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Adaptation of *Staphylococcus aureus* to host redox environments

by:

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Summary

The ability of *Staphylococcus aureus* to survive in the harsh environment of the mammalian host is central to its success as a pathogen. Understanding how *S. aureus* does this so successfully is central to improving treatment options. However, previous work has failed to adequately explore one of the key environmental factors *S. aureus* encounters. The redox potential of the environments *S. aureus* dwells in during an infection vary dramatically and this work set out to investigate how *S. aureus* adapts to these changes.

Continuous culture, transcriptomics, metabolomics and a transposon mutant library were utilised to gain a broad picture of how *S. aureus* responds to low redox potential environments. Furthermore, the role of hydrogen sulfide (H_2S) and sulfur metabolism were explored in detail, in order to improve understanding of how *S. aureus* survives in oxidative stress environments.

This work mapped the response of *S. aureus* to a low redox environment and highlighted the importance of redox as a signalling prompt. This work also upheld H_2S as a defence mechanism against oxidative stress and contributed to better understanding H_2S production, H_2S homeostasis and redox homeostasis in *S. aureus*.

These findings contribute to the understanding of how *S. aureus* is able to readily adapt to host environments. They also establish redox as a central consideration when investigating persistent *S. aureus* infections and they additionally contribute to knowledge of the mechanisms involved in *S. aureus* redox homeostasis.

Presentations and publications resulting from this work

CHRISTMAS, B.A.F., KNAFLER, H., MARU. K. & GREEN, J. 2015 (March). Understanding hydrogen sulfide: a potential mediator of antibiotic and oxidative stress resistance in *Staphylococcus aureus*. *Poster presentation at the Society of General Microbiology (SGM) annual conference, Birmingham, United Kingdom.*

CHRISTMAS, B.A.F., KNAFLER, H., MARU. K. & GREEN, J. 2015 (September). Hydrogen sulfide and reductive capacity in *Staphylococcus aureus*. *Oral and poster presentation at the Staphylococcal Great Britain and Ireland (StaphGBI) conference, Reading, United Kingdom.*

CHRISTMAS, B.A.F., KNAFLER, H., ROLFE, M. D. & GREEN, J. 2016 (August). Response of intracellular *Staphylococcus aureus* to the redox environment of host cells. *Oral and poster presentation at the International Symposium on Staphylococci and Staphylococcal Infections (ISSSI), Seoul, South Korea.*

CHRISTMAS, B.A.F., ROLFE, M. D., KNAFLER, H., MARU, K. & GREEN, J. 2017. Adaptation of aerobic cultures of *Staphylococcus aureus* USA300 to reductive stress and implications for resistance to antimicrobials. *Manuscript in preparation.*

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Abbreviations

AIP	Autoinducing peptide
AMP	Antimicrobial peptide
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CA-MRSA	Community acquired – methicillin resistance *Staphylococcus aureus*
CC	Clonal complex
CDM	Chemically defined medium
CFU	Colony forming units
CHIPs	Chemotaxis inhibitory protein of staphylococci
Cm	Chloramphenicol
CoA	CoenzymeA
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside-5'-phosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
Ery	Erythromycin
Fc	Crystallisable fragment domain of immunoglobulin molecules
FLIPr	Formyl peptide receptor-like 1 inhibitors protein
gDNA	Genomic DNA
Gen	Gentamicin
GFP	Green fluorescent protein
GIZ	Growth inhibition zone
GSH	Glutathione
HA-MRSA	Hospital acquired – methicillin resistance *Staphylococcus aureus*
IL	Interleukin
IFN- γ	Interferon gamma
Kan	Kanamycin
LB	Lysogeny broth
Lin	Lincomycin
LMW	Low molecular weight
MHC	Major histocompatibility complex
min	Minutes
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesive matrix molecules
MSSA	Methicillin sensitive *Staphylococcus aureus*
NARSA	Network on Antimicrobial Resistance in *Staphylococcus aureus*
NCBI	National Centre for Biotechnology Information
Neo	Neomycin
NETs	Neutrophil extracellular traps
NTML	Nebraska transposon mutant library
OD_{600}	Optical density at 600nm wavelength

PBS	Phosphate buffered saline
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
psi	pounds per square inch
PSM	Phenol soluble modulin
PVL	Panton-Valentine leukocidin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SCIN	Staphylococcal complement inhibitor
SCVs	Small colony variants
SNPs	Single nucleotide polymorphisms
SSS	Scalded skin syndrome
SSTI	Skin and soft tissue infection
TAE	Tris-acetate EDTA
Tet	Tetracycline
Tn	Transposon
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSP	trimethylsilyl propanoic acid
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin – 1
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRSA	Vancomycin resistant *Staphylococcus aureus*
v/v	Volume for volume
WT	Wildtype
w/v	Weight for volume

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Chapter 1: Introduction

1.1 The Staphylococci

Members of the *Staphylococcus* genus are non-motile, non-spore forming Gram-positive cocci in the phylum Firmicute and the order Bacillales. They have a diameter of around 0.5 – 1.5 µm and divide across different planes producing a clustered appearance (Tzagoloff and Novick, 1977). They were first described in the late 19th century having being isolated from human abscess pus and were ultimately given the name *Staphylococcus* (from the Greek for grape '*staphylos'* and seed or berry '*kokkos')* (Ogston, 1881, Ogston, 1882). Over 40 species or sub species have now been identified (Kloos and Bannerman, 1994).

Staphylococci are catalase-positive and oxidase-negative, key characteristics in distinguishing them from streptococci, and pathogenic strains are commonly identified by their production of coagulase (Chapman et al., 1934). Coagulase-negative Staphylococcal strains such as *Staphylococcus epidermidis* are common human commensals that tend to only cause disease in immunocompromised patients or through medical device-related infections (Otto, 2012). On the other hand, coagulase-positive strains cause more invasive disease and include the pathogenic *Staphylococcus intermedius* and *Staphylococcus hyicus* that cause disease in a range of animals including pigs, horses and pigeons. *Staphylococcus aureus* is also coagulase positive and is the primary human pathogen of the *Staphylococcus* genus. It generally causes disease in humans but can also cause disease in non-human animals including in cows, chickens and pigs; however, zoonosis of livestock associated strains of *S. aureus* into the human population is rare (Shepheard et al., 2013, Smith, 2015).

1.2 *Staphylococcus aureus*

Staphylococcus aureus is a common commensal that colonises the human nose. Around 30% of the adult population are colonised at any one time and this reservoir acts as a source for disease (von Eiff et al., 2001, Graham et al., 2006, Weidenmaier et al., 2012). It gets the name 'aureus' (derived from the Latin for gold '*aurum'*) from the

distinctive golden colour often seen when colonies are grown on solid medium. This pigmentation occasionally earns *S. aureus* the colloquial name 'golden staph' and is caused by the production of carotenoids including staphyloxanthin (Clauditz et al., 2006). The first *S. aureus* whole genome sequence was published in 2001 and it revealed that *S. aureus* has a genome of around 2.8 Mb coding for around 3000 genes (Kuroda et al., 2001).

1.3 Clinical significance of *S. aureus*

Staphylococcus aureus causes a variety of different diseases including superficial skin and soft tissue infections (SSTIs), as well as, more invasive osteomyelitis, endocarditis, necrotising pneumonia and sepsis (Lowy, 1998, Tong et al., 2015). These more severe disease manifestations often result from dissemination via the bloodstream, with bacteraemia mortality rates around 30% (Wyllie et al., 2006, Laupland et al., 2008) and over 10,000 cases reported in the UK each year (PHE, 2016).

Staphylococcus aureus can also cause severe disease through it's production of certain exotoxins: scaled skin syndrome (SSS), toxic shock syndrome (TSS) and severe *S. aureus* enterotoxin related food poisoning are all caused by *S. aureus* produced toxins. These instances of *S. aureus* related disease can induce a sudden onset of severe symptoms and be difficult to treat effectively (Balaban and Rasooly, 2000, Lina et al., 2004, Bukowski et al., 2010).

Staphylococcus aureus also causes infections around the site of implanted medical devices such as catheters, artificial heart valves, prosthetic joints and other orthopaedic implants; although such device-related infections are more commonly caused by *S. epidermidis*, those caused by *S. aureus* are harder to treat (Cunningham et al., 1996, Tong et al., 2015). Biofilm formation plays a crucial role in these device related infections allowing *S. aureus* to attach to surfaces and offering protection from the host immune system and antimicrobials (Archer et al., 2011).

Recurrent *S. aureus* infections are commonly observed, with *S. aureus* able to persist asymptomatically for months or even years following treatment before relapsing to cause a new infection (Greer et al., 1993, Proctor et al., 1995, Kipp et al., 2003). This chronic relapsing disease is most often associated with osteomyelitis but has also been observed during different disease manifestations, particularly SSTIs where around 30-50% of all *S. aureus* SSTIs have a recurrence 6 months after initial treatment (Kim et al., 2012, Fritz et al., 2013, Miller et al., 2015).

Until recently severe *S. aureus* infections were generally restricted to healthcare settings; however, in recent years there has been a shift toward strains able to cause severe disease in formerly healthy patients in the community (Moran et al., 2006, Johnson et al., 2007). This is linked to the rise of certain antibiotic resistant strains known as community acquired methicillin resistant *S. aureus* (CA-MRSA). There are several CA-MRSA lineages found across the world (McDougal et al., 2003, Limbago et al., 2009) with Clonal complex CC80 among the most prevalent in the UK (Elston and Barlow, 2009) and USA300 the most prevalent in the USA (Tenover et al., 2008).

1.4 *Staphylococcus aureus* metabolism and growth requirements

The principal ecological niche of *S. aureus* is the human nose (Miles, 1941, Weidenmaier et al., 2012). Around 30% of the healthy adult population are infected at any one time (Wertheim et al., 2005, Graham et al., 2006) with 20% of the population persistently infected, 30% intermittently infected and 50% non-carriers (Wertheim et al., 2005). This reservoir acts as a major source of opportunistic *S. aureus* infection (von Eiff et al., 2001). *Staphylococcus aureus* is also able to survive in the wide range of environments it encounters over the course of an infection.

1.4.1 Oxygen

In the aerobic environment of the human nose *S. aureus* can respire aerobically but as a facultative anaerobe it is also able to grow in low oxygen environments such as in the centre of an abscess (Park et al., 1992), inside a biofilm (Lone et al., 2015) or inside host bone marrow (Wilde et al., 2015). In these low oxygen environments *S. aureus*

can respire using nitrate and nitrite as terminal electron acceptors or, in their absence, can switch to fermentative metabolism (Burke and Lascelles, 1975, Fuchs et al., 2007). Moreover, oxygen availability has been shown to have important implications for the expression of virulence factors (Yarwood and Schlievert, 2000, Fuchs et al., 2007).

1.4.2 Carbon

As a chemoheterotroph *S. aureus* requires organic carbon in order to maintain it's metabolism and growth. Glucose is the most abundant organic carbon source in human blood (Psychogios et al., 2011) and *S. aureus* has adaptive mechanisms to increase glucose uptake during the course of an infection (Vitko et al., 2016). High concentrations of blood glucose, such as those found in diabetic patients, increase the risk of developing an *S. aureus* infection and decrease the ability to clear it (Pomposelli et al., 1998, Rich and Lee, 2005, Dunyach-Remy et al., 2016). High glucose concentrations result in decreased metabolic carbon use via positive (CcpE) (Hartmann et al., 2013) and negative (CcpA) (Seidl et al., 2009) regulation of the Kreb's cycle and increased expression of virulence factors (Seidl et al., 2006, Ding et al., 2014).

However, there are many niches in which *S. aureus* must grow on a non-preferred carbon source such as when *S. aureus* is in the centre of an abscess contained by host fibrin deposits (Kelly and O'Neill, 2015). In these situations, *S. aureus* can utilise waste products such as lactate or host peptides and free amino acids (Spahich et al., 2016). *Staphylococcus aureus* produces a number of proteases, capable of degrading host proteins (Dubin, 2002), and has many oligopeptide permeases and free amino acid transporters (Yu et al., 2014a). In particular it has been found that glutamate catabolism, particularly resulting from the breakdown of proline, is important for *S. aureus* survival in the absence of glucose, as well as, the utilisation of pyruvate derived from amino acids such as alanine and serine (Halsey et al., 2017).

1.4.3 Nitrogen

Staphylococcus aureus can utilise nitrate or nitrite as electron acceptors (Burke and Lascelles, 1975). Nitrate and nitrate transport and metabolism genes are under the

regulation of the NreABC, with NreC as the response regulator (Kamps et al., 2004). NreC also derepresses the transcription of fermentative metabolism genes such as lactate dehydrogenase (Schlag et al., 2008).

1.4.4 Amino acids

Amino acid transport and biosynthesis are key in facilitating *S. aureus*'s adaptation to different environments and can be determining factors in the intensification or attenuation of an infection (Coulter et al., 1998, Chaffin et al., 2012, Krismer et al., 2014, Valentino et al., 2014). *Staphylococcus aureus* does have the biosynthetic pathways for all amino acids (Kuroda et al., 2001); however, *in vitro* studies have frequently found that various strains of *S. aureus* behave as auxotrophs with proline, arginine, valine and cysteine the most frequently required amino acids (Mah et al., 1967, Taylor and Holland, 1989).

1.4.5 Sulfur

Sulfur compounds are required for the formation of many vital bacterial functions including the production of amino acids and redox homeostasis molecules such as thioredoxin and glutathione, as well as, the formation of any protein containing an iron-sulfur cluster (Zeller and Klug, 2006, Ayala-Castro et al., 2008). Sulfur metabolism has also been linked with bacterial virulence and proposed as a target for novel antimicrobials. This link has been suggested following the observation of: repressed toxin synthesis under high sulfur concentration, the induction of genes related to sulfur metabolism following interaction with host epithelial cells, and the decrease in virulence of sulfur metabolism mutants (Bogdan et al., 2001, Bhave et al., 2007). *Staphylococcus aureus* has also been shown to be able to acquire sulfur from a number of different sulfur sources including cysteine, cystine, glutathione, thiosulfate, sulfite, taurine and homocysteine; however, it does not appear to be able to utilise methionine and sulfate (Lithgow et al., 2004, Soutourina et al., 2009). Utilisation of host derived sulfur sources is under the control of the cysteine master regulator CymR (Section 3.1) (Figure 1.1) (Soutourina et al., 2009).

1.4.6 Metal ions

1.4.6.1 Iron

Iron is crucial for bacterial survival as it is a cofactor in enzymes involved in vital functions such as respiration and DNA biosynthesis (Andrews et al., 2003). In humans iron is at its most concentrated in liver hepatocytes but there are also large quantities stored in red blood cells, in the form of haem, and in macrophages, in the form of ferritin. There are also relatively low concentrations of free iron in the blood, due to the binding of free iron to transferrin, this reduces the ability of bacterial cells to grow in the bloodstream (Hentze et al., 2004). There are also very low concentrations of iron in secreted fluids such as tears, saliva and milk, due to the production of lactoferrin (Schaible and Kaufmann, 2004).

In order for *S. aureus* to acquire iron in these low iron environments it deploys two main mechanisms: siderophore production and heme-iron acquisition. Siderophores are low molecular weight scavenger molecules that can bind iron and remove it from transferrins and then actively transport it back into the bacterial cell (Skaar et al., 2004, Park et al., 2005). The production of siderophores is essential for *S. aureus* survival in host blood and other environments where transferrins are the only source of iron (Dale et al., 2004, Park et al., 2005). In order to access the abundance of iron found inside erythrocytes *S. aureus* first breaks them open through the production of toxins. This releases heme, which is than actively transported into the bacterial cells, and degraded from haem to iron, by the Isd system (Mazmanian et al., 2003)

Staphylococcus aureus iron metabolism is regulated by Fur. In the presence of iron, Fur represses both siderophores and the Isd system (Dale et al., 2004, Cheung et al., 2009, Torres et al., 2010) and increases production of iron storage genes (Fillat, 2014). It also increases production of immunomodulatory proteins such as coagulase and decreases activity of virulence factors such as cytotoxins (Torres et al., 2010).

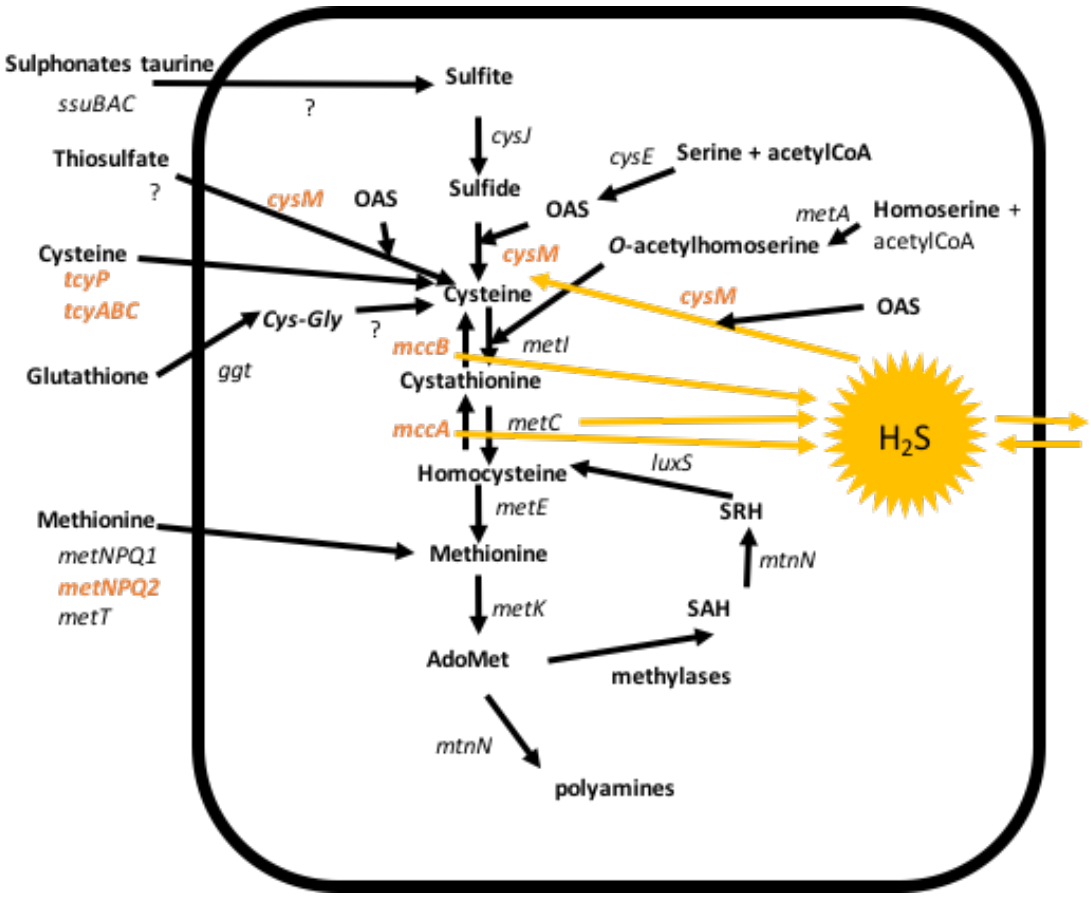


Figure 1.1 Sulfur metabolism in *S. aureus*
Regulation of sulfur metabolism and H_2S production in *S. aureus*. Genes that are targeted by the cysteine master regulator CymR are indicated in bold and orange. The yellow arrows indicate the production, diffusion or utilisation of hydrogen sulfide. Question marks indicate uncertainty. *cysE,* Serine O-acetyltransferase; *cysM,* OAS-thiol-lyase; *ggt,* γ-glutamyltranspeptidase; *metK,* methionine adenosyltransferase; *mtnN,* adenosylhomocysteine nucleosidase; *luxS*, S-ribosylhomocysteine lyase; *mccA*, cystathionine β-synthase; *mccB,* cystathionine γ-lyase; *metA*, homoserine acetyl-transferase; *metI*, cystathionine γ-synthase; *metC*, cystathionine β-lyase; *metE*, methionine synthase. H_2S, hydrogen sulfide; OAS, O-acetyl-serine; AdoMet, S-adenosyl-methionine; *SAH*, S-adenosyl-homocysteine; *SRH*, S-ribosyl-homocysteine. (Section 3.1) (Chang et al., 2006; Wolf et al., 2008 & Soutourina et al., 2009).

1.4.6.2 Copper

Copper has a number of key functions including as a cofactor in superoxide dismutase (Rensing and Grass, 2003) and it is also involved in redox reactions involved in electron transfer and oxygen transport (Linder and HazeghAzam, 1996). Copper is found incorporated as a cofactor in various different enzymes and electron transport proteins and is found in host blood in the form of ceruloplasmin (Linder and HazeghAzam, 1996). There are relatively high levels of copper in the lungs because of copper/zinc superoxide dismutase and because intracellular copper is associated with protein chaperones and chelators. *Staphylococcus aureus* is particularly prone to the toxicity caused by high concentrations of copper that disrupt respiration and damage DNA (Weaver et al., 2010).

1.4.6.3 Manganese

Manganese is another metal iron that is essential for both bacterial and mammalian cells. It is important for amino acid and carbohydrate metabolism and defence against oxidative stress (Jakubovics and Jenkinson, 2001). The two superoxide dismutase SodA and SodM require manganese in order to be able to detoxify superoxide to hydrogen peroxide, that can then be further detoxified to water by catalase (Clements et al., 1999, Valderas and Hart, 2001). *Staphylococcus aureus* acquires manganese through two transporters MntABC and MntH and is regulated by MntR and PerR (Horsburgh et al., 2001, Horsburgh et al., 2002b). Disruption of MntABC, MntH or MntR reduced intracellular survival in endothelial cells (Horsburgh et al., 2002b).

Hosts limit manganese supply by deploying a manganese transporter to phagosomes that actively removes manganese from phagosomes (Jabado et al., 2000), through the production of calprotectin in the cytoplasm and extracellular traps of neutrophils that bind and trap manganese, resulting in a limited supply of manganese particularly in abscesses (Steinbakk et al., 1990, Corbin et al., 2008, Urban et al., 2009, Kehl-Fie et al., 2011). No specific systems for overcoming calprotectin mediated limitation of manganese availability have been identified for *S. aureus* as yet; however, there is a system produced by gram-positive bacterium *Finegoldia magna*, called Protein L, that binds and disables calprotectin (Akerstrom and Bjorck, 2009). Given the ability of *S.*

aureus to grow intracellularly in neutrophils (Kapral and Shayegani, 1959, Gresham et al., 2000, Surewaard et al., 2013) it is possible that *S. aureus* produces some form of anti-calprotectin protein similar to protein L.

1.4.6.4 Zinc

Zinc is also an essential metal component required for bacterial survival; around 5% of the bacterial proteome consists of zinc-binding proteins (Andreini et al., 2006). However, zinc concentrations must be carefully controlled as zinc can have toxic effects through binding of thiols and disrupting their functions. In order to maintain zinc concentrations *S. aureus* has both zinc exporters and zinc importers. ZntA and CadA appear to be zinc exporters, under the control of ZntR (Singh et al., 1999, Nucifora et al., 1989) and MreA and MreB appear to be zinc importers under the control of the Zur regulatory element; however, the role of Zur, MreA and MreB is not clear (Lindsay and Foster, 2001). Calprotectin, produced by neutrophils, also binds zinc reducing its availability in abscesses (Corbin et al., 2008).

1.4.7 Growth conditions

Staphylococcus aureus also has a high level of resistance to many different environmental conditions. For example, it can resist high salt concentrations, it is able to grow in solutions of up to 25% (w/v) NaCl (Parfentjev and Catelli, 1964), and it can grow at temperatures ranging from 7^{o}C to 48.5^{o}C, though it grows optimally at temperatures between 30^{o}C and 37^{o}C (Schmitt et al., 1990). It also has a high resistance to desiccation (Chaibenjawong and Foster, 2011) and can grow in pHs ranging from 4.0 – 10.0, though it grows optimally in pH 6.0 – pH 7.0 (Valero et al., 2009).

1.4.7.1 Redox

Staphylococcus aureus is able to grow in environments ranging widely in their redox potential from the highly oxidative environments of host phagosomes to the reduced environments of anaerobic abscesses or the host cytosol. In order to control its own internal redox potential *S. aureus* utilises low molecular weight (LMW) thiols coenzyme

A (CoA), cysteine and bacillithiol (Perera et al., 2015). Of these both cysteine and CoA are oxidised quickly leaving bacillithiol as the key molecule for maintaining redox homeostasis, fulfilling a similar role to that played by glutathione (GSH) in eukaryotes and Gram-negative bacteria, and mycothiol in Actinobacteria including *Mycobacterium tuberculosis* (Keire et al., 1992, Park and Imlay, 2003, Newton et al., 2008, Newton et al., 2009). However, bacillithiol concentration is around 10 times lower than GSH or mycothiol, suggesting that CoA, cysteine and bacillithiol play a more equal role in maintaining redox homeostasis in *S. aureus* (Perera et al., 2015). Furthermore, some strains including *S. aureus* strain NCTC 8325, and derivatives such as SH1000, do not produce bacillithiol (Newton et al., 2012). Growth is seemingly unaffected in rich medium but these strains are more sensitive to ROS (Pothera et al., 2013, Posada et al., 2014). On the other hand, it has been found that *S. aureus* can import host derived glutathione and that it can use it for oxidative stress resistance or as a source of cysteine (Dayaram et al., 2006, Potter et al., 2012, Pothera et al., 2013).

In order for bacillithiol to be an effective redox buffer it needs a system for reducing oxidised bacillithiol; however, little is currently known about this. It is known that glutathione is returned to a reduced state in eukaryotes and Gram-negative bacteria by glutathione disulfide reductase and that a similar system operates for mycothiol in Actinobacteria (Asnis, 1955, Patel and Blanchard, 1999). There are a number of options that have been suggested for bacillithiol but no definitive system has been identified (Rajkarnikar et al., 2013).

1.5 Antibiotic resistant *S. aureus*

The introduction of antibiotics drastically improved treatment outcomes for patients infected with *S. aureus*; following the discovery of penicillin (Fleming, 1929), and it's subsequent clinical introduction in the 1940's, *S. aureus* bacteraemia mortality rate dropped dramatically from around 70% to 25% (Rubin et al., 1999). However, penicillin resistant strains were observed almost immediately (Spink et al., 1944). Penicillin is a bactericidal antibiotic that consists of a highly reactive four-membered beta-lactam

structure that binds to and inhibits transpeptidase, the enzyme that catalyses the final step in cell wall synthesis. This leads to the cell wall becoming weaker as peptidoglycan is still being hydrolysed but not cross-linked; ultimately leading to cytolysis due to the imbalance of osmotic pressure (Yocum et al., 1980). The earliest strains of *S. aureus* able to resist penicillin did so through the production of beta-lactamases (Abraham and Chain, 1940). Beta-lactamases are enzymes that are able to hydrolyse the beta-lactam ring, denaturing it and preventing it from binding to transpeptidase (Jelsch et al., 1993). The prevalence of beta-lactamase producing strains of *S. aureus* increased dramatically as resistant strains were selected for by increasing antibiotic use (Wilson and Cockcroft, 1952).

In the 1960's new antibiotics related to penicillin were developed containing a modified beta-lactam ring (Waxman and Strominger, 1983). These new semi-synthetic beta-lactam antibiotics, methicillin, oxacillin and flucloxacillin, were able to kill the penicillin resistant *S. aureus* because they remained stable following exposure to the beta-lactamase enzyme that penicillin resistant *S. aureus* produced. However, resistance to these new antibiotics was discovered only two years after their first widespread clinical use (Jevons et al., 1961). This resistance is mediated by the chromosomally localised *mecA* gene (Chambers, 1997). The *mecA* gene is found as part of a genomic island known as staphylococcal cassette chromosome *mec* (SCC*mec*) and these can vary in size from 21 to 67 kb (Hiramatsu et al., 2001). The frequency of SCC*mec* elements transferring into *S. aureus* seems to be relatively low as there are only a limited number of clones responsible for epidemic MRSA (Kreiswirth et al., 1993). The *mecA* gene is responsible for the synthesis of a modified peptidoglycan cross-linking protein, penicillin binding protein 2a (PBP2a) (Utsui and Yokota, 1985). PBP2a replaces the other PBPs and, because of its low affinity for all beta-lactam antibiotics, allows the bacteria to resist high concentrations of these antibiotics (Brown and Reynolds, 1980). PBP2s has a modified active site compared to the other PBPs resulting in a greatly reduced binding affinity for beta-lactams. This allows the transpeptidation reaction to proceed even at high concentrations of beta-lactam antibiotics (Lim and Strynadka, 2002). Strains of *S. aureus* that have the *mecA* gene and produce this modified PBP are known as methicillin resistant *Staphylococcus aureus*

(MRSA). Despite this widespread resistance to beta-lactam antibiotics, oxacillin and flucloxacillin remain the first line of treatment against most methicillin sensitive *S. aureus* (MSSA) infections.

In order to treat infections caused by MRSA strains other antibiotics with different modes of action have to be used. These can include clindamycin, the aminoglycosides neomycin and gentamicin and the glycopeptide vancomycin (Foster, 2017). However, despite each of these antibiotics having a different mechanism of action, resistant strains of *S. aureus* have evolved for each.

Clindamycin is a lincosamide antibiotic and is the preferred agent of the macrolide-lincosamide-streptogramin B (MLS_B) antibiotics used to treat *S. aureus* infections (Deotale et al., 2010). It is primarily a bacteriostatic antibiotic that inhibits protein synthesis through binding to the 50S ribosomal subunit (Wilson, 2014). Increased use of clindamycin has led to an increase in the prevalence of clindamycin resistance conferred through the methylation of the ribosomal target (Fiebelkorn et al., 2003).

Aminoglycosides, including neomycin and gentamicin, are bactericidal antibiotics that irreversibly bind the 30S subunit of the bacterial ribosome. This interrupts protein synthesis and leads to cell death (Hahn and Sarre, 1969, Yoshizawa et al., 1998). The most common mechanism conferring aminoglycoside resistance is the production of cellular enzymes, such as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (AAD) and aminoglycoside phosphotransferases (APH), that modify the aminoglycosides and thus prevent them from binding the 30S subunit of the bacterial ribosome (Freitas et al., 1999).

An effective alternative to the beta-lactam antibiotics is the glycopeptide vancomycin. Like the beta-lactam antibiotics glycopeptides are bactericidal and work by inhibiting cell wall production; unlike the beta-lactams they are unaffected by the modified PBP produced by MRSA because instead of binding PBP vancomycin binds the terminal D-alanyl-D-alanine moieties of the N-acetylmuramic acid and N-acetylglucosamine peptides that form the backbone strands of the bacterial cell wall. By binding to these

peptides vancomycin prevents them from cross-linking (Reynolds, 1989). Unfortunately, following the increased usage of vancomycin in the 1980's resistant strains were discovered. First a vancomycin-insensitive *S. aureus* (VISA) strain was discovered in the late 1990's (Hiramatsu et al., 1997, Liu and Chambers, 2003) and then during the next decade a fully vancomycin-resistant *S. aureus* (VRSA) strain was detected (Sievert et al., 2008). The VRSA strains remain rare and they contain the transposon Tn*1546* acquired from *Enterococcus faecalis*. Tn*1546* causes the D-alanyl-D-alanine moieties to be replaced by D-alanyl-D-lactate that has a lower affinity for vancomycin (Gardete and Tomasz, 2014). The VISA strains, on the other hand, seem to be produced by a number of different mutations mostly in two component sensory regulatory systems involved in cell wall synthesis. It has been hypothesised that this causes a thicker cell wall and increased production of D-alanyl-D-alanine moieties (Howden et al., 2010).

Fortunately, there are other antibiotics that can be used as last line treatments against antibiotic resistant *S. aureus*. These include bactericidal antibiotic daptomycin and bacteriostatic antibiotic linezolid, which target bacterial membranes and protein synthesis respectively (Swaney et al., 1998, Pogliano et al., 2012). However, resistance to both of these has also been observed clinically (Marty et al., 2006, Morales et al., 2010), with the potential that resistance to these last line antibiotics could also become widespread in the not too distant future.

This pattern of waves of antibiotics being introduced followed by waves of antibiotic resistance has continued unabated since the introduction of the first antibiotics (Chambers and Deleo, 2009). However, the development of new antibiotics has slowed down dramatically since the golden age of the 1940's – 1960's (Coates et al., 2011). This has resulted in fears about returning to 'the dark age of medicine' where routine treatment of infections would be severely inhibited. This would then have catastrophic implications for other clinical interventions such as surgery and chemotherapy, and would have huge economic implications (O'Neil, 2014).

1.5.1 Do antibiotics induce bacterial cell death via a common mechanism?

Despite there being different modes of action identified for each class of antibiotics it has been suggested that the actual mode of killing for bactericidal antibiotics is ultimately through the generation of damaging oxidative species (Kohanski et al., 2007, Foti et al., 2012). The original work in this area used a fluorescent reporter dye, 3'-p-hydroxyphenyl fluorescein (HPF), to detect the formation of hydroxyl radicals inside bacteria that were being challenged with antibiotics. It was observed that HPF fluorescence increased in the presence of H_2O_2 and bactericidal antibiotics but not in the presence of bacteriostatic antibiotics (Kohanski et al., 2007). It was also observed that a reduction in HPF fluorescence occurred when cells were treated with either an iron chelator (2.2'-dipidyl) or a radical scavenger (thiourea) indicating the involvement of the Fenton reaction, which oxidises ferrous ions and forms damaging hydroxyl radicals (Kohanski et al., 2007). Therefore, it was hypothesised that the bactericidal antibiotics were disrupting the Kreb's cycle leading to the over stimulation of the electron transport chain and superoxide damage to iron-sulfur clusters. This would then release ferrous iron, which would be oxidised by the Fenton reaction and lead to hydroxyl radical formation. These hydroxyl radicals would then go on to damage DNA, proteins and lipids, ultimately leading to cell death (Dwyer et al., 2009). It has also been suggested that other radical species are involved (Dwyer et al., 2014).

However, the claim that this is a universal mechanism by which bactericidal antibiotics mediate bacterial killing has some potential flaws. These include the ability of bactericidal antibiotics to kill *Streptococcus pneumoniae* even though it lacks an electron transport chain (Musher, 1992, Hoskins et al., 2001), and the comparable efficiency of bactericidal antibiotics in treating both aerobic and anaerobic infections (Brook, 2011, Liu and Imlay, 2013). Other work has also demonstrated that there is no correlation between levels of reactive oxygen species in an individual cell and the probability that particular cell will die (Keren et al., 2013). Also changes in HPF florescence are potentially more closely related to changing bacterial cell morphology than the formation of hydroxyl radicals (Paulander et al., 2014). Thus, the theory of a universal mechanism of bactericidal antibiotic cell killing remains contentious.

However, it could have wide implications for understanding *S. aureus* antibiotic defence mechanisms and for the development of new treatment options.

1.6 *Staphylococcus aureus* infection prevention strategies

1.6.1 Eradication

Because of the large proportion of the population with persistent asymptomatic colonisation full eradication of *S. aureus* is impractical. However, attempts have been made to reduce MRSA infections in healthcare settings through requiring the mandatory identification and decolonisation of asymptomatic MRSA carriers who have been admitted to hospital (Pearson et al., 2009). This has been shown to successfully reduce the number of reported MRSA bacteraemia cases but MSSA bacteraemia numbers remain relatively static (Pearson et al., 2009).

1.6.2 Hand hygiene

One of the most effective prevention strategies has been the improvement of hand hygiene in clinical settings. Simple campaigns to improve hand washing have had a positive effect on reducing both MRSA and MSSA infection rates (Stone et al., 2012).

1.6.3 Immunisation

Immunisation would be an invaluable tool in preventing *S. aureus* infection; however, an effective vaccine is yet to be developed and passive immunisation trials have also struggled to make sufficient headway (Daum and Spellberg, 2012, Fowler and Proctor, 2014). This is probably related to *S. aureus's* commensal lifestyle and the inability of hosts to produce protective immunity following exposure to *S. aureus* (van Belkum et al., 2009, David and Daum, 2010). There have been several *S. aureus* vaccine candidates that have reached various stages of clinical trial; so far, none of them have given fully satisfactory responses (Daum and Spellberg, 2012, Fowler and Proctor, 2014). One trial had particularly negative results with trial participants who had received vaccine candidate V710 being more likely to be colonised with MRSA and those that developed infections, being five times more likely to die than the control group (Fowler et al., 2013). This result came despite high antibody titres and positive

pre-clinical work. Pre-clinical vaccine work tends to place a large emphasis on the level of opsonisation and phagocytosis as indicators of a potentially successful vaccine (Fattom et al., 2004, Pozzi et al., 2012). However, *S. aureus* is known to be a successful intracellular pathogen that can survive and proliferate even inside professional phagocytes (Kapral and Shayegani, 1959, Gresham et al., 2000, Surewaard et al., 2013). This may help to explain why vaccine design focused on opsonisation is not producing successful results. A better understanding of the mechanisms underlying the interaction of *S. aureus* and host immunity, particularly intracellular survival of *S. aureus,* could help to inform better vaccine development in the future.

1.7 Host immune response

1.7.1 Innate immune response

1.7.1.1 Physical defences

The first line of defence against *S. aureus* infection is the physical barrier of the skin, *S. aureus*'s natural ecological niche is the human nose and it can readily colonise other moist areas (Weidenmaier et al., 2012); however, it generally needs to break past the skin barrier, either through a breakage, a hair follicle, the airways or via ingestion before it causes invasive disease. Not only is the skin a physical barrier to infection it is also a hostile desiccated environment with a high salt concentration, low pH and low nutrient availability. The skin also produces antibacterial molecules such as antimicrobial fatty acids, including lauric acid, capric acid, myristic acid and *cis*-6-hexadecenoic (Kelsey et al., 2006, Cartron et al., 2014), and antimicrobial peptides like cathelicidins and beta-defensins (Schauber and Gallo, 2008).

The other major physical defence encountered by *S. aureus* is the mucus membranes of the nasal cavity and the rest of the respiratory system. Here *S. aureus* is captured in mucus, which also contains AMPs such as beta-defensins, and is flushed away through the movement of cilia (Schutte and McCray, 2002).

1.7.1.2 Neutrophils

Once *S. aureus* is past the physical barriers and has entered host tissues bacterial products and cell damage induce the production of chemoattractants and cytokines that attract neutrophils to the site of infection (Spaan et al., 2013b). Neutrophils then kill or immobilise the bacteria either through phagocytosis and intracellular meditated killing or through the production of neutrophil extracellular traps (NETs). NETs can trap pathogens, thus preventing their dissemination and then damage the pathogens in association with AMPs (Brinkmann et al., 2004).

In order for neutrophils to phagocytose *S. aureus* cells the bacteria must first be opsonised by either complement or immunoglobulins. Then these opsonins are recognised by complement receptors (CRs) or Fc-receptors (FcRs), which are displayed on the surface of the neutrophils, and the bacteria are endocytosed (Amulic et al., 2012). Once *S. aureus* has been internalised it is contained within a phagosome and the neutrophil attempts to destroy the bacteria through the fusing of granules containing AMPs and antimicrobial enzymes such as lysozyme, as well as, producing damaging reactive oxygen species (ROS) (Hampton et al., 1998). ROS are formed through a chain of events starting with the action of NADPH-dependent oxidase reducing molecular oxygen into superoxide. Then the superoxide can be reduced further to form hydrogen peroxide. Then a number of different secondary reactions can produce other ROS. These include hypochlorous acid which is formed through the action of myeloperoxidase (MPO) (Babior, 1999, Klebanoff, 2005). Nitric oxide (NO) is also produced inside phagosomes, by inducible nitric oxide synthase (iNOS), resulting in the disruption of respiration, DNA replication and central metabolism (Xie et al., 1992, Fang, 2004)

This neutrophil mediated immune response is crucial for successful clearance of *S. aureus* infection. This is made very clear with individuals who have reduced neutrophil counts or malfunctioning neutrophils who tend to have high rates of *S. aureus* infection (Curnutte et al., 1974, Chemaly et al., 2010).

1.7.1.3 Complement

One of the most primitive, but also most crucial, parts of the innate immune system is the complement system (Figure 1.2). The complement system can either opsonise bacteria inducing phagocytosis by neutrophils and other phagocytes, or it can kill some bacteria directly, particularly gram-negative bacteria. It consists of over 30 proteins and is activated in three different pathways: the lectin pathway, the alternative pathway and the classical pathway (Walport, 2001, Thiel, 2007). The lectin pathway activates through the binding of mannose binding lection (MBL) to mannose residues located on the surface of *S. aureus* or on the surfaces of other pathogens. This binding leads to the activation of MBL-associated serine protease (MASP) and the activation of C4 and C2 that, in turn, form C4b2a, a C3 convertase. The alternative pathway is activated through the interaction of various factors (B, D, H and I) with each other and C3b to form C3bBb, another C3 convertase. C3bBb can activate more C3 causing a positive loop to form. This loop is inhibited by surface factors on healthy mammalian cells. The classical pathway involves the binding of antibody-antigen complexes to C1q. There is then a cascade through from C1q, C1r and C1s to C2 and C4. This leads to the formation of C4b2a as in the lectin pathway (Walport, 2001, Thiel, 2007). Ultimately, all of the complement pathways meet at the formation of C3 convertases, enzyme complexes that catalyse the cleavage of protein C3 into C3a and C3b. C3a activates mast cells and C3b forms new convertases and amplifies the process of opsonisation or is processed to form iC3b. Then once C3b concentrations reach a certain level the convertases start to cleave C5 to C5a and C5b. C5a then acts as a chemoattractant for phagocytes and C5b starts to initiate lysis, but this only occurs when deposited on Gram-negative bacteria (Figure 1.2) (Rooijakkers et al., 2009).

1.7.1.4 Abscess formation

A key part of innate immune response is the formation of abscesses containing neutrophils and macrophages that degrade infected tissue. The liquefied degraded tissue, or pus, is then encased in fibrin in order to contain the infection and prevent

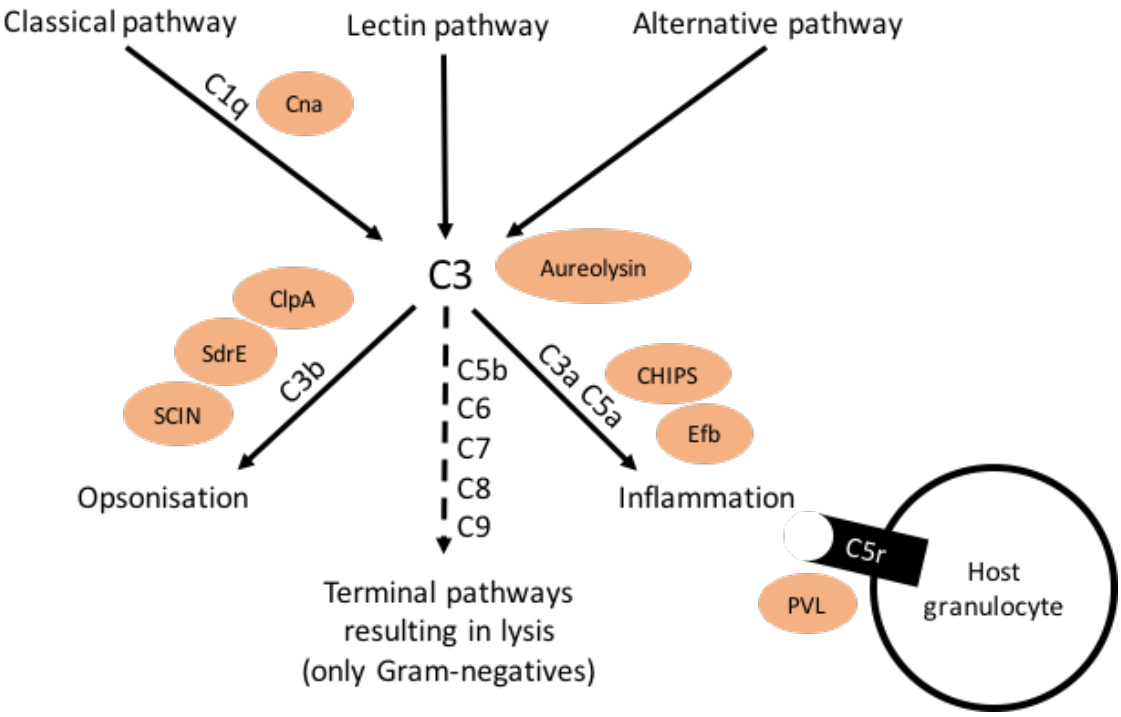


Figure 1.2 Interaction of the complement system and *S. aureus* derived molecules
The three routes to complement activation and the main downstream effects. Some of the important components of the complement system ae labeled and molecules derived by *S. aureus* that interact with the complement system are highlighted.

dissemination (Cheng et al., 2009). The abscess environment is mostly anaerobic and nutrient availability is limited, particularly of metal ions (Corbin et al., 2008); however, *S. aureus* is able to persist in this harsh environment through the production of certain surface proteins and metal scavenging proteins (Section 1.9) (Cheng et al., 2009).

1.7.2 Adaptive immune response

1.7.2.1 T-lymphocytes

The primary response to *S. aureus* infections is through the innate immune system but the adaptive immune system can assist the innate in clearing an *S. aureus* infection. The role of T-cells seems to be important in this process with patients with low CD4+ T cell counts, such as those with poorly controlled HIV, having higher rates of *S. aureus* SSTIs (Shadyab and Crum-Cianflone, 2012). T-cells play a key role in recruiting and activating phagocytes to the site of infection, facilitating antibody maturation and promoting destruction of bacteria in the phagosome through their production of IFN-γ (Bröker et al., 2016). The production of IL-17 also seems to be key in effective T-cell responses to *S. aureus* (Montgomery et al., 2014) and so in general T_H1 or T_H17 mediated responses seem to be the most effective against *S. aureus*.

1.7.2.2 B-lymphocytes

B-cells can produce antibodies that recognise various *S. aureus* surface structures and exoproteins. Binding of antibodies to the surface of a bacterium leads to mediated phagocytosis by phagocytic cells, whereas binding of antibodies to exotoxins or surface bound virulence factors inhibits function (Holtfreter et al., 2010). However, the effectiveness of antibodies in elucidating clearance of an *S. aureus* infection appears to be low with high anti-*S. aureus* antibody titres failing to be protective against recurrent *S. aureus* infection (Huang and Platt, 2003, Nguyen et al., 2005, Miller et al., 2007).

1.8 *Staphylococcus aureus* virulence factors

Staphylococcus aureus produces a wide range of virulence factors that facilitate adhesion to host tissue, immune modulation and immune evasion (Foster et al., 2014,

Otto, 2014, Li et al., 2015). These consist of surface and secreted factors and they have a wide range of functions and modes of action.

1.8.1 Surface and pigments

Staphylococcus aureus produces an array of surface based and pigment based virulence factors including carotenoid pigments, capsular polysaccharides and cell wall anchored (CWA) proteins that are attached to the peptidoglycan.

1.8.1.1 MSCRAMMs

The largest group of CWA proteins is the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) that interact with host cells or host immune system factors. These include the two fibronectin-binding proteins (Fnbp) FnbpA and FnbpB. They facilitate binding to fibrinogen and elastin (Keane et al., 2007), as well as, fibronectin (Burke et al., 2011) where they facilitate host cell invasion into non-professional phagocytes via a β-zipper mechanism (Dziewanowska et al., 1999, Sinha et al., 2000).

The group also includes the Clumping factors (Clf) ClfA and ClfB that bind to fibrinogen (Deivanayagam et al., 2002, Walsh et al., 2008) and facilitate nasal colonisation via binding to the top layer of dead (desquamated) epithelial cells (Mulcahy et al., 2012). They also have an immune evasion role through the binding of ClpA with complement factor 1 resulting in the degradation of complement component 3b (C3b) (Figure 1.2) (Hair et al., 2008). There are also a number of Serine-aspartate repeat proteins (Sdr): SdrC, SdrD and SdrE that also have a role in nasal colonisation (Corrigan et al., 2009), though this is less well defined, and SdrE degrades C3b through interaction with complement factor H (Figure 1.2) (Sharp et al., 2012). Collagen adhesion protein (Cna) is another MSCRAMM that disrupts complement activation, this time through binding to complement protein C1q (Kang et al., 2013), as well as, facilitating binding to collagen rich tissue such as cartilage and bone (Figure 1.2) (Zong et al., 2005).

1.8.1.2 Protein A

Staphylococcal protein A (SpA) is another CWA protein found in all *S. aureus* strains and is often used for typing different strains. It is characterised by tandemly repeated three-helical bundles found at the N terminus (Deisenhofer, 1981) and it can bind to a number of different ligands. These include the Fc fragment of IgG (Deisenhofer, 1981) and the Fab fragment of IgM (Graille et al., 2000). This leads to the inhibition of opsonisation and subsequent phagocytosis by the hosts professional phagocytes (Section 1.7.1.2). It also acts as a B-cell superantigen resulting in the nonspecific activation of B-cells. SpA can also bind von Willebrand factor (vWF) and this seems to play an important role in *S. aureus* induced endocarditis (O'Seaghdha et al., 2006).

1.8.1.3 Iron-regulated surface proteins

The iron-regulated surface proteins (Isd) IsdA, IsdB and IsdH are all CWA proteins that are involved in transporting haem into bacterial cells, thus enabling survival of *S. aureus* in the low iron environments found inside hosts (Mazmanian et al., 2003, Grigg et al., 2010, Hammer and Skaar, 2011). They also appear to have other roles including adherence to host epithelial cells, resistance to bactericidal lipids and resistance to lactoferrin (a part of the innate immune system responsible for sequestering iron in secreted fluids such milk and saliva) (Clarke et al., 2007, Clarke and Foster, 2008, Zapotoczna et al., 2013).

1.8.1.4 Capsular polysaccharides

Many strains of *S. aureus* also produce capsular polysaccharides that enhance virulence through preventing complement recognition, impeding phagocytosis and promoting abscess formation, as well as, promoting colonisation and persistence on mucosal surfaces (O'Riordan and Lee, 2004).

1.8.1.5 Staphyloxanthin

Staphyloxanthin is the orange carotenoid that gives *S. aureus* its characteristic golden colour and offers resistance to oxidative stress and neutrophil killing (Clauditz et al., 2006).

1.8.2 Cytolytic toxins

1.8.2.1 Alpha toxin

Staphylococcus aureus produces a vast array of secreted cytolytic toxins that lyse red blood cells (haemolysins) and/or white blood cells (neurotoxins). Of these, α-haemolysin, or alpha-toxin (hla), is the most extensively studied. It is a bi-component beta-barrel forming toxin that is lytic to red blood cells. It binds to ADAM10, a receptor on human epithelial cells, leading to the loss of epithelial barrier function; and it can even induce the apoptosis of monocytes, T-cells and B-cells, but not neutrophils (Valeva et al., 1997, Inoshima et al., 2011, Nygaard et al., 2012).

1.8.2.2 Beta toxin

β-Haemolysin, or beta toxin (hlb), which encodes a sphingomyelinase enzyme that breaks down sphingomyelin found in host cell membranes, is disrupted by a pathogenicity island in most virulent *S. aureus* strains and as such is not a very significant factor in contributing to the vast majority of severe *S. aureus* infections (Novick et al., 2001).

1.8.2.3 Leukocidins

Staphylococcus aureus secretes a number of leukocidins that are related to α-haemolysin, in that they are also bi-component beta-barrel forming toxins. These include: γ-haemolysin, or gamma-toxin (HlgA, HlgB and HlgC); Leukocidin DE (LukDE); Leukocidin AB (LukAB) and Panton-Valentine leucocidin (PVL).

PVL has gained attention through its association with CA-MRSA (Vandenesch et al., 2003); however, this association is far from clear (Otto, 2010). It binds to complement 5 receptor (C5r), found on neutrophils, other granulocytes, monocytes and dendritic cells, where it triggers a flow of cations and then lysis following the formation of a non-specific pore (Figure 1.2) (Loffler et al., 2010, Spaan et al., 2013a). PVL is also only secreted by strains that have been lysogenised with the bacteriophage containing the LukS-PV and LukF-PV genes (Kaneko et al., 1998).

Component HlgB and HlgC of gamma toxin are almost identical to the PVL LukF and LukS genes, respectively and so unsurprisingly it is also able to induce lysis in leukocytes (Cooney et al., 1993); however, unlike PVL, gamma toxin is found in almost all strains and, with the addition of HlgA, is able to induce lysis in red blood cells (Ferreras et al., 1998).

LukAB and LukDE both target different sets of leukocytes. LukAB binds cluster of differentiation molecule 11B (CC11b), a protein found on macrophages and monocytes, as well as, neutrophils, other granulocytes and NK cells (DuMont et al., 2013), and LukDE binds chemokine receptor type 5 (CCR5), a protein expressed on T-cells, macrophages and dendritic cells (Alonzo et al., 2013).

1.8.2.4 Delta toxin and PSMs

The last set of *S. aureus* associated cytolytic toxins that have been characterised are delta toxin and the related phenol-soluble modulins (PSMs). These consist of a group of small secreted peptides, that are produced by most *S. aureus* strains but that seem to play a particularly important role in CA-MRSA pathogenesis (Wang et al., 2007, Diep and Otto, 2008, Li et al., 2009). They have both hydrophobic and hydrophilic regions, with detergent like properties, and form short lived pores in a non-specific manner (Talbot et al., 2001). They also seem to play a crucial role in neutrophil lysis following phagocytosis (Geiger et al., 2012, Chatterjee et al., 2013, Surewaard et al., 2013).

1.8.3 Superantigens

Staphylococcus aureus is able to produce a number of superantigens that cause non-specific activation of T-cells, through binding MHCII, leading to full scale T-cell activation and cytokine release (Balaban and Rasooly, 2000). These include toxic shock syndrome toxin 1 (TSST-1), that causes toxic shock syndrome (TSS), and a number of staphylococcal enterotoxins (SE-A,B,C,D,E,G,H,I,J and X), that are associated with causing TSS-like food poisoning (Lina et al., 2004). They are generally encoded on mobile genetic elements with different strains expressing different combinations of

superantigens (McCormick et al., 2001). They are resistant to high heat, acids and desiccation which makes their elimination from the food chain very difficult (McCormick et al., 2001). TSST-1 has a greater capacity to penetrate mucosal surfaces than the other *S. aureus* superantigens and this probably explains its particular association with menstrual TSS (Shands et al., 1980).

1.8.4 Exoenzymes

Staphylococcus aureus also produces a whole host of exoenzymes with various virulence roles. There is a link between secretion of these enzymes and pathogenic strains of *S. aureus* particularly CA-MRSA strains (Li et al., 2009).

1.8.4.1 Proteases

Staphylococcus aureus produces a number of major secreted proteases including a metalloprotease (aureolysin *aur*), two cysteine proteases (staphopain A (ScpA) and staphopain B SspB) and a selection of serine proteases: SspA, exfoliative toxins ETA and ETB and serine like proteases SplABCDEF (Reed et al., 2001, Shaw et al., 2004, Bukowski et al., 2010). Of these, exfoliative toxins are the most clinically interesting as they are directly responsible for the symptoms associated with staphylococcal scalded skin syndrome (SSSS). Exfoliative toxins are serine proteases that specifically cleave desmosomal cadherins found in the superficial layers of the skin, leading to the widespread blister formation characteristic of SSSS (Bukowski et al., 2010).

Aureolysin is involved in immune evasion. It is able to cleave the antimicrobial peptide LL-37, found in lysosomes of macrophages, neutrophils and other granulocytes, and it can cleave complement C3, effectively reducing the complement meditated immune response (Figure 1.2) (Sieprawska-Lupa et al., 2004, Laarman et al., 2011). Furthermore, aureolysin is responsible for the activation of a serine protease SspA that subsequently activates cysteine proteases SspB and ScpA (Rice et al., 2001, Massimi et al., 2002, Nickerson et al., 2007). SspB and ScpA then go on to degrade fibrinogen, fibronectin and elastin fibres; this promotes the detachment of human keratinocytes, aiding entry of *S. aureus* past the host skin barrier (Nickerson et al., 2010).

The exact function of the related *splABCDEFG* operon is less well defined, but there is some indication that it is involved in cleavage of certain proteins found on the surface of the human lung (Paharik et al., 2016, Stentzel et al., 2017).

1.8.4.2 Nucleases

Staphylococcus aureus also produces two extracellular nucleases, Nuc and Nuc2, that are vital in the modulation of biofilm formation and degrading host neutrophil extracellular traps (NETs) (Section 1.7.1.2) (Berends et al., 2010).

1.8.4.3 Lipases

Staphylococcus aureus produces two lipases, coded by *gehA* and *gehB* that can degrade host produced bactericidal lipids (Cadieux et al., 2014). This enables *S. aureus* to breakdown the lipids secreted onto human skin by the sebaceous glands.

1.8.4.4 Hyaluronidase

Staphylococcus aureus also produces a hyaluronidase (HysA) that is able to degrade hyaluronic acid, a high molecular weight polymer found in mammalian skin, bones, lungs and heart valves (Hynes and Walton, 2000, Ibberson et al., 2014). HysA role in breaking down the hyaluronic acid polymers is important in that it enables other secreted virulence factors to penetrate host tissues (Starr and Engleberg, 2006).

1.8.4.5 Staphylokinase

Staphylococcus aureus also secretes staphylokinase (SAK) that activates host plasminogen to form plasmin, leading to the degradation of blood clotting factors and thus facilitating the dissemination of *S. aureus* in the bloodstream. SAK also binds to host defensins and prevents them from carry out their function of disrupting bacterial cell walls (Jin et al., 2004, Bokarewa et al., 2006).

1.8.5 Immune modulatory proteins

1.8.5.1 Staphylococcal complement inhibitor (SCIN) and Chemotaxis inhibitory protein of S. aureus (CHIPS)

Staphylococcus aureus also produces a number of other virulence factors that all interact with the host immune system but do not fit into the categories listed above. Two of these factors, Staphylococcal complement inhibitor (SCIN) and Chemotaxis inhibitory protein of *S. aureus* (CHIPS), are part of an innate immune evasion cluster, along with *sak* and enterotoxin A, located on a beta beta-haemolysin-converting bacteriophage (van Wamel et al., 2006). They are both small excreted proteins that are co-expressed during the early exponential phase and modulate different aspects of the early host immune response (Rooijakkers et al., 2006). SCIN has a three-helical bundle structure related to the structure of protein A (Rooijakkers et al., 2007). It functions through binding C3 convertases, preventing C3b deposition and ultimately preventing phagocytosis and neutrophil killing (Figure 1.2) (Rooijakkers et al., 2005). CHIPS, on the other hand, binds to C5a and formylated peptide receptor and prevents the chemotaxis of neutrophils and monocytes towards *S. aureus* (Figure 1.2) (de Haas et al., 2004, Postma et al., 2004).

1.8.5.2 Extracellular fibrinogen binding protein (Efb)

Extracellular fibrinogen binding protein (Efb), like ClfA and FnbpA, can bind fibrinogen and prevents platelet aggregation (Boden and Flock, 1994, Shannon and Flock, 2004). EFP also has a three helical bundle structure similar to that found in protein A and SCIN (Hammel et al., 2007); like SCIN Efb is able to prevent C3b functioning, in this case by directly binding C3 preventing it from forming C3b (Figure 1.2) (Lee et al., 2004).

1.8.5.3 Extracellular adherence protein (Eap)

Extracellular adherence protein has a diverse set of functions due to its ability to interact with a number of different host matrix molecules. These include collagen, laminin and fibronectin (McGavin et al., 1993). Therefore, Eap has important roles in cell adhesion (Palma et al., 1999), invasion of eukaryotic cells (Haggar et al., 2003) and interference with leukocyte recruitment (Chavakis et al., 2002).

1.8.5.4 Formyl peptide receptor-like 1 inhibitors protein (FLIPr)

Finally, *S. aureus* also produces formyl peptide receptor-like 1 inhibitors protein (FLIPr) that inhibits the activation of neutrophils. It does this by binding host formyl peptide receptor like protein 1 (FPRL1) and preventing it from detecting *S. aureus* (Prat et al., 2006).

1.8.6 Regulation of virulence factors

The array of virulence factors produced by *S. aureus* is regulated by a number of regulatory systems. These systems interact and form a complex regulatory network that is only partially understood (Priest et al., 2012).

1.8.6.1 agr

The two-component accessory gene regulator (Agr) is the best characterised. It is encoded by the *agrABCD* operon and main effector protein is AgrD, which is exported by AgrB, that becomes autoinducing peptide (AIP). AIP then binds to AgrC, a receptor histidine protein kinase, which in turn transfers the signal intracellularly to AgrA, the response regulator. AgrA then promotes transcription of the *agr* operon in a positive feedback loop enabling Agr to respond to cell density. AgrA also promotes the transcription of the effector molecule RNAIII that up-regulates expression of many exotoxins and down-regulates the expression of surface proteins (Figure 1.3) (Novick et al., 1993, Ziebandt et al., 2004, Traber et al., 2008).

AgrA has also been shown to be affected by redox conditions with DNA-binding being inhibited under conditions of oxidising stress due to its intramolecular dithiol-disulfide switch (Sun et al., 2012).

1.8.6.2 saeRS

Another two-component system that is important in *S. aureus* virulence regulation is the *saeRS* locus. It is responsible for the expression of alpha and beta toxins as well as coagulase and Efb (Sections 1.8.2.1, 1.8.2.2 and 1.8.5.2) (Giraudo et al., 1994).

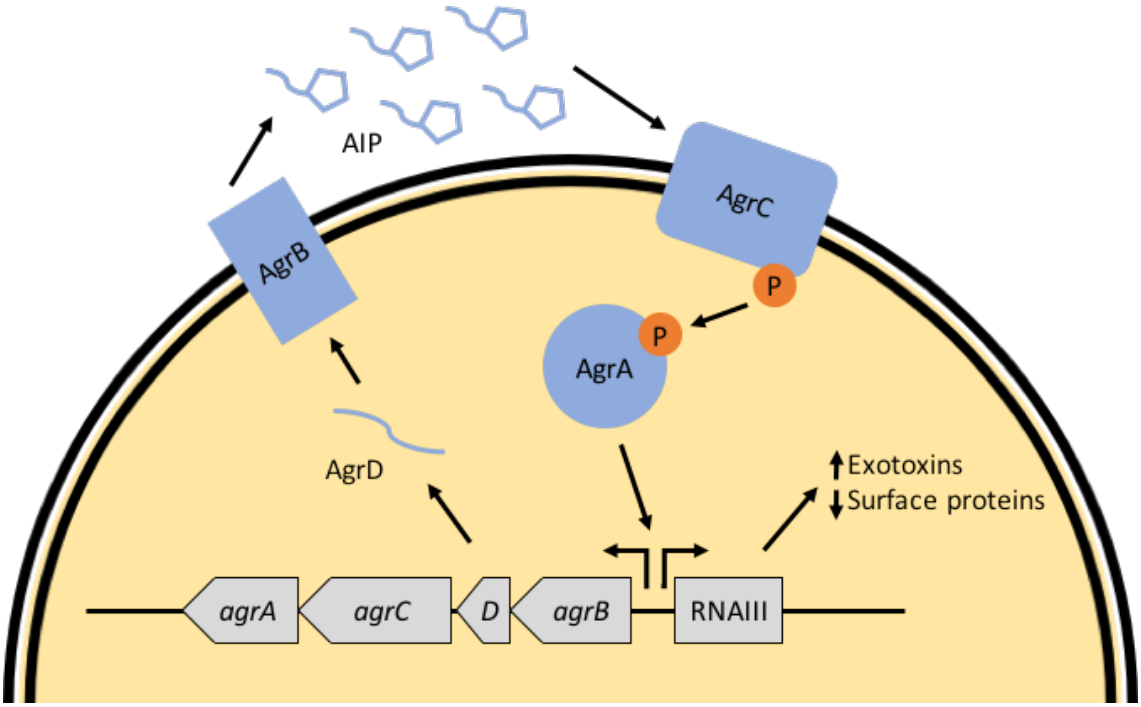


Figure 1.3 The *agr* system in *S. aureus*
The *agrBDCA* operon encoding the components of the self-regulating *agr* system that control the expression of RNAIII. The operon is shown in grey and Agr proteins are shown in blue (Novick et al., 1993; Ziebandt et al., 2004; Traber et al., 2008).

1.8.6.3 SarA and homologs

Another important set of regulators are SarA and its homologs including SarR, S, T, U, V, X and Z, and Rot. SarA upregulates the expression of capsular polysaccharides, toxins and *agr* and its homologs all act as global regulators of virulence genes (Cheung et al., 2008, Morrison et al., 2012). One of these is Rot (repressor of toxins) that represses toxin production and is down-regulated by Agr (Said-Salim et al., 2003).

1.8.6.4 Sigma B

Sigma B also plays a crucial role in controlling virulence transcription. It influences the expression of a number of cell functions, including intermediary metabolism and cell membrane synthesis, as well as, increasing the expression of adhesion molecules and decreasing the expression of toxins (Bischoff et al., 2004). It thus acts oppositely to Agr and SarA and it has been found to be an important factor in *S. aureus* intracellular survival (Tuchscherr et al., 2015).

1.9 *Staphylococcus aureus* immune evasion

1.9.1 Virulence factors

Many of the virulence factors listed above are involved in immune evasion with various factors involved in preventing phagocytosis by host phagocytes (e.g. Protein A, ClfA and SCIN), preventing phagocyte chemotaxis (e.g. CHIPs, and Eap) or directly causing damage to phagocytes (e.g. PVL and PSMs). There are also factors that can damage other immune cells (e.g. alpha toxin) or even stimulate an overzealous immune response (e.g. TSST-1 and enterotoxins). All of these factors combine to aid a successful *S. aureus* infection but *S. aureus* also employs some other important strategies to avoid killing by host immune cells.

1.9.2 Biofilm formation

One such strategy is the formation of biofilms. *Staphylococcus aureus* readily forms biofilms generally consisting of a mix of cells, teichoic acids and polysaccharide intracellular antigen (PIA) (Archer et al., 2011). Biofilm formation is a major component in persistent infections, particularly medical device related disease, osteomyelitis and

chronic wound infections. By forming this mass of cells and secreted factors *S. aureus* effectively prevents immune cells from physically reaching many of the cells in the matrix. For the same reason biofilms are also protective against antibiotics (Jones et al., 2001, Archer et al., 2011).

1.9.3 Survival in host phagosomes

Once a *S. aureus* cell is phagocytosed the host cell will try to destroy the bacteria through the production of AMPs, NO and ROS; however, *S. aureus* has a number of resistance mechanisms to each of these enabling it to survive inside the host phagosome (Schroder et al., 2006, Olivier et al., 2009).

1.9.3.1 AMP resistance

Many strains of *S. aureus* have an innate resistance to lysosome, one of the major AMPs present in neutrophil granules, through the *O*-acetylation of their peptidoglycan (Bera et al., 2005). *Staphylococcus aureus* is also able to resist host defensins and other AMPs by decreasing the negative charge on its surface. This prevents AMPs from interacting with the membrane of *S. aureus*. It achieves this through adding D-alanine to its wall teichoic acids (mediated by *dltABCD*) and through producing lysylphosphatidylglycerol by combining L-lysine and phosphatidylglycerol (mediated by multiple peptide resistance factor MprF) (Peschel et al., 2001, Collins et al., 2002).

1.9.3.2 ROS resistance

Staphylococcus aureus has a particularly high tolerance for oxidative stress. This is evident by the ability of *S. aureus* to grow on Baird-Parker agar. It does this by reducing the oxidant tellurite, that is present in the medium, to tellurium, resulting in the production of distinctive black colonies (Baird-Parker, 1962, Taylor, 1999). It appears to have a number of mechanisms for this resistance including the production of the characteristic orange antioxidant carotenoid pigment staphyloxanthin (Clauditz et al., 2006) and also the enzyme catalase, that converts hydrogen peroxide to water and oxygen. *Staphylococcus aureus* also produces superoxide dismutases (SodA and SodB)

that convert superoxide to hydrogen peroxide (Clements et al., 1999, Valderas and Hart, 2001).

Furthermore, it has been postulated that cysteine metabolism and hydrogen sulfide (H_2S) may have important roles in defence against reactive oxygen species and by extension against bactericidal antibiotics (Section 1.5.1) (Lithgow et al., 2004, Soutourina et al., 2010, Shatalin et al., 2011). However, there are still many unanswered questions surrounding the role of sulfur metabolism and H_2S production in reactive oxygen species resistance (Chapter 3).

Staphylococcus aureus can also survive in the presence of host derived NO by fermenting glucose, and using lactate dehydrogenase to maintain redox balance through NAD^+ regeneration (Richardson et al., 2008, Spahich et al., 2016).

1.9.4 Survival in host cytosol

Staphylococcus aureus is able to not only survive in the phagosome but also escape and then survive in the host cytosol (Kubica et al., 2008, Fraunholz and Sinha, 2012). This enables *S. aureus* to remain hidden from the host immune system and also avoid the harsh environment of the host phagosome. However, in order to survive in the host cytoplasm *S. aureus* must adapt to a very different environment to that of the host phagosome. Particularly, in direct contrast to the oxidative stress imposed by the phagosome the host cytosol is an environment with a low redox potential (Chapter 4). It also appears that this environment promotes the formation of small colony variants (SCVs) (Vesga et al., 1996). SCVs are slow growing with an increased expression of cell surface proteins and a decreased expression of virulence factors and toxins (Proctor et al., 2006). This enables them to effectively avoid the actions of host immunity and antibiotics, contributing to persistent and recurring infections (Proctor et al., 1995) (Chapter 4).

1.10 Areas of investigation

As is evident from the review of the characteristics of *S. aureus* and its interactions with its host provided above it is clear that *S. aureus* is an important pathogen that utilises a multitude of mechanisms allowing it to adapt to host environments. It is increasingly clear that intracellular survival is an important part of the ability of *S. aureus* too cause persistent infections. The starting point for this work was to investigate further the ability of *S. aureus* to survive inside host phagosomes, an environment with high levels of oxidative stress. In particular the claim that H_2S is a universal defence against ROS and bactericidal antibiotics (Shatalin et al., 2011) was examined (Chapter 3). *Staphylococcus aureus* is also able to also survive in the low redox environment of host cytosol. As such, the next objective was to investigate the global transcriptional and physiological response of *S. aureus* to a low redox potential environment by perturbing steady-state cultures in order to better understand how *S. aureus* adapts to intracellular survival in the host cytosol and how these adaptations might contribute to the functioning of SCVs and antibiotic resistance (Chapter 4). A transposon mutant library was also utilised in order to supplement these investigations through: identifying genes related to H_2S production, studying gene fitness in relation to low redox potential environments and exploring the links between redox and sulfur metabolism (Chapter 5).

Chapter 2: Materials and methods

2.1 Bacterial media

All media were prepared using distilled water (dH_2O) and sterilised by autoclaving at 120^{o}C and 15 psi for 20 minutes, unless otherwise specified. Solid media were made with 1.5% (w/v) Oxoid agar bacteriological (Agar No. 1), unless otherwise specified.

2.1.1 Rich media

2.1.1.1 Brain heart infusion (BHI) broth

Brain heart infusion (BHI) (Oxoid)	37 g l^{-1}

2.1.1.2 Tryptic soy broth (TSB)

Tryptic soy broth (TSB) (Oxoid)	30 g l^{-1}

2.1.1.3 Luria – Bertani (LB) broth

Tryptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
Sodium chloride (NaCl)	10 g l^{-1}

2.1.1.4 LK broth

Tryptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
Potassium Chloride (KCl)	7 g l^{-1}

2.1.1.5 2 x yeast extract and tryptone (2YT) broth

Tryptone	16 g l^{-1}
Yeast extract	10 g l^{-1}
Sodium chloride (NaCl)	2 g l^{-1}

2.1.1.6 Super optimal broth with catabolite repression (SOC)

Tryptone	20 g l^{-1}
Yeast extract	5 g l^{-1}
Sodium chloride (NaCl)	0.5 g l^{-1}
Potassium chloride (KCl)	0.186 g l^{-1}
Magnesium sulfate	2.408 g l^{-1}
Glucose	3.603 g l^{-1}

SOC medium was sterilised by filtration (0.22 µm pore size)

2.1.1.7 Columbia blood agar

Difco™ Columbia Blood Agar Base EH	39 g l^{-1}
Sterile defibrinated sheep blood	5% (v/v)

The sterile defibrinated sheep blood was added after the Columbia Blood Agar Base had been autoclaved and then cooled to 50°C.

2.1.2 Chemically defined medium (CDM)

Each of the groups listed in Table 2.1 were made up to 1 litre in dH_2O and sterilised separately. They were then added together as follows: 700 ml Group 1, 100 ml Group 2, 1 ml Group 3, 50 ml Group 4, 10 ml Group 5 and 129 ml sterilised dH_2O (Hussain et al., 1991, Lithgow et al., 2004).

Table 2.1 Chemically defined medium (CDM) recipe

Group 1	(mg l^{-1})	**Group 2**	(g l^{-1})
$Na_2HP0_4.2H_20$	7000	Glucose	27†
KH_2PO_4	3000	$MgSO_4.7H_2O$	5
L-Aspartic acid	150		
L- Alanine	100	**Group 3**	(mg l^{-1})
L- Arginine	100	Nicotinic Acid	0.02
L-Cysteine	50*	Biotin	0.4
Glycine	100	D-Pantothenic acid, Ca Salt	0.4
L-Glutamic acid	150	Pyridoxal	0.8
L-Histidine	100	Pyridoxamine dihydrochloride	0.8
L-Isoleucine	150	Riboflavin	0.4
L-Lysine	100	Thiamin hydrochloride	0.4
L-Leucine	150	*(Filter sterilised (22 µm pore size))*	
L-Methionine	100		
L-Phenylalanine	100	**Group 4**	(mg l^{-1})
L-Proline	150	Adenine Sulfate	20
L-Serine	100	Guanine Hydrochloride	20
L-Threonine	150	*(in 0.1M HCl)*	
L-Tryptophan	100		
L-Tyrosine	100	**Group 5**	(mg l^{-1})
L-Valine	150	$CaCl_2.6H_2O$	10
(pH 7.2)		$(NH_4)_2SO_4.FeSO_4.6H_2O$	6
		(in 0.1M HCl)	

*Concentration and form of sulfur source adjusted as indicated in Results sections

†Concentration of glucose reduced to 15 mM in final solution (27 g l^{-1} in stock solution), from 55.5 mM (100 g l^{-1} in stock solution) (Lithgow et al., 2004).

2.2 Antibiotics

All antibiotic solutions were made by dissolving the antibiotics in the solvents at the concentrations listed in Table 2.2. They were then filter sterilised (0.22 µm pore size) and stored at −20°C. For use in solid media, agar was cooled to 50°C before antibiotic was added.

Table 2.2 Antibiotic solutions

Antibiotic	Stock concentration (mg ml^{-1})	Solvent	Working concentration for *S. aureus* (µg ml^{-1})*
Chloramphenicol (Cm)	10	100% ethanol	10
Erythromycin (Ery)	5	100% ethanol	5
Gentamicin (Gen)	50	dH_2O	-
Kanamycin (Kan)	50	dH_2O	50
Lincomycin (Lin)	25	50% (w/v) ethanol	25
Neomycin (Neo)	50	dH_2O	50
Tetracyline (Tet)	5	50% (w/v) ethanol	5

*Unless otherwise stated

2.3 Buffers, solutions and concentrations

2.3.1 Phage Buffer

$MgSO_4$	1 mM
$CaCl_2$	4 mM
Tris-HCl pH 7.8	50 mM
NaCl	100 mM
Gelatin	0.1% (w/v)

2.3.2 Tris base, acetic acid and EDTA (TAE) Buffer (50x)

Tris base	242 g l^{-1}
Glacial acetic acid	0.57% (v/v)
EDTA	0.05 M

50 x stock was diluted 1:50 before use.

2.3.3 Instagene matrix

Chelex resin	6% (w/v)

2.3.4 QIAGEN buffers

2.3.4.1 Buffer P1

Tris-HCl, pH 8	50 mM
EDTA	10 mM
RNase A	100 µg ml^{-1}

2.3.4.2 Buffer P2

NaOH	200 mM
SDS	1% (w/v)

2.3.4.3 Buffer EB

Tris (pH 8.5 with HCl)	10 mM

2.3.4.4 Buffer AE

Tris-Cl	10 mM
EDTA	0.5 mM

2.3.4.5 Buffer AW1, AW2, AE, AL, N3, PB and PE

Supplied in QIAquick kits but with no details provided.

2.3.5 Lysis buffer

Tris-Cl	10 mM
EDTA	1 mM
Triton X-100	0.6% (v/v)

Added immediately before use:

lysozyme	10 mg ml^{-1}
lysostaphin	0.1 mg ml^{-1}

2.3.6 Other stock solutions

Some other stock solutions and concentrations are listed in Table 2.3.

Table 2.3 Stock solutions and concentrations

Solution	Stock concentration	Solvent	Storage
Lysostaphin	5 mg ml^{-1}	20 mM sodium acetate	−20°C
Bovine serum albumin (BSA)	10% (w/v)	PBS	4°C
Hydrogen peroxide (H_2O_2)	50% (w/v)	dH_2O	4°C

2.4 Bacterial strains

2.4.1 Growth and storage

Unless otherwise stated bacterial strains were grown from glycerol stock (−20°C) and cultured onto agar plates (BHI for *S. aureus* strains or LB for *E. coli* strains) containing any relevant antibiotics. For short term storage these plates were kept at 4°C and for longer term storage a single colony from solid medium was used to inoculate 5 ml BHI (with the appropriate antibiotics added) and incubated at 37°C with 250 rpm shaking overnight. In the morning the culture was centrifuged for 10 minutes at 3,380 x*g* and the supernatant discarded. The pellet was resuspended in 1.25 ml LB, 1 ml 80% (v/v) glycerol and any antibiotics. These glycerol stocks were then stored at −20°C. For bacterial growth in liquid medium a single colony from solid medium was used to inoculate 5 ml BHI (with the appropriate antibiotics added) and incubated at 37°C with 250 rpm shaking overnight. Unless otherwise stated, in the morning the culture was diluted 1:50 in 50 ml fresh medium and then cultures were incubated at 37°C with 250 rpm shaking until exponential growth phase was reached (≈ 3 hours).

2.4.2 Bacterial strains and plasmids used in this study

The *S. aureus* (Table 2.4) and *E. coli* (Table 2.5) strains, as well as the plasmids (table 2.7), used in this study are listed below.

Table 2.4 *Staphylococcus aureus* strains used in this study

Strain	Description	Reference
SH1000	Functional *rsbU*$^+$ derivative of *S. aureus* 8325-4	(Horsburgh et al., 2002a)
USA300 Je2	Plasmid cured USA300_FPR3757	(Fey et al., 2013)
J96 (SH1000)	SH1000 Δ*cysM*::Tet^R	(Lithgow et al., 2004)
J96 (Je2)	USA300 Je2 Δ*cysM*::Tet^R	This work
J106 (SH1000)	SH1000 Δ*cysM*::Tet^R pJIM80	(Lithgow et al., 2004)
J108 (SH1000)	SH1000 Δ*cysM*::Tet^R pMK4	(Lithgow et al., 2004)
J116 (SH1000)	SH1000 pMK4	(Lithgow et al., 2004)
NE60 (Je2)	*mccB*::Ery^R	This work - (Fey et al., 2013)

NE591 (Je2)	*metC*::Ery^R	This work - (Fey et al., 2013)
NE697 (Je2)	*mccA*::Ery^R	This work - (Fey et al., 2013)
NE1293 (Je2)	*cymR*::Ery^R	This work - (Fey et al., 2013)
NE100 (Je2)	*SAUSA300_1482*::Ery^R	This work - (Fey et al., 2013)
NE104 (Je2)	*SAUSA300_0641*::Ery^R	This work - (Fey et al., 2013)
NE113 (Je2)	*SAUSA300_1041*::Ery^R	This work - (Fey et al., 2013)
NE153 (Je2)	*SAUSA300_1807*::Ery^R	This work - (Fey et al., 2013)
NE181 (Je2)	*SAUSA300_1936*::Ery^R	This work - (Fey et al., 2013)
NE213 (Je2)	*glyA*::Ery^R	This work - (Fey et al., 2013)
NE231 (Je2)	*SAUSA300_0432*::Ery^R	This work - (Fey et al., 2013)
NE331 (Je2)	*rexA*::Ery^R	This work - (Fey et al., 2013)
NE346 (Je2)	*SAUSA300_1346*::Ery^R	This work - (Fey et al., 2013)
NE42 (Je2)	*SAUSA300_2212*::Ery^R	This work - (Fey et al., 2013)
NE473 (Je2)	*SAUSA300_0013*::Ery^R	This work - (Fey et al., 2013)
NE513 (Je2)	*SAUSA300_2431*::Ery^R	This work - (Fey et al., 2013)
NE525 (Je2)	*SAUSA300_1648*::Ery^R	This work - (Fey et al., 2013)
NE532 (Je2)	*SAUSA300_2473*::Ery^R	This work - (Fey et al., 2013)
NE534 (Je2)	*SAUSA300_1537*::Ery^R	This work - (Fey et al., 2013)
NE68 (Je2)	*SAUSA300_0620*::Ery^R	This work - (Fey et al., 2013)
NE751 (Je2)	*SAUSA300_1693*::Ery^R	This work - (Fey et al., 2013)
NE769 (Je2)	*ctaA*::Ery^R	This work - (Fey et al., 2013)
NE782 (Je2)	*opuCb*::Ery^R	This work - (Fey et al., 2013)
NE788 (Je2)	*trkA*::Ery^R	This work - (Fey et al., 2013)
NE885 (Je2)	*SAUSA300_1182*::Ery^R	This work - (Fey et al., 2013)
NE890 (Je2)	*SAUSA300_2560*::Ery^R	This work - (Fey et al., 2013)
NE98 (Je2)	*SAUSA300_0548*::Ery^R	This work - (Fey et al., 2013)
NE1008 (Je2)	*sdaAB*::Ery^R	This work - (Fey et al., 2013)
NE1015 (Je2)	*SAUSA300_0305*::Ery^R	This work - (Fey et al., 2013)
NE1162 (Je2)	*SAUSA300_1506*::Ery^R	This work - (Fey et al., 2013)
NE1169 (Je2)	*pepF*::Ery^R	This work - (Fey et al., 2013)
NE1219	*SAUSA300_2486*::Ery^R	This work - (Fey et al., 2013)

NE1222 (Je2)	*SAUSA300_1976*::Ery^R	This work - (Fey et al., 2013)
NE1281 (Je2)	*SAUSA300_0725*::Ery^R	This work - (Fey et al., 2013)
NE1334 (Je2)	*SAUSA300_1494*::Ery^R	This work - (Fey et al., 2013)
NE1399 (Je2)	*hlgA*::Ery^R	This work - (Fey et al., 2013)
NE1403 (Je2)	*SAUSA300_1585*::Ery^R	This work - (Fey et al., 2013)
NE1443 (Je2)	*SAUSA300_1747*::Ery^R	This work - (Fey et al., 2013)
NE1444 (Je2)	*crtM*::Ery^R	This work - (Fey et al., 2013)
NE1446 (Je2)	*SAUSA300_1141*::Ery^R	This work - (Fey et al., 2013)
NE1448 (Je2)	*SAUSA300_1947*::Ery^R	This work - (Fey et al., 2013)
NE1472 (Je2)	*rsbW*::Ery^R	This work - (Fey et al., 2013)
NE1538 (Je2)	*SAUSA300_2502*::Ery^R	This work - (Fey et al., 2013)
NE1557 (Je2)	*SAUSA300_2252*::Ery^R	This work - (Fey et al., 2013)
NE1614 (Je2)	*SAUSA300_2325*::Ery^R	This work - (Fey et al., 2013)
NE1698 (Je2)	*SAUSA300_1449*::Ery^R	This work - (Fey et al., 2013)
NE1827 (Je2)	*SAUSA300_0011*::Ery^R	This work - (Fey et al., 2013)

Tet^R – Tetracyline resistance cassette. Ery^R – erythromycin resistance cassette. All NE strains contain Tn *Bursa aurealis*.

Table 2.5 *Escherichia coli* strains used in this study

Strain	Description	Reference
K-12 BW25113	Δ(*araD-araB*)567 Δ*lacZ*4787(::*rrnB3*) λ– *rph-1* Δ(*rhaD-rhaB*)568 *hsd*R514	(Baba et al., 2006)
JRG_6031	Δ*cysM*::Kan^R	(Baba et al., 2006)

Kan^R – Kanamycin resistance cassette

Table 2.6 Plasmids used in this study

Plasmid	Description	Reference
pMK4	*E. coli – S. aureus* Cat^R shuttle vector	(Lithgow et al., 2004)
pJIM80	*cysM* locus in pMK4 for complementation	(Lithgow et al., 2004)

Cat^R – Chloramphenicol resistance cassette

2.4.3 The Nebraska Transposon Mutant Library (NTML)

The Nebraska Transposon Mutant Library (NTML) consists of 1,952 *S. aureus* USA300 LAC Je2 transposon insertion (Ery) mutants. It was stored in 20 96-well plates (containing TSB with 5 μg ml^{-1} Ery and 25 μg ml^{-1} Lin and 20% (v/v) glycerol) at −80°C. For growth and H_2S production experiments the NTML was thawed on ice and inoculated into two new sets of 96-well plates using a 96-pin replicator. The first set of plates contained fresh TSB medium (with 5 μg ml^{-1} Ery and 25 μg ml^{-1} Lin) and the other contained TSB, BHI or CDM and any supplements as specified for the relevant experiment. Both plates were incubated at 37°C for 24 hours. Glycerol was added to the first set of plates and they were stored at −80°C. The second set of plates was used for further experiments and measurements as specified.

2.5 Bacterial cell density

2.5.1 Spectrophotometric measurement (OD_{600})

To quantify bacterial growth, in term of optical density, in liquid medium spectrophotometric measurements were taken at a wavelength of 600 nm (OD_{600}) using an ATI Unicam UV/VIS spectrometer. Culture samples were diluted, when necessary, in the appropriate sterile medium.

2.5.2 Direct cell counts (CFU ml^{-1})

To quantify viable bacterial cell numbers bacterial suspensions were serially diluted 1:10 in PBS in triplicate and then 10 μl of each dilution was spotted onto BHI agar, unless otherwise stated. The agar plates were then dried and incubated at 37°C overnight and the number of colony forming units (CFUs) was counted the following day.

2.6 DNA purification

2.6.1 Genomic DNA purification

2.6.1.1 InstaGene™ Matrix (Bio-Rad)

For the quick isolation of genomic DNA from isolated bacterial colonies InstaGene™ Matrix (BIO-RAD) was used with the following protocol. An isolated bacterial colony was mixed by vortex in 1 ml of autoclaved water and centrifuged for 1 minute at 12,000 rpm. The pellet was then resuspended in 200 µl of InstaGene matrix and incubated at 56°C for 30 minutes. It was then mixed by vortex and heated to 100°C for 8 minutes. Finally, the suspension was mixed by vortex again and centrifuged at 12,000 rpm for 3 minutes. 20 µl of the resulting supernatant was used per 50 µl PCR reaction and any remainder was stored at −20°C.

2.6.1.2 DNAeasy® Blood and Tissue Kit (QIAGEN)

For the isolation of higher quality DNA, the DNAeasy® Blood and Tissue Kit (QIAGEN) was used with a modified protocol as described here. Firstly, a 3 ml overnight culture of the relevant strain was prepared and in the morning the cell density was measured and approximately 2 x 10^9 cells were centrifuged at 7500 rpm for 10 minutes. The cells were then resuspended in 180 µl of lysis buffer and incubated at 37^{o}C for 30 minutes. Next 25 µl proteinase K and 200 µl Buffer AL were added, mixed by vortex, and incubated at 56^{o}C for 30 minutes. Then 200 µl ethanol was added, and again the tube was mixed by vortex, before being placed into a DNAeasy Mini spin column, with 2 ml collection tube, and centrifuged at 8000 rpm for 1 minute. A new 2 ml collection tube was then used and 500 µl Buffer AW1 added before being centrifuged at 8000 rpm for a further minute. A final 2 ml collection tube was then used and the 500 µl Buffer AW2 added before being centrifuged at 14,000 rpm for 3 minutes to wash the DNAeasy membrane. In order to elute the DNA a 1.5 ml microcentrifuge tube was used and 200 µl buffer AE added to the DNAeasy membrane. The column was then left to incubate at room temperature for 1 minute before being centrifuged at 8000 rpm for 1 minute and the column being discarded.

2.6.2 Plasmid purification

Small scale plasmid purification was performed using the QIAprep™ Spin Miniprep Kit (QIAGEN). First, 3 ml of cells were grown overnight and centrifuged at 10,000 rpm for 1 minute and resuspended in 250 µl Buffer P1 with RNase A. 250 µl Buffer P2 was added in order to lysis the cells and the tube was inverted 5 times. After approximately 4 minutes 350 µl of Buffer N3 was added, to neutralise the lysis reaction, and the tube was inverted 5 times. The resulting suspension was centrifuged at 13,000 rpm for 10 minutes and the supernatant was transferred to a QIAprep spin column where it was centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and 750 µl buffer PE was added and centrifuged again at 13,000 rpm for 1 minute to wash the column. Then, after the supernatant had been discarded, the column was centrifuged again at 13,000 rpm for 1 minute to remove any residual buffers. To elute the DNA, the column was placed in a 1.5 ml microcentrifuge tube, 50 µl EB buffer was added to the centre of the membrane and the column was incubated for 1 minute followed by centrifugation at 13,000 rpm for 1 minute.

2.6.3 PCR purification

For the purification of DNA fragments the QIAquick spin column (QIAGEN) was used. Initially 5 parts Buffer PB were mixed with 1 part DNA sample and transferred into a QIAquick spin column, before being centrifuged at 13,000 rpm for 1 minute. The supernatant was then discarded and 750 µl Buffer PE added before being centrifuged at 13,000 rpm for 1 minute to wash the column, the supernatant was discarded again and the column was centrifuged at 13,000 rpm for a further minute to remove residual buffer. To elute the DNA the column was placed in a 1.5 ml microcentrifuge tube, 30 µl buffer EB was added to the column and it was incubated for 1 minute before being centrifuged at 13,000 rpm for 1 minute.

2.7 DNA *in-vitro* manipulation techniques

2.7.1 Polymerase chain reaction (PCR)

2.7.1.1 Primer design

A list of the primers used in this study can be found in Table 2.7. All primers were synthesized by Eurofins Scientific and stored in 100 µl stock solutions at −20^{o}C. Primers were designed and analysed using the NCBI Primer-Blast design tool at www.ncbi.nlm.nih.gov/tools/primer-blast and synthesised by Eurofins.

Table 2.7 Primers used in this study

Primer	Sequence 5' – 3'	Source
cymR_F	TCGTCTGCCATAGTGAAACCT	This work
cymR_R	TTTTGGGCGCATTCTCGATG	This work
mccB_F	TCTGAAGGTACGATTGTTGTCGT	This work
mccB_R	ACCGTGTTCTTTCGCAATTTCA	This work
metC_F	AGGTGATTGACCTAACGGTGT	This work
metC_R	TTCAGCTAACCCACCACTCT	This work
mccA_F	AAGTATGCGATGCCTGTCGT	This work
mccA_R	TCGCTGCAATAGCTAACCCT	This work
cysM_F	CGTGTTGCTAGAAAGTTTAGCGT	This work
cysM_R	TGTTTTAGTCATGTTAACCACCCT	This work
QRTPCR_mccA_F	GATGGGCGCTTCACCACT	This work
QRTPCR_mccA_R	GCCATCGCCGTACTTCCA	This work
QRTPCR_mccB_F	GGCGGTACTTATCGCGCA	This work
QRTPCR_mccB_R	TGTTGGGCGTATCGCTTGT	This work
QRTPCR_gyrB_F	ACGGATAACGGACGTGGT	This work
QRTPCR_gyrB_R	GTATCCGCCACCGCCAAA	This work

2.7.1.2 DNA amplification

DNA amplification was carried out using MyTaq™ Mix and a TC-3000 thermal cycler (Techne). MyTaq™ Mix (Bioline) is a two times concentrated master mix containing

MyTaq polymerase, MyTaq buffer, deoxynucleotides (dNTPs) and $MgCl_2$ at optimised concentrations. Reactions using MyTaq™ Mix were assembled in sterile thin-walled 0.5ml PCR tubes and contained:

MyTaq™ Mix	25 µl
Forward Primer	400 nM
Reverse Primer	400 nM
Template DNA	≈ 200 ng
Sterile RNase and DNase free water	make total volume up to 50 µl

Reaction conditions consisted of:

Initial Denaturation	95°C	1 minute

The following amplification steps repeated 30 times:

Denaturation	95°C	30 seconds
Annealing	40-65°C*	15 seconds
Extension	72°C	30 seconds per kb

And one concluding step:

Final Extension	72°C	5 minutes

Reaction products were then stored at −20°C

*Annealing temperature dependent on primers

2.7.1.3 Agarose gel electrophoresis

Agarose (500 mg) was dissolved in 50 ml TAE buffer and heated to form a 1% (w/v) agarose gel and 5 µl of 10,000 x concentrated GelRed (Biotium) was added to facilitate the visualisation of DNA. The gel was then set in a gel tray and submerged in an electrophoresis tank (Flowgen Bioscience) containing TAE buffer. DNA samples (8 µl) were mixed with 2 µl GelPilot DNA loading Dye 5x (QIAGEN) and then loaded into the agarose gel along with a DNA ladder (Hyperladder™ 1kb plus, Bioline). The DNA was electrophoresed at 100 volts for 60 minutes and then visualised using a UV transilluminater at 260 nm.

2.7.2 Restriction endonuclease digestion

Restriction enzymes were used according to the manufacturer's instructions (Promega, or New England Biolabs). The supplied buffers were used and the recommended temperature and incubation periods followed. For products that were used for further cloning the enzymes were incubated at 80^{o}C for 20 minutes.

2.7.3 DNA concentration

A Nanodrop™ (ND-1000 Spectrophotometer, Peqlab Ltd) on dsDNA mode was used to determine DNA concentration. Using 2 µl of sample for each measurement.

2.8 Transduction

2.8.1 Preparation of phage lysate using bacteriophage Φ11

A 5 ml overnight culture of donor strain was used to inoculate 5 ml BHI to an OD_{600} of 0.2 in a 30 ml universal. Phage buffer (5 ml) was then added along with 300 µl of stock lysate of bacteriophage Φ11 (Bacteriophage Φ11 is a *S. aureus* specific, temperate transducing phage of serological group B that has a genome of around 45 kb and requires Ca^{2+} ions in order to maintain infectivity (Novick, 1991)). After overnight incubation at room temperature, or until the mixture cleared, the lysate was then sterilized by filtration (0.22 µm pore size) and stored at 4^{o}C.

2.8.2 Phage transduction using bacteriophage Φ11

The recipient bacteria were grown from an isolated colony in 50 ml LK overnight, centrifuged at 5000 rpm for 10 minutes and then resuspended in 2 ml LK. An aliquot of 500 µl of these cells were then mixed with 500 µl of phage lysate and 1 ml LK containing 10 mM $CaCl_2$. A control sample with no phage lysate was also made and the two mixtures were incubated at 37^{o}C for 25 minutes without shaking followed by 15 minutes with shaking. Next 1 ml of ice-cold 0.02 M sodium citrate was added to the mixtures and they were incubated on ice for 5 minutes. They were then centrifuged at 10,000 rpm for 5 minutes and the cells were resuspended in 1 ml 0.02 M sodium citrate before a 1 hour incubation on ice. Finally, cells were spread on LK agar plates, containing 0.05% (w/v) sodium citrate and the appropriate antibiotics, and incubated

at 37^{o}C for 48 hours.

2.9 Isolation of RNA

2.9.1 Isolation of RNA from stored pellets

RNA sample pellets (Section 2.20.3.3) were resuspended in 1 ml RLT buffer (QIAGEN) with 10 µl ml^{-1} β-mercaptoethanol and transferred on ice to a FastPrep® tube containing Lysing Matrix B (MP Biomedicals). The suspensions were then mixed using a FastPrep®-24 Instrument (MP Biomedicals) at setting 6.5 for 4 x 30 seconds with a 2 minute cooling on ice between mixing. Samples were then centrifuged for 30 seconds at 20,000 x*g* and 800 µl of supernatant was transferred to a microcentrifuge tube containing 800 µl 100% (v/v) ethanol. The samples were then added to a RNAeasy Mini Spin Column (QIAGEN) and centrifuged for 30 seconds at 15,000 x*g*. After the flow through was discarded the samples were washed with 350 µl Buffer RW1 (QIAGEN) and centrifuged for 30 seconds 15,000 x*g*. Then the samples were incubated at room temperature for 15 minutes with 80µl DNase mix (10µl DNase 1 stock solution (QIAGEN) with 70 µl Buffer RDD (QIAGEN)) before being washed four times with 350 µl Buffer RW1, 500 µl Buffer RPE, 700 µl Buffer RW1 and 500 µl Buffer RPE (QIAGEN) with 30 second centrifugation at 15,000 x*g*. RNA samples were then eluted into clean microcentrifuges using 50 µl molecular biology grade water with centrifugation for 1.5 minutes at 20,000 x*g*. Samples are stored at −20^{o}C.

2.9.2 RNA concentration

A Nanodrop™ (ND-1000 Spectrophotometer, Peqlab Ltd) on RNA mode was used to determine RNA concentration. Using a 2 µl of sample for each measurement.

2.10 QRT-PCR

Quantitative real-time polymerase chain reaction (QRT-PCR) was carried out in 96-well plate format using a Stratagene MX3005P (Agilent). Results were normalised against the stably expressed gene *gyrB* (Goerke et al., 2000).

Reactions contained:

2x SYBR® Green QRT-PCR Master Mix (Agilent)	10 µl
RT/RNase block (Agilent)	1 µl
Forward Primer	300 nM
Reverse Primer	300 nM
mRNA	≈ 1.0 ng
Sterile RNase and DNase free water	to make volume up to 20 µl

Reaction conditions consisted of:

Initial annealing	50°C	10 minutes
Initial denaturation	95°C	3 minutes

The following amplification steps repeated 40 times:

Denaturation	95°C	15 seconds
Annealing and elongation	60°C	20 seconds

And one concluding step:

Final denaturation	95°C	2 minutes

2.11 Transcriptomics

2.11.1 Microarray

Microarray analysis was used with RNA samples being labelled with fluorophore Cy5 (all time points) or fluorophore Cy3 (time 0 minutes only, as a reference).

2.11.2 Array design

A custom gene expression microarray was designed using the web based application eArray (www.earray.chem.agilent.com). It contained probes for *S. aureus* USA300_FPR3757 and *S. aureus* N8325 (Agilent Design ID: 066358).

2.11.3 Direct labelling of RNA

RNA labelling as carried out using SuperScript III reverse transcriptase (Invitrogen) in 0.5 ml microcentrifuge tubes. 2.5 µg (0.9 µl of 3 mg ml^{-1} stock) of random primers (Invitrogen) was added to 8 µg of purified RNA (Section 2.9) in 6.15 µl molecular biology grade water. This mixture was incubated for 10 minutes at 72°C and then

placed on ice for 10 minutes. Then 6.25 µl reverse transcription mix was added containing:

5X First strand buffer	3.0 µl
0.1 M DTT	1.5 µl
50x dNTP mix	0.3 µl
(dNTP mix contains 25 mM dATP, dTTP, dGTP and 10 mM dCTP)	
Molecular biology grade water	1.45 µl

This was followed by the addition of 1 µl 1 mM Cy5-dCTP (or Cy3-dCTP for the time 0 minutes reference samples) (GE Healthcare) and 0.75 µl reverse transcriptase (200 U μl^{-1}, SuperScript III). The samples were incubated for 5 minutes at 25^{o}C then overnight at 50^{o}C. In the morning 7.5 µl 0.1 M NaOH was added to the samples and they were incubated for 10 minutes at 72^{o}C, to hydrolyse the RNA, before 7.5 µl of 0.1 M HCl was added to neutralise the alkali. The samples were then cleaned up using PCR purification techniques (Section 2.6.3) and the concentration of cDNA and labelling efficiency was measured using a Nanodrop™ (ND-1000 Spectrophotometer, Peqlab Ltd).

2.11.4 Hybridisation

Hybridisation of array slides and subsequent washing was carried out according to the manufacturer's instructions (Agilent "Two-Color Microarray-Based Prokaryotic Analysis (Faiplay III Labeling) Protocol"). Cy5-labelled cDNA (300 ng) from the investigative sample was hybridised in tandem with 300 ng of Cy3-labelled cDNA from the reference sample (time point 0 minutes). The array slides were incubated at 65^{o}C for 17 hours in an Agilent Hybridisation Oven (Agilent).

2.11.5 Scanning

Arrays were scanned in an Agilent Microarray Scanner (Agilent) and data was extracted from .tif files using Agilent Feature Extraction 11.5 (Agilent).

2.12 Data analysis

2.12.1 Gene expression

Array data was analysed using Agilent GeneSpring 7.3.1 (Agilent). Signal intensity data for each array was divided by the signal obtained from the control channel (Cy3-labelled time point 0 minutes, reference sample) with a median shift being applied across all samples included in each comparison.

2.12.2 Significance

A Student's t-test (2 tailed, equal variance) with Benjamini Hochberg correction was applied to the data and changes in expression were deemed significant if $p < 0.05$ and if fold change ≥ 5.

2.12.3 TFinfer

Open access TFinfer software (Asif et al., 2010) was used to analyse the transcriptional reprogramming by time-resolved transcription profiling. A matrix of genes and associated regulators was constructed for nine *S. aureus* transcription factors (Air, AgrA, CymR, MgrA, NreABC, Rex, Rot, SarA, SarZ, CtsR and CstR) and was used with the raw gene expression data used to generate transcription reprogramming profiles.

2.13 Protein

2.13.1 Protein isolation from stored pellets

Stored pellets for enzyme assays (Section 2.20.3.6) were resuspended in 10 ml 0.02 M PBS and then 1 ml transferred on ice to a FastPrep® tube containing Lysing Matrix B (MP Biomedicals). The suspensions were then mixed using a FastPrep®-24 Instrument (MP Biomedicals) at setting 6 for 3 x 30 seconds with a 5 minute cooling on ice between mixing. The tubes were then centrifuged for 30 seconds at 20,000 x*g* and 800 µl of supernatant was transferred to a microcentrifuge tube.

2.13.2 Protein concentration

Protein concentration was estimated using the Bio-Rad reagent (Bradford, 1976). A protein assay standard curve for a 2 ml reaction volume was used with absorbance measured at 595 nm using a Unicam HEλIOS spectrometer.

2.14 Growth curves

2.14.1 250ml conical flasks

A single colony from solid medium was used to inoculate 5 ml liquid medium (BHI unless otherwise stated) with the appropriate antibiotics added when necessary. The liquid culture was then incubated at 37^{o}C with 250 rpm shaking overnight. In the morning the culture was diluted 1:50 in 50 ml fresh medium and then cultures were incubated for 3 hours at 37^{o}C with 250 rpm shaking. Cultures were then diluted to OD_{600} of 0.05 in 50 ml fresh medium and incubated at 37^{o}C with 250 rpm shaking. Growth was monitored by serial OD_{600} measurements or direct cell counts until stationary phase was reached.

2.14.2 96-well plates

Growth curves were also carried out using a Sunrise™ microplate reader (TECAN.) according to the following protocol. A single colony from solid medium was used to inoculate 5 ml liquid medium (BHI unless otherwise stated) with the appropriate antibiotics added when necessary. The liquid culture was then incubated at 37^{o}C with 250 rpm shaking overnight. In the morning the culture was diluted 1:50 in 50 ml fresh medium and then cultures were incubated for 3 hours at 37^{o}C with 250 rpm shaking. Cultures when then diluted to OD_{600} of 0.0125 in 200 µl fresh medium in a 96 well plate (Thermo Scientific). Samples were then incubated at 37^{o}C in the microplate reader with shaking. The OD_{600} was measured every 15 minutes and recorded automatically using Magellan 7.1 software (TECAN.).

2.15 Hydrogen sulfide (H_2S) detection

2.15.1 Lead acetate paper

Lead acetate paper was prepared by using a 2% w/v lead acetate solution to saturate blotting paper that was then dried and placed above the level of liquid culture, either against the lid of a 96-well plate (Thermo Scientific) or the inner wall of a 250 ml conical flask. Colourless lead (II) acetate reacts with H_2S to form acetic acid and brown insoluble lead (II) sulfide, which is proportional to the concentration of H_2S. The paper was scanned and any colour change quantified using ImageJ.

2.15.2 Fourier transform infrared spectroscopy (FT-IR) detection of H_2S

A single colony from solid medium was used to inoculate 5 ml liquid medium (BHI unless otherwise stated) with the appropriate antibiotics added when necessary. The liquid culture was then incubated at 37^{o}C with 250 rpm shaking overnight. In the morning the culture was diluted 1:50 in 500 ml 2YT medium and incubated at 37^{o}C with 250 rpm shaking for 24 hours. The culture was then centrifuged at 6000 rpm for 15 minutes at 4^{o}C before being resuspended in 4 ml LB. This suspension was then used to produce a 10 ml LB solution diluted to an OD_{600} of 30 and transferred to a three-necked round bottom flask fitted with a stirrer bar. The round bottom flask was then placed in a 37^{o}C water bath and attached to the 'white cell' via an air pump. Scans were then recorded every 10 minutes for approximately 24 hours, with 600 µl 1 M cysteine hydrochloride added after 30 minutes (resolution = 0.4 cm^{-1}, gain = 4, forwards mirror velocity = 1.6 kHz). Scans were then used to produce interferograms using the FFT (Fast Fourier Transform) algorithm in WINFIRST, converted into ASCII tables and absorbance spectra generated using the Beer-Lambert law. Integrals of the curves fitted to the H_2S peaks were compared to the integral of the same peak on a known H_2S spectrum, standardized against a background spectra and then molarities of H_2S present were calculated using Henry's law.

2.16 Growth inhibition zone (GIZ) assay

A single colony from solid medium was used to inoculate 5 ml liquid medium (TSB unless otherwise stated) with the appropriate antibiotics added when necessary. The

liquid culture was then incubated at 37°C with 250 rpm shaking overnight. In the morning the culture was diluted 1:50 in 20 ml of 50°C molten medium. It was then poured on 20 ml solid medium and left to cool for 5 minutes before a sterile 6 mm filter paper disc was placed on top. Once the sterile 6 mm filter paper disc was placed on top of the agar 10 µl of the relevant antibiotic or other solution was added to the disc. The plates were then incubated at 37°C overnight and the growth inhibition zone was measured in the morning to an accuracy of 0.05 mm.

2.17 Minimum inhibitory concentration (MIC)

2.17.1 25ml universals

A single colony from solid medium was used to inoculate 5 ml liquid medium (BHI unless otherwise stated) with the appropriate antibiotics added when necessary. The liquid culture was then incubated at 37°C with 250 rpm shaking overnight. In the morning the culture was diluted to OD_{600} 0.05 and added to a series of universals containing a 5 ml serial dilution (1:2) of the appropriate antibiotic or chemical in medium (BHI, TSB or CDM as specified). These cultures were then incubated for 24 hours at 37°C with 250 rpm shaking and then the OD_{600} was measured to determine the minimum concentration of antibiotic or chemical required to prevent growth.

2.17.2 96-well plate

MIC was also determined using 96-well plates and a Sunrise™ microplate reader (TECAN.) according to the following protocol. A single colony from solid medium was used to inoculate 5 ml liquid medium (TSB unless otherwise stated) with the appropriate antibiotics added when necessary. The liquid culture was then incubated at 37°C with 250 rpm shaking overnight. In the morning the culture was diluted to OD_{600} 0.0125 and added to a series of wells in a 96-well plate containing a 120µl serial dilution (1:6) of the appropriate antibiotic or chemical in medium (BHI, TSB or CDM as specified). These cultures were then incubated for 24 hours at 37°C with 350 rpm shaking. The OD_{600} was then measured using a Sunrise™ microplate reader (TECAN.) and recorded automatically using Magellan 7.1 software (TECAN.) in order to

determine the minimum concentration of antibiotic or chemical required to prevent growth.

2.18 Survival curves

A single colony from solid medium was used to inoculate 5 ml liquid medium (BHI). The liquid culture was then incubated at 37^{o}C with 250 rpm shaking overnight. In the morning the culture was diluted to OD_{600} of 0.05 in 50 ml fresh medium (CDM containing the appropriate sole sulfur source) and then cultures were incubated for 1.5 hours at 37^{o}C with 250 rpm shaking. OD_{600} was measured and the antibiotics or chemicals were added at their stated concentrations and OD_{600} was then measured every hour. Samples were taken at each time point and serially diluted in order to measure colony forming units (CFU) (Section 2.5.2).

2.19 Small colony variant (SCV) detection

2.19.1 Solid medium

A single colony from solid medium was used to inoculate 5 ml liquid medium (TSB) and then incubated overnight at 37^{o}C with 250 rpm shaking. This culture was then used to inoculate 50 ml fresh medium (TSB) to OD_{600} 0.05 and then incubated for 3 hours at 37^{o}C with 250 rpm shaking. Then the chemical being tested was added and the culture incubated for a further hour at 37^{o}C with 250 rpm shaking. Then the culture was serially diluted 1:10 in triplicate and 10 µl spotted onto plates with/without DTT. These plates consisted of TSA plates and Columbia blood agar plates +/- 2 µg ml^{-1} gentamicin. After 48 hours the CFU were counted and the SCV were counted as small (≈ 10th size of WT colonies), light coloured (white or pale yellow as compared to orange WT colonies), gentamicin resistant colonies that produce no zone of haemolysis (Edwards, 2012, Painter et al., 2015).

2.19.2 Continuous culture

Steady state cultures were produced as for other experiments (Section 2.20) and 5 ml culture samples were taken at 0, 5, 30, 60 and 1440 minutes following the addition of

DTT. These samples were serially diluted 1:10 in triplicate and 10 µl spotted onto TSA plates and Columbia blood agar plates +/- 2 µg ml^{-1} gentamicin. After 48 hours the CFU were counted and the SCV were counted as small (≈ 10^{th} size of WT colonies), light coloured (white or pale yellow as compared to orange WT colonies), gentamicin resistant colonies that produce no zone of haemolysis (Edwards, 2012, Painter et al., 2015).

2.19.3 SCV stability assay

SCV colonies (≈ 10 colonies for each repeat) were subcultured onto TSA plates and incubated for 48 hours at 37^{o}C. Then the subcultured colonies were deemed to have either reverted to WT (large orange colonies), to have become stable mutants (remain small white colonies) or be partially stable (a mix between the two phenotypes) (Edwards, 2012, Painter et al., 2015).

2.20 Continuous culture

2.20.1 Culture conditions

A single colony from solid medium was used to inoculate 5 ml liquid medium (BHI) and then incubated overnight at 37^{o}C with 250 rpm shaking. This culture was then injected into a 2-litre Labfors chemostat vessel (Infors-HT, Switzerland) containing 1 litre of CDM medium with 15 mM glucose (unless otherwise stated) and 0.0001% (v/v) Antifoam Y-30 Emulsion (Sigma) to prevent foam and biofilm formation at the air surface interface. The culture then grew in batch for 5−20 hours before being switched to a steady state with a dilution rate of 0.2 h^{-1}. The cultures were maintained aerobically with the constant addition of filtered air at a rate of 1 l min^{-1} and constant agitation via a 400 rpm stirrer. The temperature was maintained at 37^{o}C and the pH was maintained at 7.0 throughout the experiments.

2.20.2 Direct measurements

2.20.2.1 Dissolved oxygen

Dissolved oxygen concentration was measured throughout the continuous culture experiments using a OxyProbe® polarographic dissolved oxygen sensor (Broadly

James®) according to the manufacturess instructions. One hundred percent dissolved oxygen was set by 1 l min^{-1} air flow and 400 rpm stirring and 0% oxygen was set at 1 l min^{-1} nitrogen and 400 rpm stirring.

2.20.2.2 Redox potential

Redox (Oxidation-reduction potential - ORP) was measured throughout the continuous culture experiments using an ORP FermProbe® (Broadly James®) according to the manufacturers' instructions. A redox potential of 86 mV was set by using a pH 7.0 Quinhydrone buffered solution and a redox potential of 263 mV was set by using a pH 4.0 Quinhydrone buffered solution. The potential difference between sample and a standard hydrogen electrode (E_h) can be calculated from the voltage observed with the ORP FermProbe® (E) by adding the standard potential of the reference electrode (E_R) to it. At 37°C this is 193 mV and so E_h can be calculated very simply (Equation 2.1).

Equation 2.1

$$E_h = E + E_R$$

$$E_h = E + 193\ (37^oC)$$

2.20.2.3 pH

pH was measured throughout the continuous culture experiments using a FermProbe® pH electrode (Broadly James®) according to the manufactures' instructions. The electrode was calibrated using pH 4.0 and pH 7.0 buffered solutions.

2.20.3 Sampling

Samples were taken at 0, 2, 5, 10, 15, 30, 60 and 1440 minutes following the addition of DTT, unless otherwise stated. Before any samples were taken, at each time point, 5 ml of culture was collected and discarded in order to purge the collection tubing of any older material.

2.20.3.1 Bacterial density (OD_{600})

Samples used for determining the bacterial density (OD_{600}) were collected by taking a 1 ml culture sample on ice and absorbance measurements were taken at OD_{600} using an

ATI Unicam UV/VIS spectrometer. Culture samples were diluted, when necessary, in the appropriate sterile medium (CDM).

2.20.3.2 CFU and SCV assay

Samples used for determining the viable cell count (Colony forming units – CFUs) and the number of small colony variants (SCVs) were collected, at 0, 5, 30, 60 and 1440 minutes following addition of DTT, by the taking of a 5 ml culture sample on ice. The culture sample was then serially diluted 1:10 in PBS, in triplicate and 10 µl of each sample added to the appropriate solid medium (Section 2.19.2)

2.20.3.3 RNA

Samples for RNA isolation were collected by taking a 5 ml culture sample and adding it directly to 10 ml of RNAprotect Bacteria Reagent (QIAgen). This was immediately vortexed for 5 seconds and then incubated at room temperature for 5 minutes before being centrifuged in a cooled centrifuge (4^{o}C) for 10 minutes at 3,380 x*g.* The supernatant was then poured off and the pellet resuspended in the residual solution. The resuspended sample was then transferred to a 1.5 ml microcentrifuge tube and centrifuged for 10 minutes at 20,000 x*g* before removing the supernatant and storing the pellet at −80^{o}C.

2.20.3.4 Protein

Samples for protein isolation were collected by taking a 5 ml culture sample and adding it directly to 20 µl of chloramphenicol solution (25 mg ml^{-1}) on ice. Four 500 µl aliquots were collected in microcentrifuge tubes and centrifuged for 3 minutes at 10,000 x*g*. The supernatant was removed and the pellet stored at −80^{o}C.

2.20.3.5 Supernatant

Samples of supernatant were collected by taking a 5 ml culture sample and centrifuging immediately in a cooled centrifuge (4^{o}C) for 10 minutes at 3,380 x*g*. The supernatant was then filtered using a single use 0.22 µm syringe filter and two 1.5 ml samples were stored in microcentrifuge tubes at −80^{o}C.

2.20.3.6 Enzyme assay samples

Samples for enzyme assays were collected at 0, 60 and 1440 minutes following the addition of DTT. Two 50 ml culture samples were collected in two 50 ml Falcon tubes and centrifuged in a cooled centrifuge (4^{o}C) for 10 minutes at 3,380 x*g*. The supernatant was then poured off and the samples stored at −80^{o}C.

2.20.3.7 Cell dry weight

Samples used for determining cell dry weight were taken at 0, 60 and 1440 minutes following the addition of DTT. Two 10 ml culture samples were collected in two 15 ml falcon tubes that had previously been weighed (along with two negative control falcon tubes) to a milligram degree of accuracy. The tubes were then centrifuged for 10 minutes at 3,380 x*g*. The supernatant was then poured off and the four tubes placed at 60^{o}C overnight to dry. The following day the tubes were all re-weighed; weight change of tubes containing sample was equivalent to cell dry weight after addition of observed weight loss due to heating of the two negative control tubes.

2.20.4 Addition of DTT

In continuous culture experiments where DTT (dithiothreitol) was added, a final concentration of 9.7 mM (1.5 mg ml^{-1}) was used. 1.5 g DTT was dissolved in 20 ml CDM (concentration 486 mM) and injected directly into the 1 litre culture medium at the same time that the feed supply was switched from CDM with 0mM DTT to one of CDM with 9.7 mM DTT.

2.21 NMR spectroscopy

1D – NMR (Nuclear Magnetic Resonance) spectroscopy was carried out on continuous culture supernatant samples (Section 2.20.3.5) in order to assess extracellular metabolite concentrations. Filtered (0.22 µm pore size) supernatant (450 µl) was mixed with 50 µl D_2O and 1 mM (final concentration) trimethylsilyl propanoic acid (TSP) as a standard in 5 mm NMR tubes. A Bruker DRX500 (Bruker UK) spectrometer at 298 K with 5 mm TXI probe, tuned to 1H at 500 MHz was used to acquire the spectra.

The spectra were processed and peaks quantified by integration using Topspin (Bruker UK) and metabolite concentrations were measured relative to the TSP standard.

2.22 Lactate dehydrogenase assay

Protein samples (190µl ≈ 100 ng ml^{-1}) (Sections 2.20.3.6 and 2.13.1) were added to three 2 ml cuvettes containing:

	Tube 1	Tube 2	Tube 3
Tris HCl 50 mM (pH 7.9)	700 µl	700 µl	700 µl
$MnSO_4$ 20 mM	50 µl	50 µl	50 µl
NADH 15 mM	0 µl	10 µl	10 µl
dH_2O	60 µl	0 µl	0 µl

Tube 1 was used to zero the spectrophotometer (absorbance at 340 nm). Then 50 µl dH_2O was added to Tube 2, or 50 µl pyruvate (100 mM) was added to Tube 3, and the absorbance at 340 nm measured over time. Then the specific activity of lactate dehydrogenase was calculated in µmol mg hr^{-2}.

2.23 Oxygen electrode

2.23.1 Clark-type oxygen electrode

Respiration was measured using a Clark-type oxygen electrode (Rank Bros Ltd). This consists of a chamber separated from an electrode, operating at a polarising voltage of 0.6 V, by an oxygen-permeable Teflon membrane. As the oxygen concentration was reduced at the cathode it created a potential difference, which was recorded using LabTrax-4 (World Precision Instruments) and LabScribe2 software (World Precision Instruments). The chamber temperature was maintained at 37^{o}C and stirred at a constant rate (setting 4). Calibration was to a 100% oxygen saturated buffer and 0% was determined by the addition of sodium dithionate.

2.23.2 Sample preparation

A single colony from solid medium was used to inoculate 50 ml liquid medium (TSB). The liquid culture was then incubated at 37^{o}C with 250 rpm shaking overnight. In the

morning the stationary phase culture was used or the culture was diluted 1:50 in 50 ml fresh medium incubated for 3 hours at 37^{o}C with 250 rpm shaking to produce a log phase culture. These cultures were then centrifuged in a cooled centrifuge (4^{o}C) for 10 minutes at 3,380 x*g* and the supernatant discarded. The cell pellet was then resuspended in pre-cooled 0.02 M PBS and centrifuged in a cooled centrifuge (4^{o}C) for 10 minutes at 3,380 x*g* twice before the resulting washed cell pellet was resuspended in pre-cooled 0.02 M PBS to a final cell density (OD_{600}) of 50 and put on ice.

2.23.3 Experimental procedure

Phosphate buffered saline (1950 µl 0.02 M) was added to the electrode chamber and the system was left for 15 minutes to allow the oxygen concentration to stabilise. The next 50 µl of sample (Section 2.23.2) was added to the chamber and the system was allowed to stabilise for 5 minutes before sealing the chamber with the lid. Then, after a further 5 minutes, 50 µl 1 M glucose solution was injected to begin respiration. After 1 minute either a control of 50 µl 0.02 M PBS was added or 50 µl of test compound. Respiration rates were calculated as nmolO_2 min OD_{600}^{-2}.

Chapter 3: Hydrogen sulfide: defence against ROS and antibiotics?

3.1 Introduction

3.1.1 Hydrogen sulfide as a gasotransmitter

Hydrogen sulfide (H_2S) is one of three small gaseous molecules, along with nitric oxide (NO) and carbon monoxide (CO), that have key signalling and regulatory roles in higher eukaryotes. When present at high concentrations all three gases are toxic; they inhibit oxygen consumption by cytochrome oxidase (Cooper and Brown, 2008). However, at lower concentrations they have a number of beneficial biological functions. For example NO acts as a messenger that causes vascular muscle to relax (Murad, 1999) and is involved in the destruction of macrophage-engulfed pathogens (Fang, 2004), and CO has regulatory effects in the cardiovascular system and also has a role as a neurotransmitter (Motterlini and Otterbein, 2010). H_2S has similar roles in nervous, cardiovascular and metabolic signalling (Wang, 2003, Liu et al., 2011) and it has been suggested that its presence in blood plasma may be involved with the protection of endothelium cells from oxidative stress (Bearden et al., 2010).

3.1.2 Gasotransmitters and bacteria

NO, CO and H_2S are also prominent in bacterial metabolism. NO is an intermediate product in the denitrification process (Lundberg et al., 2004), CO can be used as an alternative carbon source (King and Weber, 2007) and H_2S is a by-product of sulfate respiration in sulfate-reducing bacteria (Muyzer and Stams, 2008). Furthermore, there have been signalling type roles proposed for bacterial derived gasotransmitters. For example, NO and H_2S seem to have a role in the formation and dispersal of biofilms (Soutourina et al., 2009, Di Palma et al., 2013). One of the most interesting proposals is the suggested role that NO and H_2S may have in offering resistance to oxidative stress and certain classes of antibiotics (Gusarov and Nudler, 2005, Shatalin et al., 2008, Shatalin et al., 2011).

3.1.3 Hydrogen sulfide as a defence against oxidative stress in bacteria

It has been claimed that H_2S may be part of a universal bacterial defence mechanism against oxidative stress, and certain classes of antibiotics (Shatalin et al., 2011). Experiments supporting this hypothesis have been carried out in four genetically divergent bacterial species: *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus anthracis* and *Staphylococcus aureus* (Shatalin et al., 2011). Resistance to hydrogen peroxide (H_2O_2) and a range of antibiotics was measured for each species and either compared to mutants lacking the genes responsible for H_2S production or against strains where H_2S production had been chemically inhibited. Genes involved in the production of H_2S were knocked out in *P. aeruginosa* (Δ*cbs*Δ*cse*), *B. anthracis* (Δ*cbs*Δ*cse*) and *E. coli* (Δ*3-mst*) and these mutants were shown to produce measurably less H_2S than wildtype as well as exhibiting lower resistance to H_2O_2 and bactericidal antibiotics. However, no equivalent mutant was produced for *S. aureus.* Instead H_2S production was inhibited chemically by using aminooxyacetate (AOA) and propargylglycine (PAG), inhibitors of CSE and CBS respectively (Shatalin et al., 2011). Use of AOA and PAG did result in lower H_2S production by *S. aureus* and there was also decreased resistance to H_2O_2 and bactericidal antibiotics (Shatalin et al., 2011). However, AOA and PAG are poorly absorbed by cells and are relatively non-specific in their mode of action (Whiteman et al., 2011) making the results less convincing for *S. aureus*. As such, further studies into the role of H_2S in *S. aureus* and it's potential to protect the bacterium from oxidative stress were deemed to be needed.

3.1.4 Hydrogen sulfide biosynthesis

There are three principal enzymes, conserved across mammalian and microbial systems, that are involved in H_2S production: cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) (Shatalin et al., 2011, Kashfi and Olson, 2013). CBS and CSE produce H_2S from homocysteine and cysteine via beta-elimination reactions (Stipanuk, 2004). Whereas, 3-MST is the rate limiting step in a different cysteine to H_2S production pathway. Firstly, cysteine is converted into 3-mercaptopyruvate by cysteine amino transferase and then 3-MST converts the 3-mercaptopyruvate into 3-mercaptopyruvate-SSH, which is subsequently reduced by abundant thioredoxin and dihydrolipoic acid to

produce H_2S (Mikami et al., 2011). Homologs of CBS, CSE and 3-MST are found in many different bacteria but usually not all three in the same species. For example, *E. coli* only has a homolog of 3-MST, and no CBS or CSE homologs, whereas *S. aureus* does not appear to have a 3-MST homolog but does seem to have CBS and CSE homologs (Soutourina et al., 2009, Shatalin et al., 2011). In *S. aureus* the CBS and CSE homologs are known as *mccA* and *mccB* respectively and found together in the *mccAB* operon (Soutourina et al., 2009).

3.1.5 H_2S and cysteine metabolism in *S. aureus*

Production of H_2S is part of a complex network of enzymes regulated by the cysteine master regulator CymR (Figure 1.1) (Soutourina et al., 2009). This network includes other genes directly regulated by CymR including cysteine and methionine transporters (MetNPQ2, TcyP and TcyABC) as well as CysM which converts H_2S back into cysteine, and MetC, which has also been proposed to have a H_2S production function in *S. aureus* (Chang et al., 2006, Wolf et al., 2008). CymR acts as a transcriptional repressor for each of it's target genes and is sensitive to oxidative stress (Ji et al., 2012). It has also been suggested that CymR may form a regulatory complex with CysM in the same manner as the related proteins in *Bacillus subtilis* (Tanous et al., 2008, Soutourina et al., 2010). The role of CysM in oxidative stress has been previously investigated and this revealed that a *cysM* mutant is more sensitive to tellurite than wildtype *S. aureus* (Lithgow et al., 2004). However, the links between cysteine metabolism, H_2S, oxidative stress and antibiotic resistance are still not fully described.

3.2 Aims

The previous work summarised above led to the following research questions:

- Is the claim that H_2S is a universal defence against oxidative stress and antibiotics valid for a clinically relevant strain of *S. aureus*?
- What is the link between cysteine metabolism, H_2S production and oxidative and antibiotic stress resistance?

3.3 Results

3.3.1 Effect of excess cysteine on growth and H_2S production

Previous H_2S production experiments in bacteria were carried out with excess cysteine added to the medium (Shatalin et al., 2011). In order to investigate the effect this excess cysteine may have on growth and H_2S production *S. aureus* Je2 growth curves in the presence of different concentrations of cysteine were carried out (Figure 3.1). These growth curves showed that a concentration of cysteine of 31.25 μg ml^{-1} slightly increased the growth rate; however, concentrations above this had no positive effect on growth and once the concentration of cysteine reached 1000 μg ml^{-1} or above growth was slightly inhibited. Previous work used at most 500 μg ml^{-1} cysteine (Shatalin et al., 2011) and so was under the growth inhibition threshold for cysteine. H_2S production was also measured at each concentration of cysteine (Figure 3.1). This showed that cysteine concentrations above 250 μg ml^{-1} resulted in increased H_2S production.

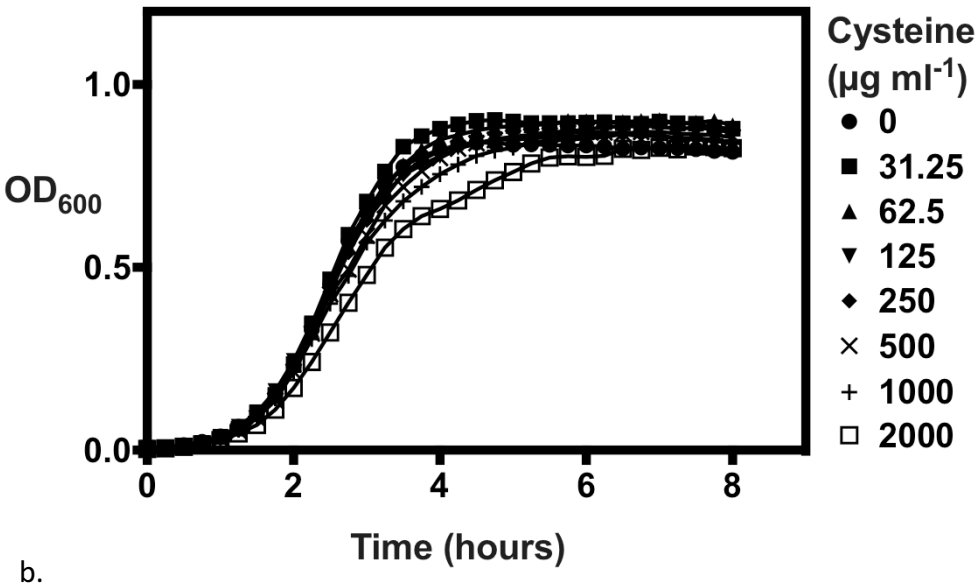
3.3.2 Production of H_2S by *S. aureus* SH1000 and Je2

In order to investigate whether there was any significant difference in the production of H_2S between laboratory and clinical strains, the production of H_2S was measured for the laboratory strain SH1000 (a derivative of 8325-4) and the highly pathogenic CA-MRSA strain USA300 Je2 (Figure 3.2). It was revealed that there was no significant difference in H_2S production between the two strains except at intermediate concentrations of external cysteine (250 μg ml^{-1} – 500 μg ml^{-1}) where the Je2 produced significantly more H_2S than SH1000. Therefore, Je2 was used for the majority of the following experiments in order to increase clinical relevance.

3.3.3 Growth and resistance to H_2O_2 and gentamicin when grown in defined medium with either cysteine or sodium thiosulfate as the sole sulfur source

Use of chemically defined medium (CDM) enabled the production of H_2S to be controlled by switching the sulfur source. Cultures were grown in CDM with either cysteine or sodium thiosulfate as the sole sulfur source at concentrations where growth was comparable (Figure 3.3). Production of H_2S was measured and there was a

a.



b.

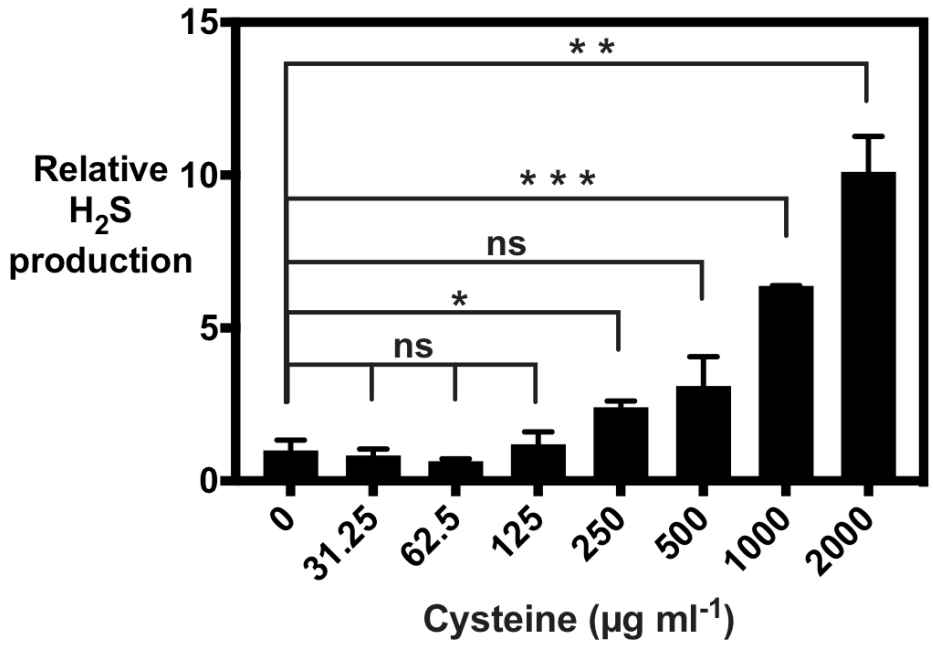


Figure 3.1 Growth and production of H_2S by *S. aureus* Je2 in the presence of excess cysteine

a) Growth of *S. aureus* Je2 in TSB supplemented with the indicated concentrations of L-cysteine. Grown under aerobic conditions at 37°C with 250 rpm shaking. Data are representative of three independent experiments.

b) Production of H_2S by *S. aureus* Je2 grown in TSB supplemented with the indicated concentrations of L-cysteine. H_2S production was measured using the lead acetate paper method (Section 2.15.1). Units represent H_2S production normalised to production of H_2S by *S. aureus* Je2 when no cysteine was added. The data are the mean values ± standard deviation obtained from three independent experiments.

ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$ ******* $p < 0.001$

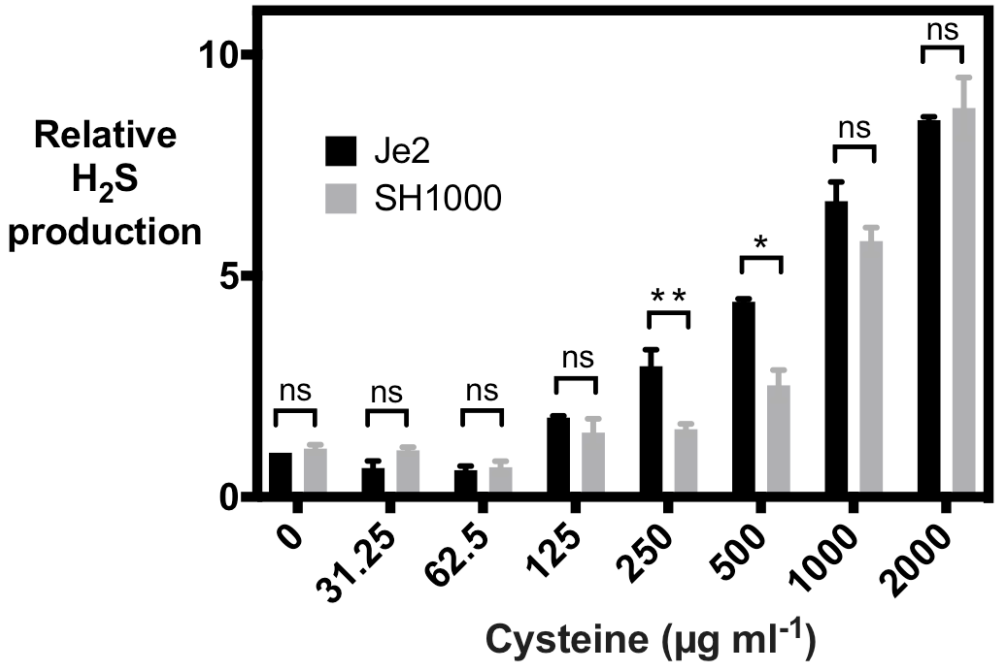


Figure 3.2 Production of H_2S by different strains of *S. aureus*
Production of H_2S by *S. aureus* Je2 and SH1000 grown in BHI medium supplemented with the indicated concentrations of L-cysteine. H_2S production was measured using the lead acetate paper method (Section 2.15.1). Units represent H_2S production normalised to production of H_2S by *S. aureus* Je2 when no cysteine was added. The data are the mean values ± standard deviation obtained from three independent experiments.
ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$

marked decrease in production of H_2S when *S. aureus* was grown in CDM with sodium thiosulfate as it's sole sulfur source as compared to when grown with cysteine as it's sole sulfur source or grown in rich medium (TSB) (Figure 3.3). Resistance to oxidative stress in the form of hydrogen peroxide (H_2O_2) and bactericidal antibiotics in the form of gentamicin was then ascertained using survival curves based on CFUs (Figure 3.3). These survival curves showed a significant decrease in resistance to both H_2O_2 and gentamicin associated with using sodium thiosulfate as a sole sulfur source, suggesting that cysteine (H_2S) might contribute to enhanced tolerance to oxidative stress.

3.3.4 Resistance to oxidative stress and antibiotics following the addition of sodium hydrosulfide (NaHS) as an external source of H_2S

Next the effect of an external source of H_2S on resistance to H_2O_2 and antibiotics was investigated. Sodium hydrosulfide (NaHS) was added to *S. aureus* Je2 cultures at increasing concentrations and the minimum inhibitory concentration (MIC) for H_2O_2 and gentamicin was measured (Figure 3.4). The results showed that above 4 mg ml^{-1} NaHS was toxic. However, some NaHS concentrations below the toxic threshold increased resistance to H_2O_2 but decreased resistance to gentamicin.

3.3.5 Cysteine metabolism mutants and production of H_2S

A number of cysteine metabolism transposon mutants were transduced from the Nebraska Transposon Mutant Library (NTML) back into the *S. aureus* Je2 background and the production of H_2S measured using the lead acetate paper method (Figure 3.6). Additionally a *cysM* mutant (Lithgow et al., 2004) was transduced from a *S. aureus* SH1000 background into *S. aureus* Je2, as there is no *cysM* transposon mutant available in the NTML. When these mutants were grown with no additional cysteine added to the medium *mccA*, *mccB* and *metC* strains showed no significant change in the production of H_2S compared to WT (Figure 3.6). However, when these three strains were grown with 500 μg ml^{-1} cysteine (Figure 3.6) *mccB* showed significantly lower production of H_2S compared to WT.

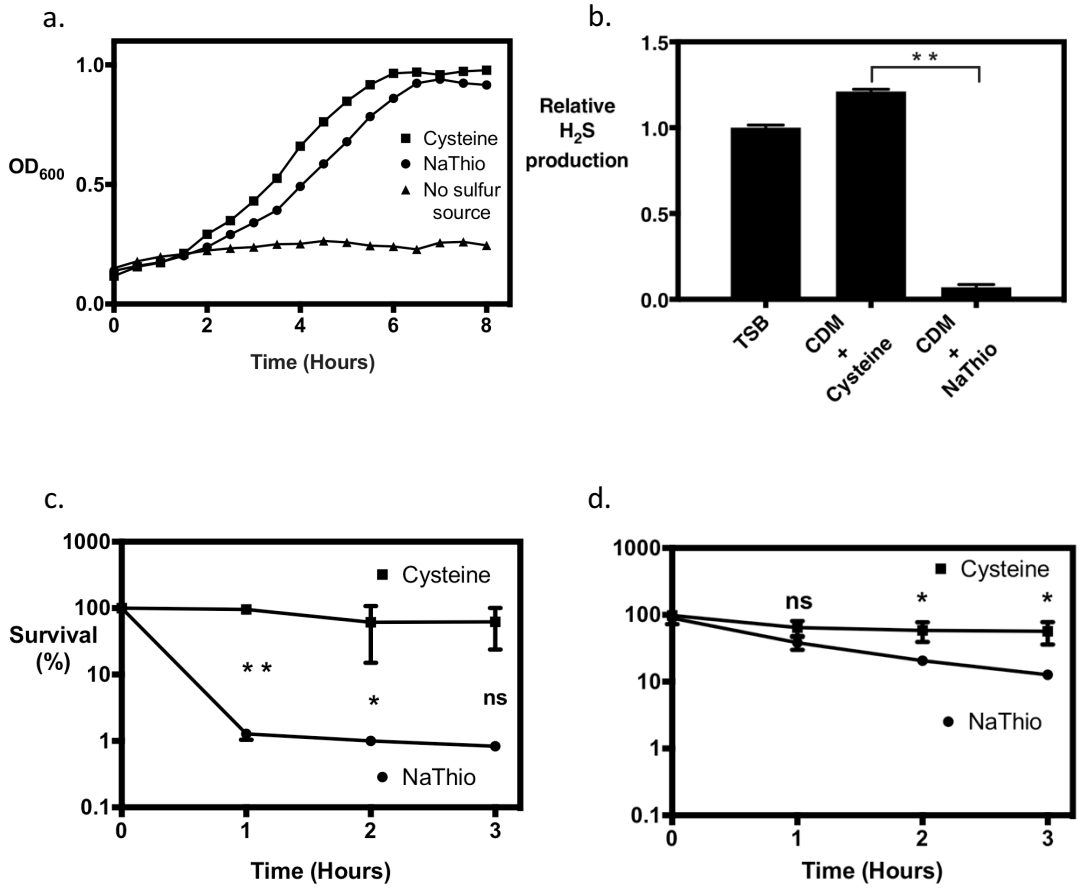


Figure 3.3 H_2S production and survival curves in CDM with cysteine and thiosulfate as sole sulfur sources

a) Growth of *S. aureus* Je2 grown in CDM (Section 2.1.2) supplemented with 2 mM sodium thiosulfate (NaThio), 500 µg ml^{-1} L-cysteine or with no sulfur source. Grown under aerobic conditions at 37°C with 250 rpm shaking. Data shown are representative of three independent experiments.

b) Production of H_2S by *S. aureus* Je2 grown in TSB or CDM supplemented with either 2 mM NaThio or 500 µg ml^{-1} L-cysteine. H_2S production was measured using the lead acetate paper method (Section 2.15.1). Units represent H_2S production normalised to production of H_2S by *S. aureus* Je2 grown in TSB. The data are the mean values ± standard deviation obtained from three independent experiments.

c and d) Survival curves for *S. aureus* Je2 grown in CDM supplemented with either 2 mM NaThio or 500 µg ml^{-1} L-cysteine. Exponentially growing cultures were challenged with either **c)** $6.25x10^{-4}$ % H_2O_2 or **d)** 2.5 µg ml^{-1} gentamicin. Survival (%) was calculated following CFU measurements. The data are the mean values ± standard deviation obtained from three independent experiments.

ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$

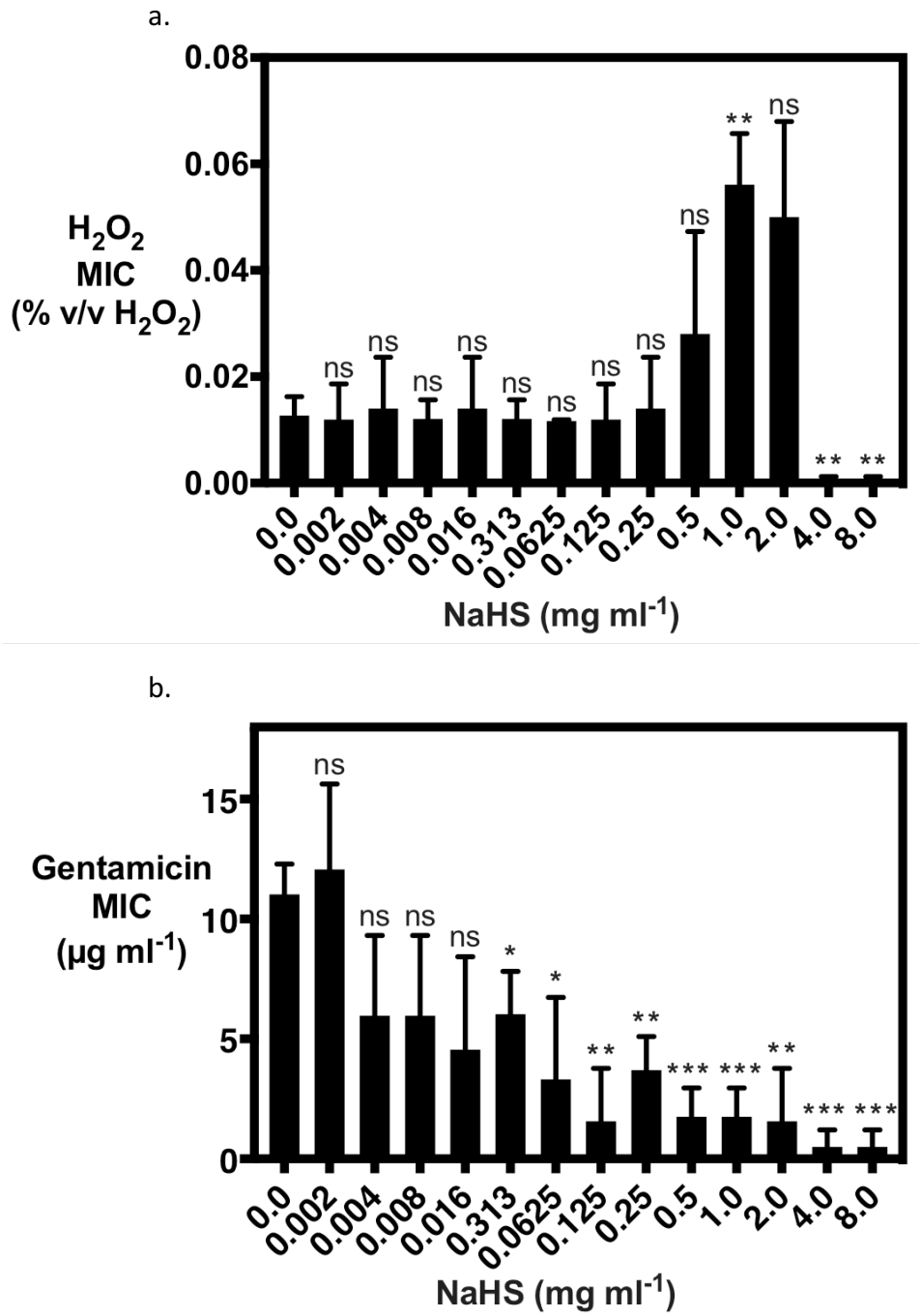
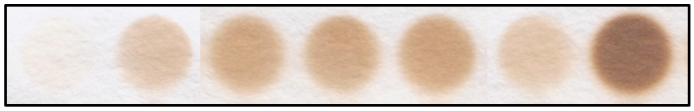


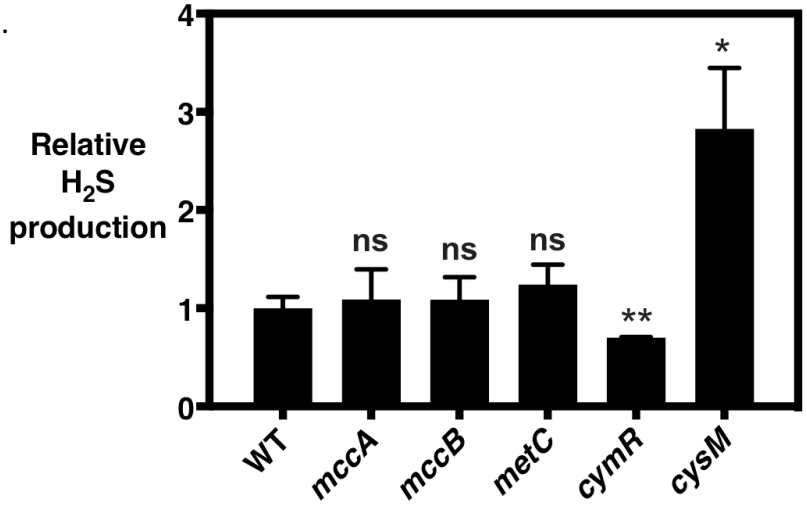
Figure 3.4 External addition of H_2S has an effect on antibiotic and peroxide resistance
The minimum inhibitory concentration (MIC) of **a)** H_2O_2 or **b)** gentamicin required to prevent growth of *S. aureus* Je2 was measured in TSB supplemented with the indicated concentrations of sodium hydrogen sulfide (NaHS), a potent producer of H_2S. Grown under aerobic conditions at 37°C with 250 rpm shaking. The data are the mean values ± standard deviation obtained from three independent experiments.
ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$ ******* $p < 0.001$

a.



Strain	Blank	WT	*mccA*	*mccB*	*metC*	*cymR*	*cysM*
Relative H_2S production	0.00	1.00	1.09	1.08	1.23	0.70	2.83

b.



c.

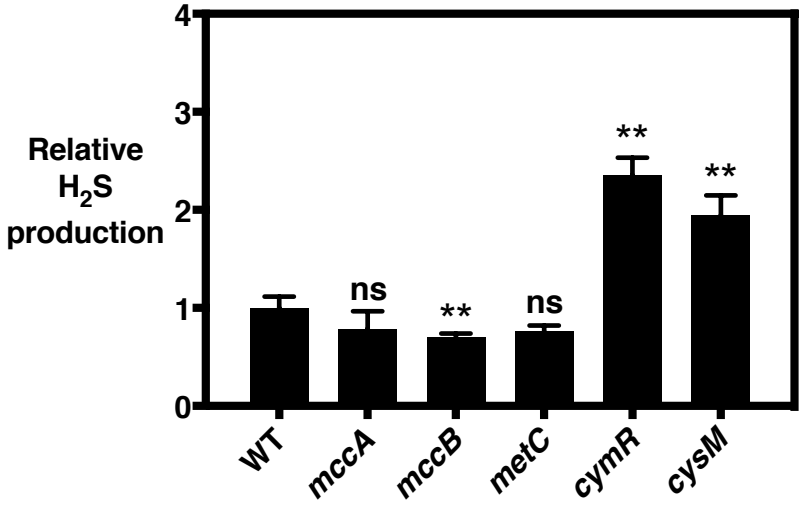


Figure 3.5 Production of H_2S by cysteine metabolism mutants
Production of H_2S by the indicated *S. aureus* Je2 mutants grown in **a and b)** TSB medium or **c)** TSB medium supplemented with 500 µg ml^{-1} L-cysteine. H_2S production was measured using the lead acetate paper method (Section 2.15.1). Units represent H_2S production normalised to production of H_2S by WT.
a) Data shown are representative of three independent experiments.
b and c) The data are the mean values ± standard deviation obtained from three independent experiments.
ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$

Interestingly the most dramatic contrast in H_2S production was observed for *cymR*: when no excess cysteine was present the production of H_2S was reduced 30% compared to WT; however, when 500 μg ml^{-1} cysteine was present the *cymR* strain exhibited enhanced H_2S production (130%). On the other hand, the *cysM* mutant had a consistent increase in H_2S production, irrespective of excess cysteine concentration, of between 100%−200% increase compared to WT (Figure 3.6).

3.3.6 Production of H_2S by *cysM* mutants measured using Fourier transform infrared spectroscopy (FT-IR)

The use of FT-IR to measure the production of H_2S more quantitatively was investigated (Figure 3.6). Preliminary experiments were carried out using *Escherichia coli* due to safety restrictions. *Escherichia coli* K12 and an equivalent *cysM* strain were used. Production of H_2S was measured over time and showed that the *cysM* mutant produced 50% more H_2S under the conditions of the experiment. Unfortunately, the FT-IR technique is not very sensitive for H_2S measurement and so high biomass suspensions were required to produce a detectable level of H_2S. Even following improvements to the technique it proved impossible to get reliable results under normal culture conditions with *S. aureus*.

3.3.7 Minimum inhibitory concentration (MIC) of cysteine metabolism mutants for H_2O_2 and gentamicin

In order to investigate the resistance of the cysteine metabolism mutants to H_2O_2 and gentamicin, MICs were measured for each mutant (Figure 3.7). The *mccA*, *mccB* and *metC* and *cymR* mutants showed no significant change in resistance to H_2O_2 and gentamicin; whereas the *cysM* mutant was significantly more resistant to both H_2O_2 and gentamicin.

3.3.8 Growth inhibition zone (GIZ) assays for investigating resistance of cysteine mutants on solid medium

Cysteine metabolism mutants were seeded as lawns on agar plates and challenged with discs containing either H_2O_2 or gentamicin (Figure 3.8). This was in order to

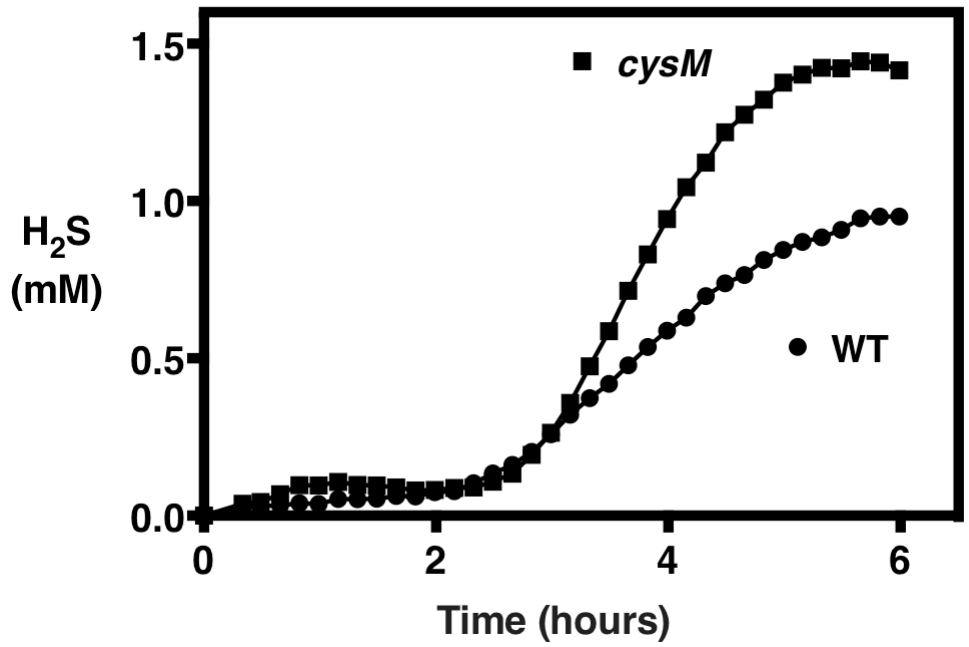


Figure 3.6 Production of H_2S by *E. coli* K12 and *cysM* mutant measured using FT-IR
Production of H_2S by *E. coli* K12 and *cysM* mutant. The strains were grown overnight under aerobic conditions at 37^{o}C with 250 rpm shaking in 2YT medium and then concentrated to an OD_{600} of 30 in LB supplemented with 130 mM cysteine hydrochloride. H_2S production was then measured using the FT-IR method over a period of 6 hours (Section 2.15.2).

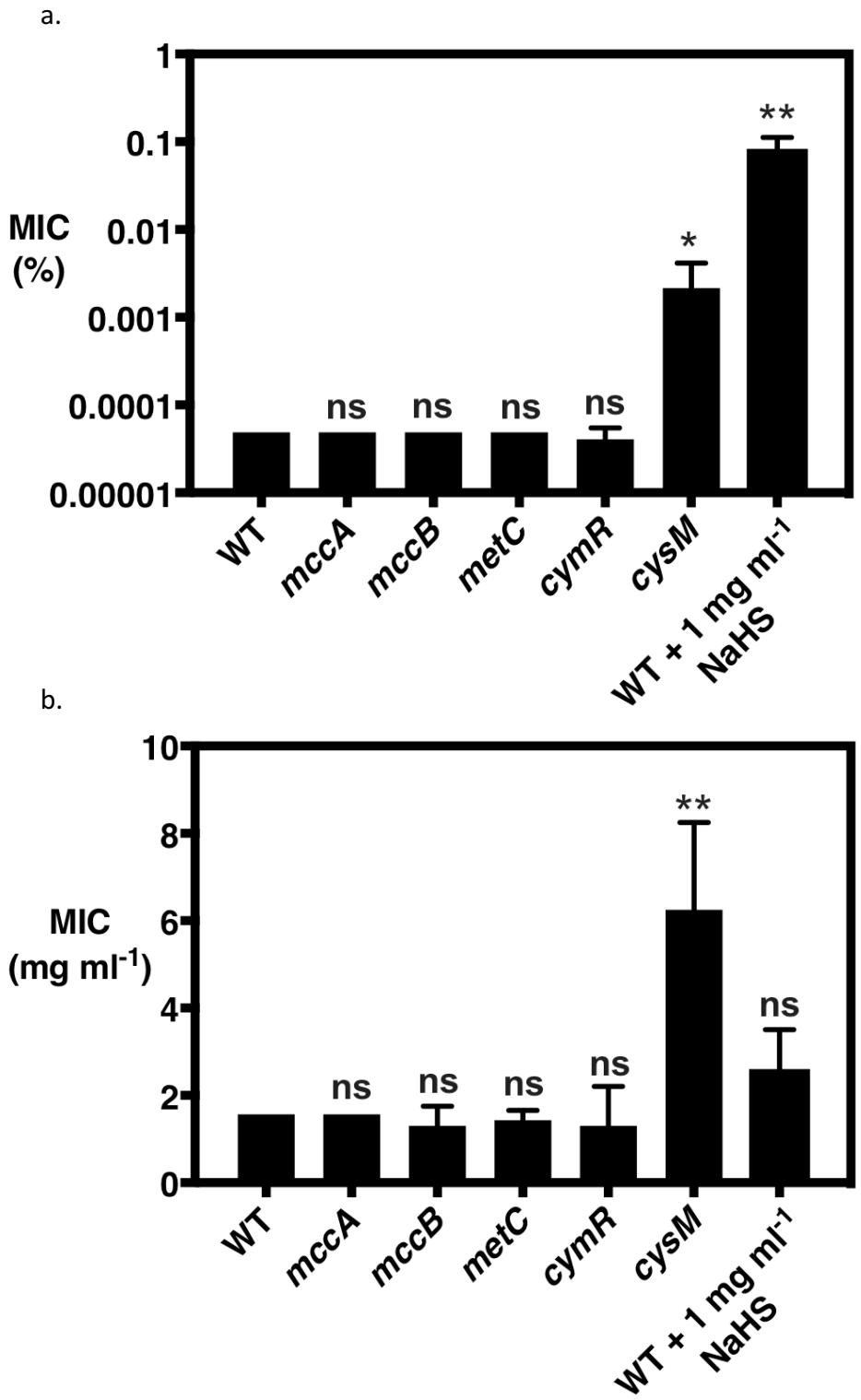
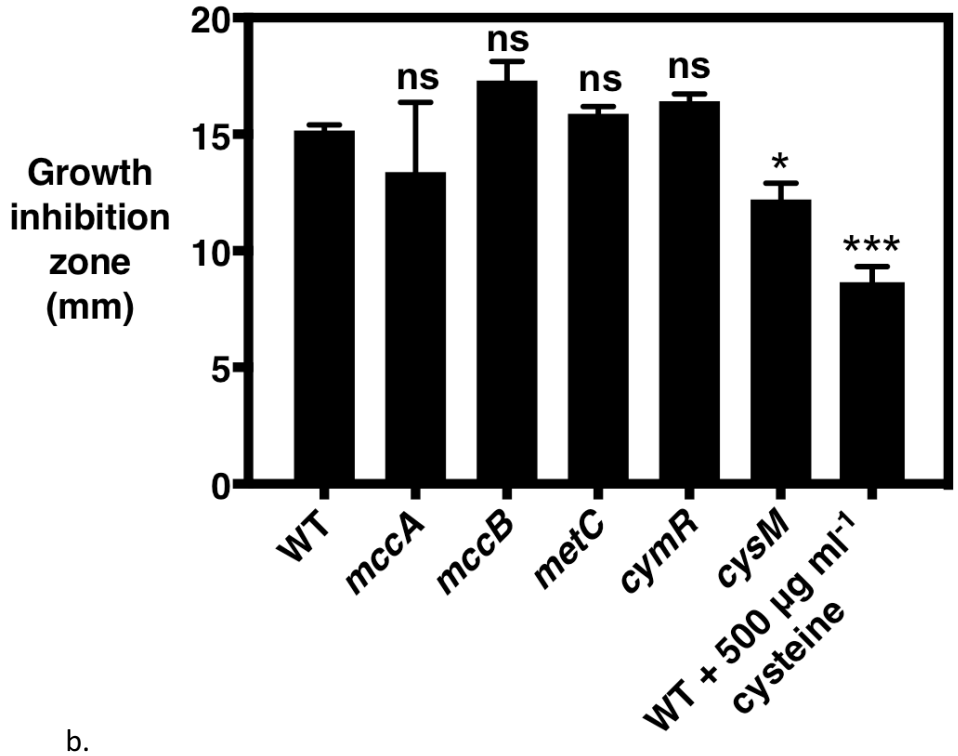


Figure 3.7 H_2O_2 and gentamicin MICs for cysteine metabolism mutants
The MIC of **a)** H_2O_2 or **b)** gentamicin required to prevent growth of the indicated *S. aureus* Je2 mutant. MICs were measured in TSB, supplemented with 1 mg ml^{-1} NaHS where indicated. The MICs were measured after 24 hours grown under aerobic conditions at 37^{o}C with 250 rpm shaking. The data are the mean values ± standard deviation obtained from three independent experiments.
ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$

a.



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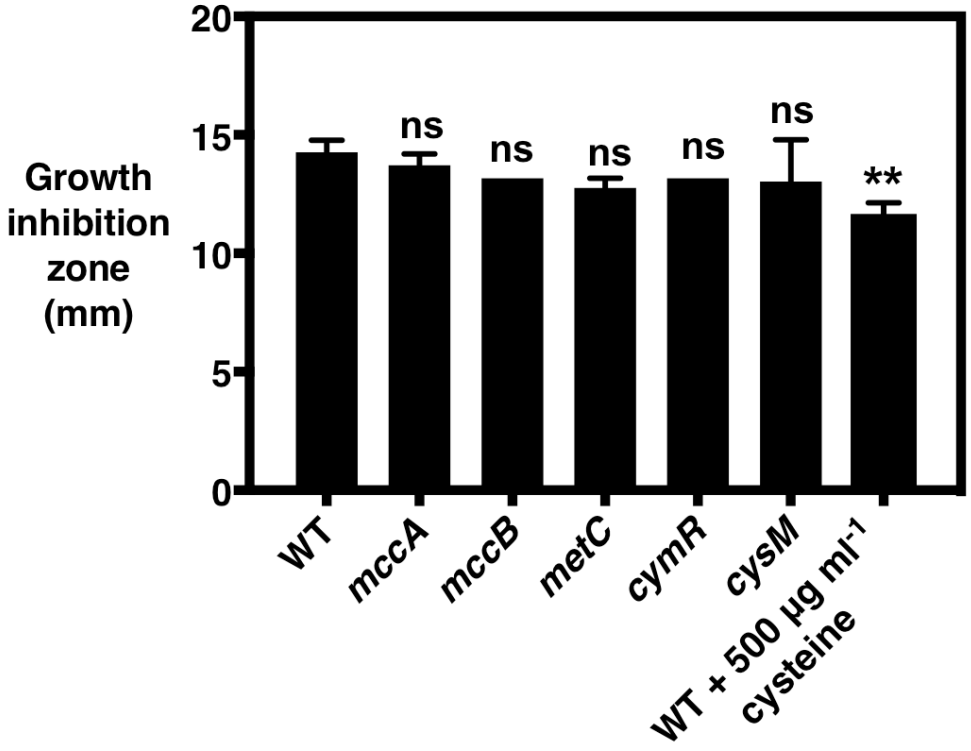


Figure 3.8 H_2O_2 and gentamicin GIZ assays for cysteine metabolism mutants
The indicated *S. aureus* Je2 mutants were seeded as lawns onto tryptic soy agar (TSA), supplemented with 500 µg ml^{-1} L-cysteine where indicated, and challenged with 6 mm paper discs containing either **a)** 10 µl 0.35% H_2O_2 or **b)** 3 mg ml^{-1} gentamicin. The growth inhibition zones were measured after 24 hours growth under aerobic conditions at 37°C. The data are the mean values ± standard deviation obtained from three independent experiments.
ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$ ******* $p < 0.001$

investigate the resistance of the cysteine metabolism mutants to H_2O_2 and gentamicin in an alternative environment. Under these conditions, the *mccA*, *mccB*, *metC* and *cymR* mutants all behaved in much the same way as in the liquid culture MIC experiments. However, the *cysM* mutant only showed significantly increased resistance to H_2O_2 but not to gentamicin.

The effect of excess cysteine under these conditions was also investigated because it was not possible to accurately measure the concentration of H_2S on solid medium (Figure 3.8). This showed that excess cysteine increased resistance to both H_2O_2 and gentamicin. This may highlight a difference between internally produced H_2S and an external source of H_2S with regards to resistance to gentamicin (Figure 3.5 and Figure 3.8).

Experiments were also carried out under anaerobic conditions for *S. aureus* Je2 and the *cysM* mutant in order to simulate more closely the centre of an abscess. Under anaerobic conditions the ability of the *cysM* mutant to display increased resistance to H_2O_2 was reversed (Figure 3.9) with the mutant actually being less resistant to H_2O_2 than the WT. Resistance to gentamicin remained the same under aerobic or anaerobic conditions.

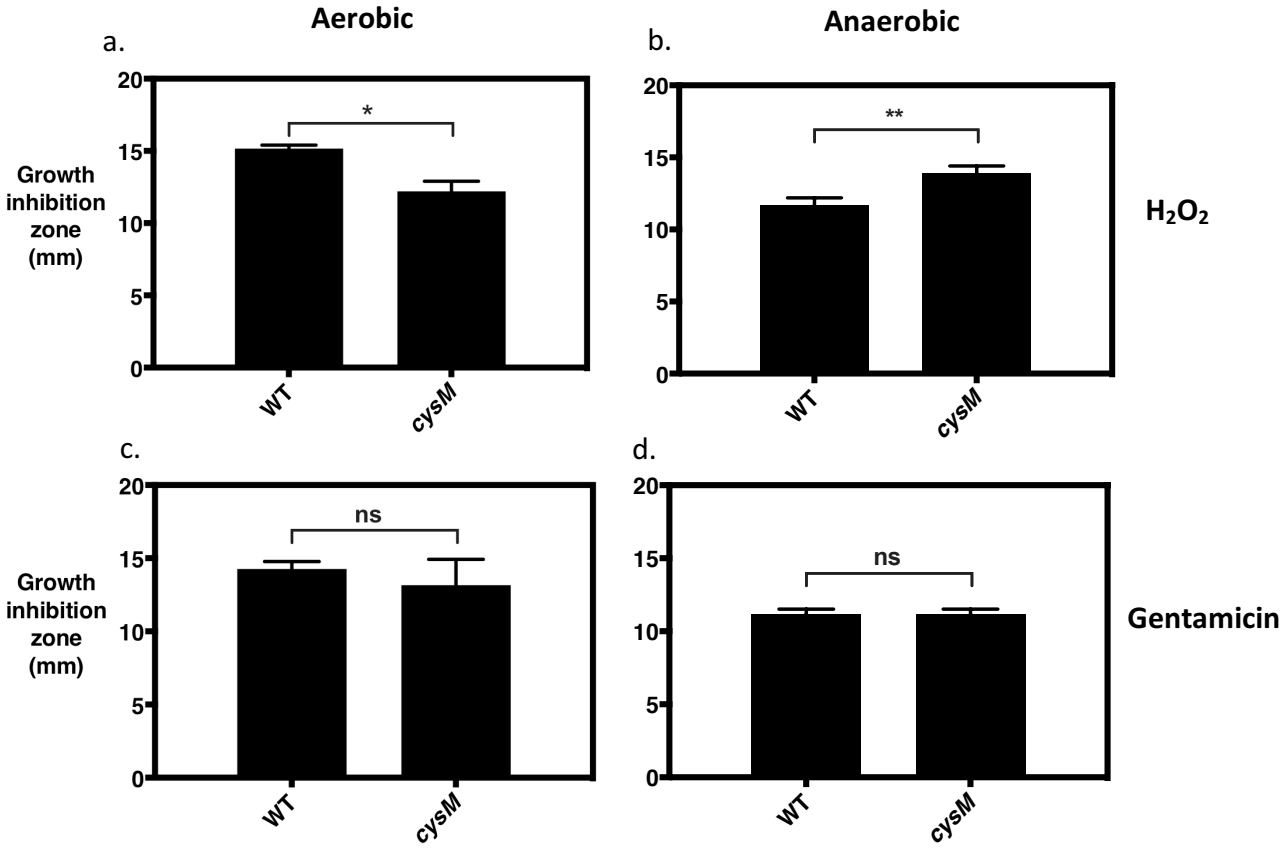


Figure 3.9 H_2O_2 and gentamicin GIZ assays for *S. aureus* Je2 and *cysM* mutant under aerobic and anaerobic conditions

Staphylococcus aureus Je2 and the *cysM* mutant were seeded as lawns onto TSA and challenged with 6 mm paper discs containing either **a and b)** 10 µl 0.35% H_2O_2 or **c and d)** 3 mg ml^{-1} gentamicin. The growth inhibition zones were measured after 24 hours grown under either **a and c)** aerobic or **b and d)** anaerobic conditions at 37^{o}C.

a) Data shown are representative of three independent experiments.

a, b, c and d) The data are the mean values ± standard deviation obtained from three independent experiments.

ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$

3.4 Discussion

Previous studies (Soutourina et al., 2009, Soutourina et al., 2010, Shatalin et al., 2011) that have investigated the role of H_2S in stress resistance have tended to supplement the medium with excess cysteine or cystine. An increasing cysteine concentration results in increased H_2S production (Figure 3.2) but might not be physiologically relevant. High concentrations of cysteine also impair bacterial growth. Therefore, the medium used in subsequent experiments did not have excess cysteine added.

It has been previously stated that MccAB is responsible for H_2S production in *S. aureus* (Shatalin et al., 2011), with some suggestion that MetC may be involved (Soutourina et al., 2010). However, *mccA, mccB* and *metC* mutants showed no significant reduction in H_2S production as compared to WT, except for a slight reduction in H_2S production for the *mccB* strain when grown in the presence of 500 μg ml^{-1} cysteine. Consequently, it is possible that there are other important genes involved in the production of H_2S that have yet to be identified. A transposon library screen is reported in Chapter 5 in attempt to identify these genes. However, it is most likely that there is a level of functional redundancy occurring for H_2S production and that a combination mutant is required in order to observe a reduction in H_2S production. Unfortunately, attempts to produce such a strain were unsuccessful during the project.

It is also possible that an alternative H_2S detection method to the very sensitive but qualitative lead acetate paper technique method could be utilised. FT-IR showed some promise but ultimately proved too cumbersome and time consuming. Methylene blue assay (Fogo and Popowsky, 1949) and H_2S probe (Li et al., 2008) were also investigated as options but proved to be too unreliable. Of them the methylene blue assay had the most promise. Therefore, future experiments could use the methylene blue assay, perhaps with a modified method, to facilitate more sensitive detection of H_2S.

The production of H_2S by the *cymR* mutant was significantly lowered in rich medium but increased when excess cysteine was used (Figure 3.6). This might simply be as a result of the de-repression of the cysteine transporters TcyP and TcyABC, resulting in a

more efficient transportation of cysteine into the bacterial cells (Figure 1.1). The *cysM* mutant reliably produced more H_2S irrespective of cysteine concentration (Figure 3.6). This is consistent with CysM being responsible for the conversion of H_2S into cysteine.

Overall there was no meaningful change in resistance to H_2O_2 and gentamicin observed for the *mccA*, *mccB*, *metC* or *cymR* mutants. However, as previously stated this may be due to functional redundancies and future work with multi-knockout strains might yield more significant results.

On the other hand, the *cysM* mutant consistently showed an increased resistance to H_2O_2 under aerobic conditions. This was consistent with the results observed when external NaHS was used and when increased H_2S production was enhanced with excess cysteine. These observations are consistent with the hypothesis that increased H_2S production can offer a defence against oxidative stress. However, the link with antibiotic resistance is not so clear because under certain conditions the *cysM* mutant was more resistant to gentamicin, such as in liquid media MICs, not in others, namely the solid media GIZ assays, it was not (Figure 3.7, Figure 3.8 and Figure 3.9). Previous work had shown the production of H_2S to be defensive against gentamicin in *S. aureus*, *Bacillus anthracis*, *Pseudomonas aeruginosa* and *Escherichia coli* using survival curves in liquid media (Shatalin et al., 2011). It would be interesting to investigate the effect of H_2S production on antibiotic resistance under different conditions for each of these other bacteria in order to see if this yields different results to the survival curves in liquid media.

When NaHS was used as an external source of H_2S there was an inverse relationship between H_2S and antibiotic resistance (Figure 3.4). This starts to question the assertion that H_2S is a universal defence against antibiotics and also undermines the theories that bactericidal antibiotics cause cell death via oxidative stress. Alternatively, this could highlight the importance of internal production of H_2S, as opposed to a high external concentration of H_2S, in promoting increased resistance to gentamicin.

3.4.1 Future work

The study of H_2S production levels and oxidative and antibiotic resistance in other strains of clinically relevant strains of *S. aureus* would be a simple way of checking how important H_2S production is to clinical resistance generally. Other follow up work *in vivo* would be invaluable for understanding the role of H_2S production in infection. Animal work with the *cysM* mutant would be valuable in ascertaining if increased production of H_2S increases pathogenicity. The creation of a full H_2S negative production strain would also be very informative for ascertaining if lowering H_2S production levels has an effect on pathogenicity. The discovery of other related H_2S production genes would also be valuable and this is explored in Chapter 5. A better understanding of sulfur metabolism and redox control could also inform studies into H_2S mediated resistance and these ideas are explored in Chapters 4 and 5.

Chapter 4: The response of *S. aureus* to a sudden reduction in redox potential

4.1 Introduction

4.1.1 Intracellular *S. aureus*

Staphylococcus aureus is able to survive and proliferate inside professional and non-professional phagocytes (Rogers and Tompsett, 1952, Kapral and Shayegani, 1959, Hudson et al., 1995, Gresham et al., 2000, Anwar et al., 2009, Fraunholz and Sinha, 2012). When *S. aureus* is engulfed by phagocytic cells the majority of the bacteria are destroyed; however, when incomplete clearance occurs *S. aureus* is able to survive inside the host cells (Garzoni and Kelley, 2011, Thwaites and Gant, 2011). Through this mechanism intracellular *S. aureus* is able to avoid the host immune system, and antibiotics, leading to wide dissemination and persistent infections (Garzoni and Kelley, 2011, Thwaites and Gant, 2011). Intracellular *S. aureus* is also responsible for persistent infections in a wide range of non-professional phagocytic cell types including in recurrent rhinosinusitis, pulmonary infections, osteomyelitis and endocarditis (Bosse et al., 2005, Clement et al., 2005, Que et al., 2005, Jarry et al., 2008). Understanding how *S. aureus* survives and proliferates intracellularly is key to preventing and treating these serious conditions.

4.1.2 The intracellular life style

Staphylococcus aureus enters cells either by engulfment by professional phagocytes or through the invasion of non-professional phagocytes via a fibronectin binding protein (FnBP) and host α5β1 integrin mediated zipper-type mechanism (Sinha et al., 2000). Other surface factors may also be involved in host phagocytosis including: wall teichoic acids (WTA) (Weidenmaier et al., 2008), clumping factor B (ClfB) (Wertheim et al., 2008, Haim et al., 2010), extracellular adherence protein (Eap) (Harraghy et al., 2003), autolysin (Atl) (Hirschhausen et al., 2010) and protein A (Claro et al., 2011); however, the extent to which each of these contribute to the engulfment of *S. aureus* is ambiguous.

Once a *S. aureus* bacterium is engulfed it must first survive in the harsh environment of the host phagosome and it has a number of mechanisms for successfully doing this (Section 1.8.3) (Schroder et al., 2006, Olivier et al., 2009).

However, in order to avoid lysosomal fusion and to carry on proliferating the *S. aureus* cells must escape from the phagosome (Schnaith et al., 2007). This has been shown to be an *agr* dependent process (Qazi et al., 2001, Shompole et al., 2003), with *agr*- strains completely unable to escape from phagosomal compartments (Schnaith et al., 2007). Phagosomal escape seems to be primarily mediated via α-toxin (Kahl et al., 2000, Jarry and Cheung, 2006) and possibly by the synergistic action of δ-toxin and β-toxin (Giese et al., 2011).

This escape into the cytoplasm can then lead to apoptotic or necrotic cell death (Bayles et al., 1998, Krut et al., 2003) or in other cases *S. aureus* survives and proliferates inside the cytoplasm (Kubica et al., 2008, Fraunholz and Sinha, 2012). Adaption to the new environment of the cytosol and the expression of toxins are likely to be key in determining the fate of the host cell. The production of very low doses of α-toxin (Bantel et al., 2001, Haslinger et al., 2003) and PVL (Genestier et al., 2005) can both induce apoptosis in leukocytes following the breakdown of mitochondrial membranes, and so in order to survive in the cytosol the expression of these toxins must be decreased. Better understanding of the factors that contribute to determining if an escape from a host phagosome results in immediate cell death or longer term intracellular survival is key in the fight against persistent *S. aureus* infections.

4.1.3 Intracellular *S. aureus* gene expression

An *in vitro* transcriptomic study of *S. aureus* 6850 internalised in lung epithelial cells has shown that there are significant changes in *S. aureus* gene expression following internalisation (Garzoni et al., 2007). Initially, two hours post-internalisation, there is a significant reduction in the transcription of genes related to metabolism, nutrient transport and cell wall synthesis and an increase in the transcription of genes related

to oxidative stress resistance and toxin production; however, the gene expression profile started to reverse 6 hours post-internalisation (Garzoni et al., 2007).

4.1.4 Small colony variants

Small colony variants (SCVs) are characterised by their having a smaller colony size than wildtype *S. aureus* and by their slower growth rate, increased expression of cell surface proteins and decreased expression of virulence factors and toxins, under aerobic conditions (Proctor et al., 2006). It has been hypothesised that SCVs may be induced by the intracellular environment (Vesga et al., 1996) and that they play a crucial role in intracellular persistence (Proctor et al., 2006, Kahl et al., 2016). SCVs can be caused by mutations in the electron transport chain, such as *hemB* mutants (von Eiff et al., 1997), but clinical SCVs mostly lack defined mutations and rapidly revert to wild-type phenotype (Kriegeskorte et al., 2011, Tuchscherr et al., 2011). It has been suggested that a SCV phenotype may be a temporary state that enables *S. aureus* to survive intracellularly and that they emerge and disappear dynamically through the course of a chronic infection (Tuchscherr et al., 2011, Horst et al., 2012)

The regulation of the SCV phenotype has been extensively investigated with a clear association between low *agr* expression and SCVs discovered (Vaudaux et al., 2002, Kohler et al., 2003, Kahl et al., 2005, Kohler et al., 2008), as well as, a clear link with SigB (Tuchscherr et al., 2015, Tuchscherr and Loffler, 2016). Better understanding the regulation of SCV phenotype formation and the links with regulators is key to preventing and treating persistent *S. aureus* infections.

4.1.5 Redox potential

Redox potential (E_h), the availability of electrons, is a key environmental parameter that can have a profound effect on bacterial phenotypes. The *E. coli* redox sensing two component ArcBA system has been shown to have profound effect on global gene expression (Bekker et al., 2010) and the *S. aureus agr* system has been shown to be down-regulated at low redox potentials (around −180 mV), with associated extensive

changes in gene expression, following co-culture with *Lactococcus lactis* (Nouaille et al., 2014).

The redox potential of an exponentially growing *S. aureus* culture is around 50 mV (Nouaille et al., 2014) and phagosomes tend to have a redox potential (Eh) of between 60 to 180 mV (Go and Jones, 2008). Whereas, when *S. aureus* enters the host cytoplasm it immediately finds itself in an aerobic environment with a low E_h (−220 to −286 mV) due to the presence of high (30:1 to 100:1) ratios of glutathione to oxidised glutathione (GSH:GSSG) (Hwang et al., 1992). Therefore, redox potential has the possibility to be an important trigger for gene expression for internal *S. aureus* as it moves between compartments with vastly different redox potentials.

4.1.6 Continuous culture

In order to investigate the effect of a sudden redox potential change, as experienced by *S. aureus* as it escapes from a phagosome into host cytoplasm, with accuracy and reliability it is important to control all other environmental factors. An effective tool to create reliable culture conditions is to use continuous culture in a chemostat (Rolfe et al., 2011, Denby et al., 2015). Under these conditions medium is added to the chemostat at a constant rate, whilst culture medium is being removed at the same rate, allowing a specific growth rate to be maintained (Novick and Szilard, 1950).

Staphylococcus aureus continuous culture has recently been used to simulate aspects of the host environment whilst maintaining a high level of control over environmental parameters. This has facilitated investigation into chronic *S. aureus* infection and SCV formation (Bui et al., 2015), as well as, creating an alternative model for testing antibiotic effectiveness against *S. aureus* infections (Udekwu and Levin, 2012)

4.2 Aims

- Develop a *S. aureus* continuous culture model in order to examine the effect of a sudden reduction in redox potential, as experienced following the escape of *S. aureus* from a phagosome in the host cytosol.
- Study the transcriptomic response of *S. aureus* to a sudden reduction in redox potential using this continuous culture model.
- Investigate the metabolomics, pathogenic and physiological responses of *S. aureus*, in response to low redox potential environments.

4.3 Results

4.3.1 Continuous culture and sudden redox shift experiment design

In order to investigate the effect of redox change, as faced by *S. aureus* as it escapes from the phagosome into the cytoplasm, a model for investigating the effect of redox potential (Eh) on *S. aureus* in aerobic chemostat continuous culture was used. A clinical relevant community acquired MRSA strain (USA300 LAC Je2) in chemically defined medium (CDM) was used and in order to reduce the effect of factors other than redox potential (Eh) confounding the results there was constant air flow (1 L min^{-1}), medium feed (0.2 L hr^{-1}), stirring (200 rpm) and pH (7.0) (Figure 4.1). Redox potential was controlled by allowing the culture to first reach a steady state with an optimal redox potential (E_h = 61.7 mV ± 14.2 (SD)) (Figure 4.2) close to the previously observed value of $E_h \approx 50$ mV (Nouaille et al., 2014)) before rapidly reducing the redox potential by adding the potent reducing agent dithiothreitol (DTT) to the medium and feedstock. DTT was used at it was able to maintain the culture at a stable redox potential over the 24 hour course of the experiment. Samples were taken during the baseline steady-state culture and then at 2, 5, 10, 15, 30, 60 and 1440 minutes (24 hours) following the addition of DTT.

The concentration of DTT used (9.7 mM) was determined to be sufficient to lower the redox potential to around that found in mammalian cytosol (E_h = −214.0 mV ± 59.6 (SD) in 10 minutes and then to maintain that redox potential for the full 24 hour experiment (at 24 hours E_h = −274 ± 40.0 (SD)) (Figure 4.2). This concentration of DTT did have an inhibitory effect on growth rate for USA300 LAC Je2 (Figure 4.3); however, the dilution rate of the chemostat (0.2 h^{-1} or a doubling time of around 3.5 hours) was lower than the maximum specific growth rate (μ_{max}) when Je2 was grown in the presence of 9.7 mM in batch (μ_{max} = 1.14 h^{-1}, which was 33% less than with 0 mM DTT where μ_{max} = 1.71 h^{-1}). Also, there was a delayed lag phase observed indicating that there was some level of adaptation taking place (Figure 4.3).

During the course of the experiment bacterial density (OD_{600}) was measured (Figure 4.4) and it showed that bacterial density stayed stable throughout the experiment

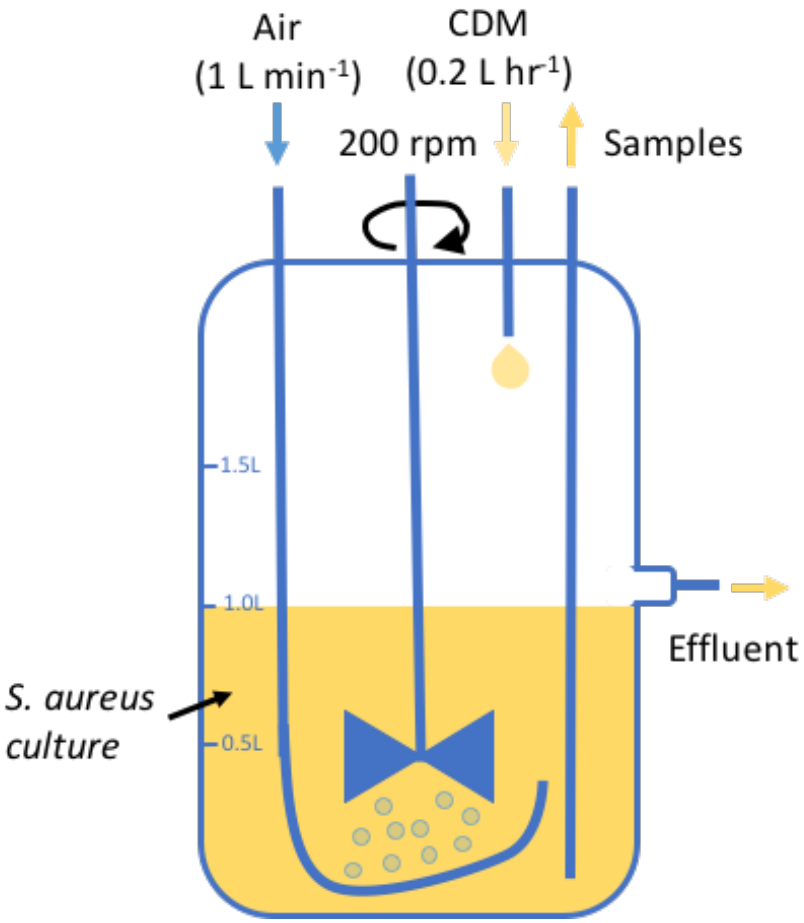


Figure 4.1 Diagram of *S. aureus* in continuous culture
The environment of the chemostat vessel was maintained at 37°C and pH 7 with 200 rpm stirring and constant aeration at 1 L min^{-1} airflow. Continuous growth was maintained through the addition of 0.2 L hr^{-1} fresh medium (CDM) to the 1 litre vessel and the draining of effluent at the same rate.

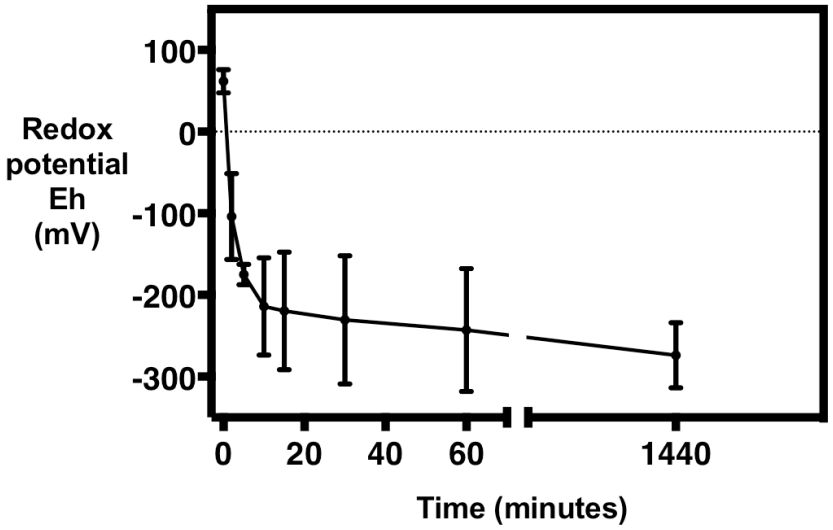


Figure 4.2 Redox potential following addition of DTT to *S. aureus* continuous culture
The redox potential of *S. aureus* grown in continuous culture following the addition of 9.7 mM DTT to *S. aureus* continuous culture. *Staphylococcus aureus* was grown in CDM at 37^{o}C and pH 7 with 200 rpm stirring and constant aeration at 1 L min^{-1} airflow. Continuous growth was maintained through the addition of 0.2 L hr^{-1} fresh medium (CDM) to the 1 litre vessel and the draining of effluent at the same rate. The data are the mean values ± standard deviation obtained from three independent experiments.

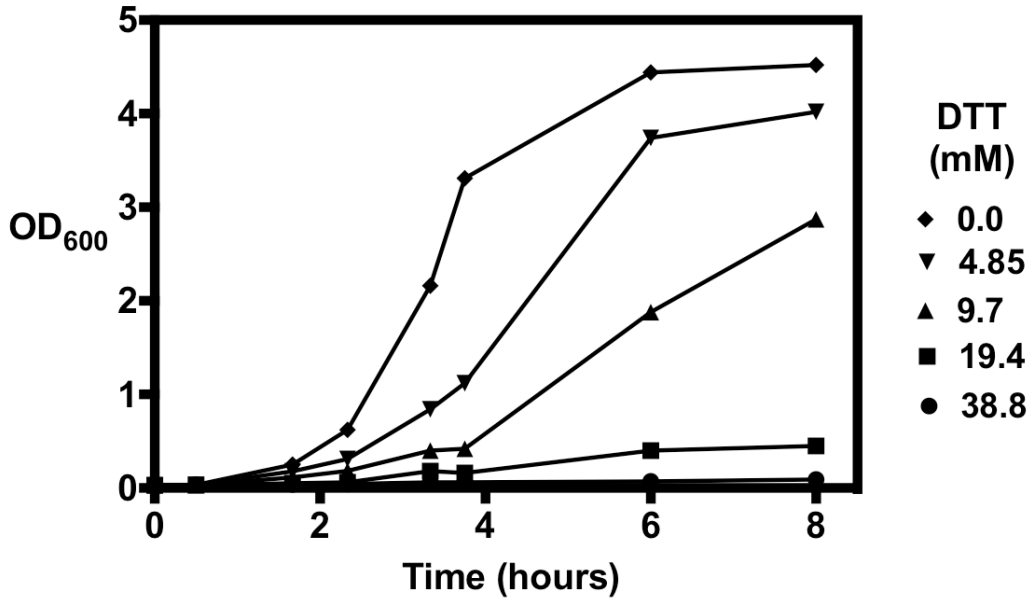
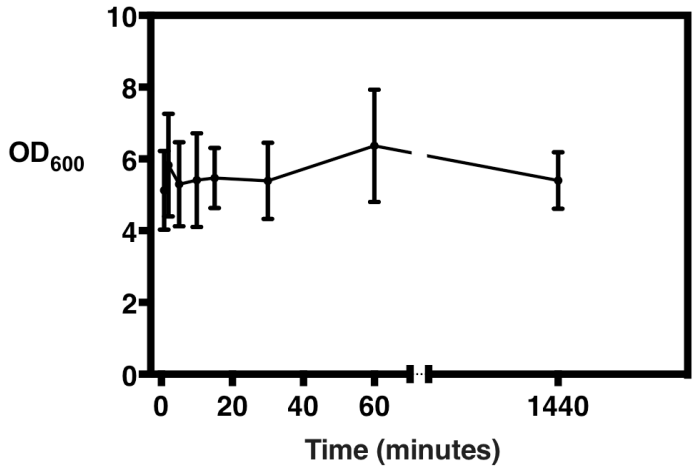


Figure 4.3 Growth of *S. aureus* in CDM supplemented with DTT
Growth of *S. aureus* grown in CDM supplemented with the indicated concentrations of DTT. Grown under aerobic conditions at 37^{o}C with 250 rpm shaking. Data shown are representative of three independent experiments.

a.



b.

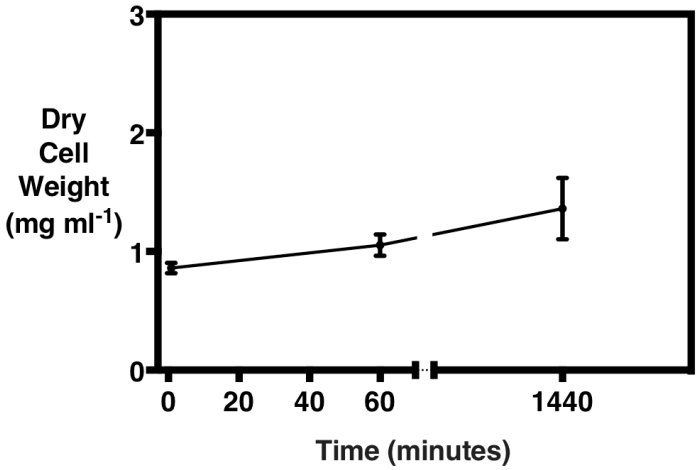


Figure 4.4 Optical density and dry cell weight (DCW) following addition of DTT to *S. aureus* continuous culture

a) Bacterial density (OD_{600}) **b)** DCW following the addition of 9.7 mM DTT to *S. aureus* continuous culture. *Staphylococcus aureus* was grown in CDM at 37^{o}C and pH 7 with 200 rpm stirring and constant aeration at 1 L min^{-1} airflow. Continuous growth was maintained through the addition of 0.2 L hr^{-1} fresh medium (CDM) to the 1 litre vessel and the draining of effluent at the same rate. The data are the mean values ± standard deviation obtained from three independent experiments.

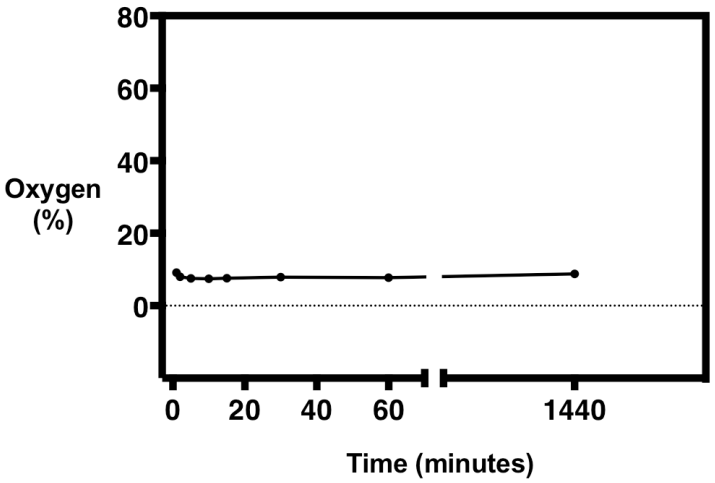
(OD_{600} = 5.5 ± 1.1 (SD)). Dry cell weight was also measured during the course of the experiment at 0, 60 and 1440 minutes following the addition of DTT and this revealed a significant increase in dry cell weight of 22% ± 11% (SD) by 60 minutes and 58% ± 30% (SD) by 24 hours (Figure 4.4).

4.3.2 Sudden redox shift and respiration

Following the addition of DTT to the steady state culture the percentage of dissolved oxygen fluctuated considerably (Figure 4.5). Before the addition of DTT the dissolved oxygen concentration was stable at around 10% but 5 minutes following the addition of DTT the dissolved oxygen concentration had increased 3-fold. Thirty minutes following the addition of DTT the dissolved oxygen concentration had returned to steady state levels (Figure 4.5). When DTT was added to the chemostat when no cells were present with aeration limited to create an initial 10% dissolved oxygen saturation, there was no change to the dissolved oxygen concentration suggesting that DTT was not affecting the oxygen probe but instead inhibiting respiration in the *S. aureus* culture when it was initial added. Furthermore, the oxygen saturation returning to, and remaining at, around 10% until the end of the 24 hour experiment (Figure 4.5), whilst the biomass increased (Figure 4.4), indicates that less oxygen was being used by each cell by the end of the experiment and suggests a switch to non-aerobic forms of respiration.

In order to investigate the hypothesis that DTT was inhibiting respiration a Clark-type oxygen electrode was used. Using this method DTT appeared to have a slight negative effect on the probe when no cells were present. Taking this into account there seemed to be a small, but insignificant, inhibition of respiration caused by DTT at concentrations similar to those used in the chemostat. When reduced glutathione (GSH), the naturally occurring reducing agent found in mammalian cytosol, was used as the reducing agent in the experiments a significant inhibition of respiration was measured (32.1% ±8.1% (SD)) (Figure 4.6).

a.



b.

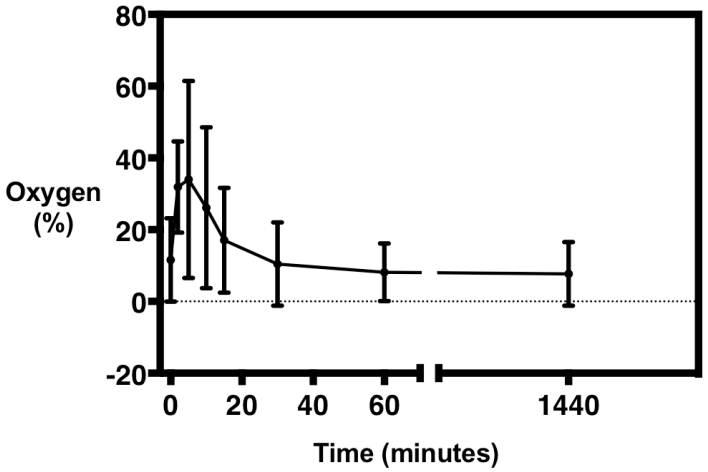


Figure 4.5 Dissolved oxygen concentration following the addition of DTT to sterile medium or *S. aureus* continuous culture

Dissolved oxygen concentration following the addition of 9.7 mM DTT to **a)** CDM with no bacteria added or **b)** *S. aureus* continuous culture. Conditions were maintained at 37^{o}C and pH 7 with 200 rpm stirring and with **a)** aeration limited to create an initial 10% dissolved oxygen saturation or **b)** constant aeration at 1 L min^{-1} airflow. Continuous growth was maintained through the addition of 0.2 L hr^{-1} fresh medium (CDM) to the 1 litre vessel and the draining of effluent at the same rate. The data are the mean values ± standard deviation obtained from three independent experiments.

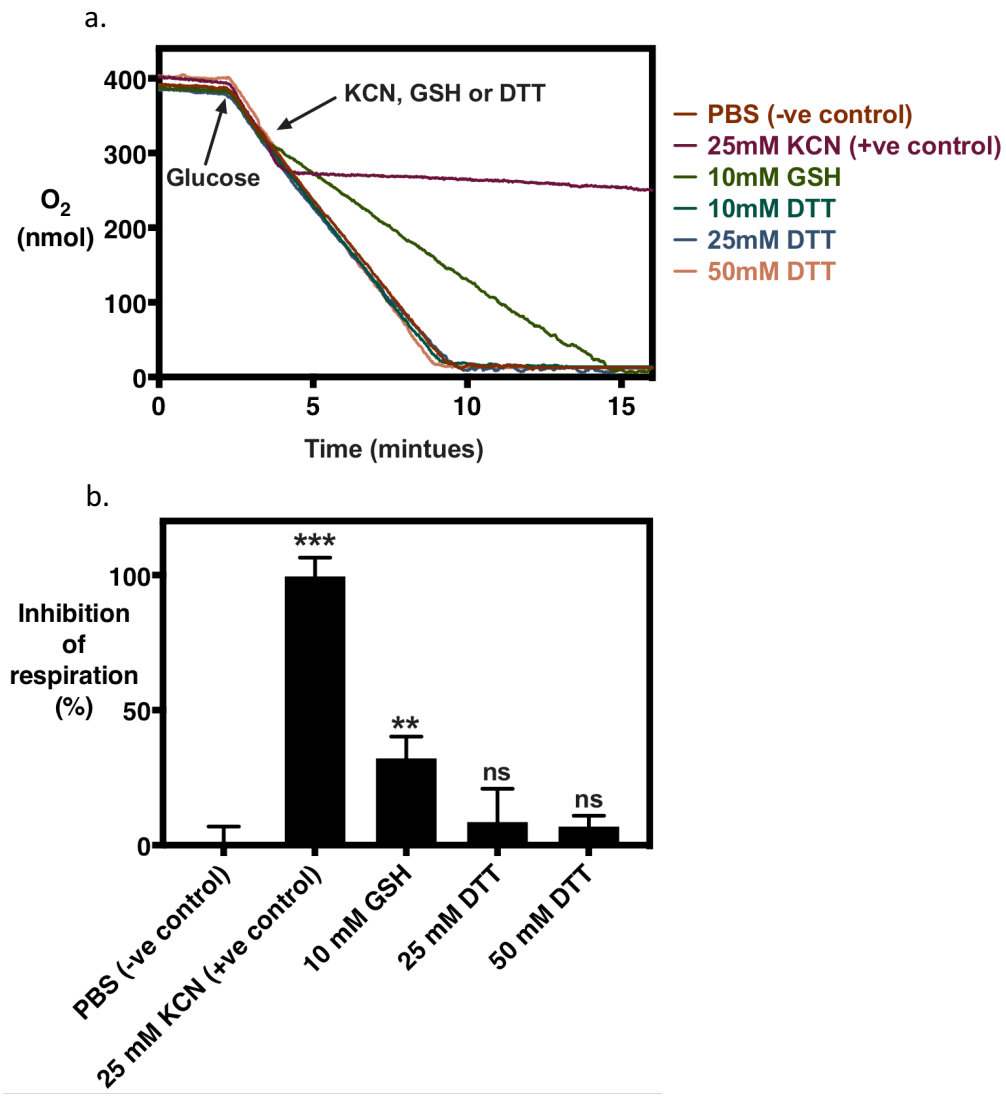


Figure 4.6 Effect of DTT and reduced glutathione (GSH) on *S. aureus* respiration, measured using a Clark-type oxygen electrode

PBS was added to the 2 ml chamber and stabilised for 2 minutes. Then 50 µl 1 M glucose was added, containing *S. aureus* in PBS, to initiate respiration. Then after a further 1 minute 50 µl of either PBS, KCN, GSH or DTT, at the indicted concentrations, was added and the effect on oxygen consumption noted. **a)** Units represent dissolved oxygen concentration (nmol) and data shown are representative of three independent experiments. **b)** Units represent the inhibition of respiration (%) and the data are the mean values ± standard deviation obtained from three independent experiments.
ns $p \geq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

4.3.3 Sudden redox shift and SCVs

In order to see if there was an effect on the proportion of SCVs following the sudden negative redox switch, samples were taken from steady state culture and 5, 30, 60 and 1440 minutes after the addition of DTT. There was no observed change in the proportion of SCV colonies (Figure 4.7). Also the stability of SCVs from each time point was determined and no significant change was observed. However there does appear to be a slight trend from stable SCVs towards unstable ones over the 24 hour experiment (Figure 4.7).

To investigate the longer term effect of a reduced redox potential on the formation of SCVs a longer term experiment was carried out on solid medium. This was due to the limitations of the chemostat culture consistently forming biofilms during longer experiments. After 48 hours on solid medium supplemented with DTT the proportion of SCVs had increased dramatically (Figure 4.8) suggesting that a switch to a SCV phenotype may occur over a longer time period than the 24 hour chemostat experiment was able to accommodate.

4.3.4 Sudden redox shift and extracellular metabolite production

Extracellular metabolite production was measured using proton-NMR. Against the complex background of CDM, it was possible to identify and measure peaks relating to DTT and glucose, as well as, key metabolites acetate and lactate (Figure 4.9). The measurement of DTT in the medium confirmed the accuracy of the NMR with the concentration of DTT being measured close to the expected level (9.7 mM) for the first 60 minutes and then falling to 6.2 mM ±0.1mM (SD) after 24 hours (Figure 4.10). There was no significant change to acetate production throughout the experiment. However, there was a sustained increase in the production of lactate, consistent with inhibited respiratory activity.

In addition to the NMR a lactate dehydrogenase enzyme assay was carried out and showed that lactate dehydrogenase activity increased following the addition of DTT (Figure 4.11). Thus, 24 hours after the addition of DTT lactate dehydrogenase activity had increased 12.6 – fold compared to before the addition of DTT.

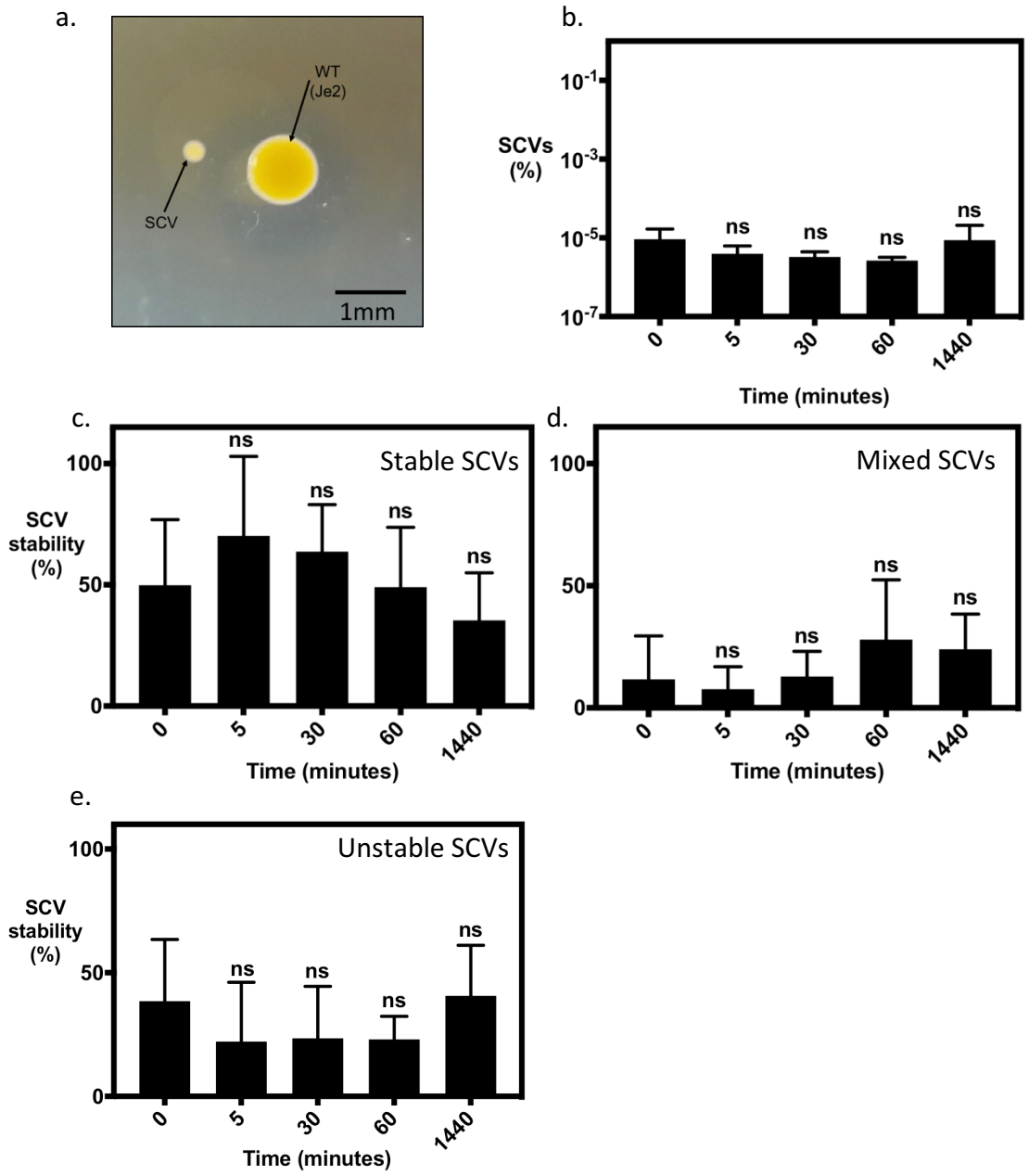


Figure 4.7 Frequency and stability of small colony variants (SCVs) following the addition of DTT to *S. aureus* continuous culture

Frequency of SCVs following addition of 9.7 mM DTT to *S. aureus* continuous culture. Conditions were maintained at 37°C and pH 7 with 200 rpm stirring and with constant aeration at 1 L min^{-1} airflow. Continuous growth was maintained through the addition of 0.2 L hr^{-1} fresh medium (CDM) to the 1 litre vessel and the draining of effluent at the same rate. Samples were taken at 0, 5, 30, 60 and 1440 minutes and diluted on MacConkey blood agar containing 2 µg ml^{-1} gentamicin before being incubated at 37°C for 24 hours. **a)** SCV were identified as being ten times smaller than WT with less pigmentation and with limited haemolytic activity. **b)** SCVs as a percentage of all cells. **c, d & e)** Around 10 SCVs from each time point were plated onto rich medium (TSB) and after 24 hours at 37°C is determined if the colony had **c)** retained a SCV phenotype **d)** partially retained a SCV phenotype but also partially reverted to WT phenotype or **e)** fully reverted to a WT phenotype. The data are the mean values ± standard deviation obtained from three independent experiments. **ns** $p \geq 0.05$

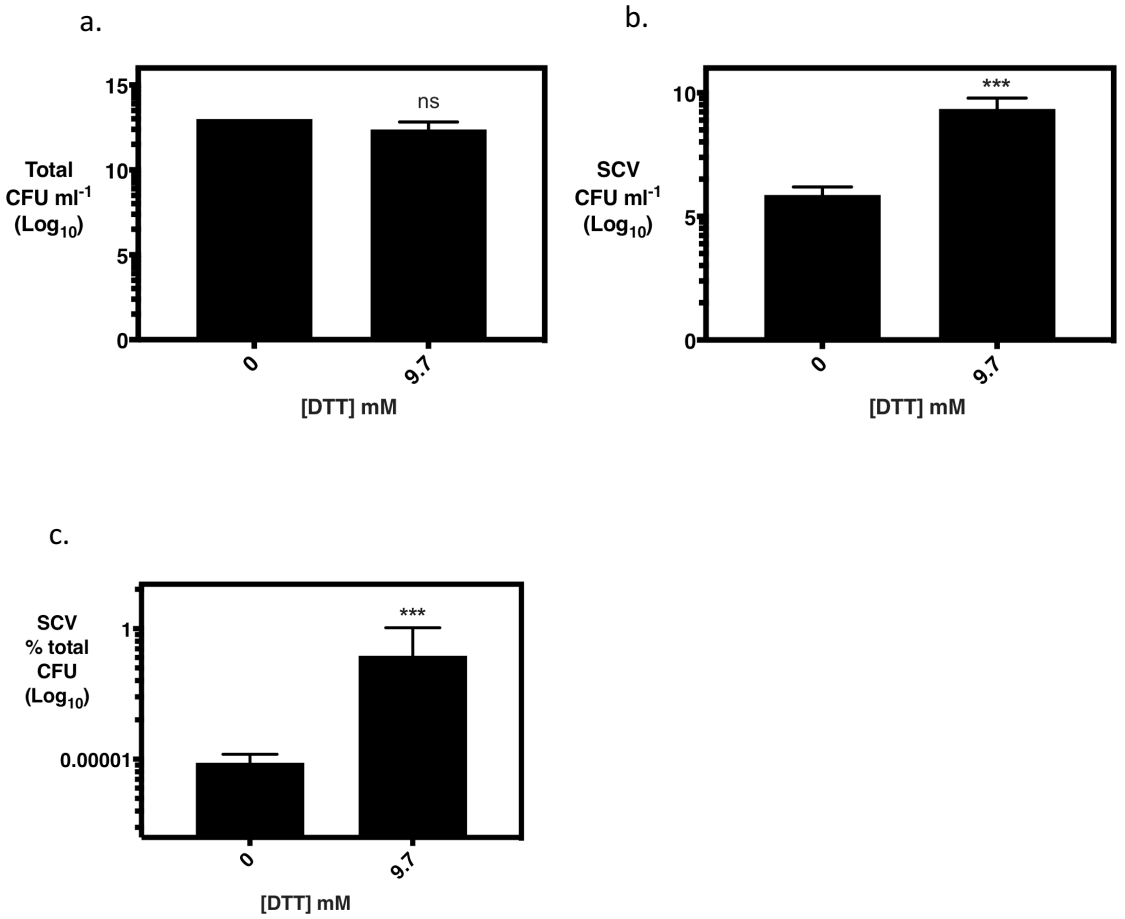


Figure 4.8 Frequency of small colony variants (SCVs) following growth on solid medium with or without the addition of DTT

Staphylococcus aureus was grown on rich medium (TSB) and MacConkey blood agar containing 2 µg ml^{-1} gentamicin. Both sets of plates either contained no DTT or were supplemented with 9.7 mM DTT as indicated. The plates were incubated at 37^{o}C for 48 hours. **a)** The total number of colony forming units (CFUs) was determined from the rich medium plates. **b)** the total number of SCVs was determined from the MacConkey blood agar plates. SCV were identified as being ten times smaller than WT with less pigmentation and with limited haemolytic activity. **c)** SCVs as a percentage of total CFUs. The data are the mean values ± standard deviation obtained from three independent experiments.

ns $p \geq 0.05$ *** $p \leq 0.001$

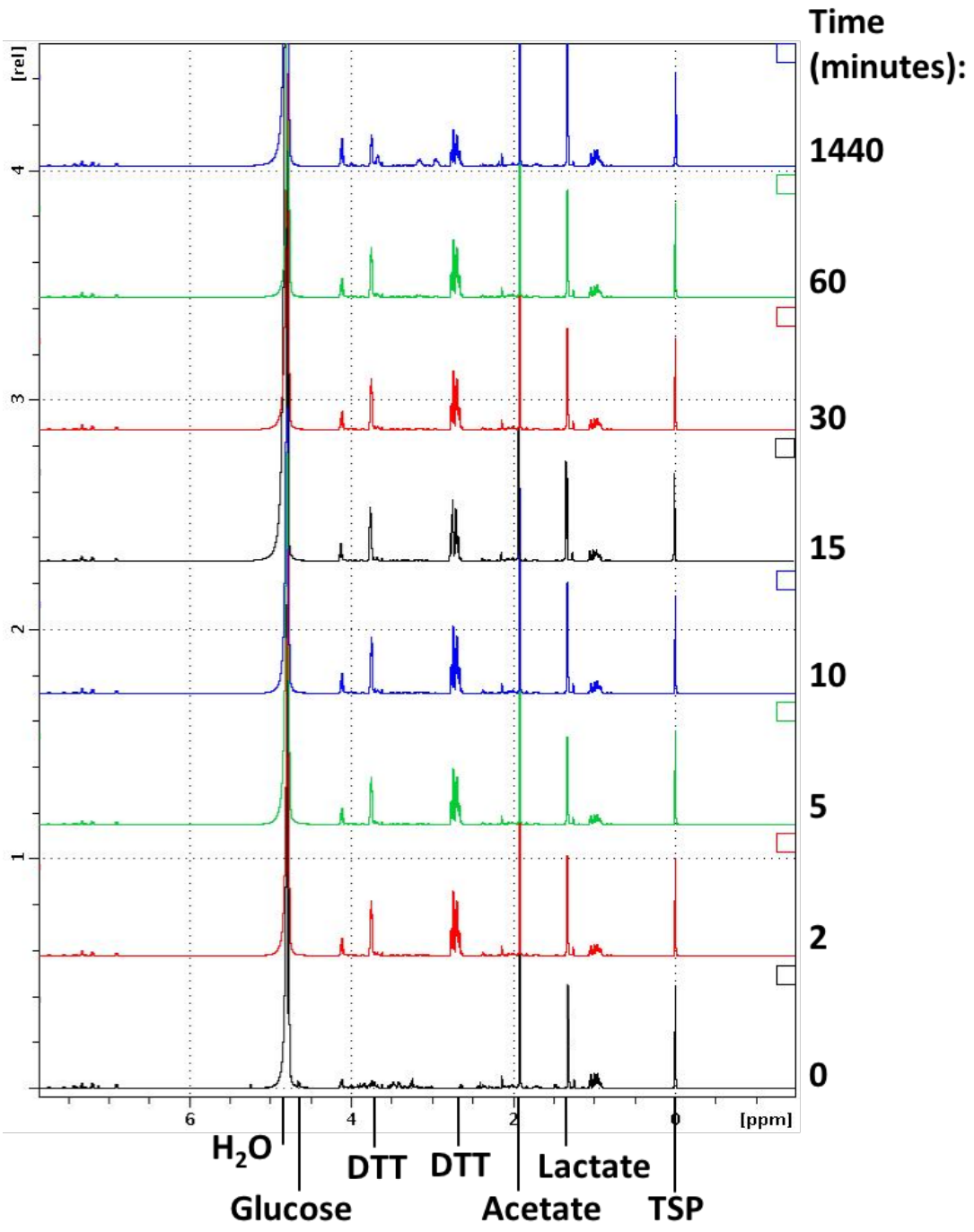


Figure 4.9 Example proton-NMR trace for *S. aureus* continuous culture samples grown in CDM following the addition of DTT

Supernatant samples from *S. aureus* continuous culture following the addition of 9.7 mM DTT were evaluated using proton NMR (Section 2.21). 1 mM trimethylislyl propionate (TSP) was used for calibration. Data shown are representative of three independent experiments.

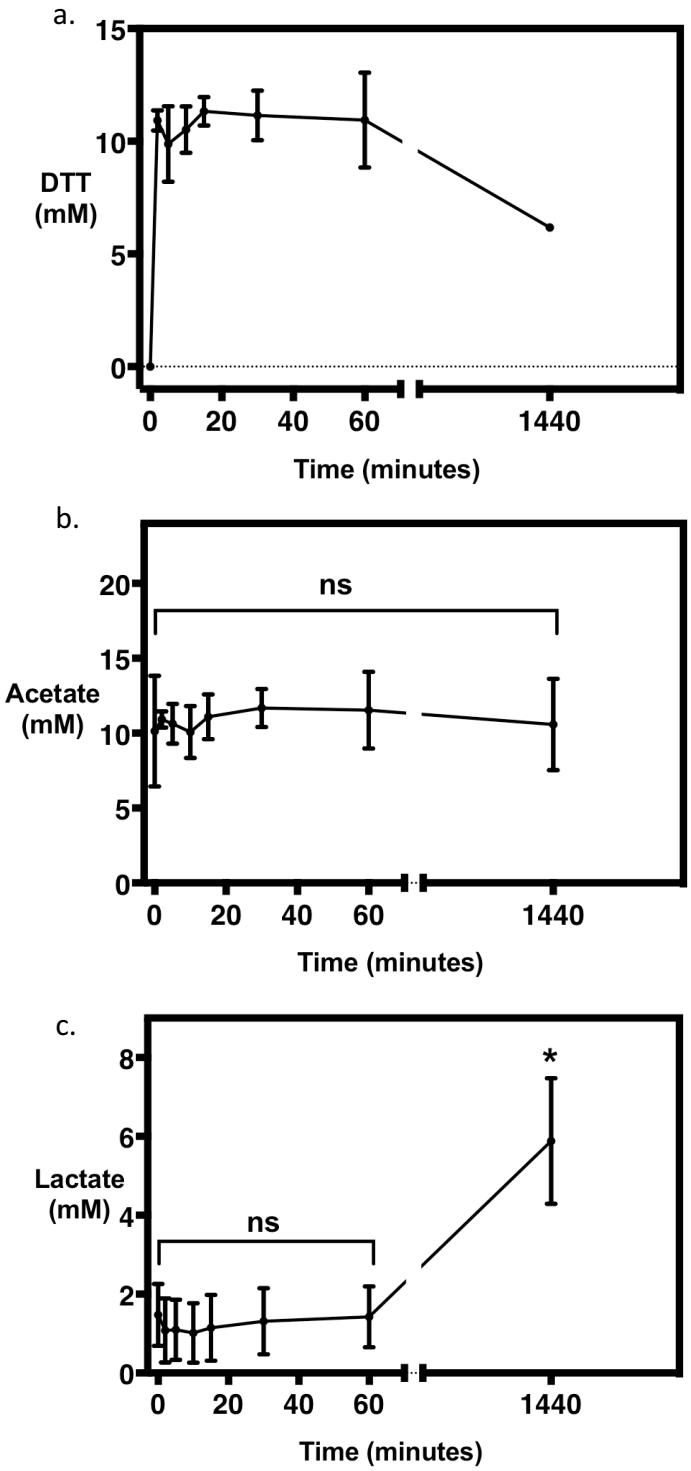


Figure 4.10 Measurement of DTT and metabolite concentration following the addition of DTT to *S. aureus* continuous culture

Proton-NMR was used to measure the concentration of metabolites in supernatant following the addition of DTT to *S. aureus* continuous culture. The concentration of **a)** DTT, **b)** acetate and **c)** lactate are shown. The data are the mean values ± standard deviation obtained from three independent experiments.

ns $p \geq 0.05$ * $p \leq 0.05$

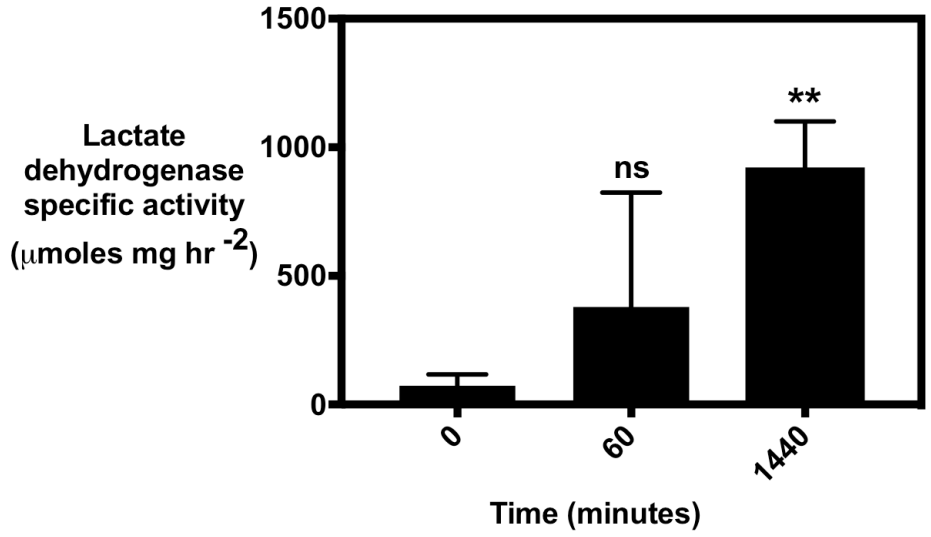


Figure 4.11 Lactate dehydrogenase activity following the addition of DTT to *S. aureus* continuous culture

The lactate dehydrogenase activity was measured in supernatant samples from *S. aureus* continuous culture following the addition of 9.7 mM DTT. The data are the mean values ± standard deviation obtained from three independent experiments.
ns $p \geq 0.05$ ** $p \leq 0.01$

4.3.5 Sudden redox shift transcriptomics and gene regulation

In order to investigate the effect a sudden negative redox shift was having on transcriptional reprogramming, microarrays were used to determine changes in transcription over the course of the experiment. Cluster analysis showed that the three repeats had similar transcriptional profiles (Figure 4.12). This was particularly clear at time 0 and 1440 minutes after the addition of DTT with more clustering around the repeats, as opposed to the intermediate samples (2, 5, 10, 15, 30 and 60 minutes after DTT addition) where the dynamics of adaptation are greatest.

4.3.6 Transcriptional regulators (TFinfer)

Before analysis of the transcription of individual genes was undertaken the entire raw dataset was analysed using the open access TFinfer programme (Asif et al., 2010). The TFinfer programme reveals changes in transcription factors that are responsible for driving transcriptional reprogramming and has been used to characterise such responses in *E. coli* (Rolfe et al., 2012, Denby et al., 2015). In order to use the TFinfer programme it is necessary to construct a matrix which contains known connections between genes and regulators. As such a matrix was constructed for *S. aureus* that contained 11 transcription factors that had previously been associated with some level of redox sensing ability. The regulators that were included were AirR, AgrA, CymR, MgrA, NreABC, Rex, Rot, SarZ, SarA, CstR and CtsR (Table 4.1).

The TFinfer software predicted four responses for these transcriptional regulators to the sudden redox drop (Figure 4.13). AgrA and Rex showed a rapid decrease in the first 2-5 minutes after the addition followed by a much slower decrease for the rest of the experiment. On the other hand, Air, CymR, CtsR and SarZ all increased drastically in the first 2-5 minutes after adding DTT before increasing at a slower rate until the end of the experiment. Rot, MgrA and SarA also increased in the first 2-5 minutes after addition of DTT; however, then decreased between 5-30 minutes before levelling off for the rest of the experiment. Finally, CstR and NreABC showed no significant change to the sudden redox potential change.

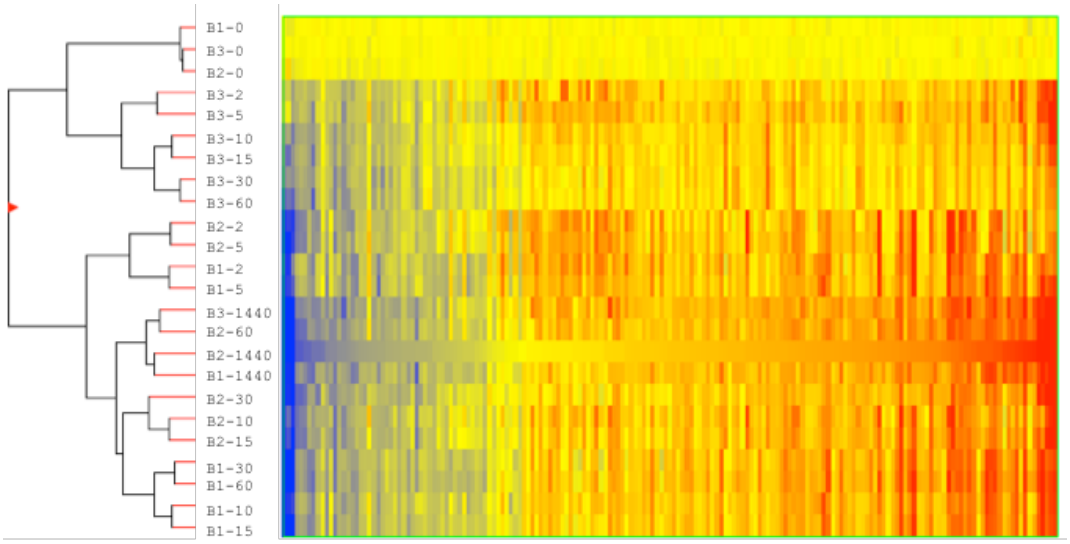


Figure 4.12 Cluster analysis of transcriptomics data
Genespring cluster analysis tool was used to compare the relationship between the different microarray data sets. Microarray data sets that show similar transcriptomic profiles are shown clustered together.
Blue) gene down-regulated relative to time 0
Red) gene up-regulated relative to time 0

Table 4.1 TFinfer transcription factors matrix

Transcription Factor	Mode of action	References
AirR	Binds DNA under reducing conditions. The sensor AirS exhibits kinase activity in reduced $[2Fe\text{-}2S]^{2+}$ but not oxidised $[2Fe\text{-}2S]^{+}$ form	(Sun et al., 2012)
AgrA	DNA binding affected by intramolecular dithiol-disulfide switch and AIPs. AIPs positively regulate Agr and inactivated by thiolysis of thiolactone ring	(Dunman et al., 2001, Wang et al., 2015)
CymR	Contains single cysteine residue that inhibits DNA binding when oxidized	(Ji et al., 2012)
MgrA	Contains single cysteine residue that inhibits DNA binding when oxidized	(Luong et al., 2006)
SarA	Contains single cysteine residue that inhibits DNA binding when oxidized	(Dunman et al., 2001)
SarZ	Contains single cysteine residue that inhibits DNA binding when oxidized	(Tamber and Cheung, 2009)
Rot	Part of SarA family but has no cysteine residues. Linked to Agr. RNAIII cleaves the *rot* transcript	(Boisset et al., 2007, Killikelly et al., 2015)
NreABC	NreC responds to oxygen through NreB [4Fe-4S] cluster. Responds to nitrate availability through action of NreA.	(Kamps et al., 2004, Schlag et al., 2008)
Rex	Binds DNA when NADH:NAD^{+} ratio is low	(Pagels et al., 2010)
CstR	Responds to sulfide stress	(Luebke et al., 2014)
CtsR	When thiols of molecular redox switch McsA are oxidised then McsB is realised, which inactivates CtsR	(Elsholz et al., 2011)

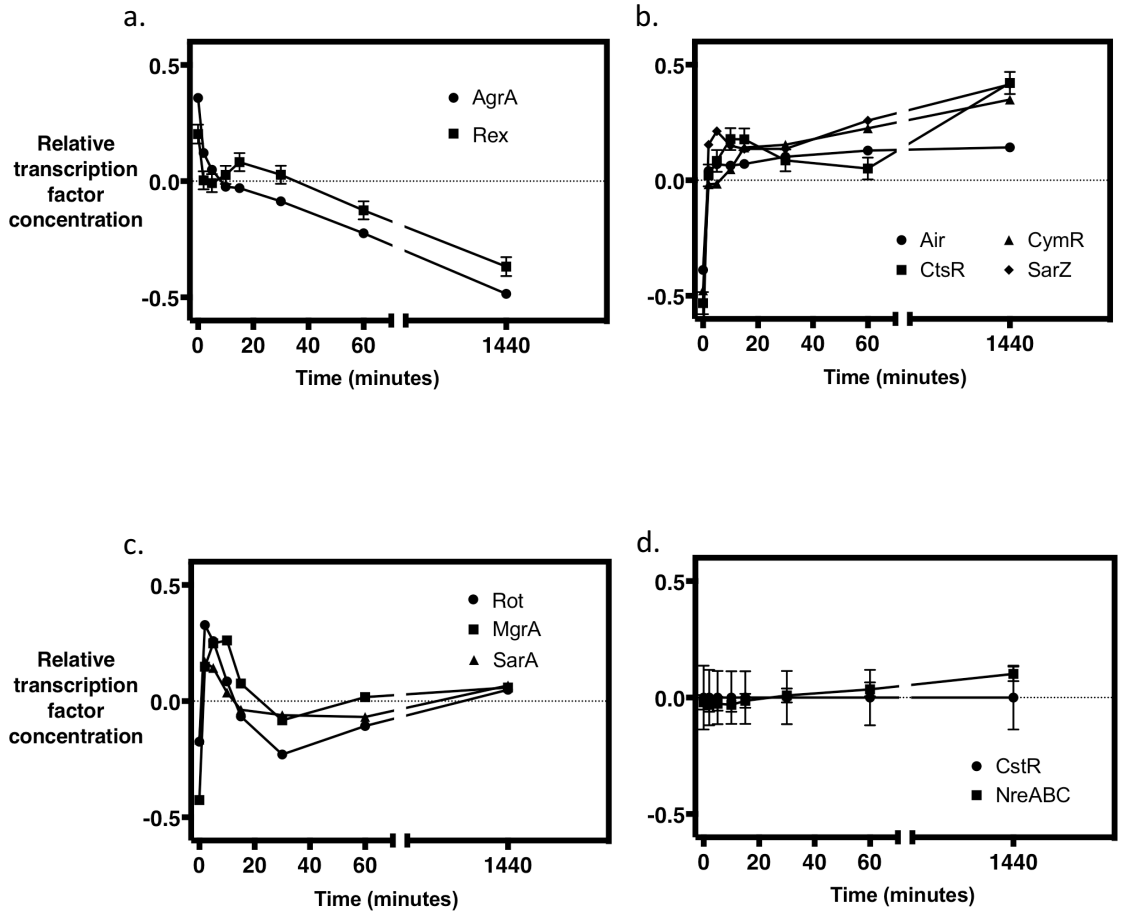
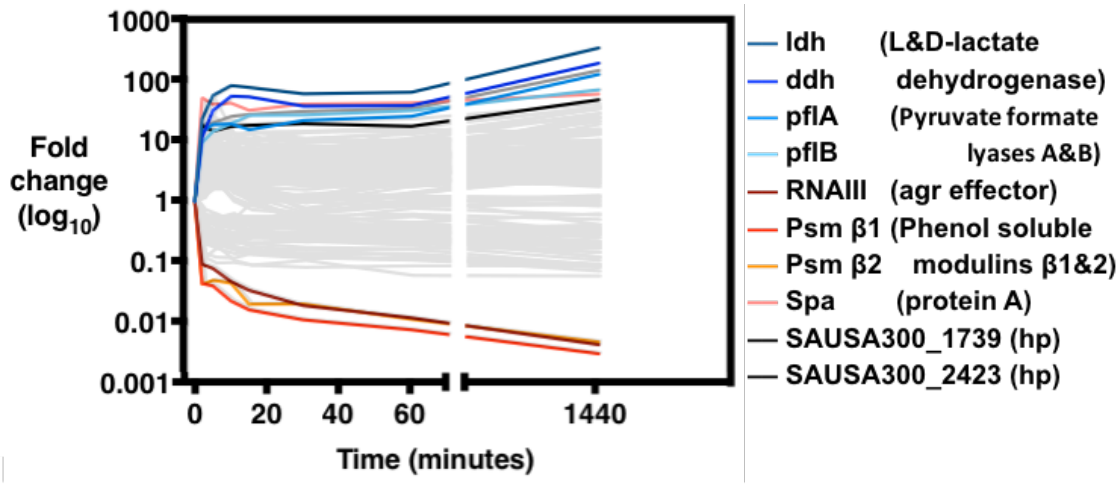


Figure 4.13 Relative concentrations of transcription factor proteins following addition of DTT to *S. aureus* continuous culture calculated using TFinfer software
The whole microarray data set was analysed using TFinfer (Section 2.12.3).
a) CstR and NreABC remain mostly unchanged. **b)** Air, CymR, CtsR and SarZ increase immediately and stay high over 24 hours. **c)** AgrA and Rex decrease immediately and continue to decrease over 24 hours **d)** MgrA, Rot and SarA increase immediately but then decrease and stabilise between 30 minutes and 24 hours.

a.



b.

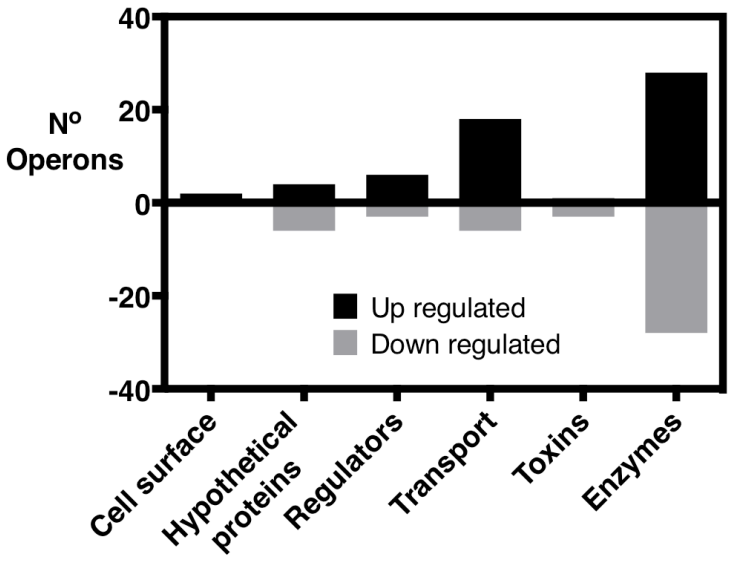


Figure 4.14 Overview of transcriptomics following the addition of DTT to *S. aureus* continuous culture

a) log_{10} fold change in genes significantly (≥ 5-fold, $p < 0.05$) up or down-regulated following the addition of 9.7 mM DTT are shown in grey and the top ten most significant results are coloured. **b)** Up or down-regulation of operons shown for 1440 minutes following DTT addition, divided by operon class (Table 4.2).

Table 4.2 Transcripts that were altered in response to the addition of dithiothreitol to aerobic respiratory steady-state cultures of *S. aureus* USA300

Transcriptional units[a]	Fold change in abundance relative to the initial aerobic steady state[b]						Function[c]
	2 min	5 min	10 min	15 min	30 min	60 min	1440 min	
Class 1: Cell surface								
0438	3.6	2.8	3.5	2.9	2.2	2.3	5.7	N-Acetylmuramoyl-L-alanine amidase
1741-1743	7.76	11.77	12.58	12.93	13.99	16.54	35.27	Putative lipoprotein; hypothetical proteins
Class 2: Hypothetical proteins								
0173	-5.4	-5.3	-6.6	-7.9	-9.0	-9.3	-14.8	Hypothetical protein
0178	-1.7	-1.8	-2.5	-3.4	-4.2	-5.1	-7.3	Hypothetical protein
0261	2.4	3.2	4.1	3.7	2.2	2.2	7.6	Hypothetical protein. Ado-Met methyltransferase superfamily
0953	-4.7	-5.2	-5.1	-4.0	-4.1	-3.9	-5.6	Fucose O-acetylase or related acetyltransferase family
1224	5.1	7.1	5.8	5.6	6.9	8.2	13.9	Hypothetical protein
1739*	17.9	14.4	17.2	17.9	19.1	17.2	47.2	Hypothetical protein
1997	-4.4	-2.8	-3.7	-3.9	-4.1	-5.2	-5.4	Hypothetical protein
1998	-4.4	-2.8	-3.7	-3.9	-4.1	-5.2	-5.4	Hypothetical protein
2132	10.7	19.2	21.7	13.7	13.7	14.1	13.8	Hypothetical protein
2164*	-3.8	-3.8	-5.7	-6.8	-8.4	-9.6	-11.8	Hypothetical protein, MAP domain (found in extracellular matrix proteins)
2423	18.2	19.7	25.2	27.0	30.4	33.8	143.6	Hypothetical protein
2448	-4.2	-4.1	-4.2	-3.7	-3.7	-4.7	-7.2	Hypothetical membrane protein
2641	2.2	2.6	2.6	2.9	3.0	4.3	6.6	Hypothetical protein, DUF3147 superfamily
Class 3: Regulators								
0114*	6.2	7.3	7.9	6.7	5.6	7.1	4.9	SarS: Staphylococcal accessory regulator
0316	3.1	2.5	2.4	2.8	3.2	3.2	6.1	ROK family DNA- and/or sugar-binding family protein
1457-1456	7.5	8.1	4.9	4.7	4.3	4.5	8.7	α-D-1,4-glucosidase; MalR: maltose operon transcriptional repressor
1708	3.2	4.0	4.0	3.0	4.8	6.7	4.0	Rot: repressor of toxins
1988	-11.0	-12.9	-21.1	-29.5	-52.7	-84.3	-232.5	RNAIII and δ-toxin
1989-1990*	-5.2	-6.2	-7.5	-7.4	-8.8	-9.7	-12.9	AgrBD: accessory gene regulator system
1991-1992*	-4.9	-3.9	-4.8	-5.8	-6.7	-6.5	-11.9	AgrCA: accessory gene regulator system
2347	7.4	6.2	4.5	4.1	4.5	7.5	23.4	NirR: nitrite reductase regulator
2444-2443	4.5	5.7	3.2	2.7	2.2	2.2	2.2	GntR: gluconate operon repressor; GntK: gluconate kinase

Class 4: Transport							
0117-0115	3.2	5.7	7.5	7.6	4.7	3.9	6.8	SirABC: ABC iron-compound transporter
0173-0177	-5.4	-5.3	-6.6	-7.9	-9.0	-9.2	-14.7	ABC transporter ATP-binding protein similar to MetN; ABC transporter Zn(II)-binding protein; ABC transporter permease; hypothetical protein, acylCoA dehydrogenase
0191*	2.0	3.2	4.4	6.4	6.7	4.7	5.1	PtsG: PTS system glucose-specific IIBC subunit;
0208-0211	5.7	7.2	5.3	4.8	4.3	4.3	4.8	Maltose ABC transporter
0216*	7.8	8.1	6.2	4.9	3.7	5.2	4.4	UhpT: sugar phosphate antiiporter
0231	-8.5	-5.3	-4.9	-5.0	-4.7	-5.5	-9.8	NikA: ABC transporter Ni(II)-binding protein (PBP_2 fold)
0333-0330	9.3	8.5	6.0	4.4	2.5	2.0	3.7	BglG family transcriptional antiterminator; PTS system, IIA component; hypothetical protein; UlaA: PTS system, IIC component 0333 numbers?
0337*	4.3	5.8	4.6	4.0	4.8	5.0	4.4	GlpT: glycerol-3-phosphate transporter
0382	-4.3	-5.0	-4.7	-4.2	-4.0	-4.5	-8.6	Sodium-dicaboxylate symporter family
0435-0437	1.3	-2.4	-4.9	-5.7	-5.3	-4.6	-4.7	ABC transporter ATP-binding protein; ABC transporter permease; dipeptide-binding NLPA lipoprotein 0435 numbers?
0448	6.1	12.1	10.8	9.1	5.6	5.7	6.6	TreP: trehalose-specifc IIBC component
0887-0891*	14.1	4.8	1.5	1.2	1.1	2.4	2.4	OppB: oligopeptide ABC transporter permease; OppC: oligopeptide ABC transporter permease; OppD: oligopeptide ABC transporter ATP-binding protein; OppE: oligopeptide ABC transporter ATP-binding protein; OppA: oligopeptide ABC transporter substrate-binding protein
0914	12.2	5.3	1.8	1.4	1.2	2.0	2.0	Alanine or glycine: cation symporter, AGCS family
0934-0936	5.4	2.1	1.0	-1.23	-1.7	-1.1	1.1	Membrane protein; Hypothetical protein; ABC transporter ATP-binding protein
1005*	-2.2	-2.0	-2.4	-3.1	-3.5	-3.3	-5.6	MntH: Manganese transporter
1191*	7.3	7.9	6.6	6.2	5.3	6.0	9.3	GlpF: glycerol uptake facilitator
2270	4.3	4.5	3.7	3.6	3.7	3.8	5.4	GlvC: arbutin-like IIBC component
2291	2.8	3.5	3.9	4.7	5.7	4.7	2.8	GltS: sodium-glutamate transporter
2313	9.2	13.9	10.1	10.3	13.4	16.1	35.2	L-lactate permease
2359-2357	-3.1	-3.0	-3.5	-3.7	-4.3	-4.6	-5.8	Cystine ABC transporter substrate-binding protein; Cystine ABC transporter permease protein; Cystine ABC transporter ATP-binding protein
2383	12.8	13.5	12.3	13.7	10.6	8.7	20.5	Amino acid permease
2442	3.4	6.7	4.5	2.7	2.4	2.1	1.9	GntP: gluconate permease

2449	8.4	11.0	6.6	4.6	4.8	5.1	5.4	Putative MFS transporter
2476*	4.0	8.1	8.6	8.7	8.1	6.1	6.7	PtsG: PTS system glucoside-specific IIABC subunit
Class 5: Toxins								
1065	2.7	2.7	2.7	2.6	2.4	3.6	6.1	Exfoliative toxin A
1067	-22.7	-25.1	-44.3	-62.2	-90.6	-132.6	-329.3	Phenol soluble modulin β1
1068	-23.5	-20.2	-22.4	-49.9	-49.5	-88.9	-210.4	Phenol soluble modulin β2
2572	-2.3	-2.3	-2.1	-2.6	-2.8	-3.1	-5.0	Aur: aureolysin (zinc metalloprotease)
Class 6: Enzymes								
0008	12.8	13.1	8.3	5.7	4.5	5.2	3.1	HutH: Histidine ammonia lyase
0009*	5.8	4.5	2.2	1.2	1.0	1.2	1.1	SerS: Seryl-tRNA synthetase
0108	2.0	3.5	3.5	3.1	2.8	4.7	10.2	Carbon-oxygen lyase (myosin-cross-reactive antigen)
0220-0221*	23.0	56.0	81.1	75.6	59.4	63.1	337.4	PflB: pyruvate formate-lyase; PflA: pyruvate formate-lyase activating enzyme
0229-0225	26.5	23.4	13.0	12.1	5.9	10.2	9.8	FadX: putative acylCoA transferase; FadE: acylCoA synthetase; FadD: acyl-CoA dehydrogenase; 3-hydroxyacylCoA dehydrogenase; FadA: putatative acylCoA acetyltransferase
0235*	13.8	18.6	18.9	15.2	21.6	25.3	123.5	L-lactate dehydrogenase
0311*	5.3	5.0	3.9	7.0	5.4	11.1	28.0	PfkB: carbohydrate kinase
0315-0314	9.9	12.7	9.8	10.4	10.7	12.5	32.7	NanA: N-acetylneuraminate lyase; sodium:solute symporter family protein
0433-0434*	-7.6	-8.7	-8.9	-7.5	-6.2	-8.5	-11.3	MccA: cysteine synthase/cystathionine-β-synthase; MccB: cystathionine-γ-synthase
0536	-4.3	-5.5	-9.9	-11.7	-12.8	-12.1	-11.9	HchA: chaperone protein and glyoxalase
0538	1.8	5.0	6.4	5.1	5.6	4.5	1.9	NAD-dependent epimerase/dehydratase family protein
0594*	2.9	5.3	5.2	4.7	4.2	8.2	20.5	Adh: alcohol dehydronase
0604*	7.0	6.8	4.5	3.3	2.8	3.6	4.1	alpha-beta fold family hydrolase
0636-0638*	1.9	2.4	2.4	2.5	2.6	2.6	5.7	DhaKL: dihydroxyacetone kinase subunits; phosphotransferase mannnose-specific family component IIA
0860*	5.3	4.0	3.2	3.2	3.3	3.6	5.7	RocD: ornithine--oxo-acid transaminase
0862	8.3	10.4	10.8	10.0	9.3	10.0	35.0	GlpQ: glycerophosphoryl diester phosphodiesterase
0963-0960*	2.6	2.8	3.7	4.0	4.8	5.7	8.7	QoxCBA: quinol oxidase
0986-0987	1.8	3.7	7.0	7.0	6.1	7.3	7.4	Cytochrome D ubiquinol oxidase, subunit I and II
1015	1.7	2.8	3.4	3.4	4.2	5.1	4.9	CtaA: cytochrome oxidase assembly protein
1016-1017*	2.3	2.9	3.7	4.0	4.2	5.2	6.3	CyoE: protoheme IX farnesyltransferase; DUF420, predicted membrane protein
1192*	4.0	6.5	4.4	4.8	4.0	5.6	5.1	GlpK: glycerol kinase

1226-1228	14.4	7.4	2.9	1.4	-1.3	1.3	3.0	Homoserine dehydrogenase; ThrC: threonine synthase; ThrB: homoserine kinase
1330-1329	1.2	1.7	1.7	1.7	2.0	4.4	23.1	IlvA: threonine dehydratase; amino acid permease
1331	1.5	1.6	1.6	1.5	2.7	6.0	27.5	Ald: alanine dehydrogenase;
1655	2.9	3.8	4.0	4.5	5.9	5.8	6.7	Ald: alanine dehydrogenase
1679	21.4	19.5	12.2	10.5	10.5	13.3	24.3	AcsA: acetyl-CoA synthetase
1680-1681	9.0	6.7	5.2	5.2	5.2	5.8	14.2	AcuA: acetoin dehydrogenase; AcuC: acetylCoA synthetase deacetylase
1711	9.2	7.8	5.7	6.0	7.4	7.8	13.4	PutA: proline dehydrogenase
1731	7.7	8.2	6.8	7.1	9.3	10.5	8.7	PckA: phosphoenolpyruvate carboxykinase
2277-2278	43.2	44.2	12.8	6.9	6.0	15.4	12.9	HutI: imidazolonepropionase; HutU: urocanate hydratase
2343	6.5	6.4	3.0	2.3	2.0	5.0	18.4	NarG: nitrate reductase subunit A
2346-2344*	2.7	3.7	2.8	2.8	2.8	4.7	7.7	NirBD: nitrite reductase; uroporphyrin-III C-methyl transferase
2463	9.2	14.2	19.0	26.2	26.5	31.2	69.1	Ddh: D-lactate dehydrogenase
2491	7.3	8.5	9.3	8.5	8.2	7.2	12.4	1-Pyrroline-5-carboxylate dehydrogenase
2537	2.6	3.1	3.9	3.4	3.1	3.7	9.1	L-lactate dehydrogenase

[a] The extent of the operons are based on the observed coordinated regulation and the information in the BioCyc database for the *S. aureus* USA300 genome (Caspi et al., 2014).

[b] The data shown are for the first gene in the operon.

[c] Function for each gene as described in the NCBI genome database for *Staphylococcus aureus* USA300 or description of homolog gene in other *S. aureus* strain.

*Indicates transcription units that exhibited similar transcriptional regulation 6 h post-internalization of *S. aureus* in human epithelial cells, a period during which *S. aureus* appears in the cytosol of infected cells (Garzoni et al., 2007; Menzies and Kourteva, 1998).

4.3.7 Global transcriptional reprogramming

Global transcriptional reprogramming was analysed and 122 operons were judged to be significantly (≥ 5-fold and $p < 0.05$) regulated. There were 91 operons up-regulated and 31 operons down-regulated (Figure 4.14 and Table 4.2). The operons were grouped into six classes according to function: cell surface, hypothetical proteins, regulators, transport, toxins and enzymes.

4.3.7.1 Class 1: Cell surface

Contains two operons that both had increased transcription 2 minutes after DTT was added and continued to increase transcription for the full 24 hour experiment. The operon SAUSA300_1741-1743, a lipoprotein and two hypothetical proteins, increased dramatically with a 35.3 – fold increase in transcription by the final time point. The up-regulation of SAUSA300_0438, an N-acetylmuramoyl-L-alanine amidase autolysin, suggests an altered peptidoglycan structure or increased turnover.

4.3.7.2 Class 2: Hypothetical proteins

Consists of 5 operons that were up-regulated and 8 operons that were down-regulated following the negative redox shift. However, they are largely uncharacterised and require further investigation before conclusions can be made about them.

4.3.7.3 Class 3: Regulators

Contains 9 operons including the various parts of the Agr system: AgrBD, AgrCA and RNAIII/ð-toxin which are all immediately and consistently down-regulated following the addition of DTT. This suggests that there is a pronounced effect of negative redox potential on Agr. This could be through the inactivation of the AIPs through the N-acetyl –cysteamine thiolysis of the thiolactone ring resulting in the breaking of the thiolactone macrocycle bond between the C-terminal carboxyl and the cysteine thiol (Wang et al., 2015). This suggests that the deactivation of Agr observed in intracellular *S. aureus* may be as a result of the low redox potential in the mammalian cytoplasm. Rot (repressor of toxins) transcription is directly related to Agr and RNAIII and so it is no surprise to observe Rot transcription go up in contrast to AgrBD, AgrCA and RNAIII.

Rot regulates a number of virulence factors and biofilm formation through repressing the secretion of certain proteases (Said-Salim et al., 2003, Mootz et al., 2015). SarS (Staphylococcal accessory regulator) is also known to be repressed by Agr (Cheung et al., 2001) and so its de-repression following sudden redox drop is expected. SarS in turn regulates a number of virulence factors including the activation of protein A production (Cheung et al., 2001); NirR is also upregulated following the addition of DTT and as NirR is a regulator of nitrate reductase it suggests that there may be a switch towards anaerobic respiration despite the constant availability of oxygen during the experiment. The final regulators that are significantly up-regulated are MalR and GntR-GntK. MalR is the maltose operon repressor and GntR is a gluconate operon repressor. As such, the increased transcription of both these perhaps indicates a change of carbon metabolism.

4.3.7.4 Class 4: Transport

Contains a number of carbon transporter proteins including 5 phosphotransferase (PTS) system transporters that are upregulated: *glcA*, *glcB*, *glvC*, *treP* and *ulaA* that transport glucose, glucoside, arbutin, trehalose and ascorbate respectively. There is also an ABC transporter for maltose (SAUSA300_0288-0211) that is upregulated and a couple of major facilitator system (MFS) transporters for glycerate (SAUSA300_2449) and glycerol (*glpF*) that are upregulated as well. There are also a number of secondary carbon transporters upregulated: *uhpT*, *glpT*, SAUSA300_2313 and *gntP* that transport sugar phosphate, glycerol-3-phosphate, lactate and gluconate. Finally, there is one carbon transporter that is down-regulated and that is SAUSA300_0382 which is a secondary transporter for dicarboxylic acid. Overall the trend is towards increased transport of carbon, in line with studies of intracellular *S. aureus* (Garzoni et al., 2007).

There also three metal transporters that have a significant transcription response. Manganese (*mntH*) and nickel (*nikA*) transporters are down-regulated whereas the iron transporter *sirABC* was up-regulated.

The other transporters to be significantly up-regulated or down-regulated were mainly amino acid transporters. ABC transporter of oligopeptides (SAUSA300_0887-0891) was

upregulated as well as the secondary transport systems for glutamate (*gltS*), alanine/glycine (SAUSA300_0914) and an amino acid permease of unknown specificity (SAUSA300_2383). Whereas, the ABC transporters for dipeptides (SAUSA300_0435-0437) and cystine were down-regulated. The down-regulation of cystine transport probably resulting from the low redox environment converting all the cystine in the environment to cysteine. Other sulfur related transport proteins were also down-regulated including a sulfonate transporter (SAUSA300_0174-0177) and a Gly-Met dipeptide-binding protein (*glmpC*).

4.3.7.5 Class 5: Toxins

Included in this group are five toxins (the four listed and ð-toxin (SAUSA300_1988) which is included in *Class 3: Regulators* because of its being encoded by RNAIII), including three phenol soluble modulins (PSMs): PSMβ1, PSMβ2 and PSMγ (ð-toxin). All three of these are regulated by Agr and are down-regulated following the addition of DTT. There is a link between ð-toxin and phagosome escape (Giese et al., 2011); whereas, PSMβ1 and PSMβ2 have no cytolytic activity but do induce neutrophil chemotaxis and calcium flux (Peschel and Otto, 2013). Aureolysin, a toxin that inhibits the activation of complement and the influx of neutrophils (Laarman et al., 2010), is also down-regulated following the addition of DTT. Finally, expression of exfoliative toxin A, a serine protease that damages desmosomal proteins in the skin (Bukowski et al., 2010), was increased following the addition of DTT.

4.3.7.6 Class 6: Enzymes

The final group consists of 35 enzyme operons that were up-regulated or down-regulated following DTT addition. Of these a number were related to respiration. There was increased expression of *qoxABCD, cyoE* and *ctaA*, which code for the major quinol oxidase (Qox) and genes involved in the synthesis of Qox heme cofactors, in order to increase flux through the electron transport chain (Figure 4.15). Expression of cytochrome D oxidase (*cyd*) was also increased further indicating a disruption of the respiratory chain. There was also increased expression of genes involve with anaerobic

fermentation. Namely: *adh, ddh, ldh* and *pflBA* coding for alcohol dehydrogenase, L and D lactate dehydrogenases and pyruvate formate-lyase respectively.

Operons encoding amino acid metabolism enzymes were also significantly up-regulated and down-regulated. Alanine (*ald*), histidine (*hutH* and *hutUI*) and proline (*putA*) degradation enzymes were upregulated; whilst, isoleucine (*ilvA*) and threonine (*thrCB*) synthesis enzymes were also upregulated. Cysteine and/or hydrogen sulfide (H_2S) metabolism (*mccAB*) on the other hand was down-regulated in keeping with the role of H_2S a reductant (Shatalin et al., 2011).

4.4 Discussion

Intracellular *S. aureus* are increasingly understood as playing a crucial role in chronic staphylococcal infections (Garzoni and Kelley, 2011, Fraunholz and Sinha, 2012). Understanding how *S. aureus* is able to adapt to the environment of host cytosol is crucial to tackling these persistent *S. aureus* infections. One area which has been potentially overlooked is the response of *S. aureus* to the changing host intracellular redox environment. Changes in redox have been shown to have a profound effect on *S. aureus* metabolism and transcription (Nouaille et al., 2014).

Here it was shown that continuous chemostat culture can be used successfully to investigate the response of *S. aureus* to redox change. The use of a chemostat allowed the parameters of 1 L min^{-1} air flow, 400 rpm stirring and pH 7 to be maintained throughout the experiment and allowed the response to redox potential to be investigated in isolation. The use of DTT to control redox potential proved a reliable mimic for the drop of redox potential experienced when bacteria escape from mammalian phagosomes into the cytosol (Figure 4.2).

Following the sudden drop in redox potential reduced by the DTT there was an increase in the oxygen concentration suggesting an inhibition of respiration. Then follow up experiments using a Clark-type oxygen electrode indicated a potential inhibition of respiration by GSH, the reducing agent found in mammalian cytosol

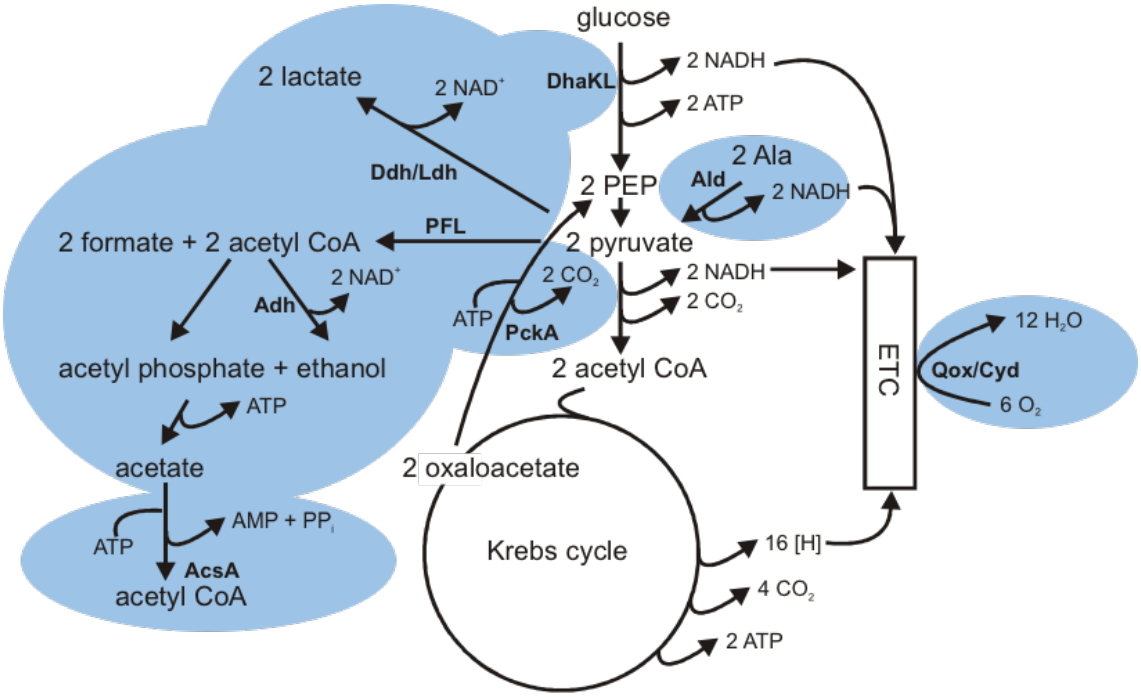


Figure 4.15 Effect of DTT on expression of genes related to respiration
Genes related to respiration that were identified as having been significantly up-regulated following the addition of DTT are highlighted in bold.

(Figure 4.6). This combined with the transcriptomics data, that revealed an increase in electron transport associated *qox* and *cyd* genes, suggests that the reducing conditions of the experiment inhibited aerobic respiration by disrupting the electron transport chain (Figure 4.15).

This inhibition of aerobic respiration, despite constant availability of oxygen, led to the initiation of anaerobic fermentation. The transcriptomics revealed the increased expression of anaerobic fermentation related genes with the production of lactate and the activity of lactate dehydrogenase also confirmed. The changes in carbon uptake may be due to this switch to a less efficient form of metabolism. It has also been shown previously that a switch to anaerobic fermentation, particularly pyruvate fermentation, can mitigate the slow growing phenotype of SCVs (Cao et al., 2017).

There was also a shift in the transport and production of amino acids. Glutamate and glycine transport increased and isoleucine and threonine synthesis increased, despite these being available in excess in the medium. Whilst, alanine, histidine and proline degradation increased, and cystine transportation and cysteine synthesis decreased. This would fit with the reduction of disulfide bonds, leading to the majority of the cystine in the medium being converted to cysteine.

Toxin production was generally decreased with PSMs β1, β2 and delta toxin specifically significantly decreased and the expression of Rot (repressor of toxins) increased. Production of beta and delta toxins has been linked to phagosomal escape (Giese et al., 2011); therefore, observing a significant decrease in their production following a reduction in redox potential aids the theory that redox is an important factor in signalling during intracellular S. *aureus* infections. It has already been shown that redox is an important signalling factor in *Listeria monoctogenes*. In *L. monoctogenes* the Crp-Fnr family transcriptional regulator PrfA is activated by binding host cell cytosol glutathione. This leads to the expression of numerous essential virulence factors including the hemolysin listeriolysin (Scortti et al., 2007, Portman et al., 2017).

An interesting exception to the pattern of decreased toxin production by *S. aureus* in the presence of DTT was the increased expression of exfoliative toxin A. Exfoliative toxin A actually increased expression in the low redox environment, with potentially intriguing consequences in regards to understanding staphylococcal scalded skin syndrome (SSSS). SSSS is generally an acute condition but there have been reports of persistent SSSS (Shelley et al., 1998) infections. This raises the possibility that SSSS could be induced by intracellular dwelling *S. aureus* following their escape into the low redox potential environment of the host cytoplasm. Production of molecules associated with the cell surface, on the other hand, was generally increased in keeping with regulation by Agr.

The decreased activity of Agr, and the associated phenotype of decreased toxin production and increased cell surface factor expression following the sudden drop in redox potential, links with the transcriptomic profile of *S. aureus* following 6 hours internalisation in lung epithelial cells (in the lung epithelial model the *S. aureus* cells had escaped from the phagosome into the host cytoplasm by 6 hours) (Garzoni et al., 2007). This further corroborates with the hypothesis that redox is a major transcriptional trigger which, following escape from the host phagosome, leads to persistent survival in the host cytosol. This work also reemphasises the importance of Agr in facilitating survival in the host cytosol. This matches with the previous research that found an *agr- S. aureus* strain cannot escape from host phagosomes and survive in host cytosol (Schnaith et al., 2007).

In keeping with the observed Agr profile following growth at a reduced redox potential; the proportion of SCVs observed on solid media containing DTT increased over a period of 48 hours. However, there was no increase in SCVs during the 24 hour course of the main experiment. Agr is known to be important for the production of SCVs and survival intracellularly. However, it may be that this link between SCV formation and Agr is incidental. Intracellular SCV formation could occur because of the disruption of aerobic metabolism by the reduced environment of host cytoplasm. SCV mutants generally have SNPs in metabolic or electron transport chain genes and a key phenotype of all these SCV mutants is a slowed metabolism. It may be the case that *S.*

aureus responds to the sudden redox drop and the subsequent inhibition of the electron transport chain by inducing anaerobic respiration and that this less efficient form of respiration is what gives the appearance of SCVs.

The sudden drop in redox potential also had a broader effect on other regulators including increased expression of SarZ (Figure 4.13). It has been postulated that SarZ may play an important role in modulating the expression of Agr, MgrA and SarA with a *sarZ* mutant having reduced Agr and MgrA expression and increased SarA expression (Tamber and Cheung, 2009, Chen et al., 2009). However, in these experiments, although MgrA expression went down, in line with what would be expected from increased SarZ expression, Agr expression went down and SarA expression increased (Figure 4.13). This questions the relationship between these four regulators and suggests that SarZ is not able to modulate the others under the conditions of the experiments detailed in this chapter.

4.4.1 Future work

Future experiments could be conducted using alternative reductants such as GSH. This would improve the accuracy of comparisons to conditions *in vivo* because GSH is an important mediator of the low reduced state of mammalian cytosol. However, because of GSH's relative instability experiments over a 24 hour, or even longer time periods, it may not stay at as stable a redox potential as when DTT was used.

Another approach to increase understanding of aerobic low redox potential environments could be to increase the length of the experiment. The continuous culture could be maintained for a number of days better mimicking a persistent infection. Continuous *S. aureus* cultures have been maintained for up to 60 days, with resulting increases in SCV numbers; however, biofilm formation is a constant problem with such long experiments (Udekwu and Levin, 2012, Bui et al., 2015).

Follow up work to investigate links between intracellular *S. aureus* and scalded skin syndrome would be interesting. Intracellular persistence is normally associated with low virulence and asymptomatic carriage; however, the production of exfoliative toxin

when *S. aureus* was exposed to a low redox environment suggests that there may be a link between intracellular *S. aureus* and scolded skin syndrome.

Chapter 5: Identification of *S. aureus* genes related to redox response

5.1 Introduction

5.1.1 Growth in low redox environment

The ability to grow in low redox potential environments is essential for the proliferation of intracellular *S. aureus* and the establishment of persistent infections (Chapter 4) (Kubica et al., 2008, Fraunholz and Sinha, 2012). Intracellular dwelling *S. aureus* is able to survive and proliferate in mammalian cytosol where the redox potential is kept low by high ratios of reduced glutathione to oxidised glutathione (Hwang et al., 1992). Identifying genes that facilitate growth in such a low redox potential environment could aid in developing new treatments for persistent intracellular *S. aureus* infections.

5.1.2 Sulfur metabolism

Another way by which *S. aureus* encounters different redox potential environments is through the uptake of different sulfur sources. Different sulfur containing compounds encountered by *S. aureus* contain sulfur in different redox states. This may have an effect on the ability of *S. aureus* to utilise these different sulfur sources. Improving understanding of which sulfur sources *S. aureus* readily utilises and identifying genes involved in the process could aid in developing a clearer idea of *S. aureus* sulfur metabolism and redox homeostasis.

5.1.3 Hydrogen sulfide production

Related to both sulfur metabolism and the response to redox environment is the production of H_2S. The production of H_2S by *S. aureus* is thought to be under the control of the master cysteine regulator (CymR) and linked to the actions of MccAB, MetC and CysM (Chapter 3) (Lithgow et al., 2004, Soutourina et al., 2009, Shatalin et al., 2011). However, a clear understanding of how these genes relate to H_2S production and the wider picture of cysteine and sulfur metabolism remains unclear. Identifying

genes that are involved in H_2S production could aid in developing a clearer idea of *S. aureus* H_2S production mechanisms, sulfur metabolism and redox homeostasis.

5.1.4 The Nebraska Transposon Mutant Library (NTML)

The study of individual genes through their disruption is particularly time consuming and costly in *S. aureus*. The construction of plasmids and homologous recombination into the chromosome is required and there is a need to use shuttle plasmids and intermediary *S. aureus* strains because of the extensive restriction modification systems (Vasu and Nagaraja, 2013).

A more efficient way to create specific genetic mutations is to use transposon mutagenesis. Using transposons to interrupt random genes throughout the genome leads to the creation of a library of mutants that can be phenotypically tested. Transposon mutation of *S. aureus* has been attempted a number of times with varied results. Transposon Tn917, delivered using the temperature sensitive plasmid pTV1*ts* was used in *S. aureus* Newman to successfully create a transposon mutant library; however, it's overall success was limited by transposon sequence preference leading to the localisation of insertions (McDevitt et al., 1994). Another transposon mutant library was created in Newman, using a two plasmid system, with more success (Bae et al., 2004). The two plasmids used were pFA545, which encodes the *mariner* transposase and ampicillin and tetracycline resistance cassettes, and pBursa, which encodes a chloramphenicol resistance cassette, a temperature sensitive plasmid replicon and a minimariner transposable element (*Bursa aurealis*). The *Bursa aurealis* element contains an erythromycin resistance cassette, a *Aequorea victoria* green fluorescent protein (*gfp*) and the mariner terminal inverted repeats (TIR) (Bae et al., 2004). This system proved to be more successful than the Tn917 and no discernible transposon sequence preference was observed.

In order to create a transposon library of more clinical relevance this *Bursa aurealis* based system was recently used to create a library in the community acquired MRSA strain USA300 LAC Je2 (Fey et al., 2013). The location of each transposon has been identified by sequencing the chromosomal DNA adjacent to the inserted transposon

and comparison with the USA300_FPR3757 genome. Only mutants with Tn's that were inserted towards the 5' end of genes were included in the final transposon mutant library. The Nebraska Transposon Mutant Library (NTML) contains 1,952 strains and has been made available as part of the Network on Antimicrobial Resistance in *S. aureus* (NARSA) strain repository to registered members (www.beiresources.org). There is also a comprehensive 'genetic toolkit' that allows users to replace the transposon with useful genetic markers or fluorescent reporters (Bose et al., 2013)

The NTML has already been used in many different phenotypic screens in order to identify genes involved in many different functions. The first set of screens looked at, protease activity, pigment formation and mannitol utilization and haemolytic activity on rabbit blood agar (Fey et al., 2013, Bose et al., 2014). These identified a number of novel genes involved in these processes including a new virulence factor regulatory operon (*vfrAB*) (Bose et al., 2014). Subsequent studies have looked at many more topics including activity of hyaluronidase as a virulence factor (Ibberson et al., 2014) and biofilm formation in the presence of low doses of amoxicillin (Mlynek et al., 2016). As such, the NTML is proving to be a valuable tool for identifying new genes involved in interesting phenotypes.

5.2 Aims

- Exploit the NTML to identify genes associated with redox homeostasis in *S. aureus*; specifically, those for H_2S production, growth in low redox potential environments and sulfur metabolism.
- Investigate the ability of *S. aureus* to utilise different sulfur sources.

5.3 Results

5.3.1 H_2S production NTML screen

In order to identify *S. aureus* genes related to the production of H_2S, the production of H_2S by each of the 1952 transposon mutants in the NTML was measured. Lead acetate paper was used to measure H_2S production relative to H_2S production by wildtype (WT). The production of H_2S relative to WT and the *p*-value from a Student's t-test was then used to select the 20 strains with the most significant increase or decrease in H_2S production relative to WT Je2 (Figure 5.1). The 20 strains selected had a *p*-value less than 0.05 and produced either 20% more H_2S than WT or 15% less than WT (Table 5.1).

There were 7 transposon mutants that were selected as having a significantly increased production of H_2S and 13 that were selected as having a significantly decreased production of H_2S. These 20 mutants were then grown from a separate frozen stock and the production of H_2S measured more accurately (Figure 5.2). Following this only two of the strains originally selected as having an increased production of H_2S, and two of the mutants originally selected as having a decreased production of H_2S, had a reproducible phenotype. None of the 4 mutants selected by the second-round screen (Table 5.2) had any previous link with H_2S production or cysteine metabolism. No *cysM* mutant was selected in the screen because there is no *cysM* transposon mutant in the NTML. The four mutants all appeared to have a slight growth defect compared to WT (Figure 5.3); however, this is in keeping with other transposon mutants from the NTML (data not shown).

The two strains that consistently produced more H_2S than WT were SAUSA300_0725 and SAUSA300_1648. SAUSA300_0725 produces twice as much H_2S as WT Je2 is a hypothetical protein that is predicted to be thioredoxin like. Thioredoxins reduce oxidised cysteine and cleave disulfide bonds, they are essential for mitigating oxidative stress and are essential for life in higher eukaryotes (Nakamura et al., 1997). Analysis using BLAST revealed that SAUSA300_0725 is identical to YtxJ and YtxJ is known to interact with the LMW thiol bacillithiol (Perera et al., 2015). Furthermore, analysis using SWISS model revealed that there is a crystal structure for this protein confirming

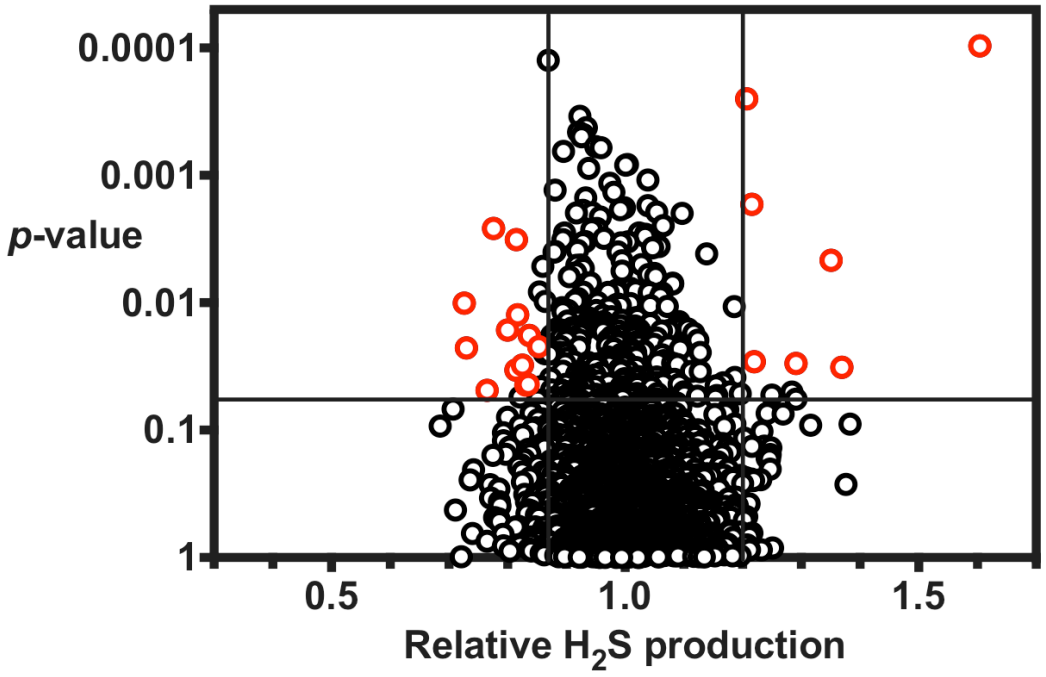


Figure 5.1 Nebraska transposon mutant library (NTML) H_2S production screen
The NTML was grown in TSB for 24 hours under aerobic conditions at 37°C with 250 rpm shaking and H_2S production was measured using the lead acetate paper method (Section 2.15.1). Units represent H_2S production normalised to production of H_2S by WT. Mutants selected for further analysis, produced 20% more or 15% less H_2S than WT and had a *p*-value of less than 0.05, are highlighted in red and listed in Table 5.1. The data are the mean values obtained from three independent experiments.

Table 5.1 Significant results from H_2S screen

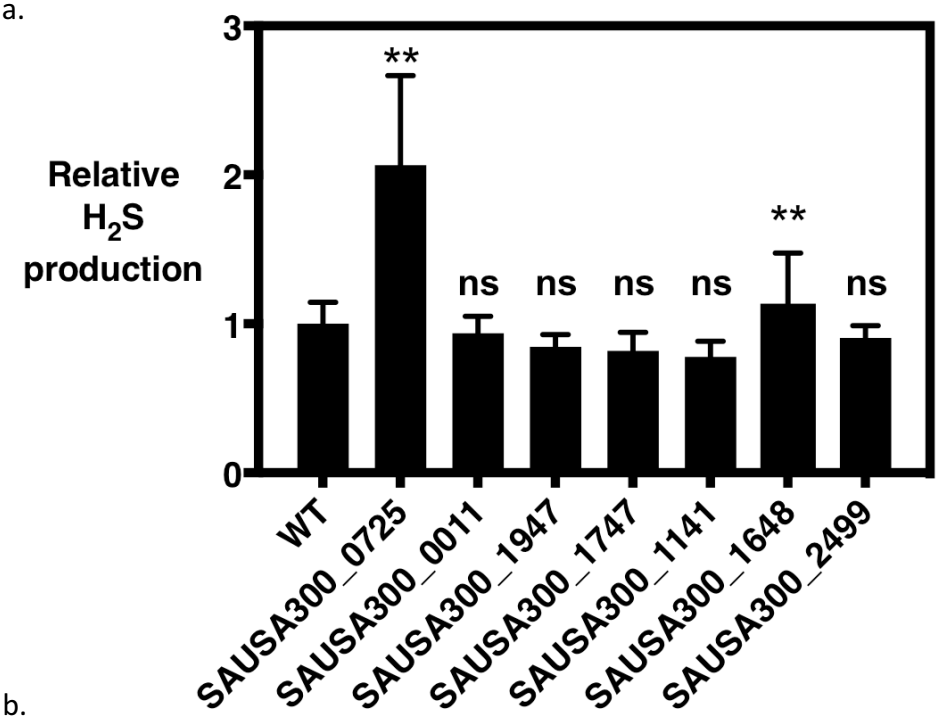
Accession number	Gene name	Description	Relative H_2S production [a,b]	*p*-value [b]
SAUSA300_0725	-	hypothetical protein	1.60	***
SAUSA300_0011	-	hypothetical protein	1.37	*
SAUSA300_1947	-	phi77 ORF031-like protein	1.35	**
SAUSA300_1747	-	hypothetical protein	1.29	*
SAUSA300_1141	-	endopeptidase resistance gene	1.22	*
SAUSA300_1648	-	putative NADP-dependent malic enzyme	1.22	**
SAUSA300_2499	*crtM*	squalene desaturase	1.21	***
SAUSA300_1346	-	putative DnaQ family exonuclease/DinG family helicase	0.85	*
SAUSA300_2212	-	conserved hypothetical protein	0.84	*
SAUSA300_0870	*rexA*	exonuclease RexA	0.83	*
SAUSA300_0013		putative membrane protein	0.83	*
SAUSA300_1449	-	MutT/nudix family protein	0.82	*
SAUSA300_1041	-	conserved hypothetical protein	0.82	*
SAUSA300_2470	*sdaAB*	L-serine dehydratase	0.82	**
SAUSA300_2431	-	putative helicase	0.81	*
SAUSA300_0902	*pepF*	oligoendopeptidase F	0.80	*
SAUSA300_1693	-	hypothetical protein	0.78	**
SAUSA300_1976	-	succinyl-diaminopimelate desuccinylase	0.76	*
SAUSA300_1537	-	hypothetical protein	0.73	*
SAUSA300_2560	-	hypothetical protein	0.73	*

[a] Units represent H_2S production normalised to production of H_2S by WT. The data are the mean values obtained from three independent experiments.

[b] Mutants selected for further analysis produced 20% more or 15% less H_2S than WT and had a *p*-value of less than 0.05.

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

a.



b.

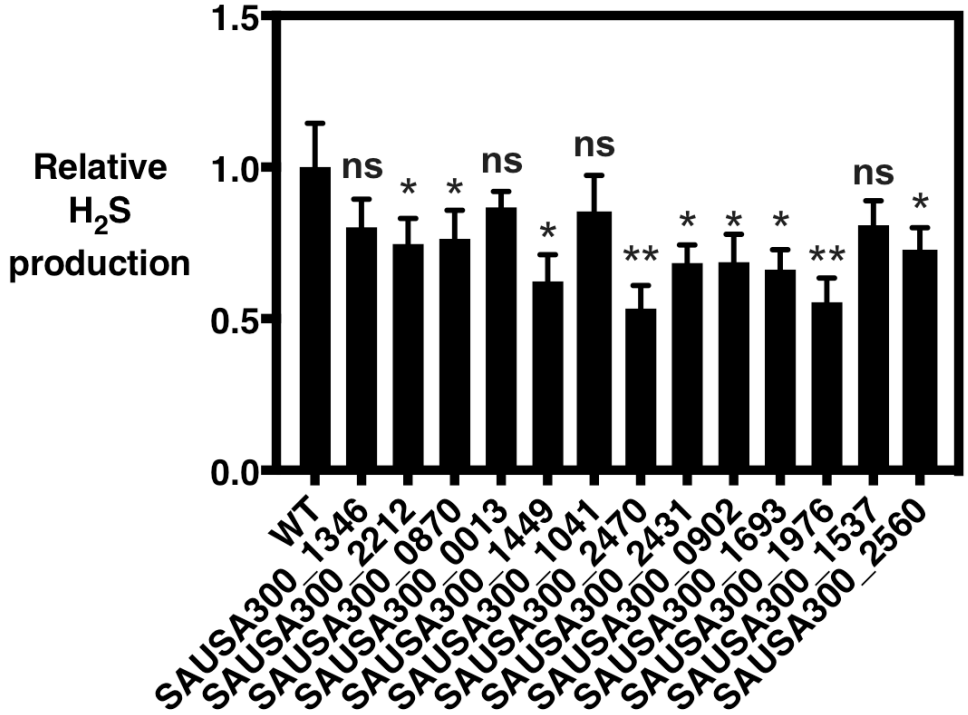


Figure 5.2 Relative production of H_2S – second round screen
Each of the strains identified in the first round screen (Table 5.1) as producing either **a)** more or **b)** less H_2S than WT were grown in TSB for 24 hours under aerobic conditions at 37°C with 250 rpm shaking and H_2S production was measured using the lead acetate paper method (Section 2.15.1). Units represent H_2S production normalised to production of H_2S by WT. Mutants selected for further analysis had a *p*-value of less than 0.01 and are listed in Table 5.2. The data are the mean values obtained from three independent experiments.
ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$

Table 5.2 Significant results from the second round H_2S production screen

Accession number	Gene name	Description	Relative H_2S production [a]	*p*-value [b]
SAUSA300_0725	-	hypothetical protein	2.06	**
SAUSA300_1648	-	putative NADP-dependent malic enzyme	1.13	**
SAUSA300_1976	-	succinyl-diaminopimelate desuccinylase	0.55	**
SAUSA300_2470	*sdaAB*	L-serine dehydratase	0.53	**

[a] Units represent H_2S production normalised to production of H_2S by WT. The data are the mean values obtained from three independent experiments.
[b] Mutants selected for further analysis had a *p*-value of less than 0.01.
** $p < 0.01$

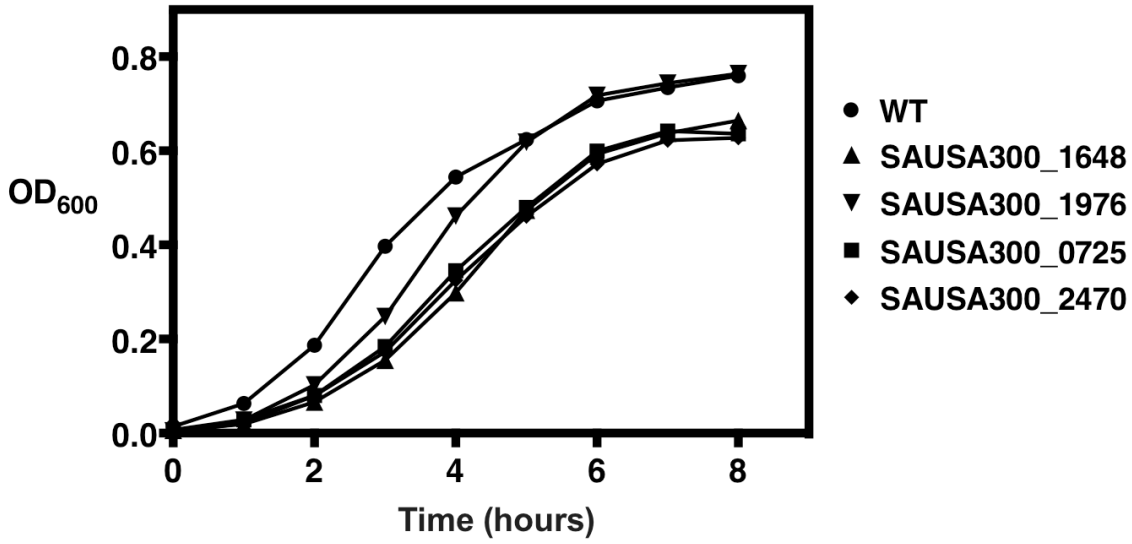


Figure 5.3 Growth of mutants identified during the second round H_2S production screen

Growth of the indicated *S. aureus* Je2 mutants grown in rich medium (TSB). Grown under aerobic conditions at 37^{o}C with 250 rpm shaking. Data shown are representative of four independent experiments.

that the structure has a thioredoxin fold (Fan et al., 2009). From this it seems that SAUSA300_0725 is important for maintaining reducing conditions inside *S. aureus*. If SAUSA300_0725 is interrupted then more H_2S seems to be produced to compensate. This corroborates previous observation about the role of H_2S in defence against oxidative stress (Chapter 3).

SAUSA300_1648 has a proposed function as a NADP-dependent malate dehydrogenase. This means that it catalyses the conversion of malate and $NADP^+$ to oxaloacetate and NADPH. Thus, it is possible that in SAUSA300_1648 there is less NADPH than WT resulting in oxidative stress. Therefore, the observed increase in H_2S production may be as a result of this and be part of the mutants attempts to maintain redox homeostasis.

On the other hand, the two mutants that consistently produced less H_2S, were SAUSA300_1976 and SAUSA300_2470 (*sdaAB*). They both appear to have a slight growth defect that may be a contributing factor (Figure 5.3) but their growth is in line with other transposon mutants from the NTML.

SAUSA300_1976 is predicated to be a succinyl-diaminopimelate desuccinylase. Succinyl-diaminopimelate desuccinylase is an enzyme that catalyses the production of succinate and LL-2,6-diaminoheptanedioate from N-succinyl-LL-2,6-diaminoheptanedioate and water. It is a step in the biosynthesis of lysine and so the SAUSA300_1976 mutant could have a shortage of lysine. This could result in a pH imbalance due to lysine's basic properties and this could affect redox homeostasis causing *S. aureus* to produce less H_2S.

SdaAB is a serine dehydrogenase and it therefore catalyses the conversion of serine to pyruvate. Therefore, *sdaAB* may have increased serine levels and decreased pyruvate levels. This increased serine concentration could be converted to O-acetylserine by CysE and then the o-acetyleserine could be combined with H_2S to form cysteine through the action of CysM. This would result in the decreased levels of H_2S observed.

In summary, it appears that H_2S does have a role in defence against oxidative stress and redox balancing but that it is part of a complex relationship involving different related factors. In order to better understand this complex network, investigation into survival in a low redox environment could be illuminating. This would also complement previous work into the adaption of *S. aureus* to low redox environments (Chapter 4).

5.3.2 Growth in a low redox potential environment - NTML screen

In order to investigate genes that might be important for *S. aureus* to grow in low redox environments, like that found in the cytoplasm of mammalian cells during *S. aureus*'s intracellular lifecycle, bacterial cell density of each mutant in the NTML was determined following 24 hour growth in rich medium (TSB) with a high concentration of DTT. DTT was used because of being a potent reducing agent that maintains a low redox potential environment over a reasonable period of time (Chapter 4). The concentration of DTT used (80 mM) was chosen as the highest concentration before growth was significantly inhibited (Figure 5.4). The NTML was then grown in TSB with 80 mM DTT at 37^{o}C and the OD_{600} measured at 24 hours. The growth of each mutant relative to WT was determined for each of the repeats and a Students t-test performed to establish significance (Figure 5.5). Strains with growth less than 20% of WT and a *p*-value of less than 0.05 were selected for further study (Table 5.3). There were 16 strains selected at this stage.

5.3.3 Second round screen - DTT MIC

A second screen was then performed in which the 16 strains that had been previously selected were grown up from individual frozen stocks and resistance to a low redox potential environment was measured using the same method as the first round screen (Figure 5.6 and Table 5.4). This narrowed the number of selected mutants down to just four. Two of these (SAUSA300_0432 and *lipM* (SAUSA300_1494)) were the two mutants from the original screen with the lowest *p*-values.

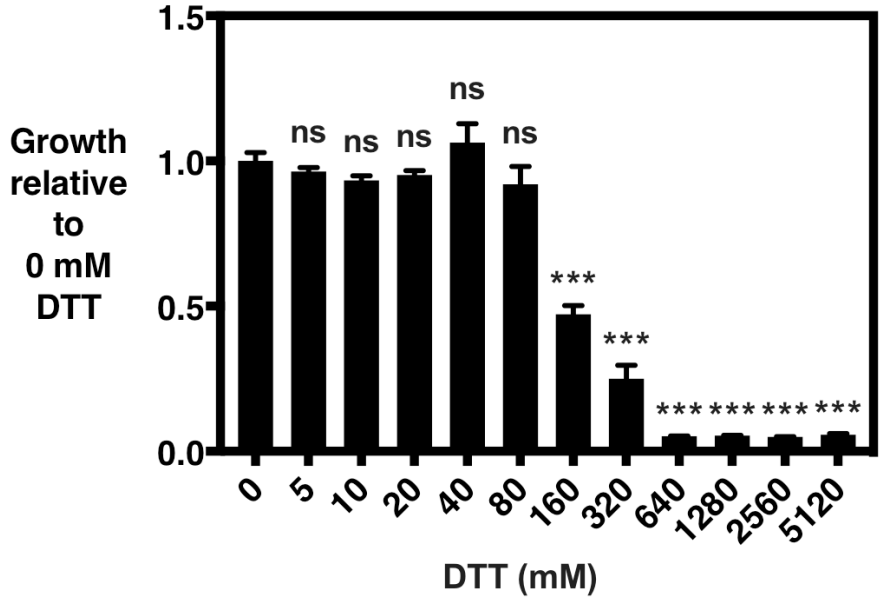


Figure 5.4 Growth of *S. aureus* in the presence of DTT
Growth of *S. aureus* Je2 grown in TSB supplemented with the indicated concentrations of DTT. Grown under aerobic conditions at 37^{o}C with 250 rpm shaking for 24 hours. Units represent growth at 24 hours normalised to growth of *S. aureus* Je2 when no DTT was added. The data are the mean values ± standard deviation obtained from three independent experiments.
ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$ ******* $p < 0.001$

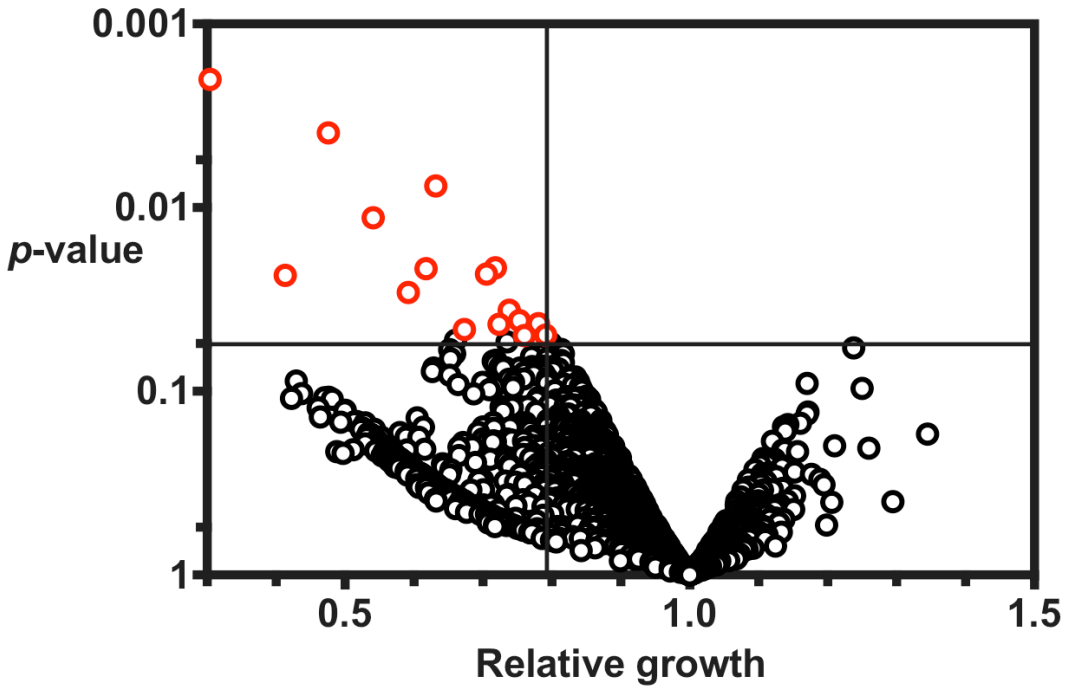


Figure 5.5 Nebraska transposon mutant library (NTML) DTT resistance screen
The NTML was grown in rich medium (TSB) with 80 mM DTT for 24 hours under aerobic conditions at 37°C with 250 rpm shaking and growth (OD_{600}) was measured. Units represent growth at 24 hours normalised to growth of WT. Mutants selected for further analysis, grew 20% less than WT and had a *p*-value of less than 0.05, are highlighted in red and listed in Table 5.3. The data are the mean values obtained from three independent experiments.

Table 5.3 Significant results from NTML DTT resistance screen

Accession Number	Gene name	Description	Relative growth [a]	*p*- value [b]
SAUSA300_1494	-	hypothetical protein (lipM)	0.30	**
SAUSA300_1807	-	amino acid ABC transporter	0.41	*
SAUSA300_0432	-	sodium-dependent transporter	0.48	**
SAUSA300_2365	*hlgA*	gamma-hemolysin component A	0.54	*
SAUSA300_2067	*glyA*	serine hydroxymethyltransferase	0.59	*
SAUSA300_0305	-	formate/nitrite transporter family protein	0.62	*
SAUSA300_1585	-	conserved hypothetical protein	0.63	**
SAUSA300_2473	-	conserved hypothetical protein	0.67	*
SAUSA300_1015	*ctaA*	cytochrome oxidase assembly protein	0.71	*
SAUSA300_0620	-	ABC transporter ATP-binding protein	0.72	*
SAUSA300_0988	*trkA*	potassium uptake protein	0.72	*
SAUSA300_1182	-	pyruvate ferredoxin oxidoreductase, alpha subunit	0.74	*
SAUSA300_2023	*rsbW*	anti-sigma-B factor, serine-protein kinase	0.75	*
SAUSA300_2325	-	hypothetical protein	0.76	*
SAUSA300_2392	*opuCb*	glycine betaine/carnitine/choline ABC transporter	0.78	*
SAUSA300_2486	-	putative ATP-dependent Clp proteinase	0.79	*

[a] Units represent growth at 24 hours normalised to growth of WT. The data are the mean values obtained from three independent experiments.

[b] Mutants selected for further analysis grew 20% less than WT and had a *p*-value of less than 0.05.

* $p < 0.05$ ** $p < 0.01$

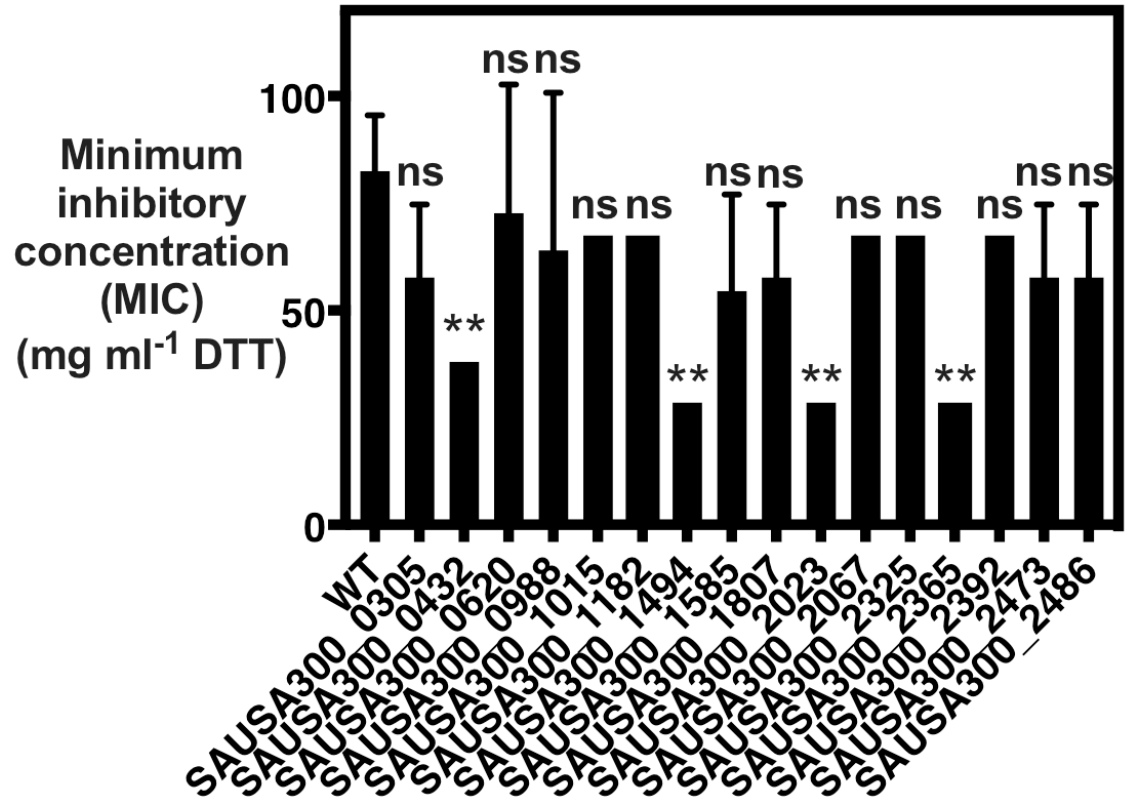


Figure 5.6 DTT MIC – second round screen

The MIC of DTT required to prevent growth of the mutants identified in the first round DTT screen (Table 5.3) were measured in rich medium (TSB). The MICs were measured after 24 hours grown under aerobic conditions at 37°C with 250 rpm shaking. Mutants selected for further analysis had a *p*-value of less than 0.01 and are listed in (Table 5.4). The data are the mean values ± standard deviation obtained from three independent experiments.

ns $p \geq 0.05$ ***** $p < 0.05$ ****** $p < 0.01$

Table 5.4 Significant results from the second round DTT MIC screen

Accession number	Gene name	Description	MIC DTT (mg ml^{-1}) [a]	*p*-value [b]
WT	-	-	82.5	-
SAUSA300_0432	-	sodium-dependent transporter	38.0	**
SAUSA300_1494	-	hypothetical protein (lipM)	28.5	**
SAUSA300_2023	*rsbW*	anti-sigma-B factor, serine-protein kinase	28.5	**
SAUSA300_2365	*hlgA*	gamma-hemolysin component A	28.5	**

[a] The data are the mean values obtained from three independent experiments.
[b] Mutants selected for further analysis had a *p*-value of less than 0.01.
** $p < 0.01$

There was a slight inhibition of growth for the *lipM* transposon mutant compared to WT; this may have resulted in the decreased growth observed in the original screen (Figure 5.7). LipM is an octanoyl transferase which is involved in the biosynthesis of lipoic acid (Zorzoli et al., 2016). Lipoic acid is an important cofactor involved in fatty acid biosynthesis, glycolysis and the Krebs cycle. There are only very low levels of lipoic acid found free in mammalian cells but *S. aureus* is relatively unique in having a diversity of enzymes involved in lipoic acid biosynthesis and salvage (Spalding and Prigge, 2010). There is also a suggested role for lipoic acid in redox defence (Rack et al., 2015).

SAUSA300_0432 is predicted to be a sodium-dependent transporter. Analysis using BLAST and Swiss Model revealed that it is related to neurotransmitter transporters and therefore possibly some form of sodium-dependent small molecule transporter. However, SAUSA300_0432 is also directly upstream of *mccAB* (Figure 5.8). As such, it was suspected that the transposon in SAUSA300_0432 may be having an effect on the transcription of *mccAB*, which is known to be involved in the redox response through cysteine and H_2S metabolism (Chapter 3). In order to investigate this qRT-PCR was used to evaluate the transcription of *mccA* and *mccB* in SAUSA300_0432 (Figure 5.9). This clearly showed a reduction in the transcription of *mccA* and *mccB* in the SAUSA300_0432 transposon mutant compared to WT. The production of H_2S was also measured for the USA300_0432 transposon mutant revealing an 18% reduction compared to WT (Figure 5.10). As such, the reduced DTT MIC of the SAUSA300_0432 transposon mutant may be due to downstream effects on cysteine and H_2S metabolism. However, even with the reduced transcription of both *mccA* and *mccB* the production of H_2S is only slightly reduced. This suggests that *mccAB* is not the primary source of H_2S in *S. aureus*. This corroborates previous results that showed single transposon mutants of *mccA* and *mccB* do not result in a reduction in H_2S production (Chapter 3) This adds weight to the possibility that there are other key sources of H_2S production in *S. aureus*. Furthermore, previous results where DTT was added to continuous *S. aureus* culture *mccAB* was down regulated (Chapter 4) yet SAUSA300_0432 is more sensitive to DTT (Figure 5.6). This suggests that the down

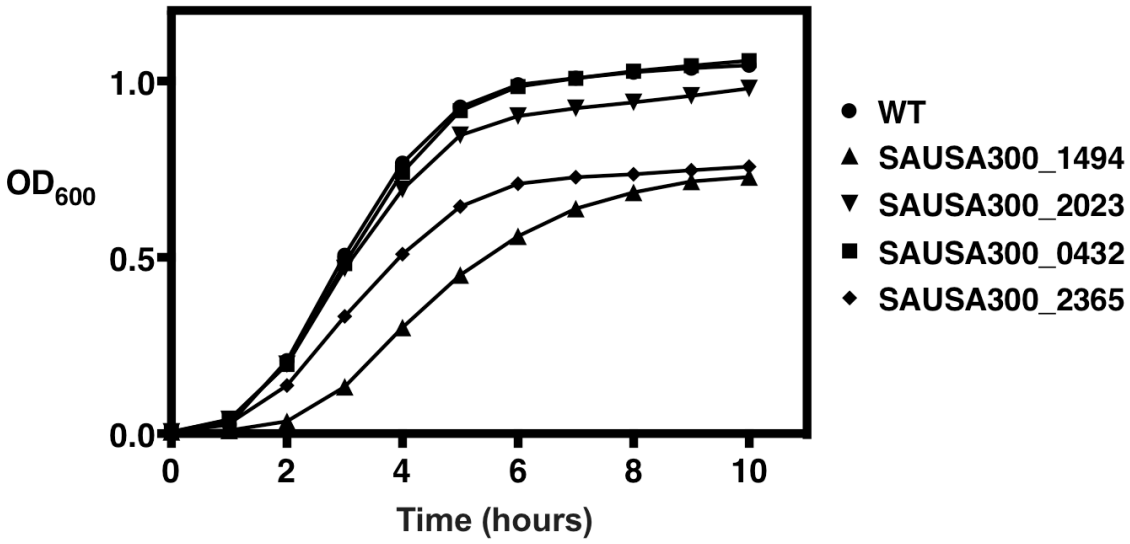


Figure 5.7 Growth of mutants identified during the second round DTT screen
Growth of the indicated *S. aureus* Je2 mutants grown in rich medium (TSB). Grown under aerobic conditions at 37^{o}C with 250 rpm shaking. Data shown are representative of four independent experiments.

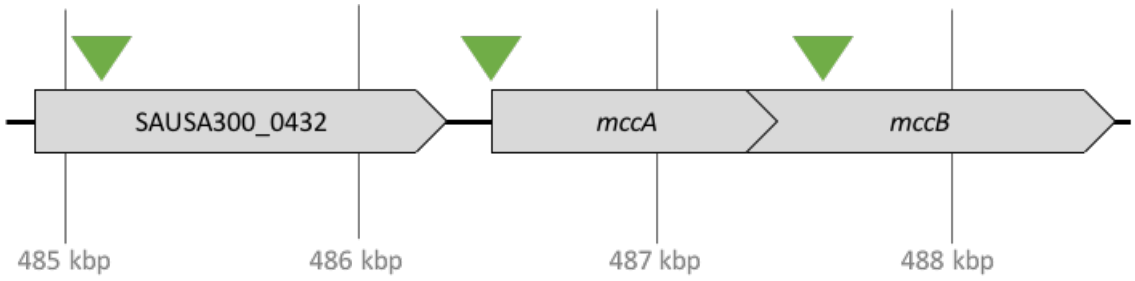


Figure 5.8 Position of SAUSA300_0432, *mccAB* and transposon mutants
Diagram showing the relative position of SAUSA300_0432 and *mccAB* in the genome of *S. aureus*. The arrows represent the direction of transcription and the green triangles indicate the position of the NTML transposons.

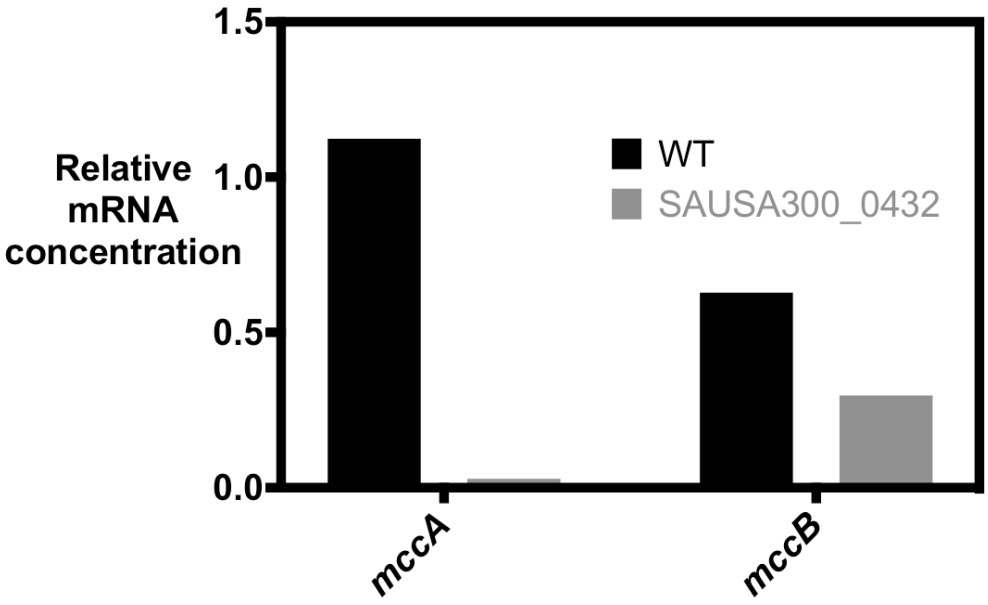


Figure 5.9 Transcription of *mccA* and *mccB* in SAUSA300_0432
mRNA was extracted from exponential phase cultures of SAUSA300_0432 and WT *S. aureus* that had been grown under aerobic conditions at 37^{o}C with 250 rpm shaking. mRNA levels were measured using qRT-PCR. Units represent mRNA concentration relative to *gyrB*.

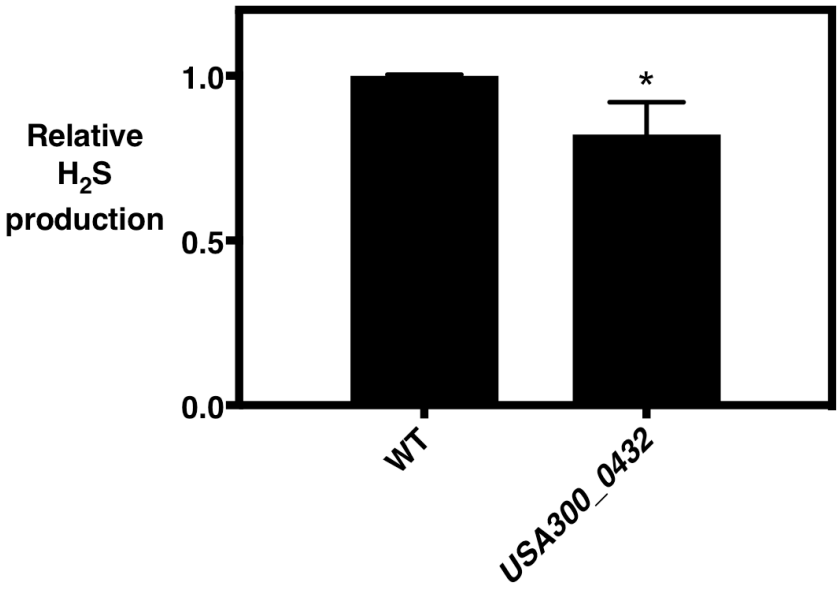


Figure 5.10 Production of H_2S by USA300_0432
Production of H_2S by WT and SAUSA300_0432 in rich medium (TSB). H_2S production was measured using the lead acetate paper method (Section 2.15.1). Units represent H_2S production normalised to production of H_2S by WT. The data are the mean values ± standard deviation obtained from three independent experiments.
* $p < 0.05$

regulation of *mccAB* in SAUSA300_0432 is not the reason for its DTT sensitive phenotype. Instead it is likely that SAUSA300_0432 codes for a transporter that is involved in the H_2S or sulfur homeostasis, or redox regulation.

The other two mutants selected by the DTT resistance screen were *rsbW* and *hlgA*. RsbW is an anti-sigma-B factor that inhibits SigB through protein sequestering (Miyazaki et al., 1999). SigB is crucial for survival of *S. aureus* intracellularly (Tuchscherr et al., 2015, Tuchscherr and Loffler, 2016) and this is usually linked to the role of promoting the formation of small colony varients (SCVs).

It appears that growth is inhibited in the *hlgA* mutant; this could account for the reduced growth observed in the original screen (Figure 5.7). HlgA codes for gamma-haemolysin component A. Gamma haemolysins are bi-component cytolysins that have an erythrocyte specificity (Sugawara et al., 1997) and there are no clear links between gamma haemolysins and redox or intracellular survival.

Not only is *S. aureus* affected by external changes in redox potential but it is also encounters changes in redox potential in many different forms. One way in which the redox homeostasis can be effected is through the intake of sulfur sources in different oxidative states.

5.3.4 Sulfur sources

Sulfur metabolism is the key process underlying H_2S production and is closely linked to redox homeostasis. It has also been shown that respiration rate is increased when *S. aureus* is grown with different sulfur sources. If the sole sulfur source is either methionine sulfoxide or S-methyl L-cysteine then respiration rate appears to be increased compared to when L-cysteine, sodium thiosulfate or a number of other sulfur sources are the sole sulfur source (Figure 5.11) (Bochner - personal correspondence). Each of these sulfur sources has a different oxidative state and this may be having an effect on the ability of *S. aureus* to utilise them. L-cysteine and S-methyl L-cysteine both have an oxidation state of -2, whereas, methionine sulfoxide has an oxidation state of 0 and sodium thiosulfate has an oxidation state of +2.

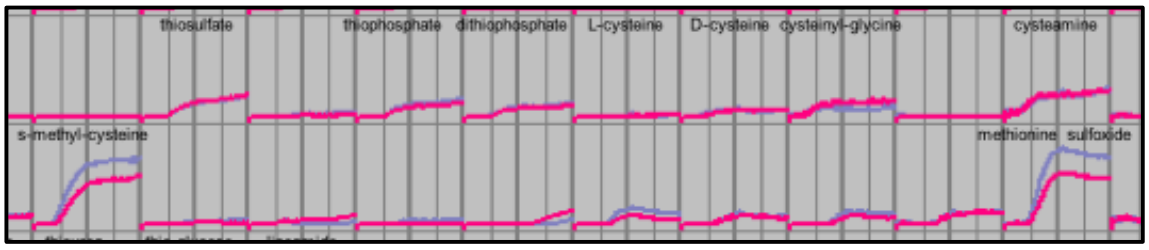


Figure 5.11 Sulfur sources Biolog assay
Staphylococcus aureus USA300 was grown in Biolog microtiter plates under various different conditions and the respiration rate recorded. Labelled here are the strains grown with different sole sulfur sources. Figure supplied by Barry Bochner (personal correspondence).

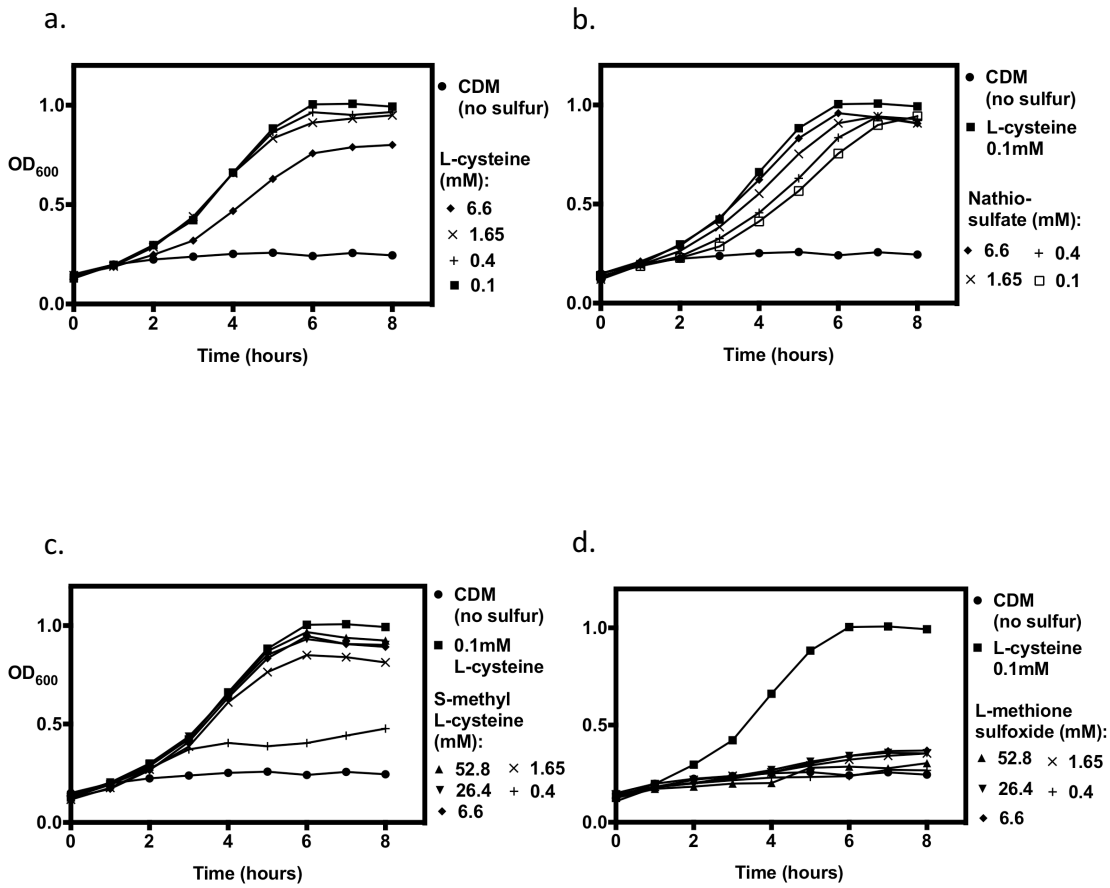


Figure 5.12 Growth of *S. aureus* in CDM with different sole sulfur sources
Growth of *S. aureus* grown in CDM (Section 2.1.2) supplemented with the indicated concentrations of **a)** L-cysteine, **b)** sodium thiosulfate (NaThio-sulfate), **c)** S-methyl L-cysteine or **d)** L-methionine sulfoxide as the sole sulfur source. Grown under aerobic conditions at 37^{o}C with 250 rpm shaking. Data shown are representative of three independent experiments.

5.3.5 Growth of *S. aureus* with different sole sulfur sources

In order to investigate this further, the growth of *S. aureus* was measured in CDM with different sole sulfur sources (Figure 5.12). Growth with L-cysteine and sodium thiosulfate were compared to S-methyl L-cysteine and L-methionine sulfoxide over a range of concentrations.

As little as 0.1 mM L-cysteine was able to produce a growth rate comparable to rich medium (TSB) with a concentration of cysteine of 6.6 mM or above being toxic. Growth on sodium thiosulfate required slightly higher concentrations to reach a maximum growth rate with 6.6 mM sodium thiosulfate being required for optimal growth. S-methyl L-cysteine also required higher concentrations than L-cysteine for optimal growth with at least 6.6 mM required. Surprisingly L-methionine sulfoxide as a sole sulfur source was unable to support significant growth of *S. aureus* at any concentration. There was no significant growth for concentrations 0.4 mM – 26.4 mM and when 52.8 mM was used it appeared to be toxic.

This suggests that L-methionine sulfoxide is most likely not preferentially used by *S. aureus* as it is unable to grow with it as a sole sulfur source. S-methyl L-cysteine, on the other hand, can be used by *S. aureus* as a sole sulfur source and so may have an interesting role in sulfur metabolism.

5.3.6 S-methyl L-cysteine as a sole sulfur source – NTML screen

In order to try and identify genes involved in the uptake, processing or utilisation of S-methyl L-cysteine a NTML screen was carried out in which the growth of the transposon mutants was measured compared to WT with a low concentration of S-methyl L-cysteine as the sole sulfur source. The NTML was grown in CDM with 1.65 mM S-methyl L-cysteine as the sole sulfur source, the concentration at which growth is slightly inhibited. The OD_{600} was measured at 24 hours post-inoculation and compared to WT. A Students t-test was performed and a *p*-value determined. Then the mutants that had a *p*-value of less than 0.1 and had an OD_{600} at least 15% less than WT Je2 were selected (Figure 5.13 and Table 5.5). There were 7 mutants selected by the screen (Table 5.5) four of which have unknown functions.

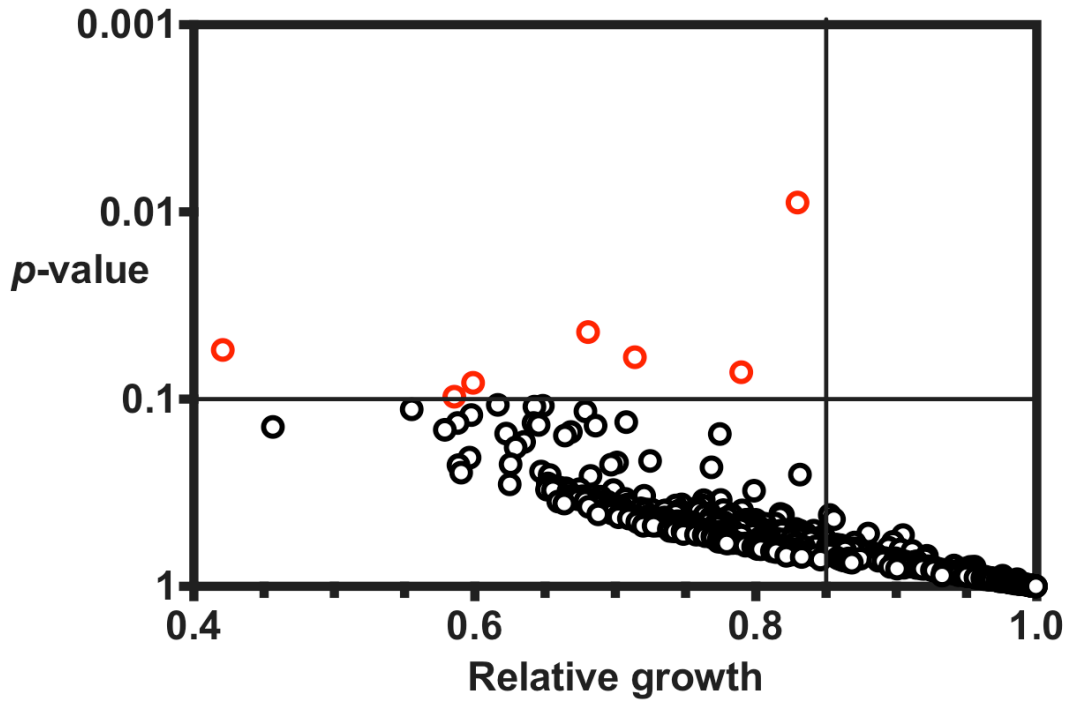


Figure 5.13 Nebraska transposon mutant library (NTML) S-methyl L-cysteine as a sole sulfur source growth screen
The NTML was grown in CDM with 1.65 mM S-methyl L-cysteine as the sole sulfur source for 24 hours under aerobic conditions at 37°C with 250 rpm shaking and growth (OD_{600}) was measured. Units represent growth at 24 hours normalised to growth of WT. Mutants selected for further analysis, grew 15% less than WT and had a *p*-value of less than 0.1, are highlighted in red and listed in Table 5.5. The data are the mean values obtained from four independent experiments.

Table 5.5 Significant results from NTML S-methyl L-cysteine as a sole sulfur source growth screen

Accession Number	Gene name	Description	Relative growth [a]	*p*-value [b]
SAUSA300_2502	-	hypothetical protein	0.83	**
SAUSA300_0548	-	SdrE protein	0.79	ns
SAUSA300_1482	-	FtsK/SpoIIIE family protein	0.71	ns
SAUSA300_0641	-	putative lipase/esterase	0.68	ns
SAUSA300_1936	-	conserved hypothetical phage protein	0.60	ns
SAUSA300_1506	-	hypothetical protein	0.59	ns
SAUSA300_2252	-	hypothetical protein	0.42	ns

[a] Units represent growth at 24 hours normalised to growth of WT. The data are the mean values obtained from four independent experiments.

[b] Mutants selected for further analysis grew 15% less than WT and had a *p*-value of less than 0.1.

ns $p \geq 0.05$ ** $p < 0.01$

5.3.7 Second round screen – growth curves with S-methyl L-cysteine or L-cysteine as the sole sulfur source

In order to investigate the ability of the mutants selected in the NTML S-methyl L-cysteine growth screen, the selected mutants were grown up from individual frozen stocks and growth curves were obtained in CDM with either L-cysteine or S-methyl L-cysteine as the sole sulfur source (Figure 5.14).

None of the mutants had a significant reduction in growth when S-methyl L-cysteine was the sole sulfur source as compared to L-cysteine. However, SAUSA300_1506 seemed to have reduced growth with L-cysteine as sole sulfur source compared to when it was grown with S-methyl L-cysteine as the sole sulfur source. The difference was not significantly different because of the large variation between repeats but could still be an interesting indication of some difference in sulfur metabolism. Comparing the amino acid sequence of SAUSA300_1506 to other genes using BLAST reveals that it contains thiamine binding protein superfamily domains.

5.4 Discussion

5.4.1 The NTML as a tool for researching redox homeostasis

The NTML can be used to successfully screen for genes involved in redox homeostasis. This was most clearly seen in the low redox environment (DTT) growth screen (Table 5.4). The four genes selected by the screen included three that already had established link with redox homeostasis LipM, RsbW and SAUSA300_0432. LipM has been shown to have a link with redox defence and RsbW (the anti-sigma-B factor) is known to link, via SigB and Agr, to a vast array of genes relating to intracellular survival. Also, having established that SAUSA300_0432 is directly upstream of *mccAB* and that the transposon mutant was effecting the transcription of *mccAB* it was found that the production of H_2S was decreased. This suggests a clear link between H_2S production and survival in low redox potential environments. These findings make the decrease in resistance to low redox potential observed for the *hlgA* transposon mutant more reliable. Perhaps a decrease in the production of gamma toxin has some unidentified

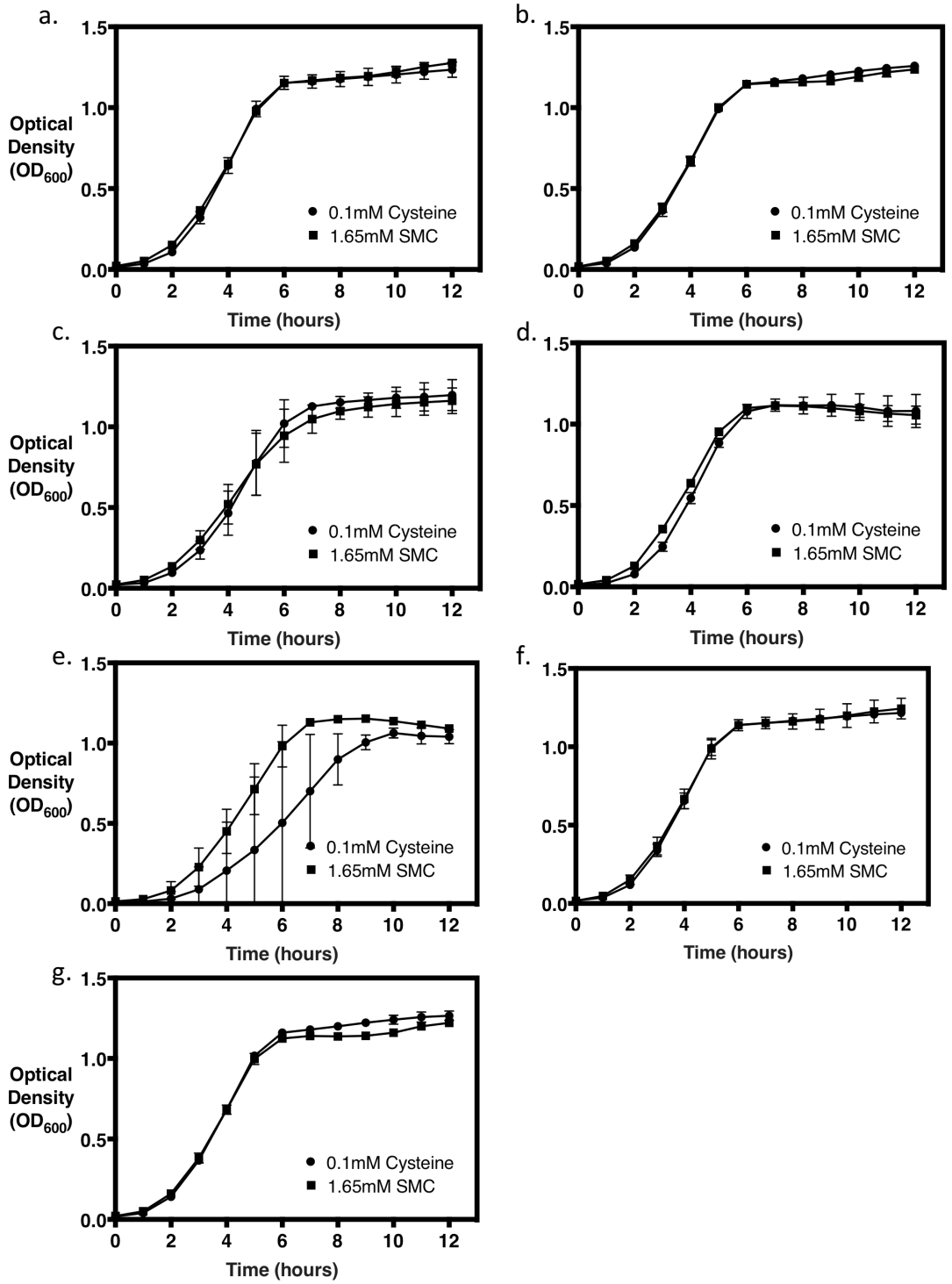


Figure 5.14 Growth of *S. aureus* mutants with L-cysteine or S-methyl L-cysteine (SMC) as the sole sulfur source - second round screen

Growth of *S. aureus* mutants identified in the first round screen (Table 5.4) **a)** SAUSA300_0548, **b)** SAUSA300_1482, **c)** SAUSA300_0641, **d)** SAUSA300_1936, **e)** SAUSA300_1506, **f)** SAUSA300_2502 and **g)** SAUSA300_2252 grown in CDM (Section 2.1.2) supplemented with either 0.1 mM L-cysteine or 1.65 mM S-methyl L-cysteine as the sole sulfur source. Grown under aerobic conditions at 37^{o}C with 250 rpm shaking. The data are the mean values ± standard deviation obtained from three independent experiments.

downstream effects that reduce the ability of *S. aureus* to survive in a low redox potential environment.

5.4.2 Genes related to H_2S production

The H_2S production NTML screen did not select for any genes that are known to be involved in H_2S production (Table 5.2). This raises doubts about the quality of the screen but it could still be useful as a first step to identifying novel genes involved in redox homeostasis. The most promising of these is SAUSA300_0725 that appears to produce twice as much H_2S as WT. It is a thioredoxin-like gene and so could be vital for maintaining redox balance in *S. aureus*. When SAUSA300_0725 was disrupted then more H_2S is produced suggesting that SAUSA300_0725 and H_2S production may be complementary. SAUSA300_1693 could also be interesting with a possible link to beta-lactamase production. A link between H_2S and antibiotic resistance has been previously suggested and SAUSA300_1693 could be part of a novel interaction between H_2S production and antimicrobial resistance.

5.4.3 Investigating sulfur metabolism

Unfortunately attempts to identify genes involved in novel cysteine source metabolism were less successful. Firstly, the BioLog results did not correspond with the growth curve data in the case of L-methionine sulfoxide (Figure 5.11 and Figure 5.12). L-methionine sulfoxide appeared to have increased respiration in the Biolog assay but *S. aureus* was completely unable to grow on L-methionine sulfoxide as a sole sulfur source. This result is particularly surprising because *S. aureus* has four sulfoxide reductases (Singh et al., 2015) and so should be able utilise methionine sulfoxide as a sulfur source. This suggests that *S. aureus* is not able to transport methionine sulfoxide across the cell membrane.

On the other hand, *S. aureus* could grow on S-methyl L-cysteine at relatively low concentrations and so could utilise this as a source of sulfur. Unfortunately, there were no genes identified in the NTML screen that could be convincingly linked to S-methyl L-cysteine transport or processing.

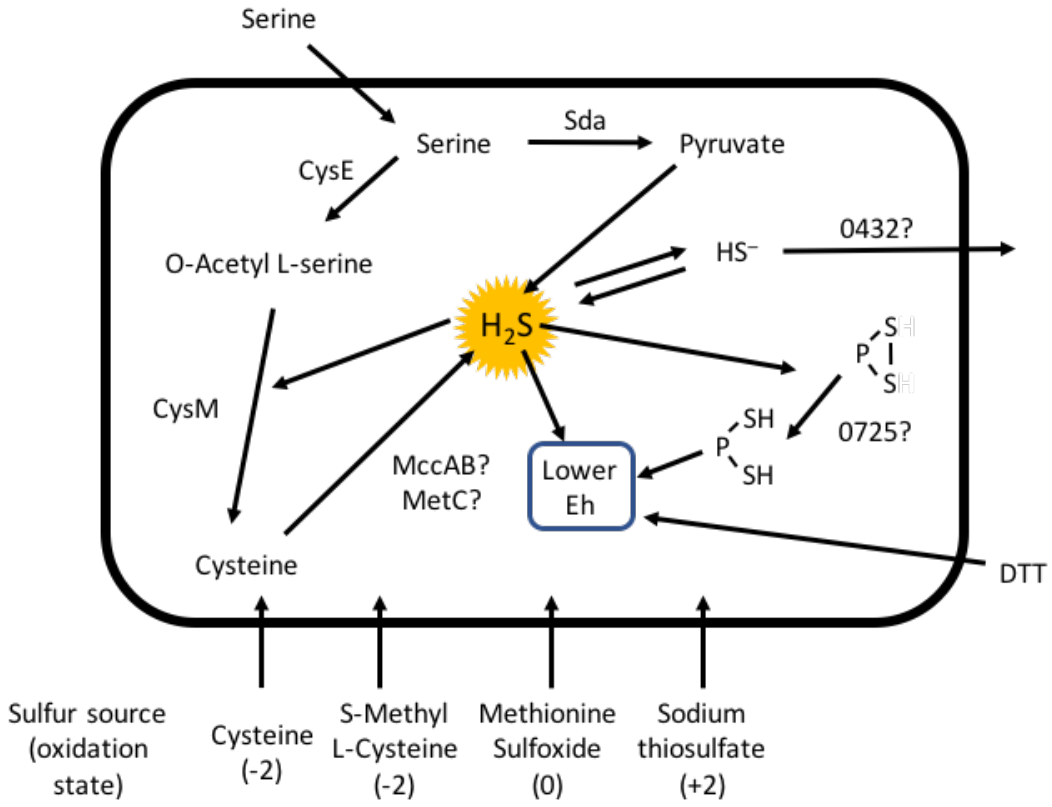


Figure 5.15 The interaction of redox potential and H_2S in *S. aureus*
Regulation of redox potential and H_2S showing genes identified through the NTML screens. SAUSA300_0432 and SAUSA300_0725 are shown as 0432 and 0725 respectively. Question marks indicate uncertainty about protein function.

5.4.4 Future work

The obvious first step for future work would be to transduce each of the transposon mutants into a number of different strain backgrounds. This would help to eliminate confounding effects of any random point mutations.

Other follow up work could be completed with SAUSA300_0725. The involvement of SAUSA300_0725 in H_2S production and the link with thioredoxin-like genes could be an interesting lead. A whole gene knockout and a complemented strain would be invaluable for confirming its H_2S production phenotype. Similar follow up experiments for the other H_2S production mutants could also be carried out, particularly SAUSA300_1693. Investigations into possible links between gamma haemolysin and redox homeostasis could also prove to be interesting.

Chapter 6: Final discussion

6.1 Discussion

6.1.1 A love-hate relationship

Staphylococcus aureus has a close relationship with humanity that has developed over their long shared history of co-evolution. Most of the time this association is friendly with up to two thirds of humans tolerating colonisation by *S. aureus* at any one time. However, *S. aureus* has a sporadic habit of becoming a very unwelcome guest. It can cause a whole host of different diseases ranging from skin and soft tissue infections (SSTIs) to life threating conditions such as bacteraemia, endocarditis and osteomyelitis. Not only that, but *S. aureus* also often refuses to leave when it has clearly outstayed its welcome. Even despite the best efforts of the host immune system and the utilisation of antibiotics, *S. aureus* sometimes simply carries on regardless leading to debilitating persistent infections.

Humans have evolved a vast array of strategies for evicting disease-causing *S. aureus.* These range from relatively nonspecific defences, such as the production of AMPs in mucus, to far more intricate ones, such as the crucial role T-cells plays in recruiting phagocytic cells to sites of infection. These phagocytic cells, mostly neutrophils, then engulf and destroy the offending bacteria. Meanwhile, *S. aureus* has also evolved effective evasion techniques and is able to produce an arsenal of virulence factors to aid it in this endeavour. The production of these virulence factors is the basis of many of *S. aureus* disease pathologies and so research into virulence factors has always been a major focus of *S. aureus* research. However, another important facet of *S. aureus'*s success is its ability to survive in different host environments.

6.1.2 A changing environment

Over the course of an infection *S. aureus* will encounter a whole range of different host environments. Key environmental factors such as nutrient availability, oxygen concentration, pH and redox potential vary dramatically between different sites of infections and are also used by the host immune system to disrupt bacterial growth. For example, high levels of reactive oxygen species (ROS) are deployed against bacteria

that have been engulfed within professional phagocytes. However, *S. aureus* is able to survive within this hostile environment. It then often escapes into the host cytosol where the environmental conditions change suddenly; including a sudden reduction in redox potential. Determining how *S. aureus* adapts to these changes is a crucial part of developing an understanding of how *S. aureus* is able to persist and why it causes different types of infection.

6.1.3 Oxidative stress

S. aureus is well adapted to surviving in conditions of oxidative stress. It is defined by its ability to produce both catalase and the golden antioxidant pigment staphyloxanthin. It also appears that *S. aureus* is able to utilise the production of H_2S as a ROS defence mechanism. Previous work demonstrated that when production of H_2S was disrupted in *S. aureus* then resistance to ROS decreased (Shatalin et al., 2011); this result has now been complemented by showing that when *S. aureus* produces more H_2S than WT there is increased resistance to H_2O_2 (Chapter 3).

Furthermore, this work revealed a possible link between H_2S production and a thioredoxin like gene SAUSA300_0725 (Chapter 5). It appears that H_2S and 0725 both have a role in lowering redox potential in *S. aureus* and that 0725 interacts with bacillithiol. Therefore, it is likely that they are both part of the wider redox regulatory system alongside cysteine, CoA and bacillithiol.

6.1.4 Production of H_2S

The production of H_2S by *S. aureus* was thought to be under the control of *mccAB*. However, previous work (Shatalin et al., 2011) and this work (Chapter 3) failed to produce a mutant strain that inhibited this operon and resulted in reduced H_2S production. Furthermore, even when USA300_0432, the gene directly before *mccAB*, was interrupted and shown to result in reduced transcription of both *mccA* and *mccB*, H_2S production was still only slightly reduced (Chapter 5). As such, it appears that *mccAB* are not the genes solely responsible for H_2S production in *S. aureus*.

6.1.5 Antibiotic resistance

It has also been suggested that H_2S is utilised by *S. aureus* as a defence against bactericidal antibiotics. However, when the bactericidal antibiotic that had shown the clearest results previously, gentamicin, was used to investigate this claim, increased production of H_2S failed to offer increased resistance (Chapter 3). Therefore, it appears that H_2S produced by *S. aureus* may not offer resistance to bactericidal antibiotics despite offering resistance to ROS. This brings into further question the claims made about the link between bactericidal antibiotics and cell death via ROS (Kohanski et al., 2007, Dwyer et al., 2009). If H_2S offers increased resistance to ROS but not to bactericidal antibiotics then it seems unlikely that ROS is the main cause of bactericidal antibiotic induced cell death. This would appear to uphold the claims made previously, that there is no direct link between ROS and antibiotic induced cell death (Keren et al., 2013).

6.1.6 Persistent infections

This work also characterised the response of *S. aureus* to a sudden drop in redox potential. It was revealed that reducing the redox potential suddenly had profound effects on virulence expression mediated through *agr*. It was also shown to inhibit the electron transport chain, leading to the initiation of less effective anaerobic fermentative metabolism.

Responses to redox environment are crucial for understanding *S. aureus* infections. The switch to a low redox potential environment may be a critical driver behind virulence factor expression and persistent infections. It has been long understood that *agr* is important for controlling virulence factor expression and that it is a quorum sensing system. However, from this work it appears that a sudden change to a low redox potential environment is enough of a switch to dramatically decrease the expression of the *agr* operon. This may be due to the effect of a low redox potential environment on AIP. It is known that oxidation of the C-terminal AIP methionine is responsible for AIP inactivation (Rothfork et al., 2004) so it is possible that a reduced environment may have the opposite effect.

6.1.7 SCVs

Studies have shown that SCVs may be a phenotype brought on by the intracellular environment. From this work, it seems that a drop in redox potential may be enough to produce SCVs through the inhibition of the electron transport chain and the promotion of anaerobic respiration resulting in the key characteristics of SCVs, i.e. slow growth, reduced toxin expression and decreased pigment production. This fits with the phenotype switching model of SCVs whereby SCVs are formed as a temporary phenotype and then generally revert to a WT phenotype. It may simply be that the SCVs are colonies that are respiring anaerobically and that once they return to aerobic respiration are able to grow at the same rate as WT. Stable SCVs that are recovered from clinical isolates tend to have SNPs in genes relating to aerobic metabolism (Proctor et al., 2006) and so it is possible that the SCV phenotype stems from their inability to respire efficiently.

6.2 Future directions of study

Future work could be carried out to investigate the relationship between redox and the changes to metabolism and virulence. In particular it would be interesting to investigate whether the speed of the change in redox is important or if there is an absolute redox potential at which key changes occur. These investigations could be carried out using the chemostat model developed in Chapter 4. The chemostat experiment could be repeated but with a gradient of different concentrations of DTT used or the rate at which the DTT is added could be varied. Understanding if there is a particular redox potential were the effect on the transcriptomic profile changes dramatically, or if the rate of redox potential change is important, could be valuable for understanding why *S. aureus* is sometimes able to persist in the cytoplasm and other times induces host cell lysis.

Another avenue for future work could include investigations *in vivo*. H_2S sensitive probes could be used to improve the understanding of how and when *S. aureus* modulates its production of H_2S as it encounters different environments during the infection lifecycle. An *in vivo* cell model for intracellular survival of *S. aureus* could be

used for example with human lung epithelial cells (Garzoni et al., 2006). There are a number of different fluorescent probes that have been developed that are suitable for the detection and imaging of intracellular H_2S (Yu et al., 2014b). Of these two-photon fluorescent probes show promise and have been used to detect and image real time endogenous H_2S in living cells (Chen et al., 2015). Developing these for use with the intracellular *S. aureus* infection model would allow for real time detection of H_2S as *S. aureus* is engulfed and survives intracellularly.

6.3 Final considerations

Staphylococcus aureus is very adept at making itself at home inside our cells and often outstays its welcome. Improved understanding of how *S. aureus* responds to the environmental conditions it encounters during this intracellular persistence is key to understanding how *S. aureus* can cause chronic infections. Focusing on one environmental condition, redox, has proven fruitful and has shown that an approach that looks closely at an individual environmental factor can be valuable. Further work with a similar approach would assist in piecing together an improved picture of the processes underlining persistent *S. aureus* infection and would aid in the development of more effective treatments.

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