. I., Lige, B., Mauk, M. R., Lelj-Garolla, B., Moore, G. R. and Mauk, A. G. (2006). Functional characterization of the dimerization domain of the ferric uptake regulator (Fur) of *Pseudomonas aeruginosa*. *Biochem. J.* **400**(3):385-392.

Baichoo, N., Wang, T., Ye, R. and Helmann, J. D. (2002). Global analysis of the *Bacillus subtilis* Fur regulon and the iron starvation stimulon. *Mol. Microbiol.* **45**(6):1613-1629.

Baker, H. M., Anderson, B. F. and Baker, E. N. (2003). Dealing with iron: common structural principles in proteins that transport iron and heme. *Proc. Natl. Acad. Sci. U.S.A.* **100**(7):3579-3583.

Barkema, H. W., Schukken, Y. H. and Zadoks, R. N. (2006). Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J. Dairy Sci.* **89**(6):1877-1895.

Bayle, D., Wängler, S., Weitzenegger, T., Steinhilber, W., Volz, J., Przybylski, M., Schäfer, K. P., Sachs, G. and Melchers, K. (1998). Properties of the P-type ATPases encoded by the *copAP* operons of *Helicobacter pylori* and *Helicobacter felis*. *J. Bacteriol.* **180**(2):317-329.

Beinert, H. (1997). Copper A of cytochrome *c* oxidase, a novel, long-embattled, biological electron-transfer site. *Eur. J. Biochem.* **245**(3):521-532.

Belitsky, B. R., Janssen, P. J. and Sonenshein, A. L. (1995). Sites required for GltC-dependent regulation of *Bacillus subtilis* glutamate synthase expression. *J. Bacteriol.* **177**(19):5686-5695.

Bender, R. A. (1991). The role of the NAC protein in the nitrogen regulation of *Klebsiella aerogenes*. *Mol. Microbiol.* **5**(11):2575-2580.

Bender, C. L. and Cooksey, D. A. (1987). Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. *J. Bacteriol.* **169**(2):470-474.

Bento, I., Peixoto, C., Zaitsev, V. N. and Lindley, P. F. (2007). Ceruloplasmin revisited: structural and functional roles of various metal cation-binding sites. *Acta Crystallogr. D. Biol. Crystallogr.* **63**(Pt. 2):240-248.

Bitoun, J. P., Wu, G. and Ding, H. (2008). *Escherichia coli* FtnA acts as an iron buffer for re-assembly of iron-sulfur clusters in response to hydrogen peroxide stress. *Biometals* **21**(6):693-703.

BLAST database: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Bollinger, C. J. and Kallio, P. T. (2007). Impact of the small RNA RyhB on growth, physiology and heterologous protein expression in *Escherichia coli*. *FEMS Microbiol. Lett.* **275**(2):221-228.

Bonnah, R. A. and Schryvers, A. B. (1998). Preparation and characterization of *Neisseria meningitidis* mutants deficient in production of the human lactoferrin-binding proteins LbpA and LbpB. *J. Bacteriol.* **180**(12):3080-3090.

Boukhalfa, H. and Crumbliss, A. L. (2002). Chemical aspects of siderophore mediated iron transport. *Biometals* **15**(4):325-339.

Boulton, I. C., Yost, M. K., Anderson, J. E. and Cornelissen, C. N. (2000). Identification of discrete domains within Gonococcal transferrin-binding protein A that are necessary for ligand binding and iron uptake functions. *Infect. Immun.* **68**(12):6988-6996.

Bradbeer, C. (1993). The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. *J. Bacteriol.* **175**(10):3146-3150.

Braun, V., Gaisser, S., Herrmann, C., Kampfenkel, K., Killmann, H., Traub, I. (1996). Energy-coupled transport across the outer membrane *of Escherichia coli*: ExbB binds ExbD and TonB in vitro, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. *J. Bacteriol.* **178**(10):2836-2845.

Braun, V., Mahren, S. and Ogierman, M. (2003). Regulation of the FecI-type ECF sigma factor by transmembrane signalling. *Curr. Opin. Microbiol.* **6**(2):173-180.

Bremner, I. (1998). Manifestations of copper excess. *Am. J. Clin. Nutr.* **67**(5 Suppl):1069S–1073S.

Bronner, S., Monteil, H. and Prévost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *FEMS Microbiol. Rev.* **28**(2):183-200.

Brown, N. L., Stoyanov, J. V., Kidd, S. P. and Hobman, J. L. (2003). The MerR family of transcriptional regulators. *FEMS Microbiol. Rev.* **27**(2-3):145-163.

Bsat, N. and Helmann, J. D. (1999). Interaction of *Bacillus subtilis* Fur (ferric uptake repressor) with the *dhb* operator in vitro and in vivo. *J. Bacteriol.* **181**(14): 4299-4307

Buchanan, S. K., Smith, B. S., Venkatramani, L., Xia, D., Esser, L., Palnitkar, M., Chakraborty, R., van der Helm, D. and Deisenhofer, J. (1999). Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. *Nat. Struct. Biol.* **6**(1):56-63.

Bull, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R. and Cox, D. W. (1993). The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nat. Genet.* **5**(4):327-337.

Burlak, C., Hammer, C. H., Robinson, M. A., Whitney, A. R., McGavin, M. J., Kreiswirth, B. N. and Deleo, F. R. (2007). Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced in vitro and during infection. *Cell. Microbiol.* **9**(5):1172-1190.

Cabrera, G., Xiong, A., Uebel, M., Singh, V. K. and Jayaswal, R. K. (2001). Molecular characterization of the iron-hydroxamate uptake system in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **67**(2):1001-1003.

Catalani, S., De Palma, G., Mangili, A. and Apostoli, P. (2008). Metallic elements in lung tissues: results of a meta-analysis. *Acta Biomed.* **79**(Suppl. 1):52-63.

Casey, A.L., Lambert, P.A., Miruszenko, L. and Elliott, T. S. J. (2008). Copper surface clinical trial; http://www.copperinfo.co.uk/antimicrobial/clinical-trial.shtml

CDC, (1999). Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*--Minnesota and North Dakota, 1997-1999. *JAMA* **282**(12):1123-1125.

Chang, W., Small, D. A., Toghrol, F. and Bentley, W. E. (2006). Global transcriptome analysis of *Staphylococcus aureus* response to hydrogen peroxide. *J. Bacteriol.* **188**(4):1648-1659.

Chasin, L. A. and Magasanik, B. (1968). Induction and repression of the histidine-degrading enzymes of *Bacillus subtilis*. *J. Biol. Chem.* **243**(19):5165-5178.

Chen, C. Y., Berish, S. A., Morse, S. A. and Mietzner, T. A. (1993). The ferric iron-binding protein of pathogenic *Neisseria* spp. functions as a periplasmic transport protein in iron acquisition from human transferrin. *Mol. Microbiol.* **10**(2):311-318.

Cheung, A. L., Bayer, A. S., Zhang, G., Gresham, H. and Xiong, Y. Q. (2004). Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunol. Med. Microbiol.* **40**(1):1-9.

Cheung, A. L., Koomey, J. M., Butler, C. A., Projan, S. J. and Fischetti, V. A. (1992). Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. *Proc. Natl. Acad. Sci. U.S.A.* **89**(14):6462-6466.

Cheung, A. L., Nishina, K. A., Trotonda, M. P. and Tamber, S. (2008). The SarA protein family of *Staphylococcus aureus*. *Int. J. Biochem. Cell Biol.* **40**(3):355-361.

Childs, C., Edwards Jones, V., Dawson, M., Davenport, P. J. (1999). Toxic shock syndrome toxin-1 (TSST-1) antibody levels in burned children. *Burns* **25**(6):473-476.

Chillappagari, S., Miethke, M., Trip, H., Kuipers, O. P. and Marahiel, M. A. (2009). Copper acquisition is mediated by YcnJ and regulated by YcnK and CsoR in *Bacillus subtilis*. *J. Bacteriol.* **191**(7):2362-2370.

Chu, V. H., Crosslin, D. R., Friedman, J. Y., Reed, S. D., Cabell, C. H., Griffiths, R. I., Masselink, L. E., Kaye, K. S., Corey, G. R., Reller, L. B., Stryjewski, M. E., Schulman, K. A. and Fowler, V. G. Jr. (2005). *Staphylococcus aureus* bacteremia in patients with prosthetic devices: costs and outcomes. *Am. J. Med.* **118**(12):1416.

Clark, N. C., Weigel, L. M., Patel, J. B. and Tenover, F. C. (2005). Comparison of Tn*1546*-like elements in vancomycin-resistant *Staphylococcus aureus* isolates from Michigan and Pennsylvania. Antimicrob. *Agents Chemother.* **49**(1):470-472.

Clarke, S. R., Wiltshire, M. D. and Foster, S. J. (2004). IsdA of *Staphylococcus aureus* is a broad spectrum, iron-regulated adhesin. *Mol. Microbiol.* **51**(5):1509-1519.

Clarke, S. R., Mohamed, R., Bian, L., Routh, A. F., Kokai-Kun, J. F., Mond, J. J., Tarkowski, A. and Foster, S. J. (2007). The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. *Cell Host Microbe.* **1**(3):199-212.

ClustalW2 website: http://www.ebi.ac.uk/Tools/clustalw2/index.html

Clements, M. O., Watson, S. P. and Foster, S. J. (1999). Characterization of the major superoxide dismutase of *Staphylococcus aureus* and its role in starvation survival, stress resistance, and pathogenicity. *J. Bacteriol.* **181**(13):3898-3903.

Cobine, P., Wickramasinghe, W. A., Harrison, M. D,, Weber, T., Solioz, M. and Dameron, C. T. (1999). The *Enterococcus hirae* copper chaperone CopZ delivers copper(I) to the CopY repressor. *FEBS Lett.* **445**(1):27-30.

Coote, J. G. and Hassall, H. (1973). The degradation of L-histidine, imidazolyl-L-lactate and imidazolylpropionate by *Pseudomonas testosteroni*. *Biochem. J.* **132**(3):409-422.

Corson, L. B., Folmer, J., Strain J. J., Culotta, V. C., Cleveland, D. W. (1999). Oxidative stress and iron are implicated in fragmenting vacuoles of *Saccharomyces cerevisiae* lacking Cu,Zn-superoxide dismutase. *J. Biol. Chem.* **274**(39):27590-27596.

Cosgrove, K., Coutts, G., Jonsson, I. M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J. and Foster, S. J. (2007). Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J. Bacteriol.* **189**(3):1025-1035.

Courcol, R. J., Trivier, D., Bissinger, M. C., Martin, G. R. and Brown, M. R. (1997). Siderophore production by *Staphylococcus aureus* and identification of iron-regulated proteins. *Infect Immun.* **65**(5):1944-1948.

Courvalin, P. (2006). Vancomycin resistance in gram-positive cocci. *Clin. Infect. Dis.* **42**(Suppl. 1):S25-34.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W. and Götz, F. (1999). The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect. Immun.* **67**(10):5427-5433.

Crichton, R. R. and Pierre, J. L. (2001). Old iron, young copper: from Mars to Venus. *Biometals* **14**(2):99-112.

Crossley, K. B. and Archer, G. L. (1997). The Staphylococci in Human Disease. W.B. Saunders Company.

Dale, S. E., Sebulsky, M. T. and Heinrichs, D. E. (2004). Involvement of SirABC in iron-siderophore import in *Staphylococcus aureus*. *J. Bacteriol.* **186**(24):8356-8362.

Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J. and Klausner, R. D. (1994). Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. *Cell* **76**(2):393-402.

Deighton, M. A., Capstick, J., Domalewski, E. and van Nguyen, T. (2001). Methods for studying biofilms produced by *Staphylococcus epidermidis*. *Methods Enzymol.* **336**:177-195.

De Lorenzo, V., Wee, S., Herrero, M. and Neilands, J. B. (1987). Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. *J. Bacteriol.* **169**(6):2624-2630.

Delany, I., Rappuoil, R., and Scarlato, V. (2004). Fur functions as an activator and as a repressor of putative virulence genes in *Neisseria meningitis*. *Mol. Microbiol.* **52**(4):1081-1090

Delany, I., Spohn, G., Rappuoli, R., and Scarlato, V., (2001). The Fur repressor controls transcription of iron-activated and –repressed genes in *Helicobacter pylori. Mol. Microbiol.* **45**(5):1297-1309.

DeRocco, A. J., Yost-Daljev, M. K., Kenney, C. D. and Cornelissen, C. N. (2009). Kinetic analysis of ligand interaction with the gonococcal transferrin-iron acquisition system. *Biometals* **22**(3):439-451.

Dinges, M. M., Orwin, P. M. and Schlievert, P. M. (2000). Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev.* **13**(1):16-34.

Djoko, K. Y., Xiao, Z. and Wedd, A. G. (2008). Copper resistance in *E. coli*: the multicopper oxidase PcoA catalyzes oxidation of copper(I) in Cu(I)Cu(II)-PcoC. *Chembiochem.* **9**(10):1579-1582.

Dunman, P. M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E. L., Zagursky, R. J., Shlaes, D. and Projan, S. J. (2001). Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *J. Bacteriol.* **183**(24):7341-7353.

Duthie, E. S. and Lorenz, L. L. (1952). Staphylococcal coagulase: mode of action and antigenicity. *J. Gen. Microbiol.* **6**(1-2):95-107.

Elguindi, J., Wagner, J. and Rensing, C. (2009). Genes involved in copper resistance influence survival of *Pseudomonas aeruginosa* on copper surfaces. *J. Appl. Microbiol.* **106**(5):1448-1455.

Ellermeier, J. R. and Slauch, J. M. (2008). Fur regulates expression of the *Salmonella* pathogenicity island 1 type III secretion system through HilD. *J. Bacteriol.* **190**(2):476-486.

Emerit, J., Beaumont, C. and Trivin, F. (2001). Iron metabolism, free radicals, and oxidative injury. *Biomed. Pharmacother.* **55**(6):333-339.

Escolar, L., Pérez-Martín, J. and de Lorenzo, V. (2000). Evidence of an unusually long operator for the Fur repressor in the aerobactin promoter of *Escherichia coli*. *J. Biol. Chem.* **275**(32):24709-24714.

Espariz, M., Checa, S. K., Audero, M. E., Pontel, L. B. and Soncini, F. C. (2007). Dissecting the *Salmonella* response to copper. *Microbiology* **153**(Pt. 9):2989-2997.

Espírito Santo, C., Taudte, N., Nies, D. H. and Grass, G. (2008). Contribution of copper ion resistance to survival of *Escherichia coli* on metallic copper surfaces. *Appl. Environ. Microbiol.* **74**(4):977-986.

Esposito, L., Seydel, A., Aiello, R., Sorrentino, G., Cendron, L., Zanotti, G. and Zagari, A. (2008). The crystal structure of the superoxide dismutase from *Helicobacter pylori* reveals a structured C-terminal extension. *Biochim. Biophys. Acta* **1784**(11):1601-1606.

Fattom, A. I., Sarwar, J., Ortiz, A. and Naso, R. (1996). A *Staphylococcus aureus* capsular polysaccharide (CP) vaccine and CP-specific antibodies protect mice against bacterial challenge. *Infect. Immun.* **64**(5):1659-1665.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**(1):6-13.

Ferguson, A. D., Hofmann, E., Coulton, J. W., Diederichs, K. and Welte, W. (1998). Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. *Science* **282**(5397):2215-2220.

Ferreirós, C., Criado, M. T. and Gómez, J. A. (1999). The neisserial 37 kDa ferric binding protein (FbpA). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **123**(1):1-7.

Field, L. S, Luk, E. and Culotta, V. C. (2002). Copper chaperones: personal escorts for metal ions. *J. Bioenerg. Biomembr.* **34**(5):373-379.

Fitzgerald, J. R., Sturdevant, D. E., Mackie, S. M., Gill, S. R. and Musser, J. M. (2001). Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc. Natl. Acad. Sci. U.S.A.* **98**(15):8821-8826.

Flint, D. H., Tuminello, J. F. and Emptage, M. H. (1993). The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J. Biol. Chem.* **268**(30):22369-22376.

Fox, L. K., Zadoks, R. N. and Gaskins, C. T. (2005). Biofilm production by *Staphylococcus aureus* associated with intramammary infection. *Vet. Microbiol.* **107**(3-4):295-299.

Francis, M. S. and Thomas, C. J. (1997). The *Listeria monocytogenes* gene *ctpA* encodes a putative P-type ATPase involved in copper transport. *Mol. Gen. Genet.* **253**(4):484-491.

Fraser, J. D. and Proft, T. (2008). The bacterial superantigen and superantigen-like proteins. *Immunol. Rev.* **225**:226-243.

Frazão, C., Aragão, D., Coelho, R., Leal, S. S., Gomes, C. M., Teixeira, M. and Carrondo, M. A. (2008). Crystallographic analysis of the intact metal centres $[3Fe\text{-}4S]^{1+/0}$ and $[4Fe\text{-}4S]^{2+/1+}$ in a Zn^{2+} -containing ferredoxin. *FEBS Lett.* **582**(5):763-767.

Friedman, D. B., Stauff, D. L., Pishchany, G., Whitwell, C. W., Torres, V. J. and Skaar, E. P. (2006). *Staphylococcus aureus* redirects central metabolism to increase iron availability. *PLoS Pathog.* **2**(8):e87.

Gaballa, A., Antelmann, H., Aguilar, C., Khakh, S. K., Song, K. B., Smaldone, G. T. and Helmann, J. D. (2008). The *Bacillus subtilis* iron-sparing response is mediated by a Fur-regulated small RNA and three small, basic proteins. *Proc. Natl. Acad. Sci. U.S.A.* **105**(33):11927-11932.

Gaballa, A. and Helmann, J. D. (2003). *Bacillus subtilis* CPx-type ATPases: characterization of Cd, Zn, Co and Cu efflux systems. *Biometals* **16**(4):497-505.

Gaetke, L. M. and Chow, C. K. (2003). Copper toxicity, oxidative stress and antioxidant nutrients. *Toxicology* **189**(1-2):147-163.

Gee, J. M., Valderas, M. W., Kovach, M. E., Grippe, V. K., Robertson, G. T., Ng, W. L., Richardson, J. M., Winkler, M. E. and Roop, R. M. 2nd. (2005). The *Brucella abortus* Cu,Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. *Infect. Immun.* **73**(5):2873-2880.

Geissmann, T. A. and Touati, D. (2004). Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J.* **23**(2):396-405.

Georgatsou, E., Mavrogiannis, L. A., Fragiadakis, G. S. and Alexandraki, D. (1997). The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *J. Biol. Chem.* **272**(21):13786-13792.

Giraudo, A. T., Calzolari, A., Cataldi, A. A., Bogni, C. and Nagel, R. (1999). The *sae* locus of *Staphylococcus aureus* encodes a two-component regulatory system. *FEMS Microbiol Lett.* **177**(1):15-22.

Giraudo, A. T., Mansilla, C., Chan, A., Raspanti, C. and Nagel, R. (2003). Studies on the expression of regulatory locus *sae* in *Staphylococcus aureus*. *Curr. Microbiol.* **46**(4):246-250.

Goji, N., Potter, A. A. and Perez-Casal, J. (2004). Characterization of two proteins of *Staphylococcus aureus* isolated from bovine clinical mastitis with homology to glyceraldehyde-3-phosphate dehydrogenase. *Vet Microbiol.* **99**(3-4):269-279.

Gold, B., Deng, H., Bryk, R., Vargas, D., Eliezer, D., Roberts, J., Jiang, X. and Nathan, C. (2008). Identification of a copper-binding metallothionein in pathogenic mycobacteria. *Nat. Chem. Biol.* **4**(10):609-616.

Götz, F., Verheij, H. M. and Rosenstein, R. (1998). Staphylococcal lipases: molecular characterisation, secretion, and processing. *Chem. Phys. Lipids* **93**(1-2):15-25.

Grass[1], G. and Rensing, C. (2001). CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **286**(5):902-908.

Grass[2], G. and Rensing, C. (2001). Genes involved in copper homeostasis in *Escherichia coli*. *J. Bacteriol.* **183**(6):2145-2147.

Grifantini, R., Sebastian, S., Frigimelica, E., Draghi, M., Bartolini, E., Muzzi, A., Rappuoli, R., Grandi, G. and Genco, C. A. (2003). Identification of iron-activated and –repressed Fur-dependant genes by transcrptome analysis of *Neisseria meningitidis* group B. *PNAS* **100**(16):9542-9547.

Grkovic, S., Brown, M. H., Hardie, K. M., Firth, N. and Skurray, R. A. (2003). Stable low-copy-number *Staphylococcus aureus* shuttle vectors. *Microbiology* **149**(Pt 3):785-795.

Grundmann, H., Aires-de-Sousa, M., Boyce, J, and Tiemersma, E. (2006). Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. *Lancet* **368**(9538):874-885.

Hagan, E. C. and Mobley, H. L. (2009). Haem acquisition is facilitated by a novel receptor Hma and required by uropathogenic *Escherichia coli* for kidney infection. *Mol. Microbiol.* **71**(1):79-91.

Hall, S. J., Hitchcock, A., Butler, C. S. and Kelly, D. J. (2008). A Multicopper oxidase (Cj1516) and a CopA homologue (Cj1161) are major components of the copper homeostasis system of *Campylobacter jejuni*. *J. Bacteriol.* **190**(24):8075-8085.

Hall, A. E., Domanski, P. J., Patel, P. R., Vernachio, J. H., Syribeys, P. J., Gorovits, E. L., Johnson, M. A., Ross, J. M., Hutchins, J. T. and Patti, J. M. (2003). Characterization of a protective monoclonal antibody recognizing *Staphylococcus aureus* MSCRAMM protein clumping factor A. *Infect. Immun.* **71**(12):6864-6870.

Hantke, K. (1981). Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. *Mol. Gen. Genet.* **182**(2):288-292.

Hansen, U., Hussain, M., Villone, D., Herrmann, M., Robenek, H., Peters, G., Sinha, B. and Bruckner, P. (2006). The anchorless adhesin Eap (extracellular adherence protein) from *Staphylococcus aureus* selectively recognizes extracellular matrix aggregates but binds promiscuously to monomeric matrix macromolecules. *Matrix Biol.* **25**(4):252-260.

Harraghy, N., Kormanec, J., Wolz, C., Homerova, D., Goerke, C., Ohlsen, K., Qazi, S., Hill, P. and Herrmann, M. (2005). *sae* is essential for expression of the staphylococcal adhesins Eap and Emp. *Microbiology* **151**(Pt 6):1789-800.

Hasman, H. (2005). The *tcrB* gene is part of the t*crYAZB* operon conferring copper resistance in *Enterococcus faecium* and *Enterococcus faecalis*. *Microbiology* **151**(Pt. 9):3019-3025.

Hasman, H. and Aarestrup, F. M. (2002). *tcrB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. *Antimicrob. Agents Chemother*. **46**(5):1410-1416.

Hassett, R., Dix, D. R., Eide, D. J. and Kosman, D. J. (2000). The Fe(II) permease Fet4p functions as a low affinity copper transporter and supports normal copper trafficking in *Saccharomyces cerevisiae*. *Biochem. J.* **351**(Pt. 2):477-484.

Heinrichs, J. H., Gatlin, L. E., Kunsch, C., Choi, G. H. and Hanson, M. S. (1999). Identification and characterization of SirA, an iron-regulated protein from *Staphylococcus aureus*. *J. Bacteriol.* **181**(5):1436-1443.

Henikoff, S., Haughn, G. W., Calvo, J. M. and Wallace, J. C. (1988). A large family of bacterial activator proteins. *Proc. Natl. Acad. Sci. U.S.A.* **85**(18):6602-6606.

Hentze, M. W., Muckenthaler, M. U. and Andrews, N. C. (2004). Balancing acts: molecular control of mammalian iron metabolism. *Cell* **117**(3):285-297.

Hernández-Lucas, I., Gallego-Hernández, A. L., Encarnación, S., Fernández-Mora, M., Martínez-Batallar, A. G., Salgado, H., Oropeza, R and Calva, E. (2006). The LysR-type transcriptional regulator LeuO controls expression of several genes in *Salmonella enterica* serovar Typhi. *J. Bacteriol.* **190**(5):1658-1670.

Hill, P. J., Cockayne, A., Landers, P., Morrissey, J. A., Sims, C. M. and Williams, P. (1998). SirR, a novel iron-dependent repressor in *Staphylococcus epidermidis*. *Infect. Immun.* **66**(9):4123-4129.

Hiniker, A., Collet, J. F. and Bardwell, J. C. (2005). Copper stress causes an *in vivo* requirement for the *Escherichia coli* disulfide isomerase DsbC. *J. Biol. Chem.* **280**(40):33785-33791.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J. Antimicrob. Chemother.* **40**(1):135-136.

Hobman, J. L. (2007). MerR family transcription activators: similar designs, different specificities. *Mol. Microbiol.* **63**(5):1275-1278.

Holden, M. T., Feil, E. J., Lindsay, J. A., Peacock, S. J., Day, N. P., Enright, M. C., Foster, T. J., Moore, C. E., Hurst, L., Atkin, R., Barron, A., Bason, N., Bentley, S. D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K. D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall, K., Ormond, D., Quail, M. A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrell, B. G., Spratt, B. G., and Parkhill. J. (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc. Natl. Acad. Sci. U.S.A.* **101**(26):9786-9791.

Horsburgh[1], M. J., Clements, M. O., Crossley, H., Ingham, E. and Foster, S. J. (2001). PerR controls Oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infect. Immun.* **69**(6): 3744-3754.

Horsburgh[2], M. J., Ingham, E. and Foster, S. J. (2001). In *Staphylococcus aureus*, Fur is an interactive regulator with PerR, contributes to virulence, and is necessary for Oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J. Bacteriol.* **183**(2):468-475.

Horsburgh[1], M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K. and Foster, S. J. (2002). σ^B modulates virulence determinant expression and stress resistance: characterization of functional *rsbU* strain derived from *Staphylococcus aureus* 8325–4. *J. Bacteriol.* **184**(19):5457-5467.

Horsburgh[2], M. J., Wharton, S. J., Cox, A. G., Ingham, E., Peacock, S. and Foster, S. J. (2002). MntR modulates expression of the PerR regulon and superoxide resistance in *Staphylococcus aureus* through control of manganese uptake. *Mol. Microbiol.* **44**(5):1269-1286.

Howlett, N. G. and Avery, S. V. (1997). Induction of lipid peroxidation during heavy metal stress in *Saccharomyces cerevisiae* and influence of plasma membrane fatty acid unsaturation. *Appl. Environ. Microbiol.* **63**(8):2971-2976.

HPA UK hospital MRSA mandatory bacteraemia reports table 1
http://www.hpa.org.uk/servlet/Satellite?c=HPAweb_C&childpagename=HPAweb%2FHPAwebStandard&cid=1233906819629&p=1191942169773&pagename=HPAwebWrapper

Hu, L. and Phillips, A. T. (1988). Organization and multiple regulation of histidine utilization genes in *Pseudomonas putida*. *J. Bacteriol.* **170**(9):4272-4279.

Huston, W. M., Jennings, M. P. and McEwan, A. G. (2002). The multicopper oxidase of *Pseudomonas aeruginosa* is a ferroxidase with a central role in iron acquisition. *Mol. Microbiol.* **45**(6):1741-1750.

Imbert, M. and Blondeau, R. (1998). On the iron requirement of lactobacilli grown in chemically defined medium. *Curr. Microbiol.* **37**(1):64-66.

Irwin, S. W., Averil, N., Cheng, C. Y. and Schryvers, A. B. (1993). Preparation and analysis of isogenic mutants in the transferrin receptor protein genes, *tbpA* and *tbpB*, from *Neisseria meningitidis*. *Mol. Microbiol.* **8**(6):1125-1133.

Ito, T., Okuma, K., Ma, X. X., Yuzawa, H. and Hiramatsu, K. (2003). Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. *Drug Resist. Updat.* **6**(1):41-52.

Iwatsuki, K., Yamasaki, O., Morizane, S. and Oono, T. (2006). Staphylococcal cutaneous infections: invasion, evasion and aggression. *J. Dermatol. Sci.* **42**(3):203-214.

Jacques, J. F., Jang, S., Prévost, K., Desnoyers, G., Desmarais, M., Imlay, J. and Massé, E. (2006). RyhB small RNA modulates the free intracellular iron pool and is essential for normal growth during iron limitation in *Escherichia coli*. *Mol. Microbiol.* **62**(4):1181-1190.

Johnson, A. P., Aucken, H. M., Cavendish, S., Ganner, M., Wale, M. C., Warner, M., Livermore, D. M. and Cookson, B. D; UK EARSS participants. (2001). Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). *J. Antimicrob. Chemother.* **48**(1):143-144.

Johnson, M., Cockayne, A. and Morrissey, J. A. (2008). Iron-regulated biofilm formation in *Staphylococcus aureus* Newman requires *ica* and the secreted protein Emp. *Infect. Immun.* **76**(4):1756-1765.

Johnson, M., Cockayne, A., Williams, P. H. and Morrissey, J. A. (2005). Iron-responsive regulation of biofilm formation in *Staphylococcus aureus* involves Fur-dependent and Fur-independent mechanisms. *J. Bacteriol.* **187**(23):8211–8215.

Kaila, V. R., Johansson, M. P., Sundholm, D., Laakkonen, L. and Wiström, M. (2009). The chemistry of the CuB site in cytochrome *c* oxidase and the importance of its unique His-Tyr bond. *Biochim. Biophys. Acta.* **1787**(4):221-233.

Kaito C, Morishita D, Matsumoto Y, Kurokawa K, Sekimizu K. (2006). Novel DNA binding protein SarZ contributes to virulence in *Staphylococcus aureus*. *Mol. Microbiol.* **62**(6):1601-1617.

Kammler, M., Schön, C. and Hantke, K. (1993). Characterization of the ferrous iron uptake system of *Escherichia coli*. *J. Bacteriol.* **175**(19):6212-6219.

Kaneko, J., **Kimura, T.**, **Narita, S.**, **Tomita, T.** and **Kamio, Y. (1998).** Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage phiPVL carrying Panton-Valentine leukocidin genes. *Gene* **215**(1):57-67.

Karavolos, M. H., Horsburgh, M. J., Ingham, E. and Foster, S. J. (2003). Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. *Microbiology* **149**(Pt 10):2749-2758.

Karjalainen, T. K., Evans, D. G., Evans, D. J. Jr., Graham, D. Y. and Lee, C. H. (1991). Iron represses the expression of CFA/I fimbriae of enterotoxigenic *E. coli*. *Microb. Pathog.* **11**(5):317-323.

Keren, N., Aurora, R. and Pakrasi, H. B. (2004). Critical roles of bacterioferritins in iron storage and proliferation of Cyanobacteria. *Plant Physiol.* **135**(3):1666-1673.

Kershaw, C. J., Brown, N. L., Constantinidou, C., Patel, M. D. and Hobman, J. L. (2005). The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. *Microbiology* **151**(Pt 4):1187-1198.

Kim, N. H. and Kang, J. H. (2006). Oxidative damage of DNA induced by the cytochrome *c* and hydrogen peroxide system. *J. Biochem. Mol. Biol.* **39**(4):452-456.

Kim, C., Lorenz, W.W., Hoopes, J.T. and Dean, J.F. (2001). Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli yacK* gene. *J. Bacteriol.* **183**(16):4866-4875.

Kim, S. J., Park, R. Y., Kang, S. M., Choi, M. H., Kim, C. M. and Shin, S. H. (2006). *Pseudomonas aeruginosa* alkaline protease can facilitate siderophore-mediated iron-uptake via the proteolytic cleavage of transferrins. *Biol. Pharm. Bull.* **29**(11):2295-2300.

Klabunde, T., Eicken, C., Sacchettini, J. C. and Krebs, B. (1998). Crystal structure of a plant catechol oxidase containing a dicopper center. *Nat. Struct. Biol.* **5**(12):1084-1090.

Knight, S. A., Labbé, S., Kwon, L. F., Kosman, D. J. and Thiele, D. J. (1996). A widespread transposable element masks expression of a yeast copper transport gene. *Genes Dev.* **10**(15):1917-1929.

Koorts, A. M. and Viljoen, M. (2007). Ferritin and ferritin isoforms I: Structure-function relationships, synthesis, degradation and secretion. *Arch. Physiol. Biochem.* **113**(1):30-54.

Kraemer, G. R. and Iandolo, J. J. (1990). High-frequency transformation of *Staphylococcus aureus* by electroporation. *Curr. Microbiol.* **21**(6):373-376.

Kumar, K. S., Dayananda, S. and Subramanyam, C. (2005). Copper alone, but not oxidative stress, induces copper-metallothionein gene in *Neurospora crassa*. *FEMS Microbiol. Lett.* **242**(1):45-50.

Kumarevel, T., Mizuno, H. and Kumar, P. K. (2005). Characterization of the metal ion binding site in the anti-terminator protein, HutP, of *Bacillus subtilis*. *Nucleic Acids Res.* **33**(17):5494-5502.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H. and Hiramatsu, K. (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357**(9264):1225-1240.

Kühlbrandt, W. (2004). Biology, structure and mechanism of P-type ATPases. *Nat. Rev. Mol. Cell Biol.* **5**(4):282-295.

Ladhani, S., Joannou, C. L., Lochrie, D. P., Evans, R. W. and Poston, S. M. (1999). Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded-skin syndrome. *Clin. Microbiol. Rev.* **12**(2):224-242.

Lange, S. J. and Que, L. Jr. (1998). Oxygen activating nonheme iron enzymes. *Curr. Opin. Chem. Biol.* **2**(2):159-172.

Ledala, N., Pearson, S. L., Wilkinson, B. J. and Jayaswal, R. K. (2007). Molecular characterization of the Fur protein of *Listeria monocytogenes*. *Microbiology* **153**(Pt. 4):1103-1111.

Lee, S. M., Grass, G., Rensing, C., Barrett, S. R., Yates, C. J., Stoyanov, J. V. and Brown, N. L. (2002). The Pco proteins are involved in periplasmic copper handling in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **295**(3):616-620.

Lee, J. H., Wang, T., Ault, K., Liu, J., Schmitt, M. P. and Holmes, R. K. (1997). Identification and characterization of three new promoter/operators from *Corynebacterium diphtheriae* that are regulated by the diphtheria toxin repressor (DtxR) and iron. *Infect. Immun.* **65**(10):4273-4280.

Lentino, J. R. (2003). Prosthetic joint infections: bane of orthopedists, challenge for infectious disease specialists. *Clin. Infect. Dis.* **36**(9):1157-1161.

Lewinson, O., Lee, A. T. and Rees, D. C. (2009). A P-type ATPase importer that discriminates between essential and toxic transition metals. *Proc. Natl. Acad. Sci. U.S.A.* **106**(12):4677-4682.

Li, J. and Fitzpatrick, P. F. (2008). Characterization of metal ligand mutants of phenylalanine hydroxylase: Insights into the plasticity of a 2-histidine-1-carboxylate triad. *Arch. Biochem. Biophys.* **475**(2):164-168.

Li, L., Vulpe, C. D. and Kaplan, J. (2003). Functional studies of hephaestin in yeast: evidence for multicopper oxidase activity in the endocytic pathway. *Biochem J.* **375**(Pt. 3):793-798.

Lim, C. K. and Cooksey, D. A. (1993). Characterization of chromosomal homologs of the plasmid-borne copper resistance operon of *Pseudomonas syringae*. *J. Bacteriol.* **175**(14):4492-4498.

Lindsay, J. (2008). *Staphylococcus*: Molecular Genetics. Caister Academic Press.

Lindsay, J. A. and Foster, S. J. (2001). *zur*: a Zn^{2+}-responsive regulatory element of *Staphylococcus aureus*. *Microbiology* **147**(Pt 5):1259-1266.

Lindsay J. A. and Holden T. G. (2004). *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol.* **12**(8):378-385.

Lindsay, J. A. and Holden, M. T. (2006). Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Funct. Integr*. Genomics **6**(3):186-201.

Lindsay, J. A. and Riley, T. V. (1994). Staphylococcal iron requirements, siderophore production, and iron-regulated protein expression. *Infect. Immun.* **62**(6):2309-2314.

Lindsay, J. A., Ruzin, A., Ross, H. F., Kurepina, N. and Novick, R. P. (1998). The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Mol. Microbiol.* **29**(2):527-543.

Linder, M. C. and Hazegh-Azam, M. (1996). Copper biochemistry and molecular biology. *Am. J. Clin. Nutr*. **63**(5):797S-811S.

Lindqvist, A., Membrillo-Hernańdez, J., Poole, R. K. and Cook, G. M. (2000). Roles of respiratory oxidases in protecting *Escherichia coli* K12 from oxidative stress. *Antonie Van Leeuwenhoek* **78**(1):23-31.

Litwin, C. M. and Calderwood, S. B. (1993). Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.* **6**(2):137-149.

Liu, T., Ramesh, A., Ma, Z., Ward, S. K., Zhang, L., George, G. N., Talaat, A. M., Sacchettini, J. C. and Giedroc, D. P. (2007). CsoR is a novel *Mycobacterium tuberculosis* copper-sensing transcriptional regulator. *Nat. Chem. Biol.* **3**(1):60-68.

Lowy, F. D. (1998). *Staphylococcus aureus* infections. *N. Engl. J. Med.* **339**(8):520-532.

Lutkenhaus, J. F. (1977). Role of a major outer membrane protein in *Escherichia coli*. *J. Bacteriol.* **131**(2):631-637.

Macomber, L. and Imlay, J. A. (2009). The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc. Natl. Acad. Sci. U.S.A.* **106**(20):8344-8349.

Macomber, L., Rensing. C. and Imlay, J. A. (2007). Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli. J. Bacteriol.* **189**(5):1616-1626.

MacPherson, I. S. and Murphy, M. E. (2007). Type-2 copper-containing enzymes. *Cell. Mol. Life Sci.* **64**(22):2887-2899.

Maddocks, S. E. and Oyston, P. C. (2008). Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology* **154**(Pt 12):3609-3623.

Magnani, D. and Solioz, M. (2005). Copper chaperone cycling and degradation in the regulation of the *cop* operon of *Enterococcus hirae*. *Biometals* **18**(4):407-412.

Maira-Litrán, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G. 3rd, Goldmann, D. A. and Pier, G. B. (2002). Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infect. Immun.* **70**(8):4433-4440.

Maltezou, H. C. and Giamarellou, H. (2006). Community-acquired methicillin-resistant *Staphylococcus aureus* infections. *Int. J. Antimicrob. Agents* **27**(2):87-96.

Maresso, A. W. and Schneewind, O. (2006). Iron acquisition and transport in *Staphylococcus aureus*. *Biometals* **19**(2):193-203.

Martins, A. and Cunha, M. de L. (2007). Methicillin resistance in *Staphylococcus aureus* and coagulase-negative staphylococci: epidemiological and molecular aspects. *Microbiol. Immunol.* **51**(9):787-795.

Massé, E. and Gottesman, S. (2002). A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **99**(7):4620-4625.

Massé, E., Vanderpool, C. K. and Gottesman, S. (2005). Effect of RyhB small RNA on global iron use in *Escherichia coli*. *J. Bacteriol.* **187**(20):6962-6971.

Mazmanian, S. K., Ton-That, H., Su, K. and Scheewind, O. (2002). An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis. *PNAS* **99**(4): 2293-2298.

Meiwes, J., Fiedler, H. P., Haag, H., Zähner, H., Konetschny-Rapp, S. and Jung, G. (1990). Isolation and characterization of staphyloferrin A, a compound with siderophore activity from *Staphylococcus hyicus* DSM 20459. *FEMS Microbiol. Lett.* **55**(1-2):201-205.

Metruccio, M. M., Fantappiè, L., Serruto, D., Muzzi, A., Roncarati, D., Donati, C., Scarlato, V. and Delany, I. (2009). The Hfq-dependent small noncoding RNA NrrF directly mediates Fur-dependent positive regulation of succinate dehydrogenase in *Neisseria meningitidis*. *J. Bacteriol.* **191**(4):1330-1342.

McAleese, F., Wu, S. W., Sieradzki, K., Dunman, P., Murphy, E., Projan, S. and Tomasz, A. (2006). Overexpression of genes of the cell wall stimulon in clinical isolates of *Staphylococcus aureus* exhibiting vancomycin-intermediate- *S. aureus*-type resistance to vancomycin. *J. Bacteriol* **188**(3):1120-1133.

Mills, S. D., Jasalavich, C. A. and Cooksey, D. A. (1993). A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. *J. Bacteriol.* **175**(6):1656-1664.

Mills, S. D., Lim, C. K. and Cooksey, D. A. (1994). Purification and characterization of CopR, a transcriptional activator protein that binds to a conserved domain (*cop* box) in copper-inducible promoters of *Pseudomonas syringae*. *Mol. Gen. Genet.* **244**(4):341-351.

Modun, B. J., Cockayne, A., Finch, R. and Williams, P. (1998). The *Staphylococcus aureus* and *Staphylococcus epidermidis* transferrin-binding proteins are expressed *in vivo* during infection. *Microbiology* **144**(Pt. 4):1005-1012.

Modun, B., Kendall, D. and Williams, P. (1994). Staphylococci express a receptor for human transferrin: identification of a 42-kilodalton cell wall transferrin-binding protein. *Infect. Immun.* **62**(9):3850-3858.

Modun, B., Morrissey, J. and Williams, P. (2000). The staphylococcal transferrin receptor: a glycolytic enzyme with novel functions. *Trends Microbiol.* **8**(5):231-237.

Modun, B. and Williams, P. (1999). The staphylococcal transferrin-binding protein is a cell wall glyceraldehydes-3-phosphate dehydrogenise. *Infect. Immun.* **67**(3):1086-1092.

Moore, P. C. and Lindsay, J. A. (2002). Molecular characterisation of the dominant UK methicillin-resistant *Staphylococcus aureus* strains, EMRSA-15 and EMRSA-16. *J. Med. Microbiol.* **51**(6):516-521.

Morikawa, K., Ohniwa, R. L., Kim, J., Maruyama, A., Ohta, T. and Takeyasu, K. (2006). Bacterial nucleoid dynamics: oxidative stress response in *Staphylococcus aureus*. *Genes Cells* **11**(4):409-423.

Morozova, O. V., Shumakovich, G. P., Gorbacheva, M. A., Shleev, S. V and Yaropolov, A. I. (2007). "Blue" laccases. *Biochemistry (Mosc.)* **72**(1):1136-1150.

Morrissey, J. A., Cockayne, A., Brummell, K., and Williams, P. (2004). The staphylococcal ferritins are differentially regulated in response to iron and manganese and via PerR and Fur. *Infect. Immun.* **72**(2):972-979.

Morrissey, J. A., Cockayne, A., Hammacott, J., Bishop, K., Denman-Johnson, A.. Hill, P. J. and Williams, P. (2002). Conservation, surface exposure, and *in vivo* expression of the Frp family of iron regulated cell wall proteins in *Staphylococcus aureus. Infect. Immun.* **70**(5):2399-2407.

Morrissey, J. A., Cockayne, A., Hill, P. J., and Williams, P. (2000). Molecular cloning and analysis of a putative siderophore ABC Transporter from *Staphylococcus aureus. Infect. Immun.* **68**(11):6281-6288.

Munson, G. P., Lam, D. L., Outten, F. W. and O'Halloran, T. V. (2000). Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. *J. Bacteriol.* **182**(20):5864-5871.

Muraoka, S., Okumura, R., Ogawa, N., Nonaka, T., Miyashita, K. and Senda, T. (2003). Crystal structure of a full-length LysR-type transcriptional regulator, CbnR: unusual combination of two subunit forms and molecular bases for causing and changing DNA bend. *J. Mol. Microbiol. Biotechnol.* **328**(3):555-566.

Murphy, E., Huwyler, L., de Freire Bastos Mdo C. (1985). Transposon Tn554: complete nucleotide sequence and isolation of transposition-defective and antibiotic-sensitive mutants. *EMBO J.* **4**(12):3357-3365.

Muryoi, N., Tiedemann, M. T., Pluym, M., Cheung, J., Heinrichs, D. E. and Stillman, M. J. (2008). Demonstration of the iron-regulated surface determinant (Isd) heme transfer pathway in *Staphylococcus aureus*. *J. Biol. Chem.* **283**(42):28125-28136.

Nadadur, S. S., Srirama, K. and Mudipalli, A. (2008). Iron transport & homeostasis mechanisms: their role in health & disease. *Indian J. Med. Res.* **128**(4):533-544.

NCBI website: http://www.ncbi.nlm.nih.gov/

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., Ganz, T. and Kaplan, J. (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306**(5704):2090-2093.

Nemeth, E., Valore, E. V., Territo, M., Schiller, G., Lichtenstein, A. and Ganz, T. (2003). Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* **101**(7):2461-2463.

Newman, D. L. and Shapiro, J. A. (1999). Differential *fiu-lacZ* fusion regulation linked to *Escherichia coli* colony development. *Mol. Microbiol.* **33**(1):18-32.

Nicholls, D. G. (2002). Mitochondrial function and dysfunction in the cell: its relevance to aging and aging-related disease. *Int. J. Biochem. Cell Biol.* **34**(11):1372-1381.

Nishiyama, Y., Massey, V., Takeda, K., Kawasaki, S., Sato, J., Watanabe, T. and Niimura, Y. (2001). Hydrogen peroxide-forming NADH oxidase belonging to the peroxiredoxin oxidoreductase family: existence and physiological role in bacteria. *J. Bacteriol.* **183**(8):2431-2438.

Noyce, J. O., Michels, H. and Keevil, C. W. (2006). Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant *Staphylococcus aureus* in the healthcare environment. *J. Hosp. Infect.* **63**(3):289-297.

Oakhill, J. S., Sutton, B. J., Gorringe, A. R. and Evans, R. W. (2005). Homology modelling of transferrin-binding protein A from *Neisseria meningitidis*. *Protein Eng. Des. Sel.* **18**(5):221-228.

Occhino, D. A., Wyckoff, E. E., Henderson, D. P., Wrona, T. J. and Payne, S. M. (1998). *Vibrio cholerae* iron transport: haem transport genes are linked to one of two sets of *tonB, exbB, exbD* genes. *Mol. Microbiol.* **29**(6):1493-1507.

Oda, M., Sugishita, A. and Furukawa, K. (1988). Cloning and nucleotide sequences of histidase and regulatory genes in *the Bacillus subtilis hut* operon and positive regulation of the operon. *J. Bacteriol.* **170**(7):3199-3205.

Odermatt, A. and Solioz, M. (1995). Two trans-acting metalloregulatory proteins controlling expression of the copper-ATPases of *Enterococcus hirae*. *J. Biol. Chem.* **270**(9):4349-4354.

Odermatt, A., Suter, H., Krapf, R. and Solioz, M. (1993). Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. *J. Biol. Chem.* **268**(17):12775-12779.

Ohsumi, Y., Kitamoto, K. and Anraku, Y. (1988). Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion. *J. Bacteriol.* **170**(6):2676-2682.

Okuma, K., Iwakawa, K., Turnidge, J. D., Grubb, W. B., Bell, J. M., O'Brien, F. G., Coombs, G. W., Pearman, J. W., Tenover, F. C., Kapi, M., Tiensasitorn, C., Ito, T. and Hiramatsu, K. (2002). Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *J. Clin. Microbiol.* **40**(11):4289-4294.

O'Riordan, K. and Lee, J. C. (2004). *Staphylococcus aureus* capsular polysaccharides. *Clin. Microbiol. Rev.* **17**(1):218-34.

Outten, F. W., Outten, C. E., Hale, J. and O'Halloran, T. V. (2000). Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, CueR. *J. Biol. Chem.* **275**(40):31024-31029.

Outten, F. W., Huffman, D. L., Hale, J. A. and O'Halloran, T. V. (2001). The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J. Biol. Chem.* **276**(33):30670-30677.

Patti, J. M., Bremell, T., Krajewska-Pietrasik, D., Abdelnour, A., Tarkowski, A., Rydén, C. and Höök, M. (1994). The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. *Infect. Immun.* **62**(1):152-161.

Palmgren, M. G and Axelsen, K. B. (1998). Evolution of P-type ATPases. *Biochim. Biophys. Acta.* **1365**(1-2):37-45.

Palmqvist, N., Foster, T., Tarkowski, A. and Josefsson, E. (2002). Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. *Microb. Pathog.* **33**(5):239-249.

Palmqvist, N., Patti, J. M., Tarkowski, A. and Josefsson, E. (2004). Expression of staphylococcal clumping factor A impedes macrophage phagocytosis. *Microbes Infect.* **6**(2):188-195.

Park, S. and Imlay, J. A. (2003). High levels of intracellular cysteine promote oxidative DNA damage by driving the fenton reaction. *J. Bacteriol.* **185**(6):1942-1950.

Peacock, S. J., de Silva, I. and Lowy, F. D. (2001). What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiol.* **9**(12):605-610.

Pecqueur, L., D'Autréaux, B., Dupuy, J., Nicolet, Y., Jacquamet, L., Brutscher, B., Michaud-Soret, I. and Bersch, B. (2006). Structural changes of *Escherichia coli* ferric uptake regulator during metal-dependent dimerization and activation explored by NMR and X-ray crystallography. *J. Biol. Chem.* **281**(30):21286-21295.

Pena, M. M., Puig, S. and Thiele, D. J. (2000). Characterization of the *Saccharomyces cerevisiae* high affinity copper transporter Ctr3. *J. Biol. Chem.* **275**(43):33244-33251.

Pettersson, A., Prinz, T., Umar, A., van der Biezen, J. and Tommassen, J. (1998). Molecular characterization of LbpB, the second lactoferrin-binding protein of *Neisseria meningitidis*. *Mol. Microbiol.* **27**(3):599-610.

Pichon, C. and Felden, B. (2005). Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. *Proc. Natl. Acad. Sci. U.S.A.* **102**(40):14249-14254.

Piddington, D. L., **Fang, F. C.**, **Laessig, T.**, **Cooper, A. M.**, **Orme, I. M.** and **Buchmeier, N. A. (2001).** Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. *Infect. Immun.* **69**(8):4980-4987.

Pohl, E., Holmes, R. K. and Hol, W. G. (1999). Crystal structure of the iron-dependent regulator (IdeR) from *Mycobacterium tuberculosis* shows both metal binding sites fully occupied. *J. Mol. Biol.* **285**(3):1145-1156.

Pontel, L. B. and Soncini, F. C. (2009). Alternative periplasmic copper-resistance mechanisms in Gram negative bacteria. *Mol. Microbiol.* **73**(2):212-225.

Porrúa, O., García-Jaramillo, M., Santero, E. and Govantes, F. (2007). The LysR-type regulator AtzR binding site: DNA sequences involved in activation, repression and cyanuric acid-dependent repositioning. *Mol. Microbiol.* **66**(2):410-427.

Portmann, R., Magnani, D., Stoyanov, J. V., Schmechel, A., Multhaup, G. and Solioz, M. (2004). Interaction kinetics of the copper-responsive CopY repressor with the *cop* promoter of *Enterococcus hirae*. *J. Biol. Inorg. Chem.* **9**(4):396-402.

Pozdnyakova, I., Guidry, J. and Wittung-Stafshede, P. (2001). Copper stabilizes azurin by decreasing the unfolding rate. *Arch. Biochem. Biophys.* **390**(1):146-148.

Prince, R. C. and George, G. N. (1995). Cytochrome *f* revealed. *Trends Biochem. Sci.* **20**(6):217-218.

Rapisarda, V. A., Chehín, R. N., De Las Rivas, J., Rodríguez-Montelongo, L., Farías, R. N. and Massa, E. M. (2002). Evidence for Cu(I)-thiolate ligation and prediction of a putative copper-binding site in the *Escherichia coli* NADH dehydrogenase-2. *Arch. Biochem. Biophys.* **405**(1):87-94.

Rapisarda, V. A., Montelongo, L. R., Farías, R. N. and Massa, E. M. (1999). Characterization of an NADH-linked cupric reductase activity from the *Escherichia coli* respiratory chain. *Arch. Biochem. Biophys.* **370**(2):143-150.

Rea, R. B., Gahan, C. G. and Hill, C. (2004). Disruption of putative regulatory loci in *Listeria monocytogenes* demonstrates a significant role for Fur and PerR in virulence. *Infect. Immun.* **72**(2):717-727.

Renauld-Mongénie, G., Latour, M., Poncet, D., Naville, S. and Quentin-Millet, M. J. (1998). Both the full-length and the N-terminal domain of the meningococcal transferrin-binding protein B discriminate between human iron-loaded and apo-transferrin. *FEMS Microbiol. Lett.* **169**(1):171-177.

Rensing, C., Fan, B., Sharma, R., Mitra, B, and Rosen, B. P. (2000). CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. *Proc. Natl. Acad. Sci. U.S.A.* **97**(2):652-656.

Rensing, C. and Grass, G. (2003). *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *FEMS Microbial. Rev.* **27**(2-3):197-213.

Rohde, H., Burandt, E. C., Siemssen, N., Frommelt, L., Burdelski, C., Wurster, S., Scherpe, S., Davies, A. P., Harris, L. G., Horstkotte, M. A., Knobloch, J. K., Ragunath, C., Kaplan, J. B. and Mack D. (2007). Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials* **28**(9):1711-1720.

Rodríguez-Montelongo, L., Volentini, S. I., Farías, R. N., Massa, E. M. and Rapisarda, V. A. (2006). The Cu(II)-reductase NADH dehydrogenase-2 of *Escherichia coli* improves the bacterial growth in extreme copper concentrations and increases the resistance to the damage caused by copper and hydroperoxide. *Arch. Biochem. Biophys.* **451**(1):1-7.

Rohrbach, M. R., Braun, V. and Köster, W. (1995). Ferrichrome transport in *Escherichia coli* K-12: altered substrate specificity of mutated periplasmic FhuD and interaction of FhuD with the integral membrane protein FhuB. *J. Bacteriol.* **177**(24):7186-7193.

Roulhac, P. L., Weaver, K. D., Adhikari, P., Anderson, D. S., DeArmond, P. D., Mietzner, T. A., Crumbliss, A. L. and Fitzgerald, M. C. (2008). Ex vivo analysis of synergistic anion binding to FbpA in Gram-negative bacteria. *Biochemistry* **47**(14):4298-4305.

RSAT data base: http://rsat.ulb.ac.be/rsat/

Ryu, S. Y., Jeong, K. S., Kang, B. N., Park, S. J., Yoon, W. K., Kim, S. H. and Kim, T. H. (2000). Modulation of transferrin synthesis, transferrin receptor expression, iNOS expression and NO production in mouse macrophages by cytokines, either alone or in combination. *Anticancer Res.* **20**(5A):3331-3338.

Saïd-Salim, B., Dunman, P. M., McAleese, F. M., Macapagal, D., Murphy, E., McNamara, P. J., Arvidson, S., Foster, T. J., Projan, S. J. and Kreiswirth, B. N. (2003). Global regulation of *Staphylococcus aureus* genes by Rot. *J. Bacteriol.* 185(2):610-619.

Sakurai, T. and Kataoka, K. (2007). Basic and applied features of multicopper oxidases, CueO, bilirubin oxidase, and laccase. *Chem. Rec.* **7**(4):220-229.

Schaible, U. E. and Kaufmann, S. H. (2004). Iron and microbial infection. *Nat. Rev. Microbiol.* **2**(12):946-953.

Sansone, A., Watson, P. R., Wallis, T. S., Langford, P. R. and Kroll, J. S. (2002). The role of two periplasmic copper- and zinc-cofactored superoxide dismutases in the virulence of *Salmonella choleraesuis*. *Microbiology* **148**(Pt. 3):719-726.

Schaffer, A. C. and Lee, J. C. (2009). Staphylococcal vaccines and immunotherapies. *Infect. Dis. Clin. North Am.* **23**(1):153-171.

Schell, M. A. (1993). Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.* **47**:597-626.

Schultz-Hauser, G., Köster, W., Schwarz, H. and Braun, V. (1992). Iron(III) hydroxamate transport in *Escherichia coli* K-12: FhuB-mediated membrane association of the FhuC protein and negative complementation of *fhuC* mutants. *J. Bacteriol.* **174**(7):2305-2311.

Schmitt, M. E., **Brown, T. A. and Trumpower, B. L. (1990).** A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic acids res.* **18**(10):3091-3092.

Schmitt, M. P., Talley, B. G. and Holmes, R. K. (1997). Characterization of lipoprotein IRP1 from *Corynebacterium diphtheriae*, which is regulated by the diphtheria toxin repressor (DtxR) and iron. *Infect. Immun.* **65**(12):5364-5367.

Schwacha, A. and Bender, R. A. (1990). Nucleotide sequence of the gene encoding the repressor for the histidine utilization genes of *Klebsiella aerogenes*. *J. Bacteriol.* **172**(9):5477-5481.

Searle, A. J. and Willson, R. L. (1983). Stimulation of microsomal lipid peroxidation by iron and cysteine. Characterization and the role of free radicals. *Biochem. J.* **212**(3):549-554.

Sebulsky, M. T. and Heinrichs, D. E. (2001). Identification and characterization of *fhuD1* and *fhuD2*, two genes involved in iron-hydroxamate uptake in *Staphylococcus aureus*. *J. Bacteriol.* **183**(17):4994-5000.

Shigematsu, T., Fukushima, J., Oyama, M., Tsuda, M., Kawamoto, S. and Okuda, K. (2001). Iron-Mediated regulation of alkaline proteinase production in *Pseudomonas aeruginosa*. *Microbiol. Immunol.* **45**(8):579-590.

Sievert, D. M., Rudrik, J. T., Patel, J. B., McDonald, L. C., Wilkins, M. J. and Hageman, J. C. (2008). Vancomycin-resistant *Staphylococcus aureus* in the United States, 2002-2006. *Clin. Infect. Dis.* **46**(5):668-674.

Simpsonv, W., Olczak, T. and Genco, C. A. (2004). Lysine-specific gingipain K and heme/hemoglobin receptor HmuR are involved in heme utilization in *Porphyromonas gingivalis*. *Acta Biochim. Pol.* **51**(1):253-262.

Singh, S. K., Grass, G., Rensing, C. and Montfort, W. R. (2004). Cuprous oxidase activity of CueO from *Escherichia coli*. *J. Bacteriol.* **186**(22):7815-7817.

Sipe, D. M. and Murphy, R. F. (1991). Binding to cellular receptors results in increased iron release from transferrin at mildly acidic pH. *J. Biol. Chem.* **266**(13):8002-8007.

Sitthisak, S., Howieson, K., Amezola, C. and Jayaswal, R. K. (2005). Characterization of a multicopper oxidase gene from *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **71**(9):5650-5653.

Sitthisak, S., Knutsson, L., Webb, J. W. and Jayaswal, R. K. (2007). Molecular characterization of the copper transport system in *Staphylococcus aureus. Microbiology* **153**(Pt. 12):4274-4283.

Skaar, E. P., Gaspar, A. H. and Schneewind, O. (2004). IsdG and IsdI, heme-degrading enzymes in the cytoplasm of *Staphylococcus aureus*. *J. Biol. Chem.* **279**(1):436-443.

Smaldone, G. T. and Helmann, J. D. (2007). CsoR regulates the copper efflux operon *copZA* in *Bacillus subtilis*. *Microbiology* **153**(Pt. 12):4123-4128.

Smith, G. R. and Magasanik, B. (1971). Nature and self-regulated synthesis of the repressor of the *hut* operons in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. U.S.A.* **68**(7):1493-1497.

Smulevich, G., Jakopitsch, C., Droghetti, E. and Obinger, C. (2006). Probing the structure and bifunctionality of catalase-peroxidase (KatG). *J. Inorg. Biochem.* **100**(4):568-585.

Solioz, M. and Odermatt, A. (1995). Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. *J. Biol. Chem.* **270**(16):9217-9221.

Solioz, M. and Stoyanov, J. V. (2003). Copper homeostasis in *Enterococcus hirae. FEMS Microbiol. Rev.* **27**(2-3):183-195.

Speziali, C. D., Dale, S. E., Henderson, J. A., Vinés, E. D. and Heinrichs, D. E. (2006). Requirement of *Staphylococcus aureus* ATP-binding cassette-ATPase FhuC for iron-restricted growth and evidence that it functions with more than one iron transporter. *J. Bacteriol.* **188**(6):2048-2055.

Spizzo, T., Byersdorfer, C., Duesterhoeft, S. and Eide, D. (1997). The yeast FET5 gene encodes a FET3-related multicopper oxidase implicated in iron transport. *Mol. Gen. Genet.* **256**(5):547-556.

Stauff, D. L., Bagaley, D., Torres, V. J., Joyce, R., Anderson, K. L., Kuechenmeister, L., Dunman, P. M. and Skaar, E. P. (2008). *Staphylococcus aureus* HrtA is an ATPase required for protection against heme toxicity and prevention of a transcriptional heme stress response. *J. Bacteriol.* **190**(10):3588-3596.

Stapleton, P. D. and Taylor, P. W. (2005). Methicillin resistance in *Staphylococcus aureus*: mechanisms and modulation. *Sci. Prog.* 85(Pt 1):57-72.

Stern, B. R., Solioz, M., Krewski, D., Aggett, P., Aw, T. C., Baker, S., Crump, K., Dourson, M., Haber, L., Hertzberg, R., Keen, C., Meek, B., Rudenko, L., Schoeny, R., Slob, W. and Starr, T. (2007). Copper and human health: biochemistry, genetics, and strategies for modeling dose-response relationships. *J. Toxicol. Environ. Health. B Crit. Rev.* **10**(3):157-222.

Stojiljkovic, I. and Hantke, K. (1995). Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). *Mol. Gen. Genet.* **247**(2):199-205.

Stoyanov, J. V., Hobman, J. L. and Brown, N. L. (2001). CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. *Mol. Microbiol.* **39**(2):502-511.

Strausak, D. and Solioz, M. (1997). CopY is a copper-inducible repressor of the *Enterococcus hirae* copper ATPases. *J. Biol. Chem.* **272**(14):8932-8936.

Taylor, J. M. and Heinrichs D. E. (2002). Transferrin binding in *Staphylococcus aureus*: involvement of a cell wall-anchored protein. *Mol. Microbiol.* **43**(6):1603-1614.

Teitzel, G. M., Geddie, A., De Long, S. K., Kirisits, M. J., Whiteley, M. and Parsek, M. R. (2006). Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *J. Bacteriol*. **188**(20):7242-7256.

Tegmark, K., Karlsson, A. and Arvidson, S. (2000). Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. *Mol. Microbiol.* **37**(2):398-409.

Tetaz, T. J and Luke, R. K. (1983). Plasmid-controlled resistance to copper in *Escherichia coli*. *J. Bacteriol.* **154**(3):1263-1268.

Thieme, D. and Grass, G. (2009). The Dps protein of *Escherichia coli* is involved in copper homeostasis. *Microbiol. Res.* [Epub ahead of print].

Tiburzi, F., Imperi, F. and Visca, P. (2008). Intracellular levels and activity of PvdS, the major iron starvation sigma factor of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **67**(1):213-227.

Toledo-Arana A, Merino N, Vergara-Irigaray M, Débarbouillé M, Penadés JR, Lasa I. (2005). *Staphylococcus aureus* develops an alternative, *ica*-independent biofilm in the absence of the *arlRS* two-component system. *J. Bacteriol.* **187**(15):5318-5329.

Torres, A. G. and Payne, S. M. (1997). Haem iron-transport system in enterohaemorrhagic *Escherichia coli* O157:H7. *Mol. Microbiol.* **23**(4):825-833.

Torres, V. J., Pishchany, G., Humayun, M., Schneewind, O., Skaar, E. P. (2006). *Staphylococcus aureus* IsdB is a hemoglobin receptor required for heme iron utilization. *J. Bacteriol.* **188**(24):8421-8429.

Touati, D. (2000). Iron and oxidative stress in bacteria. *Arch. Biochem. Biophys*. **373**(1):1-6.

Tree, J. J., Stephen, P. K., Jennings, M. P. and McEwan, A. G. (2005). Copper sensitivity of *cueO* mutants of *Escherichia coli* K-12 and the biochemical suppression of this phenotype. *Biochem. Biophys. Res. Commun.* **328**(4):1205-1210.

Tree, J. J., Ulett, G. C., Ong, C. L., Trott, D. J., McEwan, A. G. and Schembri, M. A. (2008). Trade-off between iron uptake and protection against oxidative stress: deletion of *cueO* promotes uropathogenic *Escherichia coli* virulence in a mouse model of urinary tract infection. *J. Bacteriol.* **190**(20):6909-6912.

Tuazon, C. U. (1984). Skin and skin structure infections in the patient at risk: carrier state of *Staphylococcus aureus*. *Am. J. Med.* **76**(5A):166-171.

Udo, E.E., Jacob, L.E. and Mathew, B. (2000). A cadmium resistance plasmid, pXU5, in *Staphylococcus aureus*, strain ATCC25923. *FEMS Microbiol. Lett.* **189**(1):79-80.

Uğur, A. and Ceylan, Ö. (2003). Occurrence of resistance to antibiotics, metal and plasmids in clinical strains of *Staphylococcus* spp. *Arch. Med. Res.* **34**(2):130-136.

Valderas, M. W. and Hart, M. E. (2001). Identification and characterization of a second superoxide dismutase gene (*sodM*) from *Staphylococcus aureus*. *J. Bacteriol.* **183**(11**)**:3399-3407.

Vaz-Moreira, I., Silva, M. E., Manaia, C. M. and Nunes, O.C. (2008). Diversity of bacterial isolates from commercial and homemade composts. *Microb. Ecol.* **55**(4):714-722.

Velayudhan, J., Castor, M., Richardson, A., Main-Hester, K. L. and Fang, F. C. (2007). The role of ferritins in the physiology of *Salmonella enterica* sv. Typhimurium: a unique role for ferritin B in iron-sulphur cluster repair and virulence. *Mol. Microbiol.* **63**(5):1495-1507.

Vermeiren, C. L., Pluym, M., Mack, J., Heinrichs, D. E., Stillman, M. J. (2006). Characterization of the heme binding properties of *Staphylococcus aureus* IsdA. *Biochemistry* **45**(42):12867-12875.

Vyoral, D. and Petrák, J. (2005). Hepcidin: a direct link between iron metabolism and immunity. *Int. J. Biochem. Cell Biol.* **37**(9):1768-1773.

Waidner, B., Melchers, K., Ivanov, I., Loferer, H., Bensch, K. W., Kist, M. and Bereswill, S. (2002). Identification by RNA profiling and mutational analysis of the novel copper resistance determinants CrdA (HP1326), CrdB (HP1327), and CzcB (HP1328) in *Helicobacter pylori*. *J. Bacteriol.* **184**(23):6700-6708.

Waidner, B., Melchers, K., Stähler, F. N., Kist, M. and Bereswill, S. (2005). The *Helicobacter pylori* CrdRS two-component regulation system (HP1364/HP1365) is required for copper-mediated induction of the copper resistance determinant CrdA. *J. Bacteriol*. **187**(13):4683-4688.

Wang, T. P., Quintanar, L., Severance, S., Solomon, E. I. and Kosman, D. J. (2003). Targeted suppression of the ferroxidase and iron trafficking activities of the multicopper oxidase Fet3p from *Saccharomyces cerevisiae*. *J. Biol. Inorg. Chem.* **8**(6):611-620.

Weaver, L., Michels, H. T. and Keevil, C. W. (2008). Survival of *Clostridium difficile* on copper and steel: futuristic options for hospital hygiene. *J. Hosp. Infect.* **68**(2):145-151.

Weems, J. J. Jr. (2001). The many faces of *Staphylococcus aureus* infection. Recognizing and managing its life-threatening manifestations. *Postgrad. Med.* **110**(4):24-6, 29-31, 35-6.

Weeratunga, S. K., Gee, C. E., Lovell, S., Zeng, Y., Woodin, C. L. and Rivera, M. (2009). Binding of *Pseudomonas aeruginosa* apo-bacterioferritin associated ferredoxin to bacterioferritin B promotes heme mediation of electron delivery and mobilization of core iron. *Biochemistry* **48**(31):7420-7431.

Weigel, L. M., Clewell, D. B., Gill, S. R., Clark, N. C., McDougal, L. K., Flannagan, S. E., Kolonay, J. F., Shetty, J., Killgore, G. E. and Tenover, F. C. (2003). Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* **302**(5650):1569-1571.

Wheeldon, L. J., Worthington, T., Lambert, P. A., Hilton, A.C., Lowden, C. J and Elliott, T. S. (2008). Antimicrobial efficacy of copper surfaces against spores and vegetative cells of *Clostridium difficile*: the germination theory. *J. Antimicrob. Chemother.* **62**(3):522-525.

Winkelmann, G. (2007). Ecology of siderophores with special reference to the fungi. *Biometals* **20**(3-4):379-392.

Woese, C. R. (1987). Bacterial evolution. *Microbiol. Rev.* **51**(2):221-271.

Woodmansee, A. N. and Imlay, J. A. (2002). Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. *J. Biol. Chem.* **277**(37):34055-34066.

Wooldridge, K. G., Morrissey, J. A. and Williams, P. H. (1992). Transport of ferric-aerobactin into the periplasm and cytoplasm of *Escherichia coli* K12: role of envelope-associated proteins and effect of endogenous siderophores. *J. Gen. Microbiol.* **138**(3):597-603.

Wray, L. V. Jr and Fisher, S. H. (1994). Analysis of *Bacillus subtilis hut* operon expression indicates that histidine-dependent induction is mediated primarily by transcriptional antitermination and that amino acid repression is mediated by two mechanisms: regulation of transcription initiation and inhibition of histidine transport. *J. Bacteriol.* **176**(17):5466-5473.

Wren, B. W., Henderson, J. and Ketley, J. M. (1994). A PCR-based strategy for the rapid construction of defined bacterial deletion mutants. *Biotechniques* **16**(6):994-996.

Xiong, A., Singh, V. K., Cabrera, G. and Jayaswal, R. K. (2000). Molecular characterization of the ferric-uptake regulator, *fur*, from *Staphylococcus aureus*. *Microbiology* **146**(Pt.3):659-668.

Yamamoto, K. and Kawanishi, S. (1989). Hydroxyl free radical is not the main active species in site-specific DNA damage induced by copper (II) ion and hydrogen peroxide. *J. Biol. Chem.* **264**(26):15435-15440.

York, J. T., Bar-Nahum, I. and Tolman, W. B. (2008). Copper-Sulfur Complexes Supported by N-Donor Ligands: Towards Models of the Cu_Z Site in Nitrous Oxide Reductase. *Inorganica Chim. Acta* **361**(4):885-893.

Zastawny, T. H., Altman, S. A., Randers-Eichhorn, L., Madurawe, R., Lumpkin, J. A., Dizdaroglu, M. and Rao, G. (1995). DNA base modifications and membrane damage in cultured mammalian cells treated with iron ions. *Free Radic. Biol. Med.* **18**(6):1013-1022.

Ziebandt, A. K., Becher, D., Ohlsen, K., Hacker, J., Hecker, M. and Engelmann, S. (2004). The influence of *agr* and σ^B in growth phase dependent regulation of virulence factors in *Staphylococcus aureus*. *Proteomics* **4**(10):3034-3047.

Zhang, X. X. and Rainey, P. B. (2008). Regulation of copper homeostasis in *Pseudomonas fluorescens* SBW25. *Environ. Microbiol.* **10**(12):3284-3294.

Zhao, G., Ceci, P., Ilari, A., Giangiacomo, L., Laue, T. M., Chiancone, E. and Chasteen, N. D. (2002). Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. *J. Biol. Chem.* **277**(31):27689-27696.

Zhu, H., Xie, G., Liu, M., Olson, J. S., Fabian, M., Dooley, D. M. and Lei, B. (2008). Pathway for heme uptake from human methemoglobin by the iron-regulated surface determinants system of *Staphylococcus aureus*. *J. Biol. Chem.* **283**(26):18450-18460.