A study into two pillars of staphylococcal pathogenicity: biofilm and antibiotic resistance

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Christopher Campbell, B.Sc

Microbiology, School of Natural Sciences,
National University of Ireland, Galway

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Research supervisor

Prof. James P. O'Gara

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Summary

Biofilm formation on inserted medical devices by the opportunistic pathogen, *Staphylococcus epidermidis,* continues to be constant threat in clinical health-care settings. Once established, biofilms are almost impossible to treat therapeutically, resulting in the need to remove and replace the device. In cases where a device cannot be easily replaced, such as artificial joints or prosthetic heart valves, surgical intervention is often the last resort. As a leading cause of nosocomial infections, *S. epidermidis* biofilm formation has been the subject of much research. The *ica* operon is responsible for synthesising polysaccharide intercellular adhesion (PIA) / poly-N-acetylglucosamine (PNAG), the primary composite of *S. epidermidis* biofilm. In *Staphylococcus aureus,* the transcription factor, Rbf, has been found to regulate *ica* expression indirectly via a second regulator, SarX. Despite being closely related, we show that in *S. epidermidis,* Rbf regulates *ica* expression via a second member of the Sar family of transcription factors, SarR. We propose a novel regulatory pathway consisting of two negative regulators, Rbf and SarR, under conditions of osmotic stress. This data provides an insight into how *S. epidermidis* copes with osmotically challenging conditions on human skin, and potentially aid in therapeutic design in the future.

The tricarboxylic acid (TCA) cycle is an important source of energy and metabolic intermediates for *S. aureus*. To survive β-lactam exposure the cell requires increased peptidoglycan synthesis, and increased TCA cycle activity has been reported in homogenously-resistant MRSA strains. We have identified a single enzyme within the TCA cycle that when disrupted, dramatically decreases oxacillin resistance. Succinyl-CoA synthetase converts succinyl-CoA into succinate in the TCA cycle, and transposon mutagenesis of either of its catalytic subunits increases sensitivity to oxacillin. This phenotype appears to be independent of an altered growth phenotype and colony morphology. We suspect that an accumulation of succinyl-CoA, caused by blockage of the TCA cycle at this juncture may be having post-translational effects on enzymes involved in cell wall synthesis, thereby reducing the cells ability to resist β-lactam antibiotics. Lysine-succinylation has been recently implicated in modifying proteins in several other pathogenic organisms, but to date has not been characterised in *S. aureus*. The prospect of over-

succinylating peptidoglycan-biosynthesis proteins and thereby sensitising MRSA to β-lactams, could make succinyl-CoA synthetase an attractive target for novel therapeutics.

Purine metabolism has been shown to play an important role in modulating β-lactam resistance. Mutations in both the purine *de novo* and salvage pathways have been associated with the emergence of high-level, homogenously-resistant MRSA strains. A common element noted in the literature is the overall reduction in GTP levels within the cell, either through the synthesis of the stringent response alarmone, ppGpp, using GTP as a substrate, or via mutations in various enzymes in the biosynthetic pathways such as HprT and GuaB. In a screen of the Nebraska Transposon Mutant Library (NTML) we have identified two genes encoding for elements of the purine salvage pathway that when mutated via transposon insertion, exhibit increased resistance to oxacillin. *deoD* encodes a purine nucleoside phosphorylase, and *nupG* encodes a guanosine permease. Both mutants displayed decreased levels of intracellular GTP, similar to the response found during amino acid starvation. Although the link between reduced purine metabolism and increased β-lactam resistance has not been fully elucidated, our data both complements existing literature and adds two new elements to this area of research.

Declaration

This thesis is comprised of three manuscripts (in preparation or submitted for publication). I am the first author on two papers and second author on one. The involvement of authors is outlined in the Author Contributions section. I declare that this thesis is my own and that my research described herein has not been previously submitted in part fulfilment of a degree to the National University of Ireland, Galway or to any other University. National University of Ireland, Galway library may lend or copy this thesis upon request.

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Author Contributions

Chapter 2. AraC-type regulator Rbf controls the Staphylococcus epidermidis biofilm phenotype by negatively regulating the icaADBC repressor SarR.

Conceived and designed the experiments: SER, CC, PDF JPOG

Performed the experiments: Figure 2.1 A: CC, STOD (constructed *rbf* mutant). Figure 2.1 B: CL. Figure 2.1 C: SER. Figure 2.1 D: SER. Figure 2.2 A – D: SER. Figure 2.3 A: SER. Figure 2.3 B: SER, MEO, JKL, EMW (constructed *sarR* mutant). Figure 2.3 C: SER. Figure 2.4 A: CC. Figure 2.4 B: CL. Figure 2.4 C: CC. Figure 2.5 CC. Figure 2.6: CC, JPOG.

Analysed the data: SR, CC, PDF, CL, STOD, JPOG

Wrote the paper: SR, CC, JPOG

Chapter 3. The TCA cycle enzyme succinyl-CoA synthetase influences resistance to β-lactam antibiotics in MRSA.

Conceived and designed the experiments: CC, LAG, JPOG.

Performed the experiments: All figures CC.

Analysed the data: CC, JPOG.

Wrote the paper: CC, JPOG.

Whole-genome sequencing performed by Microbes NG, Birmingham.

Chapter 4. Disruption of the purine salvage pathway in MRSA increases resistance to β-lactams.

Conceived and designed the experiments: CC, LAG, JPOG.

Performed the experiments: CC (except figure 3D – LAG).

Analysed the data: CC, JPOG.

Wrote the paper: CC, JPOG.

UPLC-MRM/MS targeted metabolomics performed by MtoZ Biolabs, Boston.

Whole-genome sequencing performed by Microbes NG, Birmingham.

Chapter 1

Introduction

1.1 Clinical importance

Staphylococci are a group of low GC content, Gram-positive bacteria, of the Firmicute phyla, first discovered by Sir Alexander Ogston in 1884[1]. The genus comprises over 30 species, many of which are either permanent or transient colonisers of human skin. Recognised as the most virulent species, *Staphylococcus aureus* is a permanent coloniser of the anterior nares and the epithelial and mucosal surfaces in approximately 20% of the population, while 60% are transiently colonised[2]. Closely related to *S. aureus*, *Staphylococcus epidermidis* is a ubiquitous coloniser of human skin. Although other staphylococci possess the ability to cause infection, usually due to host factors such as immune-suppression, *S. aureus* and *S. epidermidis* are by far the most clinically relevant pathogens of this genus of bacteria[3]. Colonisation alone has been shown to be a risk factor for infection[4], [5]; however, staphylococcal infections are typically associated with breakages in the natural epithelial barrier such as surgical wounds or central venous catheter placement.

Although both species occupy similar niches and are closely related, sharing 1,681 open reading frames[6], *S. aureus* is the more important human pathogen. Genome islands in non-syntenic regions of *S. aureus*, but not *S. epidermidis*, help explain why this species is the more invasive pathogen[6]–[8]. These genome islands harbour virulence factors such as leukocidins, enterotoxins and Toxic Shock Syndrome Toxin (TSST), allowing *S. aureus* to both damage host tissue and evade the host immune system. These genome islands also point towards horizontal gene transfer as the cause of divergent evolution between the two species. As a result, *S. aureus* has the ability to cause an array of diseases, from benign skin infections in the form of localised abscesses, to life threatening conditions such as sepsis, toxic shock syndrome and endocarditis[9].

S. epidermidis does not possess the invasive virulence factors of *S. aureus*. Nonetheless, there is a growing appreciation that *S. epidermidis* is an under-represented cause of many nosocomial infections[10]–[12]. Its ability to adhere to indwelling medical devices and form a recalcitrant extracellular matrix known as a biofilm limits the effectiveness of antimicrobial therapies and host defences. Bacterial dissemination from the biofilm allows colonisation of secondary sites and the promotion of chronic infection. The use of indwelling medical

devices is expected to increase as medical therapies advance and as Western populations age, and therefore the risk of staphylococcal infection will also increase[13].

In 2008 it was estimated that *S. aureus* infections caused more deaths in the US than AIDS, tuberculosis and viral hepatitis combined[14]. A more recent review of methicillin-resistant *Staphylococcus aureus* (MRSA) infections found that although there had been a reduction in overall infections in the US, MRSA was still responsible for over 70,000 incidents, with the majority being associated with health-care settings[15]. The same report also highlights the worrying trend of resistance to last-resort antibiotics, such as vancomycin and daptomycin, in clinical *S. aureus* isolates. Despite the introduction of new antibiotics over the last 70 years, *S. aureus* remains on the World Health Organisations list of pathogens for which there is an urgent need for new antibiotics[16].

1.2 Hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA)

As much as 60% of the population is transiently colonised by *S. aureus*[2]. In a hospital setting this pathogen takes advantage of the immuno-compromised population to establish infection. The high usage of antibiotics in intensive care units promotes the selection and transmission of resistance genes. Clinical settings allow pathogens such as MRSA to thrive in vulnerable patients, while constant exposure to antibiotics promote the emergence and acquisition of antibiotic resistance determinants.

Prior to the 1990's, staphylococcal infections were most commonly hospital-acquired (HA), causing infections in the immune-compromised clinical population, often after invasive treatments such as intravenous catheter insertion or surgery. However, in the 1990's a new isolate of *S. aureus* emerged as a cause of infection in the community, first in Western Australia followed by the US and Canada[17]. Significantly, no prior exposure to health-care settings was associated with these infections. Community-acquired MRSA (CA-MRSA) predominantly cause skin and soft tissue infections such as abscesses and cellulitis[2], [18]. They are typically more virulent than their HA-MRSA counterparts[19], [20], causing fulminant necrotizing fasciitis and necrotizing pneumonia, as well as bacteraemia and

endocarditis[21]. Aside from the presence of the methicillin resistance element, *mecA*, CA-MRSA tends to be more susceptible to antibiotic treatment, not possessing the extensive antibiotic resistance profiles associated with HA-MRSA isolates. However, studies have shown that this reduced fitness cost afforded to CA-MRSA is the reason behind their increased virulence[19], [20]. Recently it has been reported that these phenotypic lines have become blurred as CA-MRSA begin to find their way into clinical settings[22].

The *mecA* gene is always located on the SCCmec element and therefore allows MRSA strains to be categorised based on their SCC*mec* element[23]. There are currently 12 known SCC*mec* elements(I-XII)[24]. SCC*mec* elements are typically between 20-70 kilobases in size and all contain *mecA*[25]. Genetic analysis has shown that HA-MRSA tend to harbour SCC*mec* elements I-III, which are typically larger than other elements, whereas CA-MRSA most commonly have a type IV, or less frequently, type V SCC*mec* element which tend to be smaller[25], [26]. Larger SCC*mec* elements found in HA-MRSA usually contain several resistance elements. Type IV is the most common element type associated with CA-MRSA and unlike types I-III, it does not exert a fitness cost to the bacterium[27], [28]. This may explain the success of this SCC*mec* element in the community, and also why CA-MRSA are beginning to compete in health-care settings[29]. Methicillin-resistant *Staphylococcus epidermidis* (MRSE) are isolated in hospitals at a higher rate than MRSA[30], [31], with type IV SCC*mec* elements being the most commonly identified. Evidence suggests that *S. epidermidis*, or another coagulase-negative staphylococci (CoNS) species, was the original donor of the SCC*mec* elements to *S. aureus,* and CoNS may be an important reservoir for antibiotic resistance genes[32]–[34].

1.3 Peptidoglycan cell wall

The cell wall is a feature of almost all bacteria, with a few exceptions found in mycoplasmas and archaebacteria[35]. The cell wall plays an essential role in bacterial survival, protecting the cell from the external environment, as well as maintaining turgor pressure and cell shape rigidity. In Gram-positive bacteria the cell wall can range from 20nm to 40nm thick, compared to Gram-negative bacteria, that tend to have a much thinner cell wall of approximately 3-6nm[36]. In *S. aureus*, the cell wall can account for as much as 90% of dry cell weight[37]. The energy requirement to synthesise such a large macromolecule clearly

highlights the importance of the cell wall to *S. aureus*, and other Gram-positive species. Its essentiality to survival as well as its almost ubiquitous presence makes the cell wall an attractive target for antimicrobials; it is perhaps not surprising that the first isolated antibiotic, penicillin, was a cell wall active antibiotic[38], [39].

The primary component of the bacterial cell wall is peptidoglycan (PG), which is relatively well conserved across the bacterial kingdom. PG is composed of disaccharide repeats, a pentapeptide stem which is then cross linked by an interpeptide/glycine bridge structure. The disaccharide is composed of *N*-acetyl-glucosamine (NAG) β-1,4-linked to *N*-acetyl-muramic acid (NAM), a structure that is highly conserved across the eubacteria[36]. The initial steps of PG synthesis are carried out by the cytoplasmic MurABCDEF enzymes. The PG precursor, UDP-NAM, is generated by MurA and MurB. MurA transfers enolpyruvate from phosphoenolpyruvate to UDP-NAG[40], which is then reduced by MurB in an NADPH-dependent reaction to form UDP-NAM[41] (Fig. 1.1). MurC, MurD, MurE and MurF sequentially and specifically add the five residue amino acid side-chain to UDP-NAM, which in *S. aureus* is composed of L-alanine-D-iso-glutamine-L-lysine-D-alanine-D-alanine[36], [42]. Interestingly, MurF is responsible for the addition of two of the amino acids, D-ala-D-ala, in the form of a dipeptide, unlike MurC, MurD and MurE that only add one amino acid. This requires the action of the alanine racemase, Alr, and the ligase, Dlr, to supply the D-alanine dipeptide for PG-pentapeptide synthesis[43].

The NAM-NAG-pentapeptide unit is formed when NAM-pentapeptide is transferred from UDP to a membrane-embedded undecaprenyl-phosphate lipid carrier, Lipid I by MraY[44]. MurG adds a NAG moiety to Lipid I to form Lipid II, thereby completing the basic PG building block[45]. FemA, FemB and FemX are responsible for the addition of five glycine residues to the L-Lys residue of the pentapeptide that make up the cross linking bridge of PG[46] (Fig. 1.1). Disruption of *fem* genes can increase β-lactam susceptibility[47]. Flippases are required to translocate the Lipid II structure across the bacterial membrane. Once in the periplasm, the new PG disaccharide unit is incorporated into existing PG by a penicillin-binding protein (PBP) family of transglycosylases and transpeptidases.

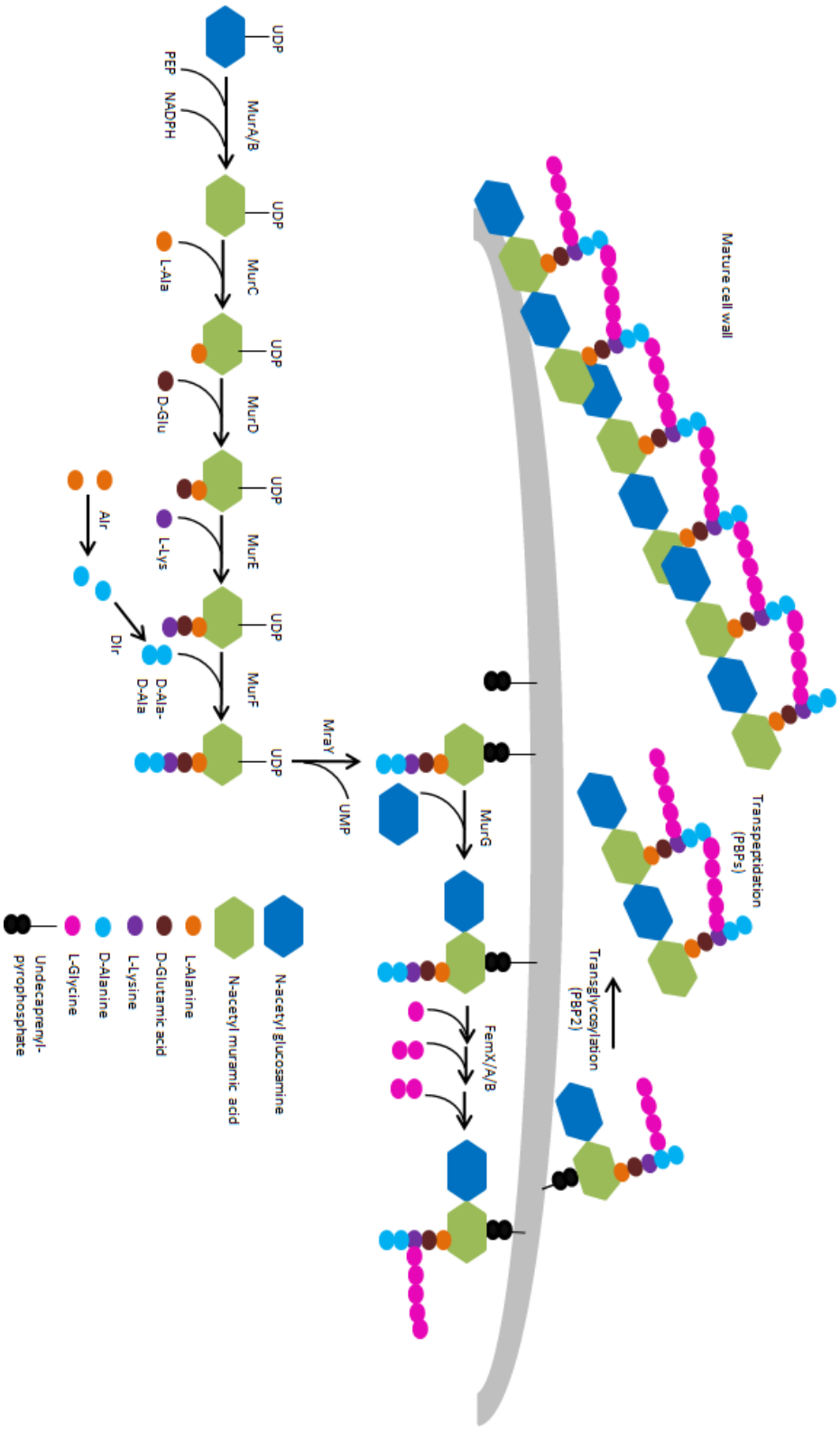

Figure 1.1. Peptidoglycan biosynthesis. A pentapeptide stem is sequentially added to UDP-N-acetylmuramic acid. MraY transfers the PG unit to membrane-bound undecaprenyl-pyrophosphate forming Lipid I. The addition of N-aetyl glucosamine forms Lipid II and Fem proteins add the five-residue glycine bridge. Flippases translocate the disaccharide unit into the periplasm (not shown) and PBP2 adds the PG unit to a growing peptidoglycan chain. PBP proteins cross-link the glycine bridges between adjacent PG units.

S. aureus has four native, membrane-bound PBPs. PBP1 is an essential transpeptidase and also plays a role in cell division and separation[48]. PBP2, uniquely, has both a transglycosylase and a transpeptidase domain. In methicillin-susceptible *S. aureus* (MSSA) PBP2 is essential; however, in MRSA it is only essential under exposure to β-lactams, due to the fact that PBP2a can rescue transpeptidase activity but not transglycosylase activity[49], [50]. The function of PBP3 is largely unknown; evidence suggests that it is associated with septum development and cell division[51]. PBP4, like PBP3 is non-essential to *S. aureus*. However, in addition to its transpeptidation domain, it also has a carboxypeptidase domain, and is thought to play a role in *mecA*-independent (MSSA) β-lactam resistance as a penicillinase[52]. PBPs are responsible for incorporating new PG units into the cell wall and cross linking them via their glycine bridge structure. *S. aureus* PG is extensively cross linked with 80 – 90% of its PG subunits being covalently linked via the glycine bridge[53].

1.4 MRSA – Methicillin-Resistant *Staphylococcus aureus*

Methicillin resistance in *S. aureus* is conferred by the alternative penicillin-binding protein, PBP2a, encoded by *mecA*[54]. *mecA* is located on a mobile genetic element, the Staphylococcal chromosome cassette (SCC*mec*) and encodes the 76kDa alternative penicillin-binding protein, PBP2a. As mentioned in the previous section, penicillin-binding proteins (PBPs) are a group of transpeptidases responsible for the final stages of cell wall biosynthesis in which they cross-link the polypeptide chain in peptidoglycan (Fig. 1.1). The mechanism of action of β-lactam antibiotics is to bind and inhibit transpeptidases, therefore preventing the completion of new PG. Cell death is brought about through the normal autolytic activity of the cell during division in the presence of β-lactams[55]. PBP2a confers resistance by having a lower binding affinity for β-lactams than other PBPs[47] and therefore facilitating continued cell wall turnover even in the presence of β-lactams. However PBP2a is unable to fully compensate for the inhibition of native PBPs, and requires the transglycosylation activity of PBP2 in order to confer resistance[50].

Regulation of *mecA* is achieved through two sets of regulatory systems, the MecI/MecR1 and BlaI/BlaR1 two component systems[56]. *mecR1* and *mecI* are located adjacent to *mecA* on the SCC*mec* element. MecR1 is a transmembrane signal transducer that becomes

activated upon exposure to β-lactams. MecI is a repressor of *mecA,* binding to its promoter region[56]. Upon β-lactam activation , MecR1 cleaves MecI, lifting repression and allowing expression of *mecA*[57], [58]. The β-lactamase (Bla) expression system is analogous to the PBP2a system. The *S. aureus* β-lactamase is encoded by the *blaZ* gene, and regulated by BlaR1 and BlaI, analogous to MecR1 and MecI, respectively[56]. The two systems are capable of cross-talk, and there is evidence of MecI/BlaI heterodimers being capable of *mecA* repression[59]. In general, the MecR1/MecI system is slow at inducing *mecA* expression due to MecI binding tightly to the *mecA* promoter, as well as the inefficient activation of MecR1 by β-lactams[60].

1.5 Heterogeneous and homogenous resistance in MRSA

MRSA exhibit a characteristic heterogeneous resistance (HeR) phenotype to β-lactams[61], [62]. Upon antibiotic exposure, the majority of the population (>99.9%) display a level of resistance on par with MSSA (1-2µg/ml oxacillin) (Fig 2). Within this susceptible/borderline susceptible population, a subset of approximately 0.1%, expresses homogeneous resistance (HoR) and survives exposure to β-lactams. The new population now has the ability to grow at high concentrations of antibiotic (>100µg/ml oxacillin) (Fig. 1.2). The HeR/HoR switch can be reproducibly performed *in vitro* and the frequency at which HoRs are selected for is strain specific and usually stable[63]. The emergence of HoRs may also indicate why β-lactam treatment often fails despite strains initially showing susceptibility to the drug.

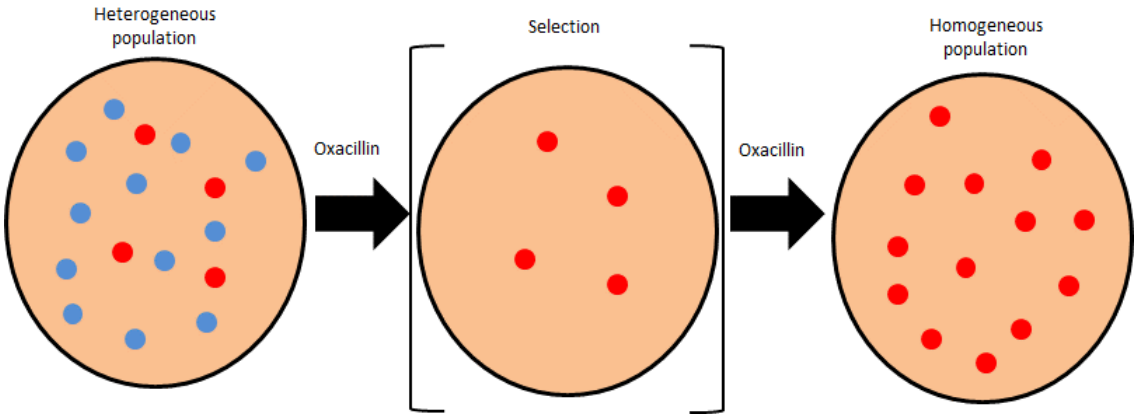

Figure 1.2. Selection of homogenous resistance in MRSA populations. In MRSA populations exposed to oxacillin, the majority exhibit low-level resistance. A sub-population of homogeneously resistance MRSA emerges with accessory mutations allowing for high-level resistance.

The mechanisms behind the transition from a HeR to HoR phenotype are not fully understood. Increased levels of PBP2a do not correlate with increased resistance[64] suggesting that other genetic elements may be involved[65]. Indeed several mutations, outside of the *mecA* regulon[64], have been associated with the HoR phenotype and increased antibiotic resistance; examples from both the TCA cycle and purine metabolic pathway are detailed in sections 1.6 and 1.7. Identifying additional elements that are influencing β-lactam resistance with PBP2a raises the possibility of novel targets for antimicrobial therapy.

1.6 The Tricarboxylic Acid (TCA) Cycle

The TCA cycle is an important cellular pathway, not only in generating reducing potential in the form of NADH and FADH, but also for the formation of metabolic intermediates utilised in various other pathways. All macromolecules required by bacteria for growth can be synthesised from 13 precursor metabolites synthesised from the Embden-Meyerhof-Parnas (glycolytic) pathway, the pentose phosphate pathway and the TCA cycle[66]. *Staphylococcaceae* possess all three pathways. Glycolysis breaks down glucose in stepwise oxidation reactions to produce two molecules of pyruvate, while reducing two molecules of NAD^+ to NADH. The genes encoding enzymes for the TCA cycle are repressed while nutrients remain available in the surrounding medium[67], [68].

The fate of pyruvate is dependent on environmental conditions. Under anaerobic conditions, pyruvate is reduced to lactic acid, using NADH as the electron donor[69]. The replenishment of NAD^+ allows for glycolysis to continue in the absence of oxidative phosphorylation. Under aerobic conditions pyruvate is oxidised to form acetyl-CoA which can then form ATP via substrate-level phosphorylation, as it is first converted to acetyl-phosphate and then acetic acid. The exponential phase growth of *S. aureus* is typically associated with a decrease in the pH of the surrounding medium as the bacteria exports either lactic acid or acetic acid[70].

Entry into post-exponential growth occurs when preferred nutrients, such as glucose, are depleted. At this point *S. aureus* will de-repress TCA cycle gene expression and import

alternative carbon sources such as acetate[71]. Acetate is coupled to coenzyme A via a thioester bond, utilising ATP, forming acetyl-CoA[72]. Acetyl-CoA can enter the TCA cycle, using the energy from thioester hydrolysis and oxaloacetate to form citrate. Citrate undergoes several decarboxylation and oxidative steps, resulting in the reformation of oxaloacetate. Within the cycle, precursors for the synthesis of a complete set of amino acids are generated i.e. α-ketoglutarate, succinyl-CoA and oxaloacetate, as well as ATP, and reduced enzyme cofactors (NADH, FADH). The reoxidation of these cofactors, required for both the continuation of the TCA cycle, and several biosynthetic pathways, requires oxidative phosphorylation. Oxidation of NADH/FADH is coupled with the generation of a proton gradient across the cell membrane which can then be used to generate ATP via ATP synthase complex. Unlike other pathogens such as *Mycobacteria tuberculosis* and *Escherichia coli*, *S. aureus* does not possess a glyoxylate shunt, leading to two carbons being lost for every two that enter the cycle. As a result, amino acids must be used as carbon donors in order to keep the cycle going[71]. Although the TCA cycle produces metabolic precursors, ATP and reducing potential, it also consumes amino acids in the process.

S. aureus uses intracellular concentrations of metabolites as a gauge of nutrient availability in its surroundings, using intermediates to allosterically control metabolic regulators[66]. An example of this signal transduction is the stringent response, which senses amino acid depletion and responds by drastically changing the transcription profile of *S. aureus*[73]. CcpA repression of TCA cycle genes is also an example of this metabolite-controlled regulation[74]. Early glycolysis intermediates glucose-6-phosphate and fructose-1,6-bisphosphate modulate CcpA repression, preventing expression of TCA cycle genes while glucose levels remains available[75].

1.6.1 TCA cycle and β-lactam resistance

High-level β-lactam resistance exerts a fitness cost on the cell[76], [77]. Increased cell wall turnover, as well as cell wall thickness, demands increased levels of ATP to provide energy for biosynthesis[78]. In 2013, Keaton *et al.* used DNA microarrays to show that, compared to its isogenic HeR parent, SA13011-HoR upregulated genes involved in peptidoglycan biosynthesis, carbohydrate transport and energy production[79]. In the same study it was found that by disrupting the TCA cycle via mutations in *acn* and *citZ*, the cells did not develop the HoR phenotype. Keaton argues that the radical oxygen species (ROS) produced

due to increased TCA cycle activity is promoting mutations that confer high-level resistance to MRSA[79].

Kohanski *et al.* suggested that there exists a common mechanism for antibiotic-dependent killing that is reliant on hydroxyl radical formation[80]. They proposed that various classes of antibiotic interact with iron-sulfur clusters to promote Fenton-mediated hydroxyl radical formation[80], [81]. This reaction depends on the transient depletion of NADH as it is oxidised by the electron transport chain, again implicating the TCA cycle in the cells response to antibiotic exposure. Other studies have also supported this hypothesis of antibiotic-induced ROS cell death[82]–[86]; although, several other research groups have refuted this common mechanism of killing hypothesis[87], [88]. It is possible that the levels of ROS produced are important in the promotion of either cell death, or high-level resistance, whereby sub-inhibitory levels of ROS are actually beneficial under oxacillin pressure, but remain lethal at higher concentrations. More research into this phenomenon is required to fully understand it.

1.7 Purine metabolism

The availability of nucleotides for the synthesis of both DNA and RNA is essential to all life. Purines hold particular importance in that, not only are they utilised in the synthesis of nucleosides, but once phosphorylated, adenosine and guanosine make up the "energy currency" for a cell. Concentrations of adenosine-triphosphate (ATP) and guanosine-triphosphate (GTP) are closely monitored by the cell. A decrease in intracellular ATP accompanies a cells entry into stationary phase[89] and can cause a highly resistant state of dormancy. GTP also acts as an internal messenger, with a drop in GTP concentration alerting the cell to a depletion of intracellular free amino acids. Both signals alert the cell to potentially dangerous nutritional limitations; as a result, the synthesis of purines must be tightly controlled.

There are two relatively well conserved mechanisms by which cells can obtain purine nucleosides; *de novo* biosynthesis, and the salvage pathway. Purine metabolism in *S. aureus* has been for the most part inferred by extensive work done on the model organism *Bacillus subtilis*[90]–[92], and by earlier studies on purine metabolism in *E. coli*[93]–[95].

1.7.1 *De novo* purine synthesis

De novo purine synthesis involves synthesising inosine-monophosphate (IMP) in a ten step pathway carried out by the *purEKCSQLFMNHD* operon (Fig. 1.3)[91]. IMP can be converted to either GTP or ATP in subsequent reactions by GuaB/GuaA and PurA/PurB, respectively, depending on metabolic requirements[96]. The *pur* operon, as well as *purA* and *purB*, are regulated by the PurR repressor[97], [98]. PurR binds to a conserved Pur-box motif, upstream of the *pur* start codon, blocking transcription. The purine precursor, PRPP (phosphoribosylpyrophosphate) is used in both initiating *de novo* purine synthesis, and in salvaging adenine and guanine. At high concentrations PRPP also allosterically inhibits PurR-DNA binding. Upon import, adenine is converted to adenine 5' nucleotides, consuming PRPP. A decrease in PRPP levels relieves PurR, allowing it bind to the Pur-box and repress transcription. In this way the cell will favour metabolising exogenous adenine over the energy expensive *de* novo pathway[97]. Interestingly, guanine can also influence *de novo* purine synthesis, independent of adenine. Unlike with adenine, where *pur* transcription is inhibited, excess guanine and/or hypoxanthine causes premature termination of the *pur* mRNA transcript[91]. It is believed that guanine or hypoxanthine can bind to a "G-box" aptamer structure in the 5'UTR of the *pur* transcript, preventing elongation of the transcript[99]. In *B. subtilis* there is also a G-box located in the 5'UTR in an operon containing the *xpt-pbuX* genes, both of which are used in the salvage pathway (see section 1.6.2)[90]. The riboswitch is specific for guanine and not adenine. The overlapping, yet differential nature of metabolite regulation in purine synthesis allows the cell to manage and direct its resources to produce the required metabolites.

1.7.2 Purine salvage pathway

The salvage pathway allows the cells to recover exogenous purine from the environment, as well as recycle nucleotides following DNA turnover. The salvage and *de novo* pathways cross-talk via the metabolite PRPP with excess adenine depleting PRPP levels, allowing PurR to repress the *pur* operon[97]. Free guanine can also bind to the G-box riboswitch terminating *pur* mRNA transcription[99]. Several studies have shown that the regulation of purine metabolism is important to virulence, whereby pathogens favour *de novo* synthesis over salvage, presumably due to the lack of availability of free purines *in vivo*[100]–[102]. One notable exception is *Helicobacter pylori*, believed to have coevolved with humans as a life-long inhabitant of the stomach[103]. *H. pylori* is missing most of the purine biosynthetic

genes, and appears to rely solely on salvaging nucleotides from its immediate environment[104].

The purine salvage pathway has two components; 1) import of exogenous nucleotides/nucleosides, 2) processing and integrating the exogenous or recycled nucleosides into the purine pathway (Fig. 1.3). Several purine transporters have been identified in *B. subtilis*, some of which have staphylococcal homologues. PbuX transports xanthine across the cell membrane and is subject to both G-box and PurR regulation[90], [105]. *pbuX* in *S. aureus* appears to be indirectly activated when CodY is repressed[106]. *pbuG* encodes is a high-affinity hypoxanthine-guanine permease, under PurR and guanine-concentration dependent regulation[105], [107]. *pbuO* has no staphylococcal homologue, but is believed to take a secondary transport role to *pbuG* mutants in transporting guanine into the cell[105]. *nupG* is purine transporter, with a high-affinity for guanosine, and to a lesser extent, inosine, and is under control of intracellular guanine via the G-box riboswitch[99], [108], [109]. Further redundancy in purine transport is demonstrated by the CodY-regulated *nupNOPQ* operon in *B. subtilis*[109]. A *nupN/nupG* double mutant is almost completely defective in importing guanosine. *S. aureus* does not have a *nupNOPQ* homologue, which may indicate the importance of *nupG* as a transporter. While *S. aureus* has genetic homologues of several of these *B. subtilis* purine transporters (*pbuX, pbuG, nupG*), experimental evidence for their respective functions is lacking.

Both imported nucleosides and recycled deoxynucleosides must first be processed before being integrated back into purine metabolism (Fig. 1.3). Crucial to this is the purine nucleoside phosphorylase family of proteins (PNPs). In *B. subtilis* there are two PNPs, one specific for (deoxy)adenosine, *pupA*, and the other specific for (deoxy)guanosine and (deoxy)inosine, *pupG*[92]. In *S. aureus*, there appears to be two PNPs, of the *deoD* family of phosphorylases, inferred by their homology to *E. coli* hexameric purine nucleoside phosphorylase[110]. Regardless of their origin, PNPs have a conserved function to remove the ribose (or deoxyribose), utilising free phosphate, resulting in ribose-1-phosphate and a purine base. Free bases are then phosphorylated to their respective monophosphate-nucleotides by purine ribosyltransferases, using PRPP as a phosphate donor.

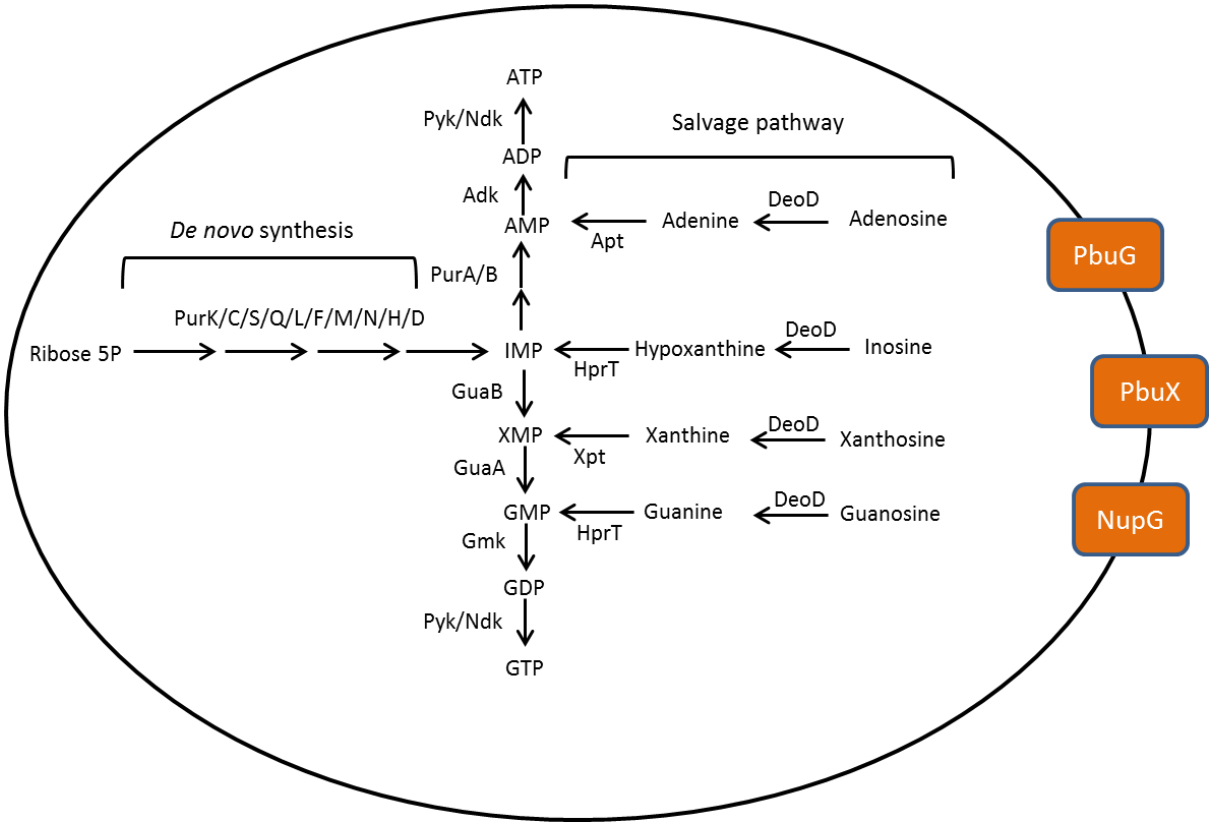

Figure 1.3. Purine biosynthetic pathway. *De novo* synthesis is carried out by the *pur* operon in a sequential, ten step pathway. The purine salvage pathway consists of purine transporters (PbuG, PbuX and NupG), purine nucleoside phosphorylases (DeoD) and purine ribosyltransferases (Xpt, Hprt and Apt).

1.7.3 Purine derivatives and β-lactam resistance

The purine pathway is an important metabolic cross-road for the cell. Several purine derivatives act as secondary messengers within the cell, with the accumulation or depletion of these messengers potentially causing large transcriptional changes in order to adapt to nutrient limitation or cell envelope stress. The intracellular concentration of nucleotide messengers such as c-di-AMP and (p)ppGpp, have also been associated with high-level β-lactam resistance[111], [112]. Furthermore, in these studies, an overlapping trend has emerged whereby GTP levels are reduced in MRSA strains with high-level resistance, either via ppGpp inhibition of biosynthetic proteins such as Gmk or HprT, or through mutations emerging in genes such as *guaB* and *gmk*[113], [114]. This dampening of the purine biosynthetic machinery ultimately lead to reduced GTP synthesis, the concentration of which is intricately linked to the activity of the metabolic repressor, CodY[106].

1.7.3.1 Cyclic-diadenylate-monophosphate (c-di-AMP)

c-di-AMP is found throughout Gram-positives and was first identified in *S. aureus* in 2011[111], [115]. In *S. aureus,* c-di-AMP was discovered by identifying compensatory mutations in a lipoteichoic acid (LTA)-negative strain that resulted in an accumulation of the nucleotide[111]. LTA is a membrane-bound chain of glycerolphosphate residues[116]; it plays an important role in normal cell growth, division, and maintaining cell shape[117], [118], in addition to biofilm formation and resistance to cationic peptides and other antimicrobials[111], [119], [120]. Corrigan *et al.* found that the morphological changes caused by LTA deficiency could be rescued by mutations in *gdpP* (GGDEF-domain protein containing phosphodiesterase)[111]. GdpP is responsible for hydrolysing c-di-AMP. The resulting increase in c-di-AMP levels in a *gdpP* mutant produced more cross-linking muropeptides in the cell wall and increased resistance to cell wall targeting antibiotics. Further evidence for c-di-AMPs involvement in high-level resistance came from Dengler *et al.*, who showed that when diadenylate cyclase, *dacA*, the gene that synthesises c-di-AMP, is mutated by one single nucleotide polymorphism (SNP), a HoR MRSA strain reverts to its HeR phenotype[121].

c-di-AMP is synthesised as *S. aureus* enters late exponential/early stationary phase[122], and can bind to and regulate certain ion pumps[123], [124]. The link between c-di-AMP targets and increased resistance remains to be fully understood. A recent study utilised a *gdpP* mutant and DNA microarrays to investigate the global transcriptional changes caused by increased concentrations of c-di-AMP[122]. Over three hundred genes were found to be either up or down-regulated in the *gdpP* mutant, many of which were involved in cellular metabolism, amino acid starvation and transport (Fig. 1.4). The overlapping regulation with the amino acid starvation is significant because it implicates “cross-talk” between c-di-AMP and another essential *S. aureus* secondary messenger, (p)ppGpp[122]. Increased levels of c-di-AMP has been shown to indirectly induce (p)ppGpp synthesis[122] (Fig. 1.4).

1.7.3.2 Guanine tetra/pentaphosphate – (p)ppGpp

Guanosine tetra/pentaphosphate (p)ppGpp is a small signalling molecule responsible for metabolic adaptations during periods of metabolic stress[125]. The primary role of (p)ppGpp in the cell is to reduce unnecessary metabolism, cell division, and protein synthesis when amino acids become scarce. The metabolic signal that stimulates (p)ppGpp

synthesis is primarily amino acid starvation. This is sensed via the presence of deacylated tRNA in the ribosomal A site[126], which alerts the cell to a deficiency in free amino acids and that it is unable to keep up with the current demands of protein synthesis. Upon sensing this signal (p)ppGpp is synthesised by the bifunctional RelA enzyme using GTP. RelA is a RelA/SpoT homologue (RSH) of *E. coli.* In *S. aureus* and other Gram-positives, the RelA N-domain contains the Rel domain, which is a synthase only domain, and a SpoT domain which has both hydrolase and weak synthase activity[127]. RelA interacts with ribosome-associated deacylated tRNA, activating its synthesis domain which then goes on to produce (p)ppGpp using ATP and GDP/GTP[128], [129]. Two additional (p)ppGpp synthases have also been identified in *S. aureus*, RelQ and RelP[130], although they lack the hydrolase and regulatory domain of RelA. RelP and RelQ (p)ppGpp synthase activity is essential for coping with cell envelope stress[130].

Increased levels of (p)ppGpp have global effects on cellular metabolism and gene transcription. Firstly it is paramount in altering cell metabolism to cope with amino acid limitation; this response is known as the stringent response[131]. During the stringent response the cell reprograms its metabolism utilising (p)ppGpp as an effector molecule. A recent study by Corrigan *et al.* showed that (p)ppGpp could bind to, and inhibit two enzymes involved in GTP synthesis[132], Gmk and HprT, which are found in the purine *de novo* and salvage pathways respectively. In the same study, (p)ppGpp was also found to bind specifically to four putative GTPases, RsgA, RbgA, Era, and HflX which have roles in normal ribosome assembly, and thus reduces protein synthesis. In *B. subtilis* and *Entercoccus faecalis* GTP synthesis was also found to be inhibited by (p)ppGpp in a similar fashion[133], [134]. Furthermore, (p)ppGpp can bind to, and inhibit GdpP, resulting in the accumulation of c-di-AMP (Fig. 1.4)[122]. In MRSA, induction of the stringent response was found to correlate with an increase in *mecA* expression[135], which could be induced by either amino acid starvation or by blocking peptide elongation using mupirocin[136].

1.7.3.3 GTP/CodY

ppGpp-dependent down-regulation of GTP synthesis is an important step in adjusting to amino acid scarcity. ppGpp-inhibition of Gmk and HprT, as well as the rapid consumption of GTP in synthesising ppGpp, results in a decrease in intracellular GTP[132],[137]. The reduction in intracellular GTP levels signals transcriptional reprogramming within the cell. In

B. subtilis it is believed that a loss of GTP in the cell favours the transcription of genes that use an alternative initiating nucleotide i.e. ATP. This subtle regulatory mechanism favours expression of amino acid biosynthetic genes over protein synthesis genes such as rRNAs[132], [133], [138].

A second consequence of reducing GTP levels is the derepression of the CodY regulon (Fig. 1.4). CodY is a transcriptional regulator of over 200 genes in *S. aureus*, primarily as a repressor, including genes of nitrogen and carbon metabolism and transportation, as well as virulence factors such as hemolysins and biofilm formation[73], [139], [140]. During exponential phase, or when nutrients are not scarce, high levels of GTP, and also branched-chain amino acids (BCAA), associate with CodY and promote DNA-binding to "CodY boxes" in the promoter region of genes under its repression[73], [141], [142]. A reduction of GTP and/or BCAAs in the cell causes CodY to disassociate from its DNA binding site, lifting repression. In the stringent response, CodY derepression allows the transcription of amino acid biosynthetic operons, as well as other nutrient stress genes[73].

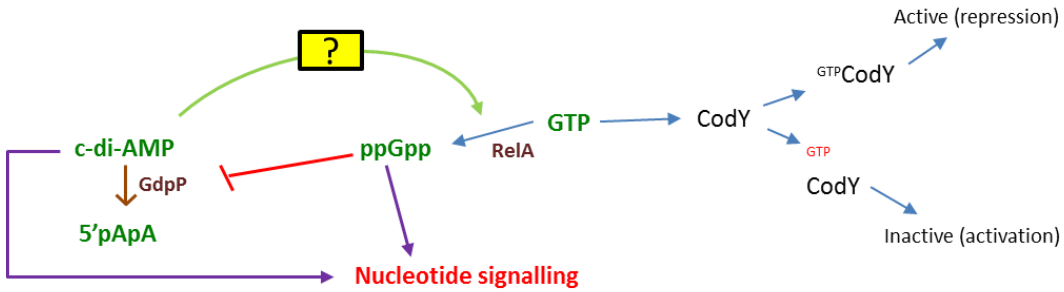

Figure 1.4. Summary of the interactions of c-di-AMP, (p)ppGpp and GTP. Entry into stationary phase or mutations in GdpP result in an accumulation c-di-AMP. C-di-AMP indirectly promotes RelA-dependent ppGpp synthesis through an unknown mechanism, causing further c-di-AMP accumulation through inhibition of GdpP. Consumption of GTP by RelA causes CodY to disassociate from its DNA binding sites, resulting in the activation of the CodY regulon.

1.7.4 Genetic loci influencing β-lactam resistance and purine metabolism

Induction of the stringent response in *S. aureus* has been associated with increased *mecA* expression, and consequently higher levels of resistance to β-lactams[135]. Further evidence for the involvement of the purine metabolism in β-lactam resistance has come from studies looking for single nucleotide polymorphisms (SNPs) in strains that have

developed high and homogenous levels of resistance upon exposure to oxacillin. The insertion of a premature stop codon in the *relA* regulatory domain caused constitutive synthesis of ppGpp, producing increased oxacillin resistance[143]. Other SNPs were found in *guaA* (IMP dehydrogenase), *guaB* (GMP synthase), and *hpt (hprT)*, all of which are found in either the purine salvage or *de novo* pathway[114]. Recently, SNPs in *gmk* and *purB* were found in strains that had switched from the HeR to HoR resistance phenotype[144].

The evidence suggests that MRSA is capable of acquiring mutations within both the purine *de novo* and salvage pathways, ultimately leading to a decrease in GTP synthesis, which may produce a similar effect to the one found when the stringent response is activated.

1.8 *Staphylococcus epidermidis* Biofilm

1.8.1 Introduction

S. epidermidis is a member of the coagulase-negative staphylococci (CoNS) and is commonly found among the natural skin flora of humans. Unlike the closely related *S. aureus*, *S. epidermidis* and other CoNS's are typically avirulent, and do not generally cause infections in the healthy immune-competent population. Indeed, the presence of *S. epidermidis* on the epidermis has been shown to be beneficial by disrupting *S. aureus* colonisation by producing the extracellular protease Esp[145]. However, it is still capable of causing infections in immune-compromised and vulnerable populations. CoNS infections are thought to be primarily caused by *S. epidermidis*[12].

Despite its distinct lack of true virulence determinants, this bacteria a leading cause of nosocomial infections[146]–[148]. *S. epidermidis* is the most common cause of central intravenous catheter infection, is isolated in 22% of patients with septicaemia, and has been found to be the causative agent of 77% of hip and knee arthroplasty infections[146], [149]–[151]. *S. epidermidis* infections often occur in immune-compromised patients (HIV, chemotherapy, etc.) or coincide with the use of implanted medical devices making it a true opportunistic pathogen[12].

Another important consideration for *S. epidermidis* colonisation is that it can act as a reservoir for antibiotic resistance genes, which may then be transmitted via horizontal gene transfer to its more virulent relative *S. aureus*[152]. Oxacillin resistance is typically conferred

via the *mecA* gene, located on the mobile genetic element SCC*mec*[54]. Among hospital isolates, oxacillin resistance has been found to be more common in *S. epidermidis* (70-80%) than in *S. aureus* (40-60%)[30], [31]. More worrying still, is the fact that the most common type of SCC*mec* element found in *S. epidermidis* was type IV[153], which is the most common *SCCmec* type in CA-MRSA strains[154], [155]. Resistance to other antibiotics such as tetracycline, erythromycin, gentamycin and fluoroquinolones have also been reported[146]. In the wider healthy population, the risk associated with carriage of antibiotic resistance genes is diluted; however, in a health-care setting such as an intensive care unit, the risk of transmission to more virulent pathogens, such as *S. aureus*, becomes a much greater threat to patient mortality.

1.8.2 Biofilm

A biofilm is an extracellular matrix, produced by a population of microorganisms that confers protection from desiccation, predation and/or dehydration[156]. A common feature of bacterial biofilms is that they are extremely difficult to remove once established. In health-care settings biofilms can be extremely destructive. One example is seen in cystic fibrosis patients who develop recalcitrant biofilms produced by *Pseudomonas aeruginosa*. The *P. aeruginosa* biofilm protects the bacteria from both oxidative bursts from neutrophils as well as antimicrobial therapy[157]. Biofilm is a key feature of both *S. aureus*, and particularly *S. epidermidis* infections. *S. epidermidis* strains that produce biofilm have been shown to be more virulent than biofilm negative strains, and resist immune-clearance much more efficiently[158]–[160].

Biofilm formation can be divided into three phases; initial attachment, maturation and dispersal (Fig 1.5). Each phase must be carefully controlled at the genetic level to ensure successful biofilm development. Unsurprisingly, many transcriptional regulators and adhesins have been identified as playing a role in the production of a biofilm.

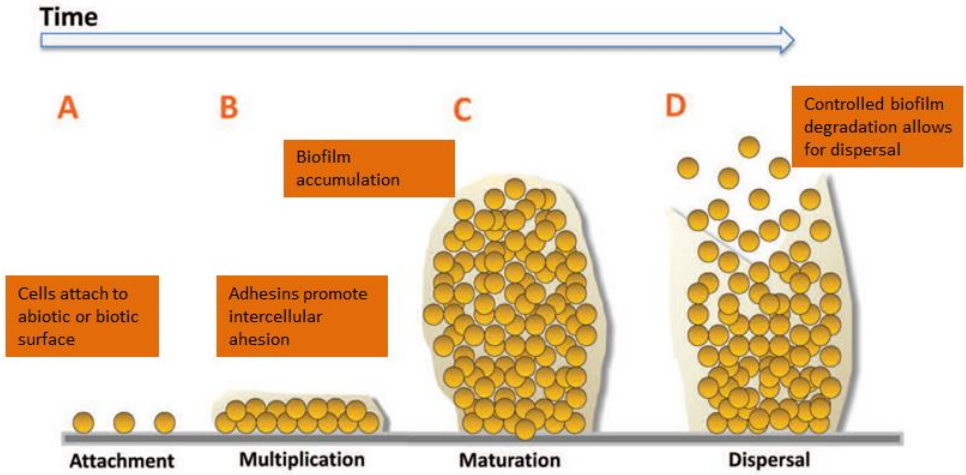

Figure 1.5. Stages of *S. epidermidis* biofilm formation. Planktonic cells attach to biotic or abiotic surfaces using either hydrophobic or specific protein-protein interactions, respectively (A). Intercellular adhesion is promoted in early stage biofilm formation (B). The accumulation of the extracellular matrix produces stalk-like structures (C). Mature biofilms utilise PSMs to break down parts of the biofilm structure, allowing for bacterial dispersal (D). Adapted: Moormeier and Bayles, 2017[161].

1.8.3 Attachment

1.8.3.1 Major autolysin – AtlE

The factors involved in attaching to a surface vary between staphylococcal species and the nature of the surface itself i.e. abiotic or biotic. The major autolysin (Atl) is part of a group of peptidoglycan hydrolases, first identified in *S. aureus*[162]. Atl in *S. aureus* plays an important role in cell division, cell wall turnover, and cell separation[163], [164]. In *S. epidermidis* an Atl homologue was identified, termed AtlE[165]. An *atlE* transposon-insertion mutant was found to be more hydrophilic that the wild type parent, suggesting a role for AtlE in controlling cell surface hydrophobicity[165]. As a result, the mutant could attach to more hydrophilic surfaces such as glass, but not hydrophobic polystyrene. Several studies have shown that *atlE* is responsible for extracellular DNA (eDNA) release during initial attachment[166], [167]. DNase treatment during the attachment phase of biofilm production severely inhibits its development[166]. In this study DNA release was attributed to the autolysin, AtlE. Interestingly, while eDNA is involved in both *S. aureus* and *S. epidermidis* biofilms, it appears to come into effect at different stages of biofilm development; in the attachment phase for *S. epidermidis*, and the transition from attachment to accumulation in *S. aureus*[168].

1.8.3.2 Autolysin/Adhesin – Aae

Aae is also an autolysin and is non-covalently bound to the cell wall[169]. First identified by Hielmann *et al.*, Aae contains a three repetitive sequence at its N terminal, homologous to other peptidoglycan hydrolases[170]. Furthermore, it was found that Aae bound to fibronectin, fibrinogen and vitronectin in a dose-dependent manner. The bi-functional Aae, as both an autolysin and adhesin, may well play an important role in establishing initial attachment on medical devices coated with host extracellular proteins[171].

1.8.4 Biofilm accumulation

1.8.4.1 *ica*-dependent biofilm

Following initial attachment, the biofilm accumulation phase sees the production of an extracellular matrix that will encapsulate the bacterial population. One of the best studied and well-understood biofilm matrix types is the staphylococcal poly-N-acetylglucosamine (PNAG) or polysaccharide intercellular adhesin (PIA)[172]. The polymeric N-glucosamine structure, which is unbranched and linked via β-1, 6 glycosidic links (Fig. 1.6B), was elucidated by Mack *et al.* in 1996[173]. PIA/PNAG has also been found in other pathogens such as *Yersinia pestis* and *E. coli*[174], [175]. Studies have also shown that the production of PIA is essential for both virulence and immune evasion in *S. epidermidis*[176].

PIA production and export is carried out by the intercellular adhesion operon *icaADBC* (Fig. 1.6A)[177], [178]. IcaA and IcaD are associated with the cell membrane and produce chains of up to twenty residues from N-acetylglucosamine monomers. Elongation is dependent on the expression of *icaC*, which is also thought to export the growing PIA chain[179]. IcaB is a non-covalently bound, cell-surface protein that is responsible for partially deacetylating the growing PIA chain (Fig. 1.6A). This partial deacetylation confers an overall positive charge to PIA, promoting its association with the negative cell surface[176]. It is also thought that deacetylation of PIA allows the biofilm to mask the native bacterial cell-surface charge from the innate immune system[176]. The importance of the *ica* operon to *S. epidermidis* virulence was highlighted by Ziebuhr *et al.* when it was shown that 85% of blood isolates harboured the *ica* locus, compared to only 6% of skin and nose isolates[180].

Directly upstream of the *ica* operon is the divergently transcribed repressor, *icaR*, that can bind to and block the *icaADBC* operon promoter site (Fig. 1.6A), in response to various environmental changes[181],[182],[183].

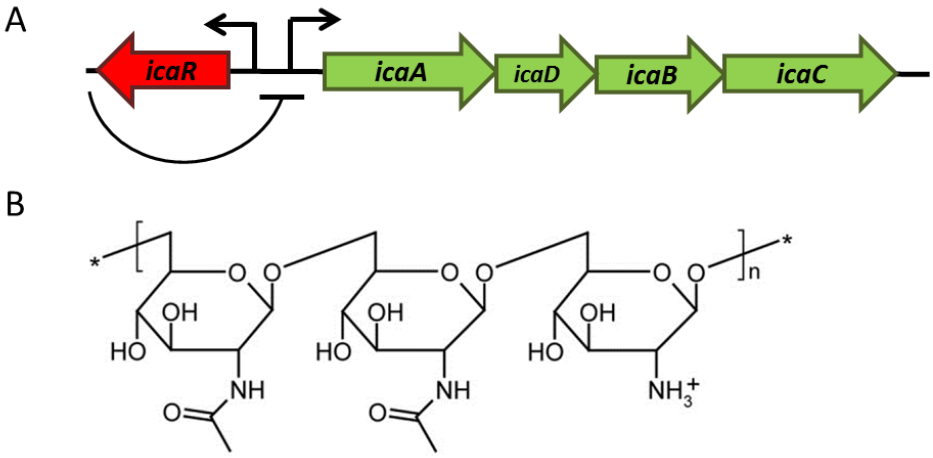

Figure 1.6. *icaADBC* operon and PIA/PNAG structure. A. Organisation of the *icaADBC* operon in *S. epidermidis*, and the divergently transcribed repressor, *icaR*. **B.** Partially deacetylated PIA/PNAG structure. Gökçen *et al*., 2013[184].

1.8.5 Biofilm Detachment

The final step in biofilm formation is the detachment phase. By controlling the breakdown of the outer-layers of a biofilm, cells can disseminate to other sites to repeat the colonisation process. In both *S. aureus* and *S. epidermidis* the *agr* quorum-sensing system is known to be upregulated in the outer layer of biofilms, and strains with a non-functional *agr* system produce thicker biofilms and do not disperse as effectively[119], [185], [186]. The dispersal mechanism required depends on the type of biofilm i.e. polysaccharide or proteinaceous. In *S. aureus* strains that form *ica*-independent biofilm, it was found that *agr* induction promoted the expression and secretion of serine proteases that were required for biofilm dispersal[187]. Although *S. epidermidis* does not appear to possess similarly dedicated proteases, it does produce and secrete exoproteases with low substrate specificity that may function to break-up protein based biofilm[188], [189]. Specific enzymes involved in PIA/PNAG degradation have not yet been identified in *S. epidermidis*, although they are present in other PIA/PNAG producing bacteria[190].

1.8.5.1 Phenol-soluble modulins

There is evidence that biofilm dispersal in *S. epidermidis* is achieved via short, amphipathic phenol-soluble modulins (PSMs)[191], [192]. These detergent-like molecules are associated

with immune activation and can have strong cytolytic activity against neutrophils[193][194]. PSMs are found ubiquitously in staphylococci and are under strict *agr* control[192]. It is believed that PSMs may disrupt the non-covalent interactions in the biofilm (electrostatic, hydrophobicity) as opposed to having specific targets[195]. Experimental evidence has linked PSMs to *S. epidermidis* dissemination in a murine catheter infection model[193].

Interestingly, a study in 2010 by Cheung *et al.* found that certain purified *S. epidermidis* PSMs (PSMδ) had the capacity to lyse neutrophils at a level equalling hemolysins from the more virulent *S. aureus*[194]. However, whole culture filtrates did not produce the same cytolytic effect. The authors speculate that *S. epidermidis* PSMs primarily play an alternative role in evasion rather than virulence, allowing the bacteria to stay anonymous to the immune system. This strategy may aid the bacteria's ability to persist in chronic infections.

1.9 Biofilm regulation

1.9.1 Accessory gene regulator - *agr*

The accessory gene regulator (*agr*) system is a two-component, quorum sensing system that has been extensively studied in both *S. aureus* and *S. epidermidis*[196]. The *agr* locus consists of two divergently transcribed mRNA transcripts, RNAII and RNAIII. RNAII is driven off the P2 promoter and contains transcripts for AgrB,D,C and A. *agrD* encodes the autoinducing peptide, AIP, eight amino acids residues in length. AgrB encodes a multifunctional transmembrane protein involved in post-translational processing of AIP, as well as its secretion. AgrC is a transmembrane histidine-kinase that binds extracellular AIP, and in turn activates AgrA. Activated AgrA promotes both P2 and P3 promoters[197]–[199], promoting the *agr* systems positive feedback loop. Studies have also shown that AgrA plays a role independent of RNAIII in directly promoting certain phenol soluble modulins (PSMα and PSMβ)[200]

The divergent P3 promoter drives expression of the RNAIII transcript and δ-toxin, a PSM. RNAIII is global regulator, controlling hundreds of gene products through antisense base pairing to 5'untranslated regions of mRNAs, including virulence factors such as toxins, proteases and hemolysins[201]. The δ-toxin (also known as PSMγ) is encoded for by the *hld*, and is a 24 amino acid peptide of the PSM family of peptides[202]. As a toxin, Hld has the ability to form pores in human erythrocytes[203] and may play a role in necrotizing enterocolitis in neonates[204]. Conversely, Cogen *et al.* (2010) found that δ-toxin from *S.*

epidermidis was not only antimicrobial towards Group A *Streptococcus,* but acted synergistically with neutrophils and antimicrobial peptides on human skin and in a murine infection model[205]. The authors speculate that this may be line with *S. epidermidis'* preferred commensal lifestyle.

The *agr* system plays a growth phase-dependent role in PIA-dependent biofilm. Vuong *et al.* showed that an *agr* mutant had stronger initial attachment to polystyrene, and that this was due to the major autolysin *atlE* being negatively regulated by RNAIII[206]. Furthermore, the same study found that δ-toxin did not affect initial attachment, indicating that the PSM plays a role in structuring the biofilm in later phases of growth or in detachment. This would be consistent with other studies that have found that *agr* expression is growth-phase dependent, whereby the system is repressed overall in early and mid-exponential phase, and then strongly expressed as cells progress into stationary phase[207]. Mutating *agr* had no effect on PIA production or *ica* expression.

1.9.2 Alternative sigma factor B (σ^B)

Sigma factors are important regulators in many bacterial species. They play a role in virulence, environmental adaptation as well as maintaining intracellular homeostasis[208]. There are four known sigma factors in staphylococci. SigmaA (σ^A) is a housekeeping-gene regulator that has been shown to be essential for growth[209]. The alternative sigma factor H (σ^H) has homology to the *B. subtilis sigH* and is involved in upregulating the *com* operons. *com* genes encode for proteins necessary for natural genetic competence[210]. Sigma S (σ^S) is the most recently discovered sigma factor in staphylococci and was found to be essential under environmental stresses such as elevated temperatures and Triton-induced lysis[211]. Further σ^S characterisation showed it to be important in responding to extracellular stress that can cause DNA damage, and neutrophil phagocytosis[212].

The alternative sigma factor B (σ^B) is a global stress response regulator in staphylococci. SigB has been implicated in regulating cellular responses to oxidative stress, antibiotic resistance, biofilm formation and adaptation to stress, as well promoting the chronic-infection-associated small colony variant (SCV) phenotype[213]–[216].

The staphylococcal σB operon is composed of four genes, rsbU, rsbV, rsbW and sigB[217], [218]. Under normal growth conditions, SigB is kept in an inactive state through the binding

of RsbW, an anti-sigma factor[219]. When exposed to environmental stress such as pH, osmolarity or heat shock, RsbU is activated, and dephosphorylates RsbV, an anti-anti-sigma factor (Fig. 1.7),[220], [221]. RsbV, now activated, competitively binds RsbW, which disassociates from SigB, allowing it to interact with RNA polymerase (Fig. 1.7)[219].

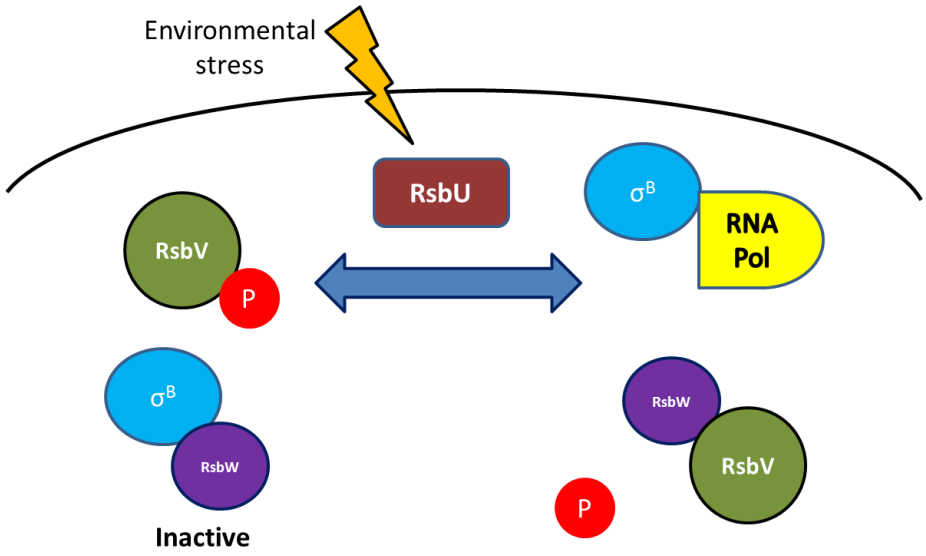

Figure 1.7. σ^B activation in staphylococci. Under environmental stress RsbU dephosphoylates RsbV. RbsV releases σB from RsbW inhibition, allowing interaction with RNA polymerase.

SigB was first linked to biofilm formation in *S. epidermidis* under certain environmental conditions[222]. The authors found that osmotic stress, induced by addition of salt to the culture media promoted biofilm formation in a *sigB*-dependent manner. In a later study, the same group discovered that this regulation was caused by repression of *icaR* by SigB, which resulted in increased *icaADBC* expression in NaCl[223]. Interestingly, exposure to ethanol also induces biofilm formation in an *icaR*-dependent pathway. However this phenotype is independent of SigB[183], [222], [223], suggesting a second regulatory pathway for *ica*-dependent biofilm. There is evidence for a third glucose-dependent regulatory pathway, independent of *icaR*[224]. Although NaCl does not promote biofilm in strains lacking *icaR*, media containing NaCl-glucose does induce biofilm[224].

1.9.3 Staphylococcus accessory regulator – SarA family

There are three groups of SarA proteins; single domain proteins, SarA, SarR, SarT, SarV and Rot (regulator of toxins), two domain proteins SarS, SarU, and SarY, and MarR homologs, MgrA and SarZ[225]. Those involved in biofilm regulation are briefly described.

1.9.3.1 SarA

SarA is a 124-residue DNA binding protein, made up of 3 overlapping transcripts with 3 separate promoters, P1, P2 and P3[226], producing the transcripts *sarA*, *sarB* and *sarC,* respectivly. SarA can promote gene expression via *agr*, by direct binding to the promoter sequence, or independently of *agr*[227]. Several studies found that a *sarA* mutant did not produce PIA/PNAG[183], [228] and was biofilm negative. Tormo *et al.* found that SarR could bind to the *ica* promoter region and promote expression of the *ica* operon, independent of *icaR*/σ^B regulation[228].

1.9.3.2 SarR

SarR is a member of the SarA family of regulators and shares homology with SarA. SarR was found to repress *sarA* expression through binding to the P1 promoter site[229]. In *S. aureus*, SarR was also found to promote *agr* expression via binding to the same promoter region as SarA[230]. Indeed, it was established that SarR had greater binding specificity to the *agr* promoter region compared to SarA[230], [231].

Although the previous work done on SarR has linked it to the regulation of the two major transcription factors, RNAIII and SarA, which can mediate biofilm formation, there are no published studies linking SarR with direct regulation of biofilm. This study investigates link between the SarR regulator and biofilm formation in *S. epidermidis*.

1.9.3.3 SarX

Identified in 2006 in *S. aureus*, SarX is a member of the SarA family of gene regulators[232]. *sarX* expression is growth-phase dependent, and maximally expressed in stationary phase. The same study also found, using gel shift assays, that purified SarX would specifically bind to the *agr* promoter region. A *sarX* mutant was found to have increased transcription of RNAII and RNAIII, indicating that SarX is a repressor of *agr*[232]. In complementing the phenotype with a plasmid-borne wild type *sarX*, the group found that a multicopy plasmid was ineffective. Complementation required a plasmid with an inducible promoter, indicating

that SarX *agr* inhibition was concentration dependent. Furthermore, a 2013 study showed that SarX could bind to, and activate, the *ica* operon[233].

In *S. epidermidis* SarX also binds to the *agr* promoter region and may act as a promoter or repressor of the operon in a concentration dependent manner. Rowe *et al.* also found that purified SarX specifically bound to the *ica* promoter region, and that a *sarX* mutant exhibited decreased biofilm production[234]. How *sarX* is regulated within *S. epidermidis* is investigated in this study.

1.9.4 Regulator of biofilm formation – Rbf

Rbf is an AraC-type transcription factor first identified in 2004[235]. The AraC-type transcriptional regulators are characterised by a region of 99 homologous amino acid residues that contain the DNA binding domain[236]. Typically, AraC-type regulators have been characterised as transcriptional activators with the exception of a few Gram-negative bacteria, where the positive or negative regulation was dependent on the presence of specific coeffectors[237]–[239]. The AraC-type regulators have diverse regulatory functions including carbon metabolism, pathogenesis and stress response[236].

Rbf is an important regulator of the *ica* operon in *S. aureus*. Rbf can bind to, and promote *sarX*, which then binds specifically to a site within the *icaA* coding region blocking repression by IcaR[233], [240]. An *rbf* transposon-mutant exhibited decreased biofilm accumulation, but not initial attachment, to both glass and polystyrene in glucose and NaCl. Complementation of the mutant was not achievable using a multicopy plasmid. In fact, over expression of *rbf* reduced biofilm production[235]. These data would suggest that the concentration of intracellular Rbf is tightly controlled, and has concentration dependent effects on biofilm formation in *S. aureus*.

Under flow conditions, a *S. aureus rbf* mutant was revealed to have delayed biofilm formation, while wild type strains harbouring *rbf* on a multicopy plasmid exhibited accelerated biofilm production[241]. It was also noticed that strains overexpressing *rbf* took longer to detach while under flow conditions, suggesting that Rbf may play a role in maintaining biofilm structure or preventing dispersal. In the same study, *rbf* mutants had decreased *in vivo* survival in a lumen catheter model in mice. Strains carrying multicopy *rbf* had increased *in vivo* survival, indicating a role for Rbf in virulence[241].

1.10 Specific aims of this study

1) Rbf has been shown to regulate biofilm formation in *S. aureus*. Here we have investigated its regulatory role in the *S. epidermidis* biofilm phenotype.

2) The TCA cycle supplies cells with energy and metabolic precursors, but has not been specifically linked to β-lactam resistance. Here we investigate the role of the TCA cycle in β-lactam resistance, specifically succinyl-CoA synthetase.

3) The intracellular concentration of purine derivatives have previously been implicated in modulating high-levels of β-lactam resistance in MRSA. We have found that disrupting the purine salvage pathway in MRSA increases β-lactam resistance.

Chapter 2

AraC-type regulator Rbf controls the *Staphylococcus epidermidis* biofilm phenotype by negatively regulating the *icaADBC* repressor SarR

Sarah E. Rowe[1,2], Christopher Campbell[1], Colm Lowry[1], Sinead T. O'Donnell[1,2], Michael E. Olson[3ǂ], Jill K. Lindgren[3], Elaine M. Waters[3], Paul D. Fey[3] and James P. O'Gara[1,2*]

[1]Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland.

[2]School of Biomolecular and Biomedical Science and Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland.

[3]Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska.

[*] Corresponding author. Tel: 353-91-492250; email: jamesp.ogara@nuigalway.ie

[ǂ] Present address: Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, Illinois.

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2.1 Abstract

Regulation of *icaADBC*-encoded polysaccharide intercellular adhesin (PIA) / poly-*N*-acetyl glucosasmine (PNAG) production in staphylococci plays an important role in biofilm-associated medical device-related infections. Here we report that the AraC-type transcriptional regulator Rbf activates *icaADBC* operon transcription and PIA production in *Staphylococcus epidermidis*. Purified recombinant Rbf did not bind to the *ica* operon promoter region in electrophoretic mobility shift assays (EMSAs), indicating that Rbf regulates *ica* transcription indirectly. To identify the putative transcription factor(s) involved in Rbf-mediated *icaADBC* regulation, the ability of recombinant Rbf to interact with the promoter sequences of known *icaADBC* regulators was investigated. Recombinant Rbf bound to the *sarR* promoter and not the *sarX, sarA, sarZ, spx* and *srrA* promoters. RT-PCR demonstrated that Rbf acts as a repressor of *sarR* transcription. PIA expression and biofilm production were restored to wild type levels in an *rbf/sarR* double mutant grown in brain heart infusion (BHI) media supplemented with NaCl, which is known to activate the *ica* locus, but not in BHI media alone. RT-PCR further demonstrated that although Rbf does not bind the *sarX* promoter, it nevertheless exerted a negative effect on *sarX* expression. Apparently direct down-regulation of the SarR repressor by Rbf has a dominant effect over indirect repression of the SarX activator by Rbf in the control of *S. epidermidis* PIA production and biofilm formation.

2.2 Introduction

Staphylococci are responsible for the majority of biofilm-mediated device-related infections[242]. Biofilms assist in the evasion of the host immune response and offer increased resistance to antimicrobial drugs. Many hospital patients undergo procedures involving the insertion of foreign biomaterials, ranging from simple intravascular catheters to more sophisticated ventricular assist devices. Vulnerable hospital patients with underlying medical conditions are particularly susceptible to device-related infections frequently caused by antibiotic-resistant pathogens. Thus, device-related infections represent a serious clinical problem, which in turn underpins the importance of understanding the mechanisms by which staphylococci form biofilms. Production of the *icaADBC* operon-encoded polysaccharide intercellular adhesin (PIA) or poly *N*-acetyl glucosasmine (PNAG) remains the best understood mechanism of biofilm production in staphylococci[242]–[244]. In *S. epidermidis*, the cell wall anchored autolysin[245] and the accumulation associated protein[158], [246], [247] have also been implicated in primary attachment and maturation phases of biofilm formation, respectively.

The contribution of many transcription factors to the regulation of biofilm formation in staphylococci underlines the importance of this phenotype. Major regulators of the *ica* operon include the IcaR repressor[181], [182], a number of the 11-member *Staphylococcus* accessory regulator (Sar) family of proteins[234], [248] and the regulator of biofilm formation, Rbf. The *rbf* gene was first identified in 2004 in *S. aureus* by transposon mutagenesis[235]. Lim *et al*. reported that *rbf* was present in 22 of 27 *S. aureus* clinical isolates tested and that inactivation of the *rbf* gene in strain 8325-4 resulted in a biofilm negative phenotype in TSB media supplemented with glucose or NaCl[235]. Rbf is a member of the AraC/XylS family of transcriptional regulators, defined by a conserved ~100 amino acid region comprising a DNA binding domain, which can positively or negative regulate gene expression[236]. Rbf controls the biofilm phenotype in *S. aureus* by increasing the production of PIA[240]. Rbf-mediated activation of the *ica* operon is indirect and is achieved by activation of *sarX*, which in turn binds upstream of *icaR* within the *icaA* coding region leading to repression of *icaR* and concomitant activation of the *ica* operon[249]. The *sarX* gene is located immediately downstream of *rbf* on the *S. aureus* and *S. epidermidis* chromosome and overexpression of *rbf* in *S. aureus* UAMS-1 led to a >50-fold increase in

sarX expression[240]. Unlike in *S. aureus*, the contribution of *sarX* to *S. epidermidis* biofilm is influenced by growth conditions, playing a regulatory role in media supplemented with glucose[248], but not salt (unpublished data) suggesting a different role for SarX in the regulation of biofilm in these two organisms. Here we report that Rbf-mediated biofilm regulation in *S. epidermidis* is mediated via SarR, which encodes a transcriptional repressor of the *ica* locus.

2.3 Materials and Methods

2.3.1 Bacterial strains, plasmids, media and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2.1. *Escherichia coli* strains were grown at 37ºC in Luria-Bertani (LB) media (Sigma) supplemented when required with kanamycin (Kan) (50µg/ml) or carbenicillin (Car) (50µg/ml) with the exception of *E. coli* strain Rosetta when used for protein expression, was grown in overnight expression media (Novagen) supplemented with chloramphenicol (Cam) (34µg/ml) and carbenicillin (50µg/ml).

S. epidermidis and *S. aureus* strains were routinely grown at 37ºC or 30ºC on brain heart infusion (BHI) media (Oxoid, UK). When required BHI was supplemented with Cam (10µg/ml), erythromycin (Em) (10µg/ml) or tetracycline (Tet) (5µg/ml). BHI was also supplemented with 4% NaCl as required.

Table 2.1. Strains and plasmids used in this study

Strain/Plasmid	Characteristic(s)	Source or reference
CSF41498	Biofilm positive, cerebrospinal fluid isolate. Beaumont Hospital, Dublin	Conlon *et al.* [181]
1457	Biofilm-positive, isolate from infected central venous catheter. Hamburg, Germany.	Mack *et al.*[250]
rbf	CSF41498 derivative, *rbf::ermB.* Em^r	This study
1457 *sarR*	1457 derivative, Δ*sarR*::*tet*. Tet^r	This study
CSF41498 *sarR*	CSF41498 derivative, Δ*sarR*::*tet* transduced from 1457. Tet^r	This study
rbf/*sarR*	CSF41498 derivative, Δ*sarR*::*tet, rbf::ermB.* Tet^r Em^r	This study
pCR-Blunt II-TOPO	PCR cloning vector. Km^r Ap^r	Invitrogen
pLI50	*Escherichia coli-Staphylococcus* cloning vector. Ap^r (*E. coli*), Cam^r (*Staphylococcus*)	Lee *et al.*[251]

pMal-c2x	*E.coli* MBP-protein expression vector. Cam^r	New England Biolabs
pET30 EK/LIC	His-tag cloning vector	Novagen
pEPSA5	*E. coli-Staphylococcus* cloning vector. Multiple cloning site downstream of xylose inducible promoter.	Forsyth *et al.*[252]
pBT2	Temperature-sensitive *E. coli*–*Staphylococcus* shuttle vector; Ap^r (*E. coli*), Cam^r(*Staphylococcus*)	Bruckner[253]
pUC19	*E. coli* cloning vector. Ap^r	NEB
pROJ6448	pRN5101::pC221 AluI-C. Temperature sensitive staphylococcal origin of replication.	Projan *et al.*[254]
pSERB1	2,445bp *rbf* fragment in pCR-Blunt II-TOPO	This study
pSERB2	*rbf* gene in pSERB1 interrupted with *ermB*	This study
pSERB3	*rbf::erm* from pSERB2 in shuttle vector pBT2	This study
pSERB5	2,800bp fragment containing *rbf* and its upstream region in pCR-Blunt II-TOPO	This study
pSERB6	*rbf* fragment from pSERB5 cloned into pLI50	This study
pEP*rbf*	*rbf* and its upstream region in pEPSA5	This study
pLI*sarR*	*sarR* and its upstream region in pLI50	This study
pTSERM6	Promoterless *rbf* in pCR-Blunt II-TOPO	This study
pSERM6	*rbf* from pTSERM6 downstream of *malE* in pMAL-c2x	This study
p*sarR*-his	*sarR* gene in pET30 EK/LIC	This study

2.3.2 Construction of *S. epidermidis rbf* and *sarR* mutants. The CSF41498 *rbf* mutant was constructed as follows. A 2,445bp fragment containing the *rbf* gene from *S. epidermidis* CSF41498 was amplified by PCR using the primers SErbf1 and SErbf3 (Table 2.2) and cloned into the pCR-Blunt II-TOPO plasmid (Invitrogen) to create pSERB1. This plasmid was then digested with *Cla*I and *Mun*I to release a 182bp fragment, which was replaced with a 1,227bp *Eco*RI-*Cla*I fragment containing the *ermB* gene from pEC5[253] to generate pSERB2. In this construct the *rbf* gene is disrupted by the *ermB* gene inserted 937bp from the *rbf* start codon. A 3,490bp *Bam*HI-*Pst*I fragment from pSERB2 containing the *rbf*::*ermB* allele was subcloned into the *Escherichia coli-Staphylococcus* shuttle vector pBT2[253]. The resulting plasmid, designated pSERB3, was transformed into *S. epidermidis* CSF41498 via RN4220 as described previously[234]. The presence of the *rbf*::*ermB* allele on the chromosome was confirmed by PCR using primers SErbf1 and SErbf2 (Table 2.2). For *rbf* over-expression and complementation experiments, a 2,800bp fragment was amplified using primers SErbf1 and SErbf2, cloned into pCR-Blunt II-TOPO plasmid (Invitrogen) to create pSERB4, before being subcloned into pLI50 to generate pSERB4. A similar fragment was amplified using primers SErbf_rbsEcoR1 and SErbf_Kpn2 and cloned into the *Eco*RI and *Kpn*I sites of pEPSA5[252], under the control of a xylose-inducible promoter to generate pEP*rbf*.

To construct a *S. epidermidis* 1457 *sarR* mutant, a 1048bp *sarR* and flanking DNA fragment was amplified from 1457 chromosomal DNA using primers 1058 (*Bam*HI tailed) and 1059 (*Xba*I tailed) and was ligated into the *Bam*HI an *Xba*I sites of pUC19. An 851bp *sarR* and flanking DNA fragment obtained from amplification with primers 1060 (*Sal*I tailed) and 1061 (*Pst*I tailed) was ligated into the corresponding sites. Next, the *tetM* gene was amplified from pJF12 into the *Sal*I site. Finally, the temperature sensitive mobilization staphylococcal replicon pR0J6448 was ligated into the *Pst*I site.

The *sarR*::Tet^r allele was transduced into *S. epidermidis* CSF41498 using phage A6C as described previously[247]. The *sarR* primers (SEsarR1 and SEsarR_comp2, Table 2.2) were used to amplify a fragment containing the *sarR* gene and upstream promoter sequences, and subsequent cloning into pLI50 (via pCR-Blunt II-TOPO) for complementation experiments.

Table 2.2. **Oligonucleotide primers used in this study**

Target	Primer	Sequence (5' - 3')
gyrB	GYRB1	TTATGGTGCTGGACAGATACA
	GYRB2	CACCGTGAAGACCGCCAGATA
icaA	KCA1	AACAAGTTGAAGGCATCTCC
	KCA2	GATGCTTGTTTGATTCCCT
P_{ica}	SEicaProm1	TGCGTTATCAATAATCTTATCTTTCAA
	SEicaProm2	GAAAAGTAAAAAGTTAAATACATGCAT
P_{sarR}	SEsarR_comp1	TATAAAACCACTCCTCTGATGCACATCTTG
	SEsarR_comp2	ATATACTAGTTTATTATGTGATATTTACAA
P_{sarX}	SEsarXPROMBio1	TGCAGTATATTTAGTTGAAATATATAAAAA
	SEsarXPROMBio2	AATCTGCACCTCCAAATATAAGTAGACAAC
P_{srrA}	SEsrr_promBio1	ACACCAAAAAGATGTAAATTACCATTAAGAT
	SEsrr_promBio2	ACTTTCTACTACCTCCTACACTTGCTGTTA
P_{sarA}	SEsarA_prom1	TAATGAAACCTCCCTATTTATATCATA
	SEsarA_prom2	AAAATGTTAGTAAAATTCTTTCCAAAA
P_{sarZ}	SEsarZpromBio1	TTTTCGTACTCCTCCATTTTTTAAAAAATT
	SEsarZpromBio2	TTAATCACTCCTTGTTAAGGTAACAATATT
P_{spx}	SEspx_promBio1	ATTAGATGCCTACTTTCTAATTAATATTGT
	SEspx_promBio2	ACATCTCACTCTCTTATAGAATGAATTTAA
*rbf (*pLI50	SErbf1	ATCAAAAAGTTGGCGAACCTTTTCA
complementation)	SErbf2	CAAAAGAGCCTGGAGAAAAGTATCA
*rbf (*pEPSA5	SErbf_rbsEcoR1	CGAGCTGAATTC*GAGGGAAAGAG*GTAAAGATA
complementation)	SErbf_Kpn2	CGAGCTGGTACCTTAAGTTGTGCTACGCCTTTTAT
rbf (protein	SErbf1	ATGGCAAATTCTTGTTTGCAT
purification)	SErbf2	TTAAGTTGTGCTACGCCTTTTATTT
sarR (protein	1589	GACGACGACAAGATGGGAAAAATTAAAGACATCA
purification)	1590	ATG

		GAGGAGAAGCCCGGTTATTTGATATAGTTTTCTAATTC
sarR (allele replacement)	1058	ATCCTAGGATCGGGTTACTTATCATTAGTG
	1059	ATCCTATCTAGACGCATTAACCAAATCATTG
sarR (allele replacement)	1060	ATCCTAGTCGACGCGTGCATGATGAAAGAACAG
	1061	ATCCTACTGCAGCCGTGTCAATGTCAACTTAG
sarR (complementation)	SEsarR1	TTATTTGATATAGTTTTCTAATTCTAAAATC
	SEsarR_comp2	ATATACTAGTTTATTATGTGATATTTACAA
sarX	SE_sarXRT2F	GCAGATTTTGAATGAGCAGAAAT
	SE_sarXRT2R	ATCTAACTCTCCTGTAGCCA

2.3.3 Biofilm assays. Semi-quantitative measurements of biofilm formation under static conditions were determined using Nunclon tissue culture treated (ΔSurface) 96-well polystyrene plates (Nunc, Denmark), as described previously[181]. Each strain was tested at least three times and average results are presented. A biofilm positive phenotype was defined as an A_{490} ≥0.17. Primary attachment assays were performed as described previously[234].

2.3.4 Biofilm flow cell experiments. The BioFlux 1000z microfluidic system (Fluxion Biosciences Inc., San Francisco, CA) was used to assess biofilm formation under shear flow conditions. Biofilms were grown in BHI and BHI supplemented with NaCl (4% w/v). The system was initiated by adding 200µl of media to the output wells of a 48-well plate and priming the channels for 5min at 5.0 dyne/cm^2. After priming, the media was aspirated from the output wells and replaced with a 50µl suspension of bacteria grown to early exponential growth phase and adjusted to an A_{600} = 0.8. A further 50µl of medium was added to the input wells and the channels were seeded by pumping from the output wells to the input wells for 3-5sec at a speed of 3 dynes/cm^2. Bacteria were allowed to attach to the surface of the plate for 1h at 37ºC. Excess inoculum solution was aspirated from the output wells and a further 1ml of medium was added to the input wells. The flow rate was set at 0.4 dyne/cm^2 for 18h and brightfield images were captured every 5min at 10× magnification.

2.3.5 RNA purification and real time RT-PCR. Cultures were grown in BHI media supplemented with 1% glucose or 4% NaCl where indicated. Cells were harvested following growth to exponential phase and immediately stored at -20ºC in RNAlater (Ambion) to ensure maintenance of RNA integrity prior to purification. RNA was extracted as described using RNA Mini-Extraction kit (Sigma). RNA integrity was examined visually by agarose gel electrophoresis and RNA concentration was determined using a Nanodrop spectrophotometer. RT-PCR was performed on a Roche LightCycler using the RNA amplification kit SYBR Green I (Roche) as previously described[234], [247]. Each experiment was performed at least three times and mean data with SDs are presented. RelQuant software (Roche Biochemicals) was used to measure relative expression of target genes. The *gyrB* gene was used as an internal standard in real-time RT-PCR experiments. Each experiment was performed at least three times and average data with standard deviations are presented. The primers used for real time RT-PCR are listed in Table 2.2.

2.3.6 PIA assays. PIA immunoblots were performed based on the method of Cramton *et al.*[255], as described previously[234], [247].

2.3.7 Purification of recombinant Rbf. To purify recombinant Rbf protein, the *rbf* gene was amplified by PCR using Phusion high-fidelity DNA polymerase and primers SErbfprot1 and SErbfprot2 and ligated into pCR-Blunt II-TOPO plasmid (Invitrogen) to create pTSERM6. Plasmid pTSERM6 was digested with *Eco*RI and the *rbf* gene was sub-cloned into the pMal-c2x vector on an *Eco*RI site downstream from the *malE* gene, resulting in plasmid pSERM6. Restriction analysis was used to confirm the correct orientation of the *rbf* gene. Plasmid pSERM6 was transformed into *E. coli* strain Rosetta (Novagen) and protein purified following a previously described protocol[256].

A single colony of *E. coli* Rosetta strain harbouring pSERM6 was used to inoculate 10ml overnight expression media (Novagen) containing Car 50µg/ml and Cam 34µg/ml. Cultures were grown overnight at 37ºC, shaking with good aeration. Cells were pelleted and resuspended in 5ml column buffer (20 mM Tris-HCL, pH 7.4, 200mM NaCl, 1mM EDTA, 1mM DTT). Cells were incubated on ice and lysed by sonification. Samples were centrifuged at 14,000 rpm for 30min at 4ºC. The soluble fraction was diluted in 50ml column buffer and passed through an amylose resin column at a speed of 1ml/min. The column was washed

and eluted with column buffer containing 10mM maltose. Purified fractions were collected in 500µl aliquots and analysed on a 10% SDS-PAGE gel and concentrations were determined using a Nanodrop spectrophotometer at A_{280}. Protein was stored at -80ºC in 50µl aliquots.

2.3.8 Purification of recombinant SarR. The *sarR* gene was amplified using primers 1589 and 1590 and cloned into pET30 EK/LIC (Novagen) using Ligation Independent Cloning methodologies. The resulting plasmid, pSarR-his, was electroporated into *E. coli* BL21-DE3 (Novagen) for protein production. BL21-DE3 containing pSarR-his was grown in 1L of 2xYT media containing 30mg kanamycin/ml. The culture was grown to an A_{600} = 0.6 and induced with 0.5mM IPTG (isopropyl-b-D-thiogalactopyranoside) and grown for an additional 2 hours. The culture was subsequently pelleted by centrifugation and resuspended in 100ml binding buffer (50mM Tris, 30mM imidazole, 500mM NaCl pH 7.4). The *E. coli* cells were then lysed by four passages through an EmulsiFlex (Abestin, Inc). Proteases were inhibited via 0.4mM phenylmethylsulfonyl fluoride (PMSF). The soluble cell extract was obtained by centrifugation at 12,000×g for 30min at 4ºC and applied to a HisTrap column (GE Healthcare) at a flow rate of 0.5ml/min and subsequently washed with 20 column washes of binding buffer. Purified SarR was eluted with elution buffer (50mM Tris, 500mM imidazole, 500mM NaCl pH 7.4) and then dialyzed against 50mM Tris (pH 7.5).

2.3.9 Electrophoretic mobility shift assays (EMSAs). The promoter regions were amplified from *S. epidermidis* strain CSF41498 genomic DNA using the biotinylated primers listed in Table 2.2 and Phusion high fidelity enzyme. The resultant PCR products were first purified from a 2% agarose gel using the QIAquick gel extraction kit (Qiagen) and subsequently purified from a 5% non-denaturing polyacrylamide gel. The DNA concentration was determined using a Nanodrop spectrophotometer.

A biotinylated DNA probe containing the promoter region of a target gene was added to increasing concentrations of recombinant Rbf or SarR protein. A 20µl binding reaction containing: varying amounts of protein, 0.2µg poly-(dI-dC) in binding buffer (100mM Tris, 500mM KCL, 10mM DTT, pH 7.5), 5% glycerol, 5mM $MgCl_2$, 4.5ng biotinylated probe unless otherwise stated. The reaction was incubated at room temperature for 20min and loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed at 100 V for 65min. DNA was transferred onto a Biodyne-B nylon membrane (Pall corporation, AGB) at 4ºC in pre-

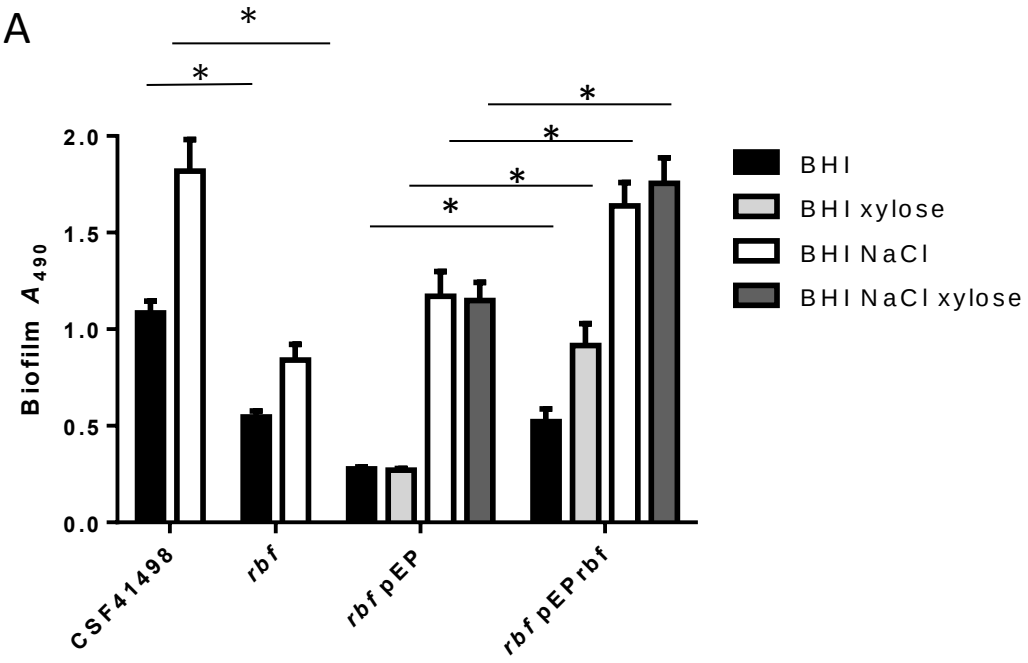
chilled 0.5% TBE and electrophoresed at 80 V for 60 min. Membrane was cross-linked under UV light for 10min. Detection of the bands was performed using a Pierce LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce Chemicals, Rockford, IL) and a FluorChem FC2 chemiluminescent unit (Alpha Innotech).

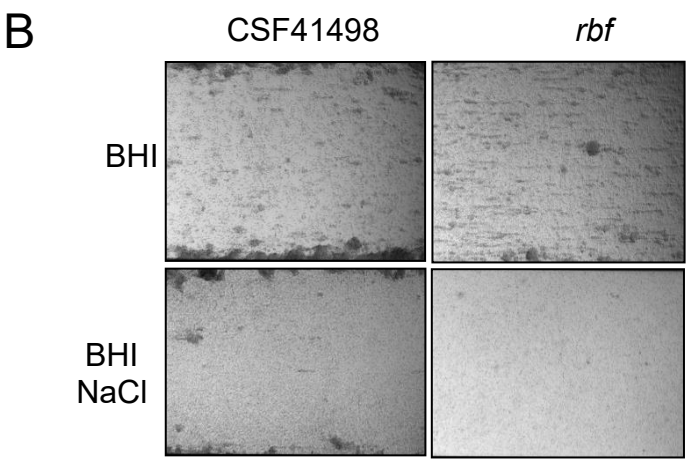
2.3.10 Statistical analysis. Two-tailed, two-sample equal variance Student's t-Tests (Microsoft Excel) were used to determine statistically significant differences in assays performed during this study. A significant difference was indicated as a *P* value <0.05.

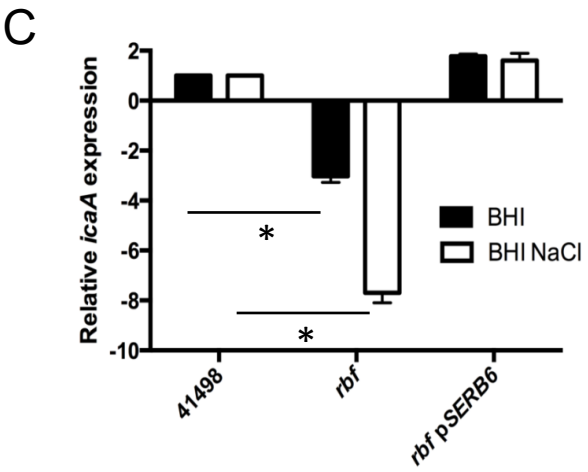
2.3 Results and discussion

2.3.1 Mutation of *rbf* impairs the biofilm phenotype of *S. epidermidis* CSF41498. An *rbf* deletion mutation was constructed in CSF41498 in which 180bp of the *rbf* gene was replaced with the erythromycin resistance gene *ermB*. No significant difference in growth rate was observed in the *rbf* mutant compared to CSF41498 (data not shown). However biofilm formation by the *rbf* mutant under static (Fig. 2.1A) or flow conditions generated using a Bioflux instrument (Fig. 2.1B) was reduced compared to CSF41498 in BHI media and BHI media supplemented with 4% NaCl (BHI NaCl) (Fig. 2.1A). Consistent with this the *rbf* mutation was associated with reduced *icaA* transcription, particularly in BHI NaCl (Fig. 2.1C) and PIA production (Fig. 2.1D). However, impaired biofilm production by the *rbf* mutant did not correlate with impaired primary attachment to polystyrene when compared to CSF41498 (data not shown).

To complement the *rbf* mutant, a 2,797bp fragment containing the *rbf* gene and its upstream regulatory sequences was cloned into the *E. coli-Staphylococcus* shuttle vector pLI50, to yield pSERB6. *icaA* transcript levels and PIA production were restored to wild type levels in the *rbf* mutant carrying pSERB6 (Fig. 2.1C & D). However, the *rbf* gene carried on pSERB6 failed to complement the biofilm effect of the *rbf* mutant (data not shown). Given that carriage of the pSERB6 plasmid restored PIA production, this observation is difficult to explain but may suggest that inappropriate *rbf* expression or carriage of pLI50 may impact Rbf-mediated regulation of the *ica* locus or other factor(s) involved in the biofilm phenotype. Efforts to complement the *rbf* mutant on the low copy plasmid pRB474 also failed because carriage of this plasmid alone significantly increased CSF41498 biofilm production (data not shown). Next, the *rbf* gene was placed under the control of a xylose inducible promoter in pEPSA5[252]. Although supplementation of the growth media with xylose had a generally positive effect on biofilm production by all strains, successful complementation of biofilm production by the *rbf* mutant in BHI (0.5% xylose), BHI NaCl (0.5% xylose) and BHI NaCl alone was observed (Fig. 2.1A). Notably, leaky expression from pEP*rbf* in the absence of xylose was sufficient to complement the *rbf* mutant in BHI supplemented with NaCl (Fig. 2.1A). Taken together these data are suggestive of a complex role for Rbf in controlling the *S. epidermidis* biofilm phenotype but support the conclusion


A
Biofilm A_{490}
2.0
1.5
1.0
0.5
0.0
CSF41498
rbf
rbf pEP
rbf pEPrbf
BHI
BHI xylose
BHI NaCl
BHI NaCl xylose


B
CSF41498
rbf
BHI
BHI NaCl


C
Relative icaA expression
2
0
-2
-4
-6
-8
-10
41498
rbf
rbf pSERB6
BHI
BHI NaCl

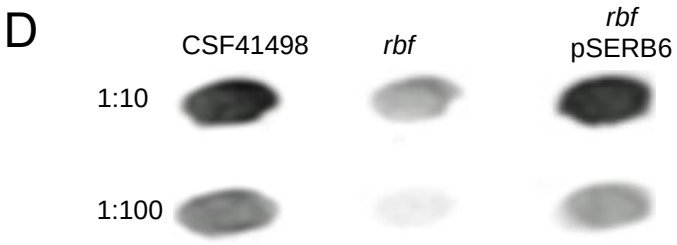

D
CSF41498
rbf
rbf
pSERB6
1:10
1:100

Figure 2.1. Rbf regulates *icaADBC* transcription, PIA expression and biofilm production in *S. epidermidis*. A. Comparative biofilm phenotypes of CSF41498 and its isogenic *rbf* mutant complemented where indicated with plasmid pEPSA5 (empty vector control) or pEP*rbf*. Semiquantitative measurements of biofilm formation under static conditions were performed in tissue culture-treated 96-well plates. All strains were grown at 37°C in BHI and BHI NaCl for 24h. BHI and BHI NaCl were also supplemented with 0.5% xylose for strains carrying pEP*rbf*. Experiments were repeated at least five times and average results are presented. Standard error of the mean is indicated and * denotes a significant difference (P<0.05) as determined by Student's t-test. **B.** CSF41498 and its isogenic *rbf* mutant grown in BHI and BHI NaCl in flow cells at 0.6 dynes/cm2 shear using a Bioflux 1000Z instrument. Brightfield images depicting biofilm accumulation after 18h were captured at 10× magnification and are representative of three independent experiments. **C.** Comparative measurement of *icaA* transcription by real-time RT-PCR in CSF41498 and its isogenic *rbf* mutant. Total RNA was extracted from cultures grown at 37°C to A_{600}=1.0. RelQuant software (Roche) was used to measure the relative expression of *icaA* compared to the constitutively expressed *gyrB* gene. Transcript levels of *icaA* in the *rbf* mutant strain were then compared to CSF41498 pLI50, which was assigned a value of 1. The data presented are the average of three separate experiments and standard deviations are indicated. Statistical significances between the *rbf* or *rbf* pEP*rbf* strains and CSF41498 as determined by Student's t-test are indicated by an * ($P \leq 0.05$). **D.** Comparative immunoblot analysis of PIA production in whole cell extracts of CSF41498, its isogenic *rbf* mutant and the *rbf* mutant complemented with pSERB6 grown overnight at 37°C in BHI. Cell extracts were diluted 1:10 and 1:100. The blot shown is representative of three independent experiments.

that Rbf influences biofilm formation in *S. epidermidis* by positively regulating *icaADBC* operon expression and PIA production.

2.3.2 Rbf does not bind to the *S. epidermidis ica* operon promoter. To investigate how Rbf regulates *ica* operon transcription, we purified recombinant Rbf protein and performed electrophoretic mobility shift assays (EMSAs) with the *ica* operon promoter. Rbf is a member of the AraC/XylS family of transcription factors, most of which are highly insoluble and difficult to purify[236]. Therefore the *rbf* gene was cloned into the pMal-c2x vector (NEB) downstream from the *malE* gene to take advantage of previous observations that tagging with maltose-binding protein (MBP) increases the solubility of recombinant proteins[257]. The resulting plasmid pSERM6 was transformed into Rosetta, a strain of *E. coli* containing pRARE, a plasmid used for expressing rare *E. coli* codons, and grown overnight in commercial OnEx media (Novagen) prior to purification of recombinant Rbf. A 218bp biotinylated oligonucleotide P*ica* probe was incubated with increasing concentrations of recombinant Rbf, separated on a 5% acrylamide gel, transferred to a positively charged

nylon membrane and the probe detected using a Pierce LightShift chemiluminescent EMSA kit (Pierce Chemicals, Rockford, IL). The recombinant Rbf protein did not bind to the *ica* operon promoter probe (Fig. 2.2A). Weak interaction between the P_{ica} probe and the highest concentration (20.37pg) of recombinant Rbf was easily disrupted with poly-(dI-dC) indicating that the complex was not specific (data not shown).

2.3.3 Rbf binds specifically to the *S. epidermidis sarR* promoter and regulates *sarR* transcription. To investigate the possibility that Rbf indirectly regulates the *ica* operon by controlling the expression of another biofilm regulator, the ability of recombinant Rbf to interact with biotinylated promoter probes comprising the promoter regions of the *sarA*[258], *sarX*[234], *sarZ*[259], *sarR* (Unpublished data Fey Laboratory), *spx*[260] and *srrA*[261] genes was next investigated. Rbf bound with high specificity to the *sarR* promoter (Fig. 2.2B & C) and not to any of the other tested promoters (Fig. 2.2B). Expression of *sarR* expression was increased approximately 2.5-fold in the CSF41498 *rbf* mutant (Fig. 2.2D), suggesting that Rbf functions as a repressor of *sarR* transcription. The *sarR* gene was first described in 2000 and encodes a 13.6KDa protein known to bind the *sarA* promoter and regulate *sarA* expression[229]. SarR can also regulate the transcriptional activity of the accessory gene regulator (*agr*) promoter[230], which is also a major target of SarA[262].

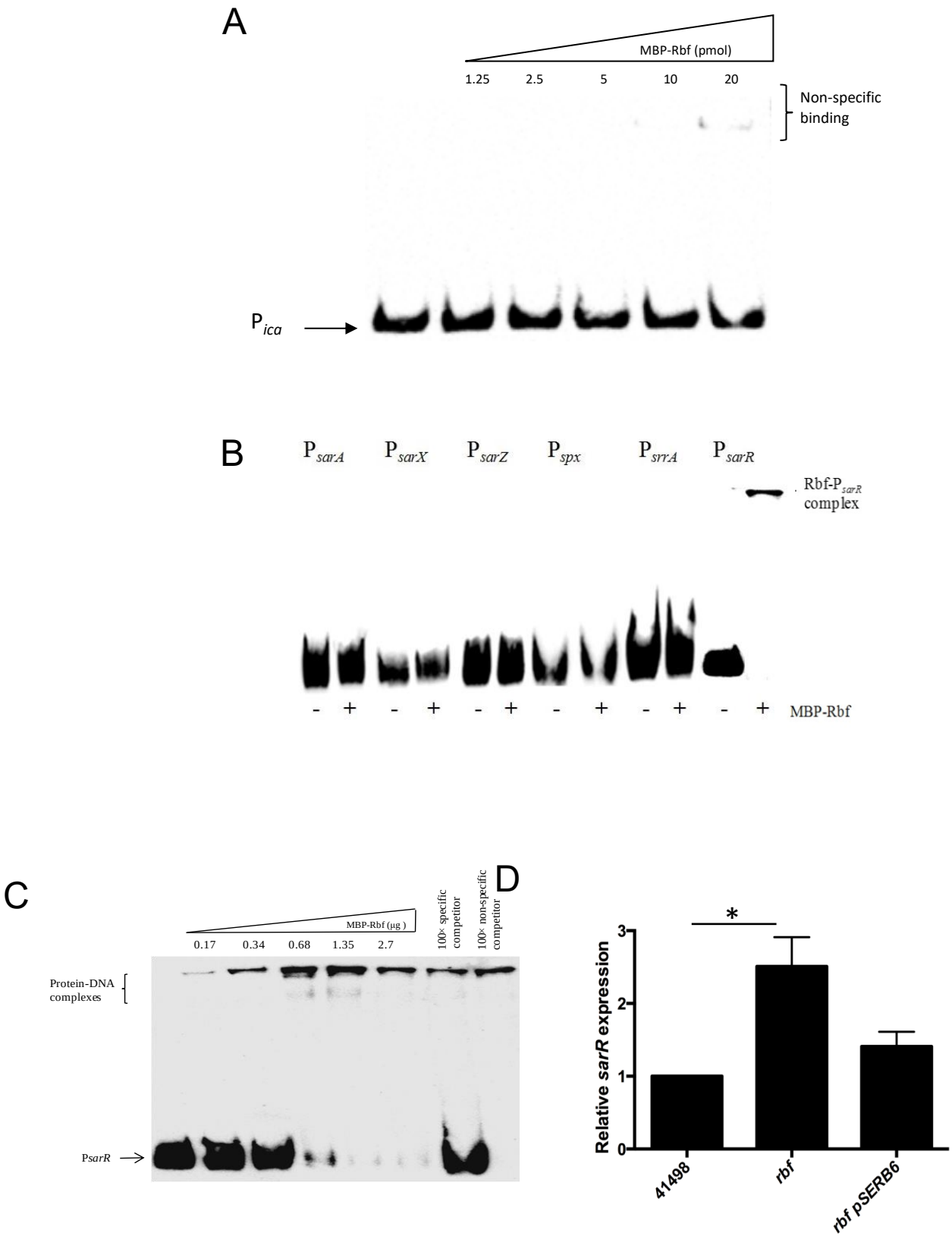

A
MBP-Rbf (pmol)
1.25
2.5
5
10
20
Non-specific binding
P ica
B
P sarA
P sarX
P sarZ
P spx
P srrA
P sarR
Rbf-P sarR complex
- + - + - + - + - + - + MBP-Rbf
C
MBP-Rbf (µg)
0.17
0.34
0.68
1.35
2.7
100× specific competitor
100× non-specific competitor
Protein-DNA complexes
PsarR
D
Relative sarR expression
*
0
1
2
3
41498
rbf
rbf pSERB6

Figure 2.2. Recombinant Rbf binds to the *sarR* promoter and regualtes *sarR* transcription. **A.** Recombinant Rbf does not bind to the *ica* promoter. Increasing concentrations (1.25 - 20pmoles) of recombinant Rbf protein were added to a biotinylated oligonucleotide P*ica* probe. **B.** Recombinant Rbf binding to the *sarA, sarX, sarZ, spx, srrA and sarR* promoters. 20pmoles of recombinant Rbf protein was added to each biotinylated oligonucleotide probe before being separated on a 5% polyacrylamide gel. **C.** Recombinant Rbf binds specifically to the *sarR* promoter. Increasing concentrations (1.25 - 20pmoles) of recombinant Rbf protein were added to a biotinylated oligonucleotide P*sarR* probe. The protein-DNA interactions were competed with 100× specific or non-specific competitor DNA. **D.** Comparative measurement of *sarR* and *gyrB* (control) transcription in *S. epidermidis* strains CSF41498 pLI50, *rbf* pLI50 and *rbf* pSERB6. RNA was prepared from cultures grown overnight to stationary phase (~16h) at 37^{o}C in BHI. RelQuant software (Roche, Switzerland) was used to compare the relative expression of *sarR* compared to the constitutively expressed *gyrB* gene. *sarR* transcript levels in all strains were compared to *sarR* transcript levels in CSF41498 pLI50 which was assigned a value of 1. The data presented are the average of three separate experiments. Standard deviations are indicated and * denotes a significant difference as determined by Student's t-test ($P \leq 0.05$).

2.3.4 SarR is a repressor of *ica* operon expression and biofilm in *S. epidermidis*. To further investigate the hypothesis that Rbf controls *ica* operon expression by regulating *sarR* expression, recombinant SarR was purified and used in EMSAs. Recombinant SarR was relatively soluble and the pET30 system (Novagen) was employed to express and purify an N-terminal His-tagged SarR recombinant protein as described in the methods. These experiments demonstrated that recombinant SarR bound specifically to the *ica* operon promoter (Fig. 2.3A). Next a *sarR::tet* deletion mutation was constructed in *S. epidermidis* 1457 using allele replacement as described in the methods. Using RT-PCR, *icaA* transcription was found to be increased 6.5-fold in the *sarR* mutant compared to 1457 (Fig. 2.3B). Inspection of the intergenic sequence upstream of the *sarR* open reading frame and downstream of SERP_RS0910 using the Softberry bacterial promoter prediction software (BPROM) identified a strong candidate promoter based on alignment to canonical -10 and -35 RNA polymerase recognition sequences (Fig. 2.3C).

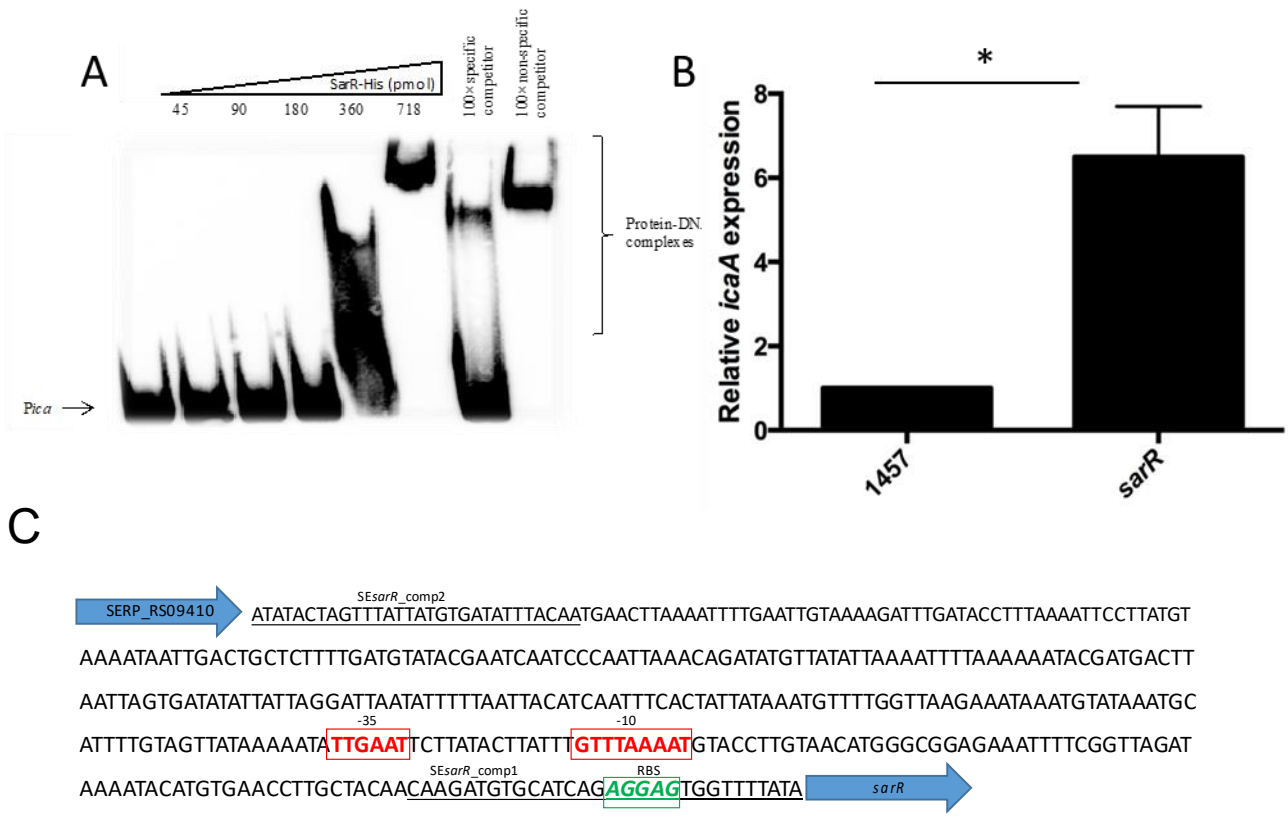


Figure 2.3. Recombinant SarR binds to the *ica* operon promoter and regulates *ica* transcription. A. Recombinant SarR binds to the *ica* promoter. Increasing concentrations (45-718pmoles) of recombinant SarR protein were added to a biotinylated oligonucleotide P*sarR* probe. The protein-DNA interactions were competed with 100× specific or non-specific competitor DNA. **B.** Comparative measurement of *icaA* and *gyrB* (control) transcription in *S. epidermidis* strains 1457 and its *sarR* mutant. RNA was prepared from cultures grown to A_{600} = 1.0 at 37°C in BHI NaCl. RelQuant software (Roche, Switzerland) was used to measure the relative expression of *icaA* against the constitutively expressed *gyrB* gene. *icaA* transcript levels in all strains were compared to *icaA* transcript levels in CSF41498 pLI50 which was assigned a value of 1. The data presented are the average of three separate experiments. Standard deviations are indicated and * denotes a significant difference as determined by Student's t-test ($P \leq 0.05$). **C.** Intergenic nucleotide sequence between SERP_RS09410 and *sarR* on the chromosome of *S. epidermidis* RP62A. The binding sites for the SEsarR_comp1 and SEsarR_comp2 oligonucleotide primers are underlined. The predicted *sarR* ribosome binding site (RBS, green text) and promoter based on canonical -10 and -35 RNA polymerase binding sites (red text), was identified using Softberry bacterial promoter prediction software (BPROM).

2.3.5 Mutation of *sarR* restores biofilm-forming capacity in an *rbf* mutant. Using phage A6C, the *sarR* mutation was transduced into CSF41498 and its isogenic *rbf* mutant and transductants confirmed by PCR of the *sarR* locus (data not shown). Immunoblots revealed

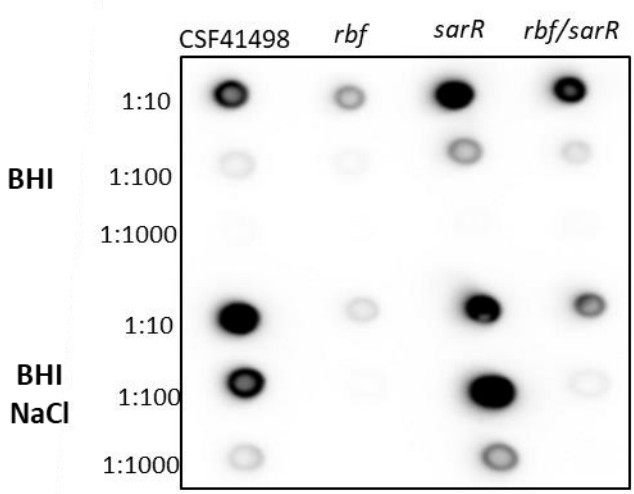
that PIA production was significantly increased in the *sarR* mutant grown in both BHI and BHI NaCl (Fig. 2.4A). Consistent with this, the *sarR* mutation promoted biofilm formation in both BHI and BHI NaCl under shear flow in the Bioflux instrument (Fig. 2.4B). In contrast, under static growth conditions the *sarR* mutation was only associated with increased biofilm formation in BHI NaCl and not BHI (Fig. 2.4C).

In the *rbf*/*sarR* double mutant PIA production was restored to wild type levels in BHI media (Fig. 2.4A), whereas in BHI NaCl PIA expression was increased but remained lower than CSF41498 (Fig. 2.4A). In the Bioflux instrument, the *rbf*/*sarR* double mutant formed robust biofilm in both BHI and BHI NaCl under shear flow (Fig. 2.4B), whereas under static growth conditions, biofilm production by the *rbf*/*sarR* double mutant was only restored to wild type levels in BHI NaCl and not in BHI media (Fig. 2.4C). Complementation of *sarR* mutants with the *sarR* gene on plasmid pLI50 significantly reduced biofilm production in BHI NaCl (Fig. 2.4C).

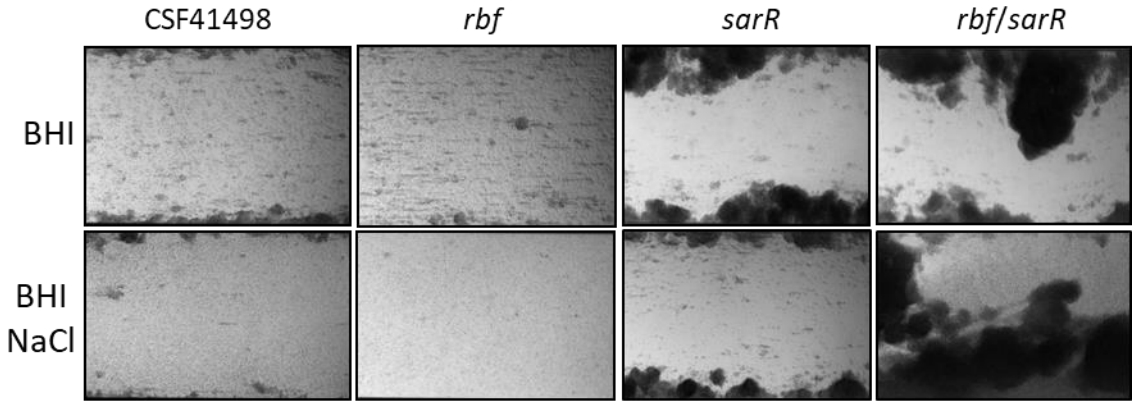
Taken together these data reveal that Rbf regulates *icaADBC* expression and biofilm, at least in part by controlling *sarR* expression. The negative impact of the *rbf* mutation *S. epidermidis* biofilm production was reversed by a second mutation in *sarR* via a mechanism that was highly dependent on growth conditions (i.e. osmotic stress and shear flow). The complex relationship between the PIA and biofilm phenotypes expressed by the *rbf*/*sarR* double mutant indicate that SarR and Rbf may also regulate both PIA production at the post-transcriptional level and/or *icaADBC*-independent biofilm factors. In this context it is noteworthy that SarR regulates expression of the two major virulence and biofilm regulators in *S. aureus,* namely *sarA* and *agr*[229], [230], which influence PIA-independent and PIA-dependent biofilm mechanisms[248], [263], [264]. Furthermore in *S. aureus* Rbf regulates >50 genes including the teichoic acid biosynthetic gene, *tagB*, and *lrgAB* and *lytS* (14), which can impact biofilm formation by influencing surface charge and cell lysis-mediated release of extracellular DNA, respectively[265], [266].

As noted above, in addition to SarA[248] and SarX[234], SarR is the third member of the Sar family known to bind the *ica* promoter. Manna and Cheung previously reported that in *S. aureus*, SarR and SarA bind to a similar target DNA sequence within the *agr* promoter, in

A


CSF41498
rbf
sarR
rbf/sarR
1:10
1:100
1:1000
BHI
1:10
1:100
1:1000
BHI
NaCl

B


CSF41498
rbf
sarR
rbf/sarR
BHI
BHI
NaCl

C

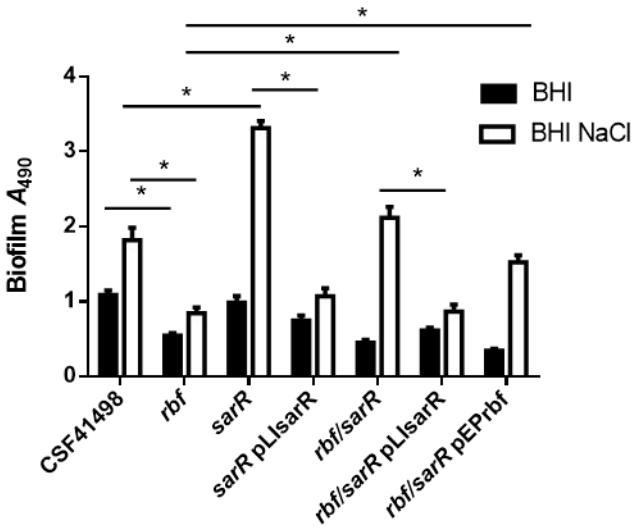

Biofilm A_{490}
0
1
2
3
4
BHI
BHI NaCl
CSF41498
rbf
sarR
sarR pLIsarR
rbf/sarR
rbf/sarR pLIsarR
rbf/sarR pEPrbf

Figure 2.4. SarR-regulated PIA production and biofilm development in *S. epidermidis*. A. Comparative immunoblot analysis of PIA production in whole cell extracts of CSF41498 and its isogenic *rbf*, *sarR* and *rbf/sarR* mutants grown overnight at 37°C in BHI and BHI NaCl. Cell extracts were diluted 1:10, 1:100 and 1:1000. The blot shown is representative of three independent experiments. **B.** CSF41498 and its isogenic *rbf*, *sarR* and *rbf/sarR* mutants grown in BHI and BHI NaCl in flow cells at 0.4 dynes/cm^2 shear using a Bioflux 1000Z instrument. Brightfield images depicting biofilm accumulation after 18h were captured at 10× magnification and are representative of three independent experiments. **C.** Comparative biofilm phenotypes of CSF41498 and its isogenic *rbf*, *sarR* and *rbf/sarR* mutants complemented where indicated with pLIsarR or pEPrbf. Semiquantitative measurements of biofilm formation under static conditions were performed in tissue culture-treated 96-well plates. All strains were grown at 37°C in BHI and BHI NaCl for 24 h. Experiments were repeated at least five times and average results are presented. Standard error of the mean is indicated and * denotes a significant difference as determined by Student's t-test ($P<0.05$).

order to activate transcription[230]. Because both SarR and SarA bind to the *sarA* promoter to repress *sarA* transcription[230], [267], it is possible that SarR and SarA may bind to the same or overlapping target DNA sequences within the *ica* promoter, to negatively or positively regulate transcription, respectively. Indeed the possibility that SarA and SarR can heterodimerise to regulate target gene expression has been proposed[225], [230], which contributes to the complex regulation of the *agr* locus and may also impact *icaADBC* expression. Our data also show that SarR primarily impacts the *S. epidermidis* biofilm phenotype in media supplemented with NaCl, which is known to repress transcription of the *icaR* repressor[181], [182]. This observation suggests that IcaR is preeminent in the hierarchy of *ica* operon regulators and that the major role of SarR and by extension Rbf is to repress and fine-tine *icaADBC* transcription primarily when IcaR levels are low. Interestingly, to date SarR has not been implicated in PIA-mediated biofilm formation in *S. aureus*, although mutation of *sarR* was shown to increase autolytic activity[268], which may impact biofilm by increasing the release of extracellular DNA[265], [269]. However as observed here, it remains possible that SarR also regulates the *ica* operon in *S. aureus* under conditions when IcaR expression is reduced.

2.3.6 Rbf negatively regulates *sarX* expression in *S. epidermidis*. In *S. aureus*, Rbf activates expression of *sarX* and Rbf-mediated regulation of the *ica* operon is dependent on SarX[249]. Recombinant SarX binds to *ica* operon regulatory sequences in both *S. aureus*

and S. epidermidis[234], [249]. LightCycler RT-PCR to measure the effect of the Rbf and SarR mutations on *sarX* expression, revealed that opposite to *S. aureus*, mutation of *rbf* was associated with

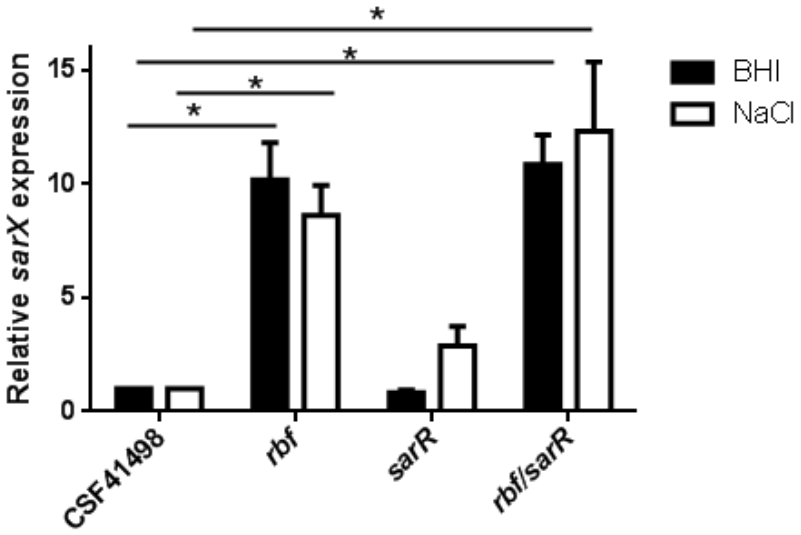


Figure 2.5. Rbf negatively regulates *sarX* transcription in *S. epidermidis*. Comparative measurement of *sarX* and *gyrB* (control) transcription in *S. epidermidis* strains CSF41498, *rbf, sarR* and *rbf/sarR*. RNA was prepared from cultures grown to A_{600} = 1.0 at 37°C in BHI and BHI NaCl. RelQuant software (Roche, Switzerland) was used to measure the relative expression of *sarX* against the constitutively expressed *gyrB* gene. *sarX* transcript levels in all strains were compared to *sarX* transcript levels in CSF41498 which was assigned a value of 1. The data presented are the average of at least three separate experiments. Standard deviations are indicated and * denotes a significant difference as determined by Student's t-test ($P \leq 0.05$).

increased *sarX* expression in both BHI and BHI NaCl (Fig. 2.5). These results were somewhat unexpected because SarX activates the *ica* operon in both *S. aureus* and *S. epidermidis*. Expression of *sarX* was also increased in the *sarR/rbf* mutant (Fig. 2.5). Increased biofilm formation by the *sarR* and *sarR/rbf* mutants can be attributed to de-repression of the *ica* operon in the absence of the SarR repressor. Mutation of *sarR* had no effect on *sarX* expression in BHI media but was associated with a 2.8-fold increase in BHI NaCl media, which was not statistically significant (p=0.8) (Fig. 2.5).

In summary, we propose an updated model for the regulation of *icaADBC* expression in *S. epidermidis* (Fig. 2.6) in which Rbf promotes biofilm formation by directly regulating expression of *sarR*. SarR and SarX exert opposite effects on *icaADBC* expression, with SarR acting as a repressor and SarX as an activator. Given that the dominant effect of an Rbf mutation is to reduce *icaADBC* expression, PIA production and biofilm (Fig. 2.1), it would appear that Rbf-mediated down-regulation of the SarR repressor is dominant over Rbf-mediated repression of the SarX activator. In the hierarchy of biofilm regulators, our data suggest that the Rbf-SarR-SarX axis appears to be subservient to IcaR, as evidenced under osmotic stress induced by NaCl, which is associated with repression of *icaR* transcription and activation of PIA production.

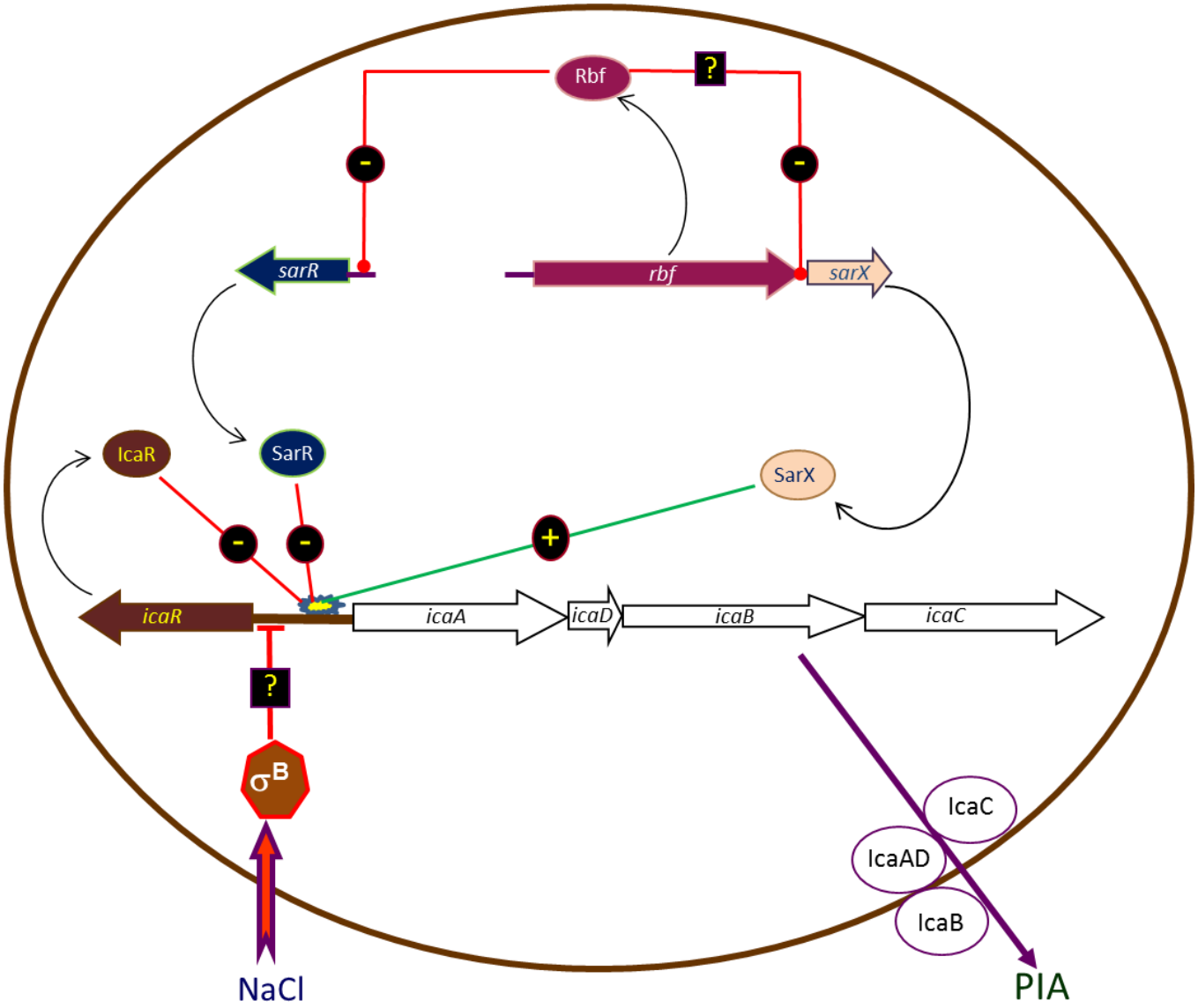


Figure 2.6. Model for *S. epidermidis icaADBC* regulation by Rbf, SarX and SarR. Rbf indirectly regulates *ica* operon expression by binding to the *sarR* promoter and negatively regulating *sarR* transcription. SarR binds to the *ica* operon promoter to repress *icaADBC* transcription. SarX acts independently of both SarR and Rbf, binding to the *ica* operon promoter to increase *icaADBC* transcription[234]. Under osmotic stress in media supplemented with NaCl, SigB-dependent repression of *icaR*[181], [182] and the concomitant activation of the *ica* operon is dominant over Rbf, SarX and SarR.

2.4 Acknowledgements

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Chapter 3

The TCA cycle enzyme succinyl-CoA synthetase influences resistance to β-lactam antibiotics in MRSA

Christopher Campbell, Laura A. Gallagher and James P. O'Gara*

Department of Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland.

*Corresponding author; jamesp.ogara@nuigalway.ie

Keywords: TCA cycle; MRSA, β-lactam resistance, succinyl-CoA synthetase

Running title: Mutations in *sucC* and *sucD* render MRSA susceptible to β-lactam antibiotics

3.1 Abstract

Expression of high-level methicillin resistance in *Staphylococcus aureus* is dependent on both the expression of the *mecA*-encoded penicillin-binding protein 2a (PBP2a), and accessory mutation(s). Here we report that mutations in the *sucC* or *sucD* genes, which encode the α and β-subunits of succinyl-CoA synthetase, respectively, were accompanied by β-lactam susceptibility in the methicillin-resistant *S. aureus* (MRSA) strain, JE2. Succinyl-CoA synthetase converts succinyl-CoA to succinate in the tricarboxylic acid (TCA) cycle, with the production of GTP via substrate level phosphorylation. The *sucC* and *sucD* mutants, as well as a number of other TCA cycle mutants, also exhibited defective growth, but only the *sucCD* mutations were associated with increased susceptibility to β-lactam antibiotics. Compensatory mutations in the ppGpp-synthase genes *relA* and *relQ* restored high-level β-lactam resistance in the *sucC* mutant indicating that β-lactam susceptibility was not associated with reduced SucCD-mediated GTP production, which is a substrate for RelA and RelQ-catalysed ppGpp production. Supplementation of the growth media with glucose, which should repress TCA cycle activity thereby abrogating the impact of *sucC* or *sucD* mutations, restored wild type levels of β-lactam resistance. Mutation of *sucA* restores wild type β-lactam resistance levels and colony morphology in a *sucC* mutant. The data presented suggest that accumulation of the TCA cycle intermediate, succinyl-CoA, may play a significant role in influencing the expression of β-lactam resistance, presenting a novel therapeutic target.

3.2 Introduction

Staphylococcus aureus is an important human pathogen, both in clinical settings and in the wider community[270] causing diseases ranging from relatively benign skin and soft tissue infections and abscesses, to more life-threatening conditions such as endocarditis and sepsis[9], [271]. A multitude of virulence factors, including immune-evasins, proteolytic enzymes and toxins allow the pathogen to gain a foothold in the host and cause serious disease[272]–[274]. Humans are constantly exposed to *S. aureus*, which is a commensal of the anterior nares in a third of the population, and a transient coloniser of 50% of the individuals[2]. Considered an opportunistic pathogen, *S. aureus* and methicillin-resistance *S. aureus* (MRSA) infections were traditionally healthcare-associated, related to the insertion of indwelling-medical devices[31], [275], [276]. In recent decades, community-acquired MRSA (CA-MRSA) has emerged, causing infections in otherwise healthy individuals[277]. Although CA-MRSA strains typically express lower levels of antimicrobial resistance, they are generally more virulent than their hospital-acquired counterparts[19], [20].

β-lactam resistance in *S. aureus* is typically expressed heterogeneously within a given population[278]. The majority of cells within a heterogeneous population exhibit susceptible or borderline susceptible resistance to β-lactams. A sub-population of approximately 0.1% can survive antibiotic treatment and upon re-exposure to the antibiotic a homogeneously resistant population emerges[278]. The mechanisms underpinning this switch from heterogeneous resistance (HeR) to homogenous resistance (HoR) are associated with accessory mutations outside *mecA*, the gene encoding the low-affinity alternative penicillin-binding protein (PBP2a)[64], [65]. Recent work has implicated several genes within the purine biosynthetic and stringent response pathways that contribute to high-level, homogeneous β-lactam resistance[114], [121], [135], [144]. The TCA cycle has also been implicated in the HoR phenotype. Rosato *et al.* reported that radical oxygen species (ROS) production, which is a by-product of TCA cycle activity, was essential in the emergence of HoR derivatives of MRSA strain SA13001[279]. However in other studies, TCA cycle-dependent ROS were implicated in susceptibility to killing by antimicrobial agents[80], [83]. One possible explanation for this apparently contradictory data is that the potentially beneficial effect of TCA-cycle derived ROS in increasing the mutation rate at accessory loci

required for production of HoR strains, needs to be balanced by the overall capacity of the cell to cope with ROS-induced stress[279].

To identify novel loci contributing to β-lactam resistance, the Nebraska Transposon Mutant Library (NTML)[280] was screened for mutants expressing altered cefoxitin resistance. Mutations in the succinyl-CoA synthetase genes, *sucC* and *sucD*, were accompanied by increased sensitivity to β-lactams. The impact of these mutations on growth and stationary phase survival was measured and compared to the impact of mutations in other TCA cycle genes. Our data suggest that among TCA cycle enzymes, succinyl-CoA synthetase is specifically required for expression of β-lactam resistance in MRSA and may represent a new therapeutic target in efforts to overcome antibiotic resistance in MRSA.

3.3 Materials and Methods

3.3.1 Bacterial strains, growth conditions and antimicrobial susceptibility testing. Bacterial strains used in this study (Table 3.1), were routinely cultured in Brain-Heart Infusion (BHI) media (Oxoid), Muller-Hinton (MH) media (Oxoid) or Luria Bertoni (LB) media (Oxoid). Where indicated erythromycin (Erm) 10µg/ml, chloramphenicol (Cm) 10µg/ml, ampicillin (Amp) 50µg/ml or kanamycin (Kan) 75µg/ml were used for antibiotic selection (all Sigma). Routine growth performed at 37ºC shaking (200rpm).

Oxacillin minimum inhibitory concentrations (MICs) were performed using oxacillin E-test strips (Oxoid), or using agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines on MH 2% NaCl agar[281]. The MSSA strain ATCC 25923 was routinely used as a control.

3.3.2 Screening of the Nebraska Transposon Mutant Library (NTML). A screen of the Nebraska Transposon Mutant Library (NTML) was carried out to identify mutants exhibiting altered susceptibility to cefoxitin (Oxoid) using the disk-diffusion method in accordance with CLSI guidelines [281]. Briefly, each library mutant was revived from a freezer stock onto BHI agar supplemented with erythromycin and grown for 18hrs at 37ºC. The following day 3-5 individual colonies were used to prepare a bacterial suspension in 0.85% saline which was standardised to 0.5 McFarland. A swab was immersed in the bacterial suspension and used to evenly inoculate the surface of MH plates before the application of a cefoxitin-impregnated disk (30µg). The plates were incubated for 24hrs at 35ºC. The zone of inhibition diameter for JE2 was 10mm, whereas the *mecA* mutant from the NTML and MSSA strain ATCC 25923 exhibited zone diameters >35mm. The entire NTML was screened in duplicate, and candidate mutants with altered cefoxitin susceptibility repeated a minimum of 5 times. Oxacillin MICs were measured using oxacillin E-tests and agar dilution assays. PCR was used to verify the transposon insertion within the gene of interest (Table 3.2). Whole genome sequencing (Microbes NG, UK) was used to ensure that there were no additional mutations contributing to the antibiotic susceptibility phenotype. Briefly, following quantification of purified gDNA, the sequencing library was prepared using Nextera XT Library Prep Kit (Illumina, San Diego) followed by sequencing on an Illumina HiSeq.

Table 3.1. **Strains and plasmids used in this study with relevant details.**

Strain/Plasmid	Relevant details
JE2	Derivative of USA300 LAC cured of plasmids p01 and p03. Parent strain of the Nebraska Transposon Mutant Library (NTML).
NE569	JE2 *sucC*::Tn. Erm^r
NE1770	JE2 *sucD*::Tn. Erm^r
NE1724	JE2 *pdhA*::Tn. Erm^r
NE1758	JE2 *pdhB*::Tn. Erm^r
NE594	JE2 *gltA*::Tn. Erm^r
NE861	JE2 *acn*::Tn. Erm^r
NE491	JE2 *icd*::Tn. Erm^r
NE547	JE2 *sucA*::Tn. Erm^r
NE1391	JE2 *sucB*::Tn. Erm^r
NE626	JE2 *sdhA*::Tn. Erm^r
NE808	JE2 *sdhB*::Tn. Erm^r
NE427	JE2 *fumC*::Tn. Erm^r
NE1003	JE2 *mqo* E1::Tn. Erm^r
NE1381	JE2 *mqo* E2::Tn. Erm^r
NE1714	JE2 *relA*::Tn. Erm^r
8325-4	MSSA. SigB-deficient, PIA biofilm positive.
RN4220	Restriction-deficient *S. aureus*.
BH1CC	MRSA clinical isolate.
E. coli	*E. coli* HST08.
MW2	CA-MRSA USA400-derivative.
ATCC 29213	MSSA strain for MIC susceptibility testing.
ATCC 25923	MSSA strain for disk diffusion susceptibility testing.
Plasmids	
pLI50	*E. coil-Staphylococcus* shuttle vector. Amp^r (*E. coli*), Cm^r (*Staphylococcus*).
p*sucC*	pLI50 carrying *sucC* from JE2.
p*sucCD*	pLI50 carrying the *sucCD* genes from JE2.
p*sucD*	p*sucCD* with a 627 bp deletion at the 5’ end of the *sucC* gene.

3.3.3 Genetic techniques. Genomic and plasmid extractions were carried out using Wizard genomic DNA purification (Promega) and plasmid extraction miniprep kits (Sigma). *S. aureus* DNA extraction was preceded by pre-treatment with 10µg/ml lysostaphin (Ambi Products, New York. All restriction enzymes were supplied by Roche, and used as per manufactures guidelines.

Table 3.2. Oligonucleotides used in this study.

Target gene	Primer name	Primer sequence (5'-3')
sucC	sucC_F	TACTCAAATCGCCATGCAGC
	sucC_R	AATGACTGAAACCGTTGCCC
sucCD	sucCD_R	CGCACGACAAATAGCCCATT
mecA	mecA_F	CATATCGTGAGCAATGAACTGA
	mecA_R	CATCGTTACGGATTGCTTCA
relA	relA_F	TGGCTTTGCACCTGTTAGAA
	relA_R	TTTTGCCGTCCTGACTTTCA
sucA	sucA_F	GGCGGTAATGGACTCGGATT
	sucA_R	TCTACGCTATCCCCTACGTT
sdhA	sdhA_F	TGGGGTGGACTTCAATCTCC
	sdhA_R	TGTGTTTCATGTTGTGGAGTGT
Infusion primers		
sucC	INF_sucC_F	TCGTCTTCAAGAATTGACGCTTGATAATGCACTG
	INF_sucC_R	TACCGAGCTCGAATTCCTTTACCAGGCGTCACA
sucCD	INF_sucCD_F	TCGTCTTCAAGAATTTACTCAAATCGCCATGCAGC
	INF_sucCD_R	TACCGAGCTCGAATTCGCACGACAAATAGCCCATT
RT-PCR primers		
mecA	mecA1_Fwd	TGCTCAATATAAAATTAAAACAAACTACGGTAAC
	mecA1_Rev	GAATAATGACGCTATGATCCCAA
gyrB	gyrB_Fwd	CCAGGTAAATTAGCCGATTGC
	gyrB_Rev	AAATCGCCTGCGTTCTAGAG

A 1,680bp fragment encompassing the *sucC* gene and a 2,610bp fragment including both *sucC* and *sucD* were PCR amplified from JE2 genomic DNA using primers INF_sucC_F / INF_sucC_R and INF_sucCD_F / INF_sucCD_R, respectively (Table 3.2) and cloned into the *E. coli-Staphylococcus* shuttle vector, pLI50, using the Clontech Infusion Cloning kit 2. The p*sucCD* plasmid was digested with *HpaI* and *SpeI*, treated with T4 DNA polymerase (Roche) and dNTPs to create a blunted ended fragment that was religated using T4 ligase (Roche). The resulting plasmid, designated p*sucD,* contained a 627bp deletion at the 5' end of the *sucC* gene with only the *sucD* gene remaining intact. Recombinant plasmids were first

transformed into cold-competent *E. coli* HST08 (supplied with Clontech Infusion Cloning kit) before being transformed by electroporation into the restriction-deficient *S. aureus* strain RN4220 and finally into NE569 and NE1770.

A *sucC*/*relA* double mutant was generated by first swapping the erythromycin resistance cassette in NE569, via allelic exchange, using the tool kit and method described by Bose *et al*.[282]. Phage transduction was used to move the *sucC*::Kan transposon into the *relA* library mutant, NE1714. PCR was used to confirm both the allelic exchange and transduction.

3.3.4 Phage Transductions. Phage 80α was used to transduce the transposon insertion from the NTML *sucC* mutant in a fresh JE2 and MW2 background, to ensure background mutations were not responsible for the antibiotic resistance phenotype. Briefly, phage 80α was propagated in soft agar with NE569 or NE1770 as required, to a titre of 10^8 plaque-forming units (PFU). Following transduction, candidates were selected for on sodium citrate TSA supplemented with erythromycin 10µg/ml or kanamycin 75µg/ml, as required. Transposon insertions were verified using PCR amplification of target loci.

3.3.5 RNA purification and real time RT-PCR. Cultures were grown in BHI media to mid-exponential phase. Harvested cells were pelleted and immediately stored at -20ºC in RNAlater (Ambion) to ensure maintenance of RNA integrity prior to purification. RNA was extracted as per manufacturers guidelines using RNA Mini-Extraction kit (Sigma). RNA integrity was examined visually by agarose gel electrophoresis and RNA concentration was determined using a Quibit Fluorometer 4 (Quigin). Quantitative reverse transcription PCR (RT-qPCR) was used to measure *mecA* transcription on the Roche LightCycler 480 instrument using the LightCycler 480 Sybr Green Kit (Roche) with primers mecA1_Fwd and mecA1_Rev (Table 3.2). The following cycling conditions were used: 95ºC for 5 minutes and followed by 45 cycles of 95ºC for 10 seconds, 58ºC for 20 seconds and 72ºC for 20 seconds. Melt curve analysis was performed at 95ºC for 5 seconds followed by 65ºC for one minute up to 97ºC at a ramp rate of 0.11c/sec with five readings taken for every degree of temperature increase. The *gyrB* gene amplified with primers gyrB_Fwd and gyrB_Rev (Table 3.2) served as an internal standard for all reactions. For each reaction, the ratio of *mecA* and *gyrB* transcript number was calculated as follows: $2^{(Ct\ gyrB\ -\ Ct\ mecA)}$. Each RT-qPCR experiment was performed three times and presented as average data with standard errors.

3.3.6 Growth assays and stationary phase survival assays. For growth assays, cultures were grown in 50ml of BHI or MH broth in a 250ml flask, with a starting A_{600}=0.01. Culture aliquots were collected at 30min - 1 hour intervals and cell density measured at A_{600}.

For stationary phase survival assays, stationary phase cultures grown for 24 h in 50ml flasks were incubated at 37ºC for 6 days. Viability was measured by enumerating the number of CFUs in culture aliquots collected every 24 hours. The growth and stationary phase survival experiments were repeated at least 3 times and standard error of the mean calculated.

3.3.7 Biofilm assays. Semi-quantitative biofilm assays were performed in static conditions using Nunc Hydrophilic 96 well tissue-culture treated polystyrene plates (Nunc, Denmark) as previously described[181]. All biofilms assays were repeated a minimum of 3 times and average data are presented including the standard error of the mean.

3.3.8 Population analysis profile. Characterisation of population resistance profiles was carried out as previously described[278]. Briefly, overnight cultures were standardised to an A_{600}=1, before being serially diluted and the number of CFUs enumerated on BHI agar plates supplemented with a range of oxacillin concentrations, from 0.25µg/ml to 100µg/ml. Population analysis profiles (PAPs) were generated from the average data of at least three independent experiments and the standard error of the mean calculated**.**

3.3.9 Isolation of homogeneously resistant (HoR) mutants. Overnight MRSA cultures were adjusted to A_{600}=1, serially diluted and plated onto both BHI and BHI agar supplemented with oxacillin 100µg/ml to isolate homogeneously resistant (HoR) mutants. The cultures were also plated onto BHI agar lacking antibiotic to enumerate the number of viable CFUs and to calculate the rate of HoR mutant production. The experiments were performed twice with standard error of the mean shown.

3.4 Results

3.4.1 Mutation of the succinyl-CoA synthetase genes (*sucC* or *sucD*) in MRSA increases susceptibility to β-lactam antibiotics. A screen of the NTML was used to identify novel genetic loci that contribute to β-lactam resistance in MRSA. In this screen mutants exhibiting altered susceptibility to cefoxitin were identified using a cefoxitin disk diffusion assay. Antibiotic susceptibility was measured a minimum of 5 times to verify candidate mutants exhibiting an altered resistance phenotype. NTML mutants NE569 and NE1770 exhibited increased susceptibility to cefoxitin (Fig. 3.1A). The cefoxitin zone diameter for JE2 was 11mm compared to 24mm for the *sucC* mutant (Fig. 3.1A). Using E-test strips, the oxacillin MICs of the *sucC* and *sucD* mutants were 0.5µg/ml, compared to 32µg/ml for JE2 (Fig. 3.1B). NE569 and NE1770 contain transposon insertions in the β and α-subunits of succinyl-CoA synthetase, respectively. The *sucC* and *sucD* genes are separated by only 21bp suggesting that they are organised in an operon (Fig. 3.1C) and that the α and β subunits of succinyl-CoA synthetase are co-expressed. CLSI interpretive guidelines[281] indicate that strains exhibiting cefoxitin zone diameters >22mm can be considered methicillin-susceptible.

Reassuringly, NE1714 (*relA*) and NE1447 (*ispA*) were among the candidate mutants exhibiting altered susceptibility to cefoxitin. The RelA-mediated stringent response has previously been implicated in methicillin resistance and several recent studies have implicated activation of the stringent response and constitutive (p)ppGpp production in homogeneous methicillin resistance[79], [113], [132]. NE1714 exhibited increased resistance to cefoxitin (data not shown), suggesting that the transposon insertion increased (p)ppGpp synthase activity in this mutant. The increased susceptibility of the NE1447 *ispA* mutant to cefoxitin (data not shown) was consistent with a recent study demonstrating that increased susceptibility to cell wall active antibiotics was among the pleiotrophic effects of disrupted IspA-dependent prenylation[283].

NE569 also exhibited increased susceptibility to cloxacillin and imipenem in disk diffusion assays (data not shown). However, the zone diameters for cloxacillin and imipenem did not cross CLSI susceptibility breakpoints, with both the parent JE2 and the *sucC* mutant exhibiting resistance to cloxacillin (<28mm) and susceptibility to imipenem (≥29mm) (data not shown). The impact of the *sucC* mutation on antibiotic susceptibility appears to be confined to β-lactam antibiotics. Using broth microdilution assays, NE569 did not exhibit

altered susceptibility to the DNA gyrase inhibitor, ciprofloxacin, or the folate biosynthesis inhibitor combination, trimethoprim/sulfamethoxazole (Supp. Table 6.1).

Comparison of the NE569 genome with the parent strain JE2 confirmed the absence of additional mutations that might contribute to increased β-lactam susceptibility. SNP calling was performed on the trimmed reads by Breseq using JE2 reference genome (NCBI RefSeq: NZ_CP020619.1). A 69bp duplication at position 2,699,017 and an A to T substitution at position 1,844,130 were identified in NE569. However, both genetic changes occur in intergenic regions and are also present in other mutants from the NTML (unpublished data) suggesting that they arose during construction of the library. PCR amplification and Sanger sequencing revealed that the *mecA* locus was intact in the *sucC* and *sucD* mutants (data not shown). Phage 80α-mediated backcross of the *sucC* and *sucD* transposon mutations into the parent strain JE2 and a second CA-MRSA USA400 strain, MW2, was accompanied by the same increase in β-lactam susceptibility observed in NE569 and NE1770 (Supp. Table 6.2). Genetic complementation experiments were further used to confirm the role of *sucC* and *sucD* in cefoxitin resistance. Given that the transposon insertion in *sucC* is likely to have polar effects on expression of *sucD*, PCR fragments containing *sucC* alone and both *sucCD* and their upstream regulator sequences was cloned into pLI50 to generate p*sucC* and p*sucCD.* To facilitate complementation with the *sucD* gene alone, a 627bp fragment at the 5' end of the *sucC* gene was deleted from the p*sucCD* plasmid to generate p*sucD* (Fig. 3.1C). Using cefoxitin disk diffusion assays and oxacillin E-test measurements, the *sucC* mutant phenotype was shown to be complemented by p*sucCD* but not p*sucC* or p*sucD* alone, whereas the *sucD* mutant was complemented partially by *psucC* and fully by *psucCD* (Table 3.3). Wild type levels of resistance to cefoxitin and oxacillin are only restored when both subunits are expressed (Table 3.3). These data further suggest that *sucCD* are organised as an operon under the control of a single promoter and that both the α and β subunits of succinyl-CoA synthetase are required for expression of wild type levels of β-lactam resistance in MRSA. Lightcycler RT-qPCR analysis revealed that expression of *mecA* was not significantly affected in NE569 compared to JE2 in BHI media or in BHI media supplemented with oxacillin 0.5μg/ml (Fig. 3.1D).

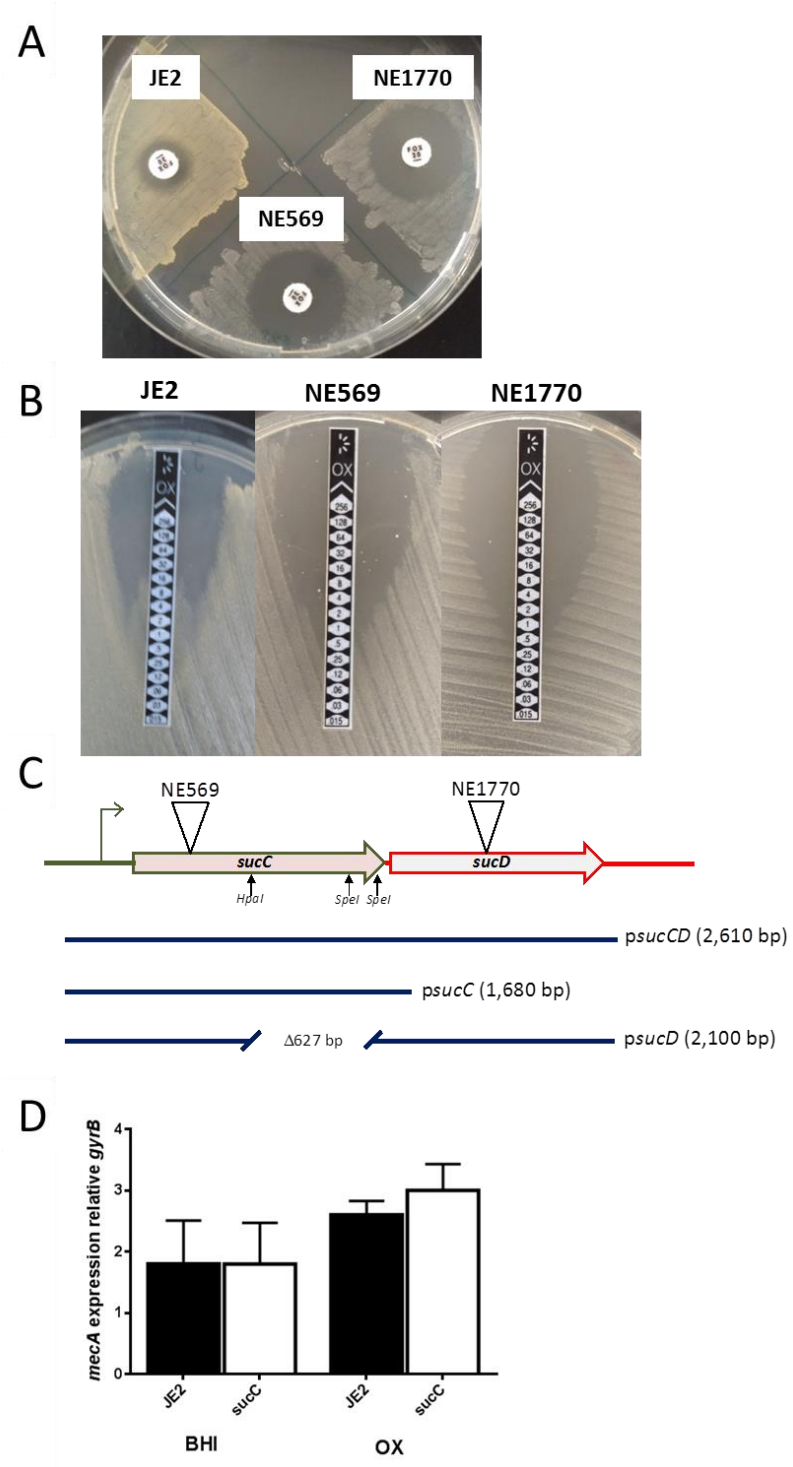

Figure 3.1. Mutation of *sucC* or *sucD* increases β-lactam susceptibility in MRSA. A. Susceptibility of JE2, NE569 (*sucC)* and NE1770 (*sucD)* strains grown on MH agar to cefoxitin (FOX, 30µg disks). **B.** E-test measurement of oxacillin minimum inhibitory concentrations (MICs; µg/ml) in JE2, NE569 and NE1770. **C.** Structure of the *sucCD* locus including location of transposon insertions in NE569 (*sucC*) and NE1770 (*sucD*). The parts of the *sucCD* operon carried on the complementation plasmids p*sucCD, psucC* and p*sucD* are indicated. **D.** Comparison of *mecA* transcription by LightCycler RT-qPCR in JE2 and NE569 (*sucC*) grown to exponential phase in BHI or BHI supplemented with 0.5µg/ml oxacillin. Experiments were repeated at least three times and standard error of the mean shown.

Table 3.3. Summary of cefoxitin disk diffusion assay (mm) and oxacillin E-test MIC (μg/ml) results for JE2, NE569 (*sucC*), NE1774 (*sucD*) and mutants complemented with *sucC* or *sucD*.

Strain	FOX (mm)*	Ox. MIC (μg/ml)**
JE2	11	32
NE569 (sucC)	24	0.5
NE569 psucC	22	0.5
NE569 psucD	25	1
NE569 psucCD	11	32
NE1770 (sucD)	26	0.5
NE1770 psucC	27	0.5
NE1770 psucD	12	16
NE1770 psucCD	11	32

* Cefoxitin (FOX) susceptibility was determined by disk diffusion assays on MH agar and expressed as zone diameters (mm) around FOX (30 μg/ml) disks.
** Oxacillin (Ox) minimum inhibitory concentration (μg/ml) was determined by agar dilution assay.

3.4.2 Succinyl-CoA synthetase mutations impair growth. The succinyl-CoA synthetase mutants grew as smaller, less pigmented colonies on Muller-Hinton (MH) agar (Fig. 3.2A). Consistent with the small colony phenotype, comparison of NE569 (*sucC*), NE1770 (*sucD*) and JE2 growth in liquid culture using both viability (CFU) counts (Supp. Fig. 6.1) and cell density (absorbance) measurements revealed impaired growth in the *sucC* and *sucD* mutants (Fig. 3.2B). The specific growth rate of NE569 was 0.067 h-1 (s=0.004), compared to 1.405 h-1 (s=0.023) for JE2, and NE569 appears to enter stationary phase early, a phenotype previously reported for TCA cycle mutants by Somerville *et al*.[284]. The specific growth rate of the NE1770 *sucD* mutant, 0.0962 h-1, was similar to that of NE569. A number of previous studies have reported that survival in stationary phase is increased in TCA cycle mutants [284], [285]. Consistent with these previous findings, both NE569 (*sucC*) and NE1770 (*sucD*) exhibited increased survival in stationary phase (Fig. 3.2C). The growth and stationary phase survival phenotypes were restored to wild type levels in the complemented *sucC* mutant.

Taken together these data show that both NE569 and NE1770 exhibited the same growth defects and increased susceptibility to β-lactam antibiotics.

Previous studies have found that the genes encoding TCA cycle enzymes are upregulated in *S. aureus* biofilm[286], [287]. To investigate whether biofilm production was affected in NE569 biofilm assays were performed in BHI and BHI 4% NaCl. No significant difference in biofilm formation was detected (Supp. Fig. 6.2). Because JE2 is a relatively weak biofilm producing strain, the *sucC*::Tn allele from NE569 was transduced into the strong biofilm forming MSSA strain, 8325-4, using phage 80α. Similarly, the disruption of *sucC* in 8325-4 appeared to not have any impact on the biofilm formation (Supp. Fig. 6.2).

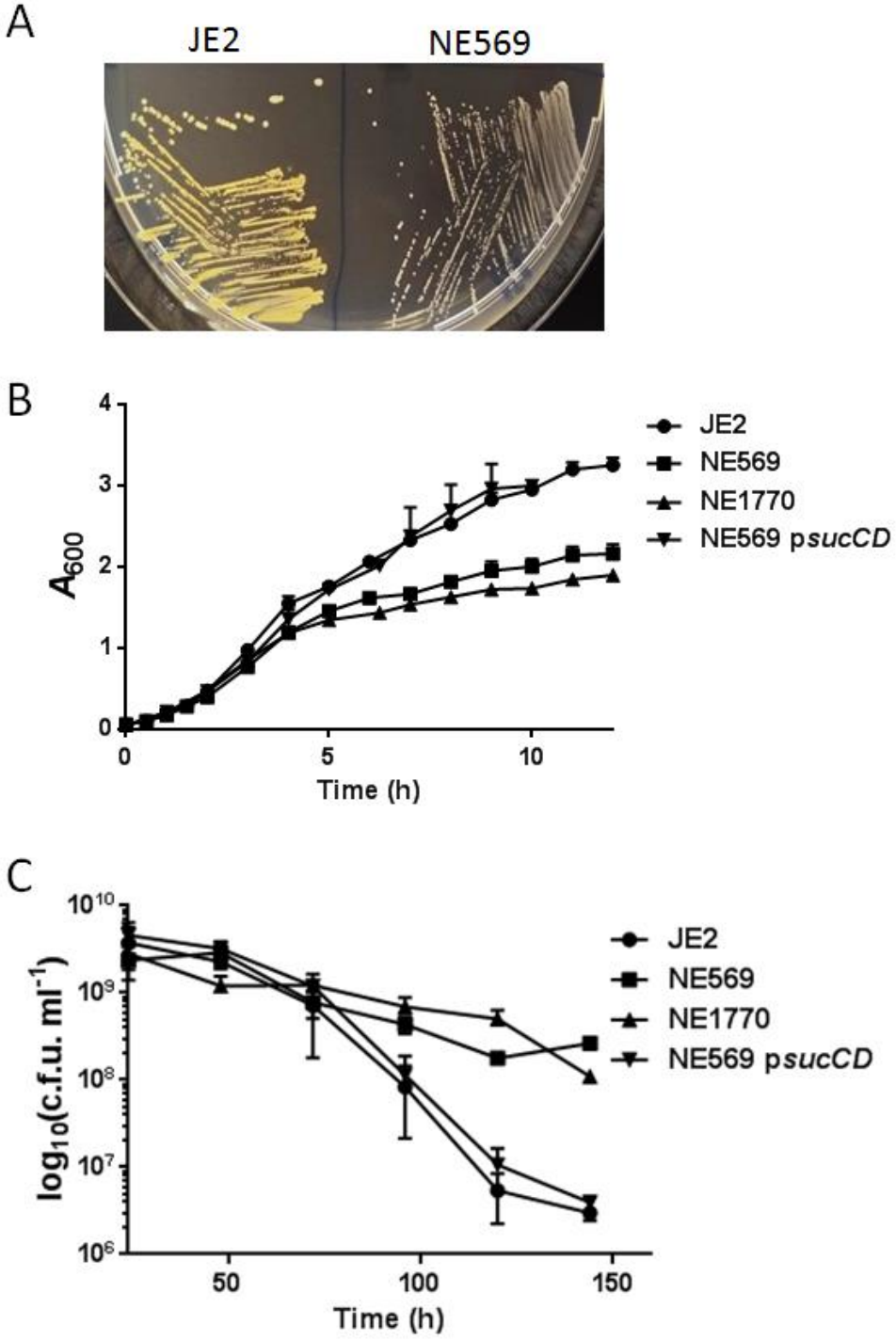

Figure 3.2. Growth of succinyl-CoA synthetase mutants is impaired. A. JE2 and NE569 colonies grown on MH agar. **B.** Growth of JE2, NE569 (*sucC*), NE569 p*sucCD* and NE1770 (*sucD*) in MH broth. Culture aliquots were collected every hour for 12 hours and cell density measured at A_{600} Growth experiments were repeated at least 3 times and standard error of the mean shown. **C.** Stationary phase survival of JE2, NE569 (*sucC*), NE569 p*sucCD* and NE1770 (*sucD*) cell suspensions over 6 days. All strains were grown to stationary phase for 24hrs and survival/viability measured by collecting culture aliquots every 24hr and enumerating colony forming units (CFUs). Stationary phase survival experiments were repeated three times and standard error of the mean is shown.

In *S. aureus*, when glucose levels are high the TCA cycle is repressed by the carbon-catabolite repressor, CcpA [66], [288]. Under CcpA repression glucose is metabolised via the glycolytic pathway to acetate, which is then excreted. To investigate the impact of glucose on *sucC*-associated changes in β-lactam resistance, cefoxitin susceptibility was measured in MH agar supplemented with increasing concentrations of glucose. Resistance of the *sucC* and *sucD* mutants to cefoxitin, as determined by disk diffusion assay, were significantly increased at glucose concentrations > 0.2%, from clinically susceptible to resistant, and fully restored to wild type levels in MH agar supplemented with 10% glucose (w/v) (Fig. 3.3A). Oxacillin susceptibility in both NE569 (*sucC*) and NE1770 (*sucD*), as determined agar dilution, was also increased from 0.25 μg/ml on MH agar to 32 μg/ml on MH agar supplemented with 5% glucose. The susceptibility of the parent strain JE2 and the complemented NE569 mutant to cefoxitin was unaffected by glucose (Fig. 3.3A). For control purposes, supplementation of MH agar with 5% glucose was shown to have no effect on oxacillin and cefoxitin susceptibility in the β-lactam susceptible *ispA* mutant, which like the *sucC* and *sucD* mutants is also susceptible to β-lactam antibiotics (data not shown). The growth defect of NE569 compared to JE2 grown in MH broth (Fig. 3.2B) was not evident in MH supplemented with 5% glucose (Fig. 3.3B). Collectively these data indicate that glucose-induced repression of the TCA cycle negates the impact of the *sucC* mutation on both growth and susceptibility to β-lactam antibiotics.

3.4.3 Mutations in other TCA cycle enzymes do not increase β-lactam susceptibility. In the NTML, there are TCA cycle mutants for all genes except for *sdhC*, which encodes succinate dehydrogenase cytochrome β-558 subunit. Apart from *sucC* and *sucD*, none of the other mutants exhibited increased susceptibility to cefoxitin or oxacillin (Table 3.4). The fumarate hydratase (*fumC*) mutant exhibited a modest increase in β-lactam resistance, but this is unlikely to be significant given that the oxacillin MIC of this mutant falls within the range that can be measured for JE2 (32-64μg/ml)[289], [290].

Growth measurements revealed that, like the *sucC* and *sucD* mutations (Fig. 2B), mutations in the oxoglutarate dehydrogenase *sucA* and *sucB* genes, and the succinate dehydrogenase *sdhA* and *sdhB* genes also impaired growth in MH media (Fig. 4.4), but as noted above, did not impact oxacillin susceptibility. Apparently impaired growth associated with TCA cycle

mutations is not necessarily associated with increased susceptibility to β-lactam antibiotics indicating that the SucCD succinyl CoA synthase plays a specific role in this phenotype.

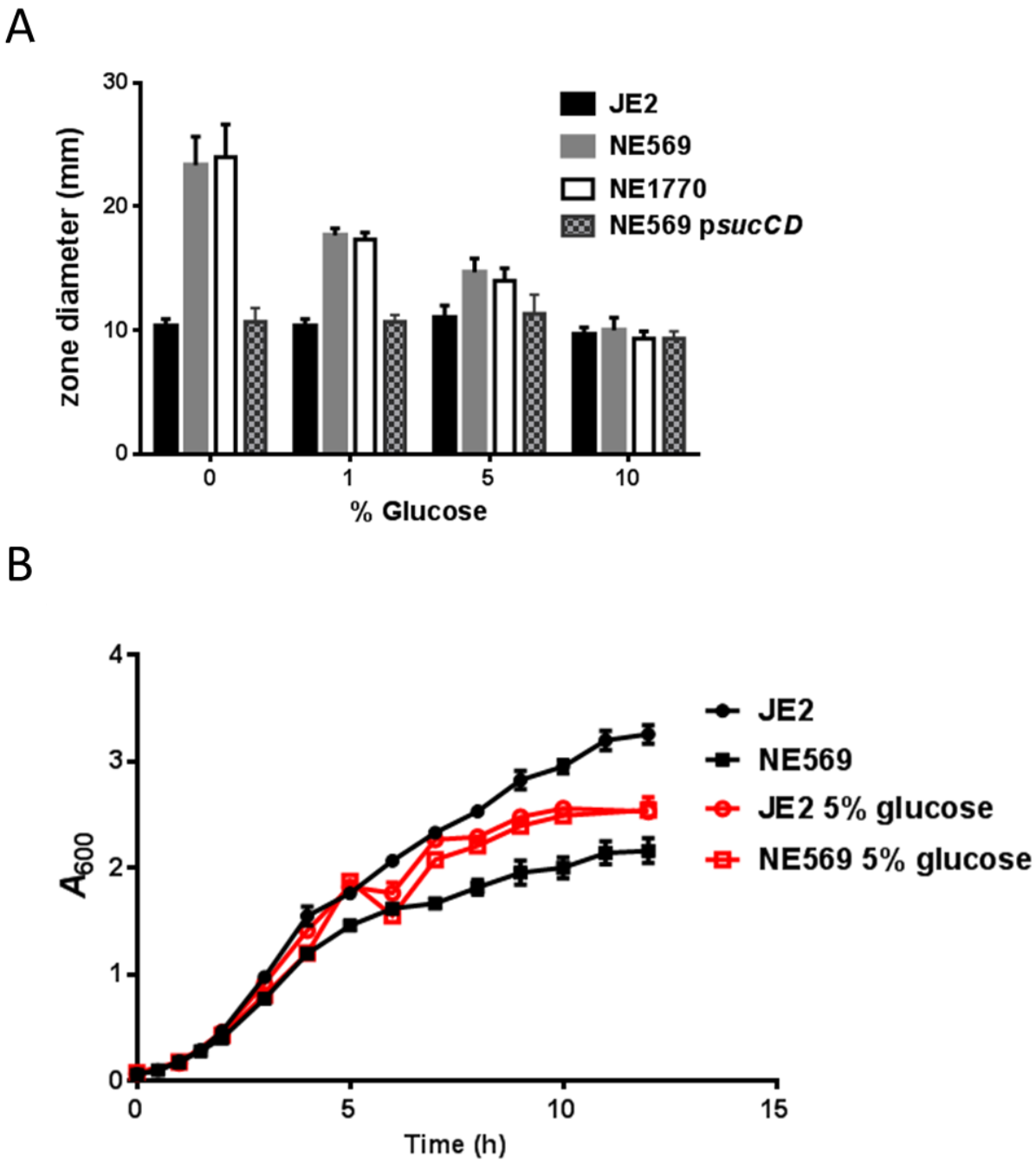

Figure 3.3. Supplementation of MH media with glucose negates the impact of the *sucC* mutation on β-lactam resistance and growth. A. Measurement of cefoxitin zone diameters on MH agar and MH agar supplemented with glucose (1, 5, 10% w/v). Data shown are the average of 3 independent experiments. Error bars reflect standard deviation. **B.** Growth of JE2 and NE569 (*sucC*) in MH broth or MH broth supplemented with 5% glucose. Cell density was measured at A_{600} and the experiments repeated three times with standard error of the mean shown.

Table 3.4. Susceptibility of JE2 and TCA cycle mutants to cefoxitin (FOX) and oxacillin (OX)

Strain	FOX (mm)*	Ox. MIC (µg/ml)**
JE2	11	32
NE569 (sucC)	24	0.25
NE1770 (sucD)	25	0.25
NE594 (gltA)	10	32
NE861 (acn)	11	32
NE491 (icd)	10	32
NE547 (sucA)	10	32
NE1391 (sucB)	10	32
sdhC	N/A	N/A
NE626 (sdhA)	10	32
NE808 (sdhB)	10	32
NE427 (fumC)	8	64
NE1003 (mqo E1)	10	32
NE1381 (mqo E2)	10	32

* Cefoxitin (FOX) susceptibility was determined by disk diffusion assays on MH agar and expressed as zone diameters (mm) around FOX (30 µg/ml) disks.
** Oxacillin (OX) minimum inhibitory concentration (µg/ml) was determined by agar dilution.

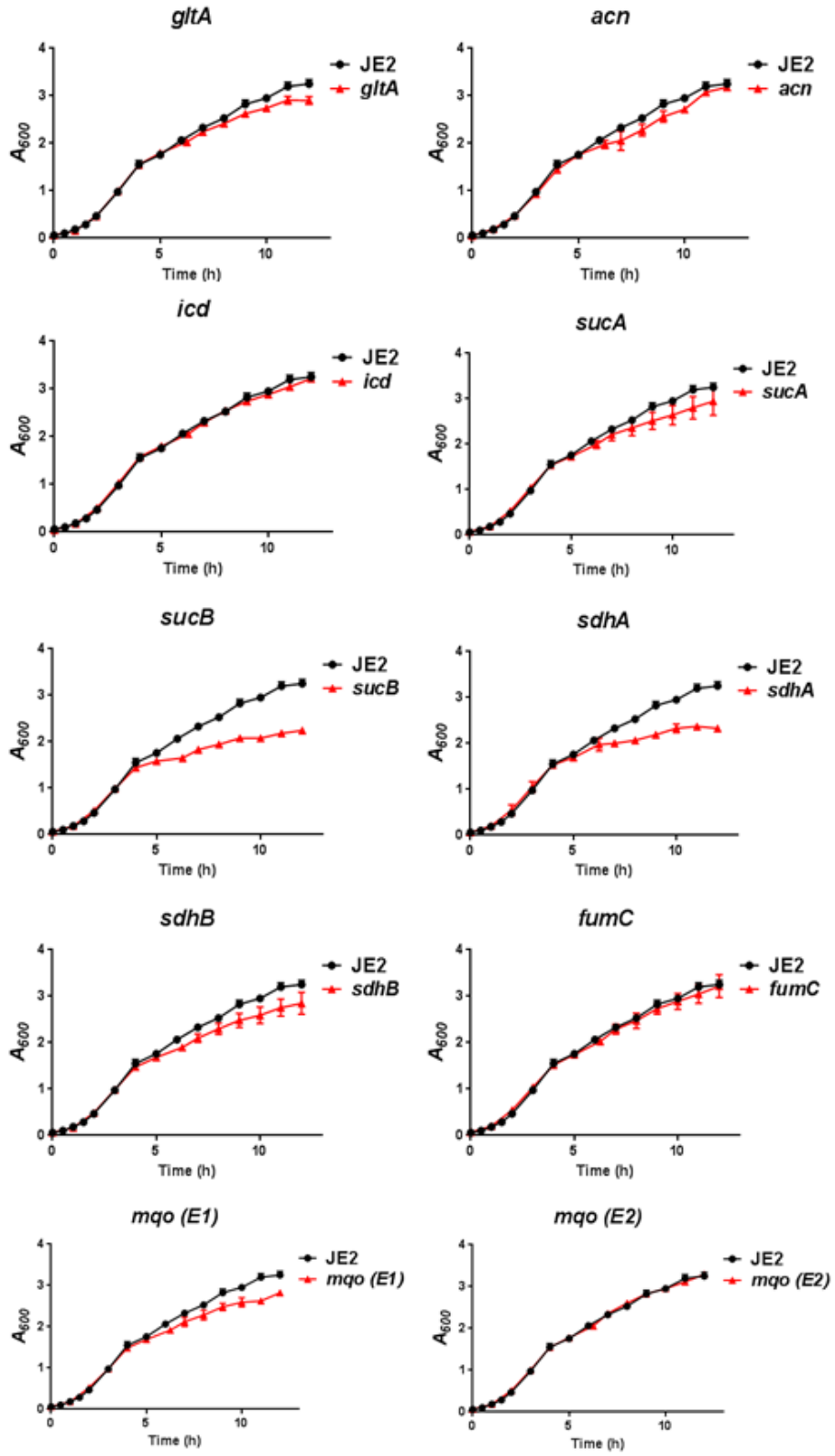

Figure 3.4. Growth of TCA cycle mutants in Muller-Hinton broth. JE2, *glt, acn, icd, fumC, sucA, sucB, sdhA, shdB, mqo(E1)* and *mqo(E2)* were grown in MH both at 37°C growth was measured by collecting culture aliquots every hour for 12 hours and measuring cell density at A_{600}. The experiments were repeated three times and standard error of the mean is shown.

3.4.4 Reduced β-lactam resistance in the *sucC* and *sucD* mutants is not related to an impaired stringent response. Expression of high-level, homogeneous β-lactam resistance is dependent on activation of the ppGpp-mediated stringent response[19], [111], [121], [291]. The SucCD complex produces GTP, via substrate-level phosphorylation, which is a substrate for the RelA, RelP and RelQ ppGpp synthases raising the possibility the NE569 (*sucC*) or NE1770 (*sucD*) mutations may negatively affect the activation of a normal stringent response required for β-lactam resistance. To investigate the possible relationship between SucCD and the stringent response, we measured the ability of the *sucC* and *sucD* mutants to express a HoR phenotype. First, we compared the rate at which HoR mutants were produced by JE2, *sucC*, *sucD* and other TCA cycle mutants by plating overnight cultures onto BHI agar and BHI supplemented with oxacillin 100μg/ml. The wild type and all TCA cycle mutants produced HoR mutants at similar rates (Fig. 3.5A). This observation contrasted with a previous study from Adriana Rosato's group with a different HeR MRSA strain, SA13011, showing that TCA cycle activity was required for HoR mutant production[279]. Our experimental approach differed in that we selected HoR strains on a high concentration of oxacillin (100μg/ml) whereas the Rosato study used a very low oxacillin concentration (0.5μg/ml). Further work is needed to determine if HoR mutants can be selected from strain SA13011 using our experimental approach.

HoR mutants were passaged for 10 days in BHI broth and oxacillin agar dilution measurements used to verify that the increased β-lactam resistance phenotypes were stable. Two NE569 HoR mutants with oxacillin MICs >256μg/ml (data not shown) were chosen for further analysis. Both *sucC* HoR1 and *sucC* HoR2 exhibited homogeneous population analysis profiles (PAPs), whereas JE2 showed a heterogeneous PAP (Fig. 3.5B). Whole-genome Illumina sequencing revealed that both *sucC* HoR strains had acquired point mutations in genes involved in (p)ppGpp synthesis. *sucC* HoR1 had a single base pair deletion at position 1,250 of the Rel/SpoT Homologue (RSH) gene, *relA* (Fig. 3.5C). In *sucC* HoR2, sequencing revealed a single, nonsynonymous point mutation, $A_{178}V$ in the GTP pyrophosphokinase, *relQ* (Fig. 3.5D). The Rel/SpoT homologue (RSH) in *S. aureus* is a bifunctional (p)ppGpp synthase/hydrolase, which controls the concentration of the stringent response alarmone ppGpp within the cell[292]. The N-terminal encodes hydrolase and synthase domains, while the C-terminal contains TGS and ACT domains predicted to regulate synthase and hydrolase activity[293]. Disruption of, or mutations in, the C-terminal

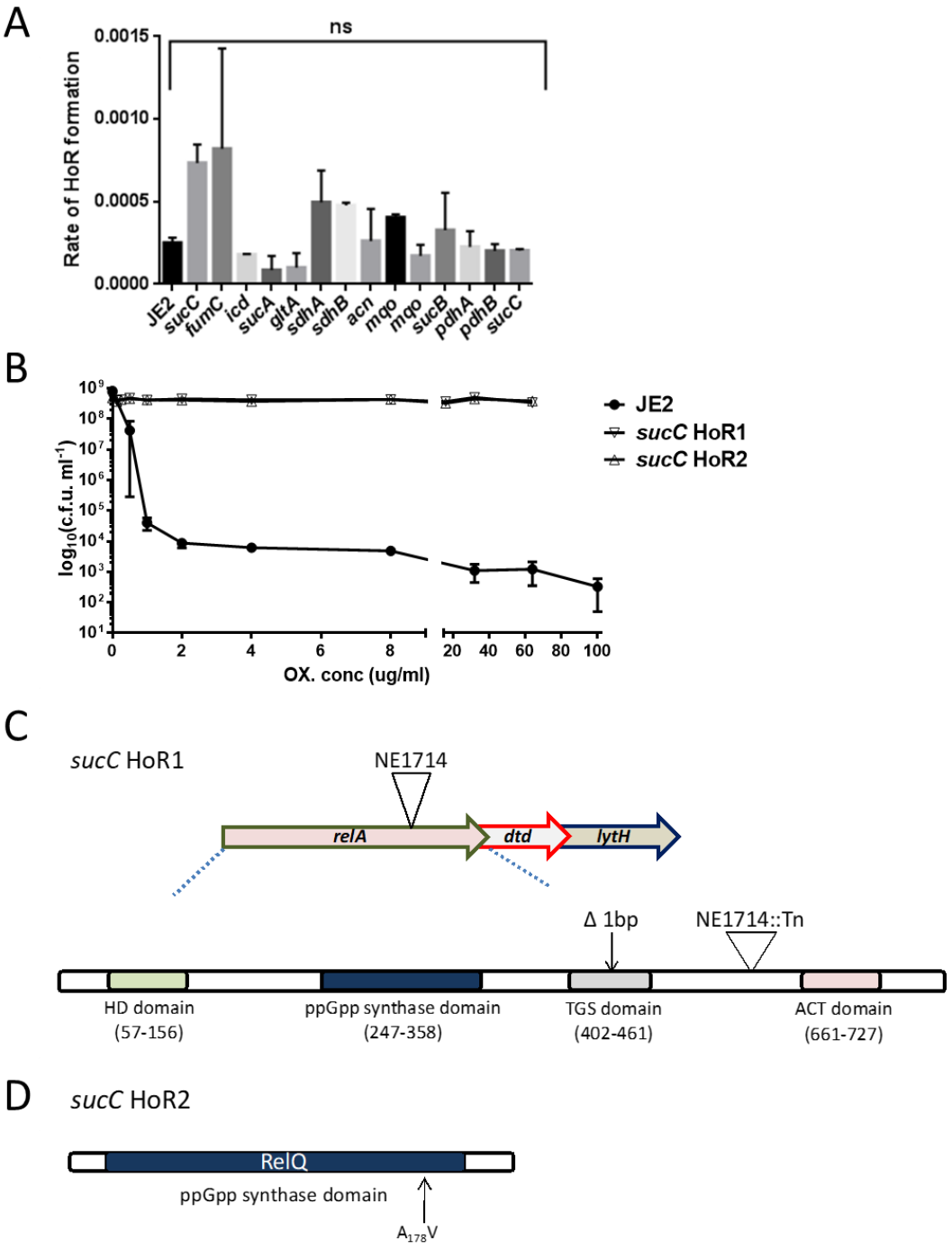

Figure 3.5. Production of HoR derivatives by TCA cycle mutants. A. Rate of HoR production by JE2 and TCA cycle mutants on oxacillin 100µg/ml plates. Experiments were repeated twice and standard deviations are shown. Significance was measured using Student t-test. **B.** Population analysis profile of JE2, *sucC* HoR1 and *sucC* HoR2 grown in BHI media to exponential phase. Cultures were standardised and serially diluted before plating onto BHI agar supplemented with increasing concentrations of oxacillin for enumeration of survivors. **C.** *relA-dtd-lytH* operon in *S. aureus* and RelA domain architecture including location of 1bp deletion in TGS domain of strain *sucC* HoR1. Also shown is the location of Tn insertion in *relA* (NE1714). **D.** Location of $A_{178}V$ mutation in ppGpp synthase domain of RelQ in strain *sucC* HoR2.

domain have been linked to deregulation of the synthase domain and an increase in intracellular (p)ppGpp[112], [293].

A single, nonsynonymous point mutation, $A_{178}V$, in RelQ was identified in *sucC* HoR2 (Fig. 3.5D). RelQ contains a (p)ppGpp synthase domain only, previously implicated in adaptation to cell wall stress induced by vancomycin and ampicillin[130]. A *relQ* mutant is not available in the NTML indicating that it may be essential. The point mutation in RelQ is consistent with previous findings if this substitution results in a gain of synthase function. Future work is required to fully characterise the effects of this mutation on ppGpp levels within the cell. These preliminary data suggest that the positive effect of *relA* or *relQ* mutations on ppGpp synthesis and an active stringent response is dominant over the negative impact of *sucCD* mutations on β-lactam susceptibility. Furthermore, GTP production associated with succinyl-CoA synthetase activity is not required for expression of a HoR phenotype, and by extension ppGpp synthesis. Quantitative measurements of intracellular ppGpp in these mutants will be required in the future.

Table 3.5. Susceptibility of JE2, NE569, NE1714 and a *sucC*/*relA* double mutant to cefoxitin (FOX) and oxacillin (OX)

	Fox (mm)*	OX MIC (μg/ml)**
JE2	11	32
NE569 (*sucC*)	24	0.25
NE1714 (*relA*)	7	256
sucC/relA	7	256

* Cefoxitin (FOX) susceptibility was determined by disk diffusion assays on MH agar and expressed as zone diameters (mm) around FOX (30 μg/ml) disks.
** Oxacillin (OX) minimum inhibitory concentration (μg/ml) was determined by agar dilution.

The *relA* mutant in the NTML, NE1714, contains a transposon insertion in the regulatory C-terminal domain, and is associated with increased β-lactam resistance, presumably due to increased (p)ppGpp synthase activity (Table 3.5). To investigate if the *relA*::Tn mutation could also reverse the β-lactam sensitivity of NE569, a *relA*/*sucC* double mutant was constructed. To achieve this, the erythromycin resistance marker in NE569 was first swapped for a kanamycin marker and the *sucC*::Kn allele then transduced into the NE1714 *relA* mutant using phage 80α. Cefoxitin disk diffusion and agar dilution assays demonstrated

that, like the *sucC* HoR1 and *sucC* HoR2 mutants, the *relA/sucC* mutant also exhibited high level β-lactam resistance (Table 3.5).

Taken together these data demonstrate that the *sucC* and *sucD* mutants have retained the capacity to express a HoR phenotype associated with mutations in *relA* and *relQ*, suggesting that their negative impact on β-lactam resistance is not related to an impaired GTP/ppGpp-mediated stringent response.

3.4.5 Wild type resistance and colony morphology could be restored in a *sucC/sucA* double mutant but not a *sucC/sdhA* mutant. In an effort to determine whether the change in β-lactam resistance observed in NE569 was due to a lack of succinyl-CoA synthetase activity or the accumulation of succinyl-CoA, a *sucC/sucA* double mutant was constructed. The *sucA* gene, which encodes 2-oxoglutarate dehydrogenase E1 subunit, is found in a two-gene operon with *sucB*, which encodes dihydrolipoyl succinyltransferase E2 subunit. The NE547 (*sucA*) and NE1391 (*sucB*) mutants do not exhibit any change in oxacillin susceptibility (Table 3.4). Using phage 80α the *sucC::*Kan transposon was transduced into NE547 to generate a *sucC/sucA* double mutant. Transposon insertions were confirmed using PCR to amplify both loci.

As hypothesised, disruption of *sucA* in a *sucC* mutant restored wild type colony morphology (Fig. 3.6). Wild type sensitivity to cefoxitin and oxacillin were also restored in the double mutant (Supp. Table 6.3). This data suggests that by removing the influence of a succinyl-CoA synthetase mutation β-lactam resistance levels return to wild type. To confirm this line of investigation a second double mutant was generated in the enzyme following succinyl-CoA synthetase in the TCA cycle, succinate dehydrogenase (NE626 - *sdhA*). NE626 did not exhibit a change in cefoxitin sensitivity or oxacillin MIC (Table 3.4) and had wild type colony morphology (Fig. 3.6). In a *sucC/sdhA* double mutant the *sucC* phenotypes were dominant with regards both β-lactam sensitivity and colony morphology (Supp. Table 6.3, Fig. 3.6).

This data confirms that the increased sensitivity of NE569 to β-lactams is specific to disruption of succinyl-CoA synthetase, and not as a result of TCA cycle disruption. It is likely that the accumulation of succinyl-CoA or the loss of SuCD enzymatic activity in another metabolic pathway is producing this susceptible phenotype.

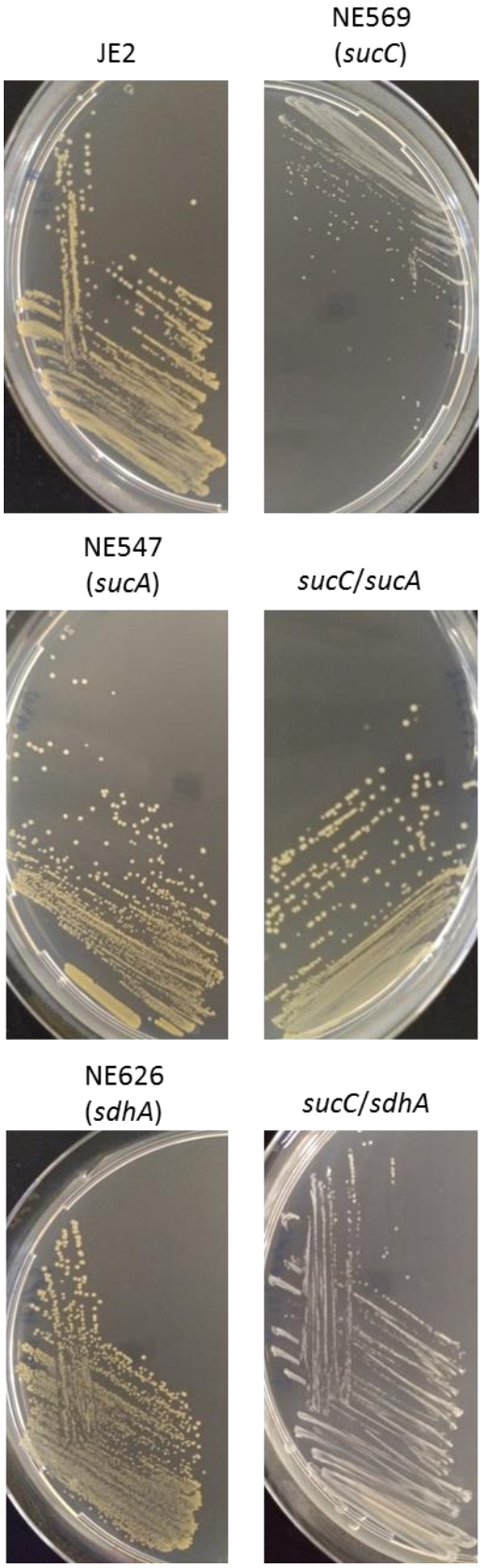

Figure 3.6. Mutation of *sucA* in a *sucC* mutant restores wild type colony morphology. JE2, NE547 (*sucA*), NE569 (*sucC*), NE626 (*sdhA*), *sucA*/*sucC* and *sucC/sdhA* double mutants colonies morphology after 24hr growth on MH agar at 37^{o}C.

3.6 Discussion

Succinyl-CoA synthetase reversibly converts succinyl-CoA into succinate and, using the phosphoryl-transfer potential of the thiol-bond, also produces GTP via substrate level phosphorylation. The NTML mutants NE569 (*sucC*) and NE1770 (*sucD*) exhibit susceptibility to cefoxitin and oxacillin characteristic of MSSA strains (Fig. 3.1A & B). The increased susceptibility of the *sucC* and *sucD* mutants was independent of altered *mecA* transcription (Fig. 3.1D). The NE569 mutant grew as smaller, less pigmented colonies on solid agar, compared to JE2, a phenotype more evident on MH agar (Fig. 3.2A). Both mutants displayed increased stationary phase survival compared to JE2 (Fig. 3.2C), a phenotype previously reported for TCA cycle mutants [284], [285]. NE569 and NE1770 also displayed a reduced specific growth rate compared to JE2 in MH broth, a difference not observed in BHI media, (data not shown). Muller-Hinton media is recommended for antibiotic susceptibility testing [281], and contains less glucose than BHI media. Supplementation of MH with 10% glucose (w/v) restored wild type levels of resistance to cefoxitin in NE569 and NE1770, while 5% glucose was sufficient to restore oxacillin MICs to wild type levels. Glucose had no effect on β-lactam susceptibility in the wild type. Similarly, the growth defect in MH broth could be overcome when the media was supplemented with 5% glucose (w/v) (Fig. 3.3B). Interestingly, in 5% glucose (w/v), growth of the parent and *sucC* mutant is similar, although it should be noted that the growth of JE2 was retarded under these conditions. This observation may be explained by repression of TCA cycle gene expression when preferred carbon sources such as glucose are freely available [74], [294].

Interestingly, disruption of other TCA cycle enzymes had no effect on β-lactam susceptibility (Table 3.4, Fig 3.7). However, in terms of growth defects, NE1391 (*sucB*), NE626 (*sdhA*) and NE1381 (*mqo* E1) were similar to NE569 (*sucC*) and NE1770 (*sucD*). NE547 (*sucA*), NE808 (*sdhB*) and NE1003 (*mqo* E1) exhibited growth profiles that fall between JE2 and the *sucC*/*sucD* mutants (Fig. 3.4). These data suggest that mutations in the TCA cycle can impact growth, in a manner specific to the mutation and not as a result of overall TCA cycle disruption. The TCA cycle in staphylococci involves the net loss of 2 carbons and requires anapleuretic reactions, typically from amino acid catabolism, to maintain continuous carbon cycling. Thus, disruption of a single TCA cycle enzymatic step can likely be compensated by input from another metabolic pathway. Although the impact of individual TCA cycle

mutations on growth is complex, these data show that a number of TCA cycle mutations do affect growth under the conditions tested, but that only the *sucC* and *sucD* mutations impact β-lactam susceptibility.

Expression of β-lactam resistance in MRSA populations is heterogeneous, with only a subset of the population expressing high levels of resistance[278]. On exposure to β-lactams, such as oxacillin, the sub-population (>0.1%) expressing high-level resistance are selected for, giving rise to a homogenously resistant population. This switch from heterogeneous resistance (HeR) to homogenous resistance (HoR) has been the subject of much research[135], [143], [144], [279], [295] and is associated with so-called accessory mutations outside the SCC*mec* locus[62], [65]. The TCA cycle has been implicated in the generation of sub-lethal concentrations of radical oxygen species, which under oxacillin pressure, may increase the mutation rate and selection of HoR strains[83], [279]. Rosato *et al.* reported that the TCA cycle was indispensible for production of HoR strains under oxacillin selective pressure[279][279]. In contrast, our data show that, despite the 7-fold increase in oxacillin susceptibility, HoRs were produced at a similar rate to the wild type (Fig. 3.5A). Furthermore, HoRs were also produced by other TCA cycle mutants at similar rates. Further experiments are required to explain this discrepancy, but our data does not support the idea that TCA cycle activity is important for the transition from the HeR to HoR phenotypes.

Whole genome sequencing of two HoR mutants isolated from NE569 was used to further investigate how the increased susceptibility of the *sucC* mutant could be reversed. Mutations in the Rel/SpoT homologue, *relA,* and the GTP pyrophosphokinase, *relQ,* which presumably increase the activity of both enzymes, and therefore ppGpp production, were identified (Fig. 3.5C & D). An activated stringent response and subsequent ppGpp production, associated with *relA*, *relP* and *relQ* mutations have previously been implicated in the *S. aureus* HoR phenotype[112], [293]. An earlier study also showed that RelQ activity was induced by vancomycin and ampicillin exposure[130], indicating that its ppGpp synthase activity may contribute to cell survival during cell wall stress. Future experiments to complement the *relA* and *relQ* mutations in the *sucC* HoR mutants are required to further verify their contributions to increased β-lactam resistance, as well as quantitative measurements of ppGpp.

A pyruvate dehydrogenase mutant (NE1724) was also identified with increased susceptibility to oxacillin, with growth and morphological phenotypes similar to NE569 and NE1770 (data not shown). Pyruvate dehydrogenase links glycolysis and the TCA cycle by converting pyruvate into acetyl-CoA. Unlike succinyl-CoA synthetase, where disruption of either subunit caused reduced oxacillin resistance, only the transposon insertion in the alpha subunit gene (*pdhA*), and not the beta subunit gene (*pdhB*), caused induced susceptibility to β-lactams. Although there is no direct metabolic link between SucCD and PdhA, the similarities in their phenotypes may be significant. Despite several attempts to compliment the *pdhA* mutant, we were unable transform a plasmid carrying either *pdhA* or *pdhAB* genes into NE1724. However, backcross of the *pdhA* mutation into JE2 was accompanied by increased susceptibility. This data raised the possibility that over-expression of *pdhA* may be lethal to the cell. Phosphoenolpyruvate is dephosphorylated, by pyruvate kinase, to pyruvate. Zoraghi *et al.* found that the specific inhibition of pyruvate kinase in MRSA was lethal[46]. This report would suggest that the intermediate pathway between glycolysis and the TCA cycle is important to overall cell survival. Whether or not the *pdhA* phenotype is linked to *sucCD* susceptibility to oxacillin requires further research.

The resistance phenotype of NE560 and NE1770 raises the question about whether it is the lack of succinyl-CoA synthetase enzymatic activity, or the accumulation and/or depletion of one of its substrates that is affecting resistance. To investigate this, we generated *sucC/sucA* and *sucC/sdhA* double mutants. The *sucC/sucA* double mutant exhibited wild type colony morphology and susceptibility to β-lactams, whereas in a *sucC/sdhA* double mutant the *sucC* phenotype was dominant (Fig. 3.6). This result suggests that the *sucCD* colony and resistance phenotype can be overcome by disrupting the previous metabolic step in the TCA cycle. Similarly, in NE626 (*sdhA*), which does not exhibit decreased resistance or altered colony growth, the *sucC* phenotype is dominant (Fig. 3.6). Our data indicates that the increased β-lactam sensitivity is specific to succinyl-CoA synthetase disruption, and perhaps the accumulation of succinyl-CoA in NE569 and NE1770. Comparisons between intracellular levels of succinyl-CoA in JE2 and NE569 will shed more light on the metabolic differences between this mutant and the parent.

Our data suggests a specific role for succinyl-CoA synthetase that is most likely independent of its energy role in the TCA cycle. Succinyl-CoA contains a high-energy thiol-bond that

drives several other pathways. In β-oxidation of odd-numbered fatty acids, the end products are acetyl-CoA and propionyl-CoA, of which the latter can be further converted to succinyl-CoA for entry into the TCA cycle. In *S. aureus* the major fatty acids are odd numbered branched-chain fatty acids[296]. The carotenoid staphyloxanthin is responsible for the strong yellow pigment associated with *S. aureus*, and is made up of a glucose residue esterified with a 30-carbon carboxylic acid chain and a 15-carbon fatty acid[297]. If an accumulation of succinyl-CoA impacts fatty acid metabolism and consequently staphyloxanthin synthesis, this may somewhat explain the pale colony morphology of *sucC* and *sucD* mutants.

Succinyl-CoA is also used in the biosynthesis of lysine from aspartate. Succinylation, using succinyl-CoA as the donor (Fig 3.7), is coupled to the amidation of tetrahydrodipicolinate to form succinyl-2-amino-6-ketopimelate[298], before the succinyl group is removed in subsequent reactions to eventually form lysine. In the Gram-positive bacterium, *Corynebacterium glutamicum*, disruption of the *sucCD* locus, and the resulting accumulation of succinyl-CoA was accompanied by lysine overproduction. Lysine is a critical component of the *S. aureus* cell wall [36], [299]. However, is difficult to envisage how enhanced production of this amino acid would have a negative effect on cell wall biosynthesis and susceptibility to β-lactam antibiotics. An analysis of peptidoglycan structure in NE569 and NE1770 will help to determine the mechanistic basis for increased β-lactam susceptibility in these mutants.

A third possibility is that the accumulation of succinyl-CoA is promoting widespread post-translational lysine-succinylation of proteins and enzymes, including some that are playing a role in cell envelope biosynthesis and susceptibility to β-lactam antibiotics (Fig 3.7). Post-translational succinylation of lysine residues was first characterised by Zhang *et al.* in *E. coli*[300] and has since been identified in mammals, fungi and other bacteria[301]–[303]. Protein succinylation was promoted by the accumulation of succinyl-CoA in a *Saccharomyces cerevisiae* mutant with its *sucCD* homologues disrupted[301]. Early studies have indicated that succinylation tends to inhibit protein and enzyme function, altering the native lysine charge from +1 to -1[300]. Succinylation also appears to exert a global effect, modifying proteins involved in a range of cellular functions including carbon metabolism, protein synthesis and also antibiotic resistance[301], [303], [304]. The functional consequences of protein succinylation remain largely unknown; however, it seems plausible

that the accumulation of succinyl-CoA in NE569 and NE1770 may negatively impact the activity of one or more proteins involved in β-lactam resistance. Post-translational modification of proteins by succinylation remains entirely unexplored in *S. aureus* and progress in this area may identify new therapeutic targets for the treatment of *S. aureus* infections. Future work will aim to show that succinylation is a form of posttranslational modification occurring in *S. aureus*, and whether or not disruption of *sucCD* impacts this process.

In terms of targeting the SucCD complex, the FDA approved anti-cancer drug streptozotocin specifically targets succinyl-CoA synthase in human cells to limit proliferation [305], and is used primarily to treat tumours that cannot be surgically removed. Although toxic, the known mechanism of action of streptozotocin shows that succinyl-CoA synthetase inhibitors exist and that the development of novel therapeutic approaches to inhibit SucCD may help overcome β-lactam resistance in *S. aureus* infections.

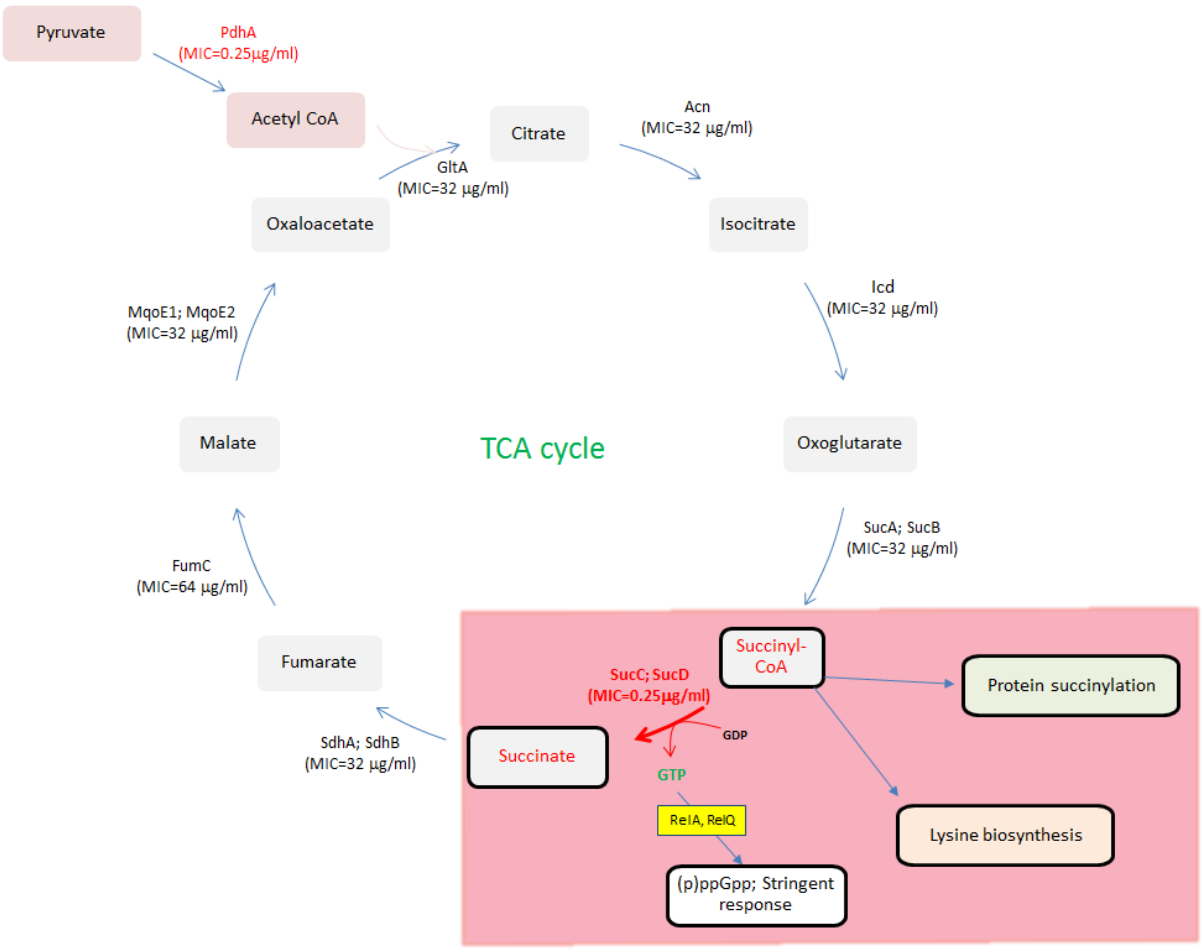

Figure 3.7. The TCA cycle of *S. aureus,* indicating the impact of mutations on susceptibility to oxacillin (Minimum inhibitory concentration (MIC); µg/ml). Mutations in either *sucC* or *sucD* render strains JE2 and MW2 susceptible to β-lactam antibiotics and are presumably associated with the intracellular accumulation of succinyl-CoA, which is a driver of the lysine biosynthetic pathway. Lysine is an important component of the Gram-positive cell wall, however, how the accumulation of succinyl-CoA impacts on lysine synthesis, and subsequent incorporation in peptidoglycan remains unclear. The accumulation of succinyl CoA may also impact global patterns of protein succinylation and the activity of proteins that are modified in this way. SucCD-mediated conversion of succinyl CoA to succinate is also a major source of GTP within the cell, which may in turn impact RelA-dependent production of the stringent response alarmone (p)ppGpp. Apart from *sucC* and *sucD*, mutations in other genes encoding TCA cycle enzymes do not increase susceptibility to β-lactams. Preliminary data indicate that mutation of the pyruvate dehydrogenase alpha subunit gene *phdA*, but not the beta subunit gene *pdhB*, also increases susceptibility to β-lactams.

Chapter 4

Disruption of the purine salvage pathway in MRSA increases resistance to β-lactams

Christopher Campbell, Laura A. Gallagher and James P. O'Gara*

Department of Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland.

*Corresponding author; jamesp.ogara@nuigalway.ie

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Running title: Depleting intracellular guanine increases β-lactam resistance in MRSA

4.1 Abstract

Expression of high-level β-lactam resistance in MRSA requires both *mecA* and so-called accessory factors. Identification and elucidation of the mechanisms through which the accessory factors control the β-lactam resistance phenotype in MRSA is important to the development of novel and effective treatments for infections caused by this pathogen. Using a cefoxitin disk diffusion assay to screen the Nebraska Transposon Mutant Library, two mutants, NE650 and NE1419 were found to have increased β-lactam resistance compared to JE2. NE650 harbours a transposon insertion in a purine nucleoside phosphorylase gene designated *deoD*, and NE1419 contains a transposon insertion in a putative nucleoside transporter with homology to *nupG*. DeoD and NupG are part of the purine salvage pathway in *Staphylococcus aureus*, which allows cells to utilise exogenous purines for the formation of deoxynucleotides, as well as the synthesis of other purine derivatives such ATP, GTP and secondary messengers such as (p)ppGpp and c-di-AMP. The intracellular concentrations of numerous nucleotides were reduced in both the *deoD* and *nupG* mutants, suggestive of pleiotrophic effects on nucleotide metabolism. The effects of the *deoD* and *nupG* mutations on β-lactam resistance were successfully complemented. Furthermore, transduction of both mutations into strain MW2 was accompanied by increased β-lactam resistance. These data suggest that disruption of nucleotide homeostasis increases β-lactam resistance in MRSA further supporting an important role for purine metabolism in this phenotype.

4.2 Introduction

Nucleotide messengers are found in all kingdoms of life and function to link deviations in homeostasis with an appropriate cellular response. A prime example is guanosine tetraphosphate (ppGpp) the stringent response alarmone. Under conditions of amino acid limitation, *S. aureus* responds by rapidly synthesising (p)ppGpp using the GTP pyrophosphokinase, RelA, with GTP as a substrate. The accumulation of ppGpp and the concomitant reduction in GTP levels have pleiotrophic effects on the cell. Firstly, GTP binding to the metabolic repressor CodY, is reduced, resulting in reduced CodY activity and activation of the CodY regulon which includes genes involved in amino acid synthesis, nitrogen assimilation and carbon transport[106], [306]. A second, related effect, of (p)ppGpp accumulation is the inhibition of enzymes that produce GTP. Both guanylate kinase (Gmk) and Hpt, which converts guanine to GMP, are inhibited by ppGpp[132], [133], [307], essentially cutting off the GTP-producing arm of purine metabolism, and driving ATP synthesis.

Purine biosynthesis is a crucial pathway in *S. aureus*, not only for its role in supplying the building blocks for DNA and RNA synthesis, but also in regulating cellular responses to environmental stresses such as nutrient limitation and antibiotic stress. Bacteria possess two convergent pathways to supply the cell with purines, the *de novo* pathway and the salvage pathway. The *de novo* pathway synthesises inosine monophosphate (IMP) from phosphoribosyl-diphosphate (PRPP) and glutamine in an energy-expensive, 11-step pathway, utilising the *purEKCSQIFMNHDB* gene products[308]. The purine salvage pathway allows cells to down-regulate *de novo* synthesis when exogenous nucleotides or nucleosides are available[107]. Internalised nucleosides and deoxynucleotides from DNA turnover can be incorporated via the salvage pathway. (Deoxy)nucleosides are first dephosphorylated by purine nucleoside phosphorylases (PNPs) to their respective bases. Phosphoribosyltransferases use PRPP to convert nucleotide bases into monophosphate-nucleosides, GMP or AMP, which themselves are interconvertible via IMP, depending on the requirements of the cell[309].

Several stages of purine metabolism have been implicated in high-level β-lactam resistance in MRSA. The ppGpp-dependent inhibition of the RsgA GTPase was found to increase resistance to both β-lactams and fluoroquinolones[132]. Mutation of the c-di-AMP phosphodiesterase gene *gdpP* results in the accumulation of the secondary messenger c-di-

AMP, and is associated with increased peptidoglycan cross-linking and decreased susceptibility to β-lactam antibiotics[111]. Whole genome sequencing of homogeneously resistant MRSA strains identified mutations in *guaA, guaB, prsA, hpt (hprT) and relA* [114], all of which result in a dampening of purine metabolism or an increase in concentrations of ppGpp, which itself inhibits enzymes of the purine pathway[132]. ppGpp has also been shown to inhibit GdpP, while c-di-AMP, though an unknown mechanism increases ppGpp synthesis[122].

Previous research suggests a strong link between purine metabolism and the ability of a bacterial cell to respond to β-lactam challenge. We have identified two mutants of the purine salvage pathway, NE650 and NE1419, which exhibited increased resistance to oxacillin. NE650 contains a transposon insertion in a purine nucleoside phosphorylase, *deoD*, while NE1419 contains a transposon insertion in the putative purine transporter *nupG*. Our evidence suggests that these mutants mimic the effect of ppGpp accumulation, in that they result in a reduction of purine intermediates, and may causing downstream activation of the CodY regulon, thus promoting β-lactam resistance.

4.3 Materials and Methods

4.3.1 Bacterial strains, growth conditions and antimicrobial susceptibility testing. Bacterial strains used in this study (Table 4.1), were routinely cultured in Brain-Heart Infusion (BHI) media (Oxoid), Muller-Hinton (MH) media (Oxoid) or Luria Bertoni (LB) media (Oxoid). Where indicated erythromycin (Erm) 10µg/ml, chloramphenicol (Cm) 10µg/ml, ampicillin (Ap) 50µg/ml or kanamycin (Kn) 75µg/ml were used for antibiotic selection (all Sigma). Routine growth was performed at 37ºC shaking (200rpm).

Oxacillin minimum inhibitory concentrations (MICs) were performed using oxacillin E-test strips (Oxoid), or using agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines on MH 2% NaCl agar[281]. The MSSA strain ATCC 29213 was routinely included as a control. Chemically defined media (CDM) was made up as previously described by Hussain *et al.*[310]. CDM lacking guanine (-Gua) was the same formula but without the addition of guanine.

4.3.2 Screening of the Nebraska Transposon Mutant Library (NTML). A screen of the Nebraska Transposon Mutant Library (NTML) was carried out to identify mutants exhibiting altered susceptibility to cefoxitin (Oxoid) using the disk-diffusion method in accordance with CLSI guidelines[281]. Briefly, each library mutant was revived from a freezer stock onto BHI agar supplemented with erythromycin and grown for 18hrs at 37^{o}C. The following day 3-5 individual colonies were used to prepare a bacterial suspension in 0.85% saline which was standardised to 0.5 McFarland. A swab was immersed in the bacterial suspension and used to evenly inoculate the surface of MH agar plates before the application of a cefoxitin-impregnated disk (30µg). The plates were incubated for 24hrs at 35^{o}C. The zone of inhibition diameter for JE2 was 16mm, whereas the *mecA* mutant from the NTML and MSSA strain ATCC 29213 exhibited zone diameters >35mm. The entire NTML was screened in duplicate, with candidate mutants with altered cefoxitin susceptibility repeated at least 5 times. Oxacillin MICs were measured using oxacillin E-tests and agar dilution assays. PCR was used to verify the transposon insertion within the gene of interest (Table 4.2). Whole genome sequencing (Microbes NG, UK) was used to ensure that there were no additional mutations contributing to the antibiotic susceptibility phenotype.

Table 4.1. Strains and relevant plasmids used in this study with relevant details

Strain/Plasmid	Relevant details
JE2	Derivative of USA300 LAC cured of plasmids p01 and p03. Parent strain of the Nebraska Transposon Mutant Library (NTML).
NE650	JE2 *deoD*::Tn. Erm^r
NE1419	JE2 *nupG*::Tn. Erm^r
NE280	JE2 *pbuX*::Tn. Erm^r
NE283	JE2 xanthine permease::Tn. Erm^r
NE353	JE2 *purH*::Tn. Erm^r
NE424	JE2 *pdp*::Tn. Erm^r
NE477	JE2 *deoD*::Tn. Erm^r
NE494	JE2 *purQ*::Tn. Erm^r
NE522	JE2 *purB*::Tn. Erm^r
NE544	JE2 *nupC*::Tn. Erm^r
NE581	JE2 *purF*::Tn. Erm^r
NE622	JE2 putative *nupC*::Tn. Erm^r
NE744	JE2 *purK*. Erm^r
NE750	JE2 *purD*::Tn. Erm^r
NE932	JE2 SAUSA300_0147::Tn. Erm^r
NE1101	JE2 *purM*::Tn. Erm^r
NE1134	JE2 *purS*::Tn. Erm^r
NE1237	JE2 *purR*::Tn. Erm^r
NE1331	JE2 *ndk*::Tn. Erm^r
NE1407	JE2 *pyk*::Tn. Erm^r
NE1464	JE2 *purL*::Tn. Erm^r
NE1583	JE2 SAUSA300_2234::Tn. Erm^r
NE1637	JE2 SAUSA300_0237::Tn. Erm^r
NE1785	JE2 *purN*::Tn. Erm^r
8325-4	MSSA. SigB-deficient, PIA biofilm positive.
RN4220	Chemically mutated, restriction-deficient *S. aureus* derived from 8325 used as an intermediate host for cloning and plasmid mobilisation into other *S. aureus* strains
BH1CC	MRSA clinical isolate
E. coli	*E. coli* HST08
MW2	CA-MRSA USA400-derivative
ATCC 295213	MSSA strain for MIC susceptibility testing
ATCC 29213	MSSA strain for cefoxitin susceptibility testing
Plasmids	
pLI50	*E. coil-Staphylococcus* shuttle vector. Ap^r (*E. coli*), Cm^r (*Staphylococcus*)
p*deoD*	pLI50 carrying *deoD* from JE2
p*nupG*	pLI50 carrying *nupG* from JE

4.3.3 Genetic techniques. Genomic and plasmid extractions were carried out using Wizard genomic DNA purification (Promega) and plasmid extraction miniprep kits (Sigma). For *S. aureus* DNA extraction was preceded by pre-treatment with 10µg/ml lysostaphin (Ambi Products, New York) as described previously. All restriction enzymes were supplied by Roche, and used as per manufactures guidelines.

Table 4.2. Oligonucleotides used in this study.

Target gene	Primer name	Primer sequence (5'-3')
deoD	deoD_F	ACATGCGTTGGATTCACACA
	deoD_R	TGGTAATGCTGGCGATGATG
nupG	nupG_F	AGCCACGAGTCATAATTCTTCG
	nupG_R	ATAGACGACATGCCTGGACA
mecA	mecA_F	CATATCGTGAGCAATGAACTGA
	mecA_R	CATCGTTACGGATTGCTTCA
Infusion primers		
deoD	INF_deoD_F	TCGTCTTCAAGAATTACATGCGTTGGATTCACACA
	INF_deoD_R	TACCGAGCTCGAATTTGGTAATGCTGGCGATGATG
nupG	INF_nupG_F	TCGTCTTCAAGAATTGGTAACAGGTAAAGGTACGCA
	INF_nupG_R	TACCGAGCTCGAATTATAGACGACATGCCTGGACA
RT-PCR primers		
mecA	mecA1_Fwd	TGCTCAATATAAAATTAAAACAAACTACGGTAAC
	mecA1_Rev	GAATAATGACGCTATGATCCCAA
gyrB	gyrB_Fwd	CCAGGTAAATTAGCCGATTGC
	gyrB_Rev	AAATCGCCTGCGTTCTAGAG

To complement NE650 and NE1419, 1,387bp and 1,740bp PCR fragments were amplified in JE2 using the primers INF_deoD_F / INF_deoD_R and INF_nupG_F / INF_nupG_R, respectively (Table 4.2). The Clontech Infusion Cloning kit was used to insert the fragments into the EcoR1 restriction site of pLI50. The plasmid was then transformed into *E. coli* HST08, extracted and electroporated into restriction-deficient *S. aureus* strain, RN4220, before being extracted and electroporated into NE650 and NE1419.

4.3.4 Phage Transductions. Phage 80α was used to transduce the transposon insertion from the NE650 and NE1419 mutants into a fresh JE2, MW2 or 8325-4 background, to ensure background mutations were not responsible for the antibiotic resistance phenotype. Briefly, phage 80α was propagated in soft agar with NE650 or NE1419 to a titre of 10^8 plaque-forming units (PFU). Following transduction, candidates were selected for on sodium citrate

TSA agar supplemented with erythromycin 10µg/ml or kanamycin 75µg/ml, as required. Transposon insertion was verified using PCR.

4.3.5 RNA purification and real time RT-PCR. Cultures were grown in BHI media to mid-exponential phase. Harvested cells were pelleted and immediately stored at -20ºC in RNAlater (Ambion) to ensure maintenance of RNA integrity prior to purification. RNA was extracted as per manufacturer's guidelines using RNA Mini-Extraction kit (Sigma). RNA integrity was examined visually by agarose gel electrophoresis and RNA concentration was determined using a Quibit Fluorometer 4 (Qiagen). Quantitative reverse transcription PCR (RT-qPCR) was used to measure *mecA* transcription on the Roche LightCycler 480 instrument using the LightCycler 480 Sybr Green Kit (Roche) with primers mecA1_Fwd and mecA1_Rev (Table 4.2). The following cycling conditions were used: 95ºC for 5 minutes and followed by 45 cycles of 95ºC for 10 seconds, 58ºC for 20 seconds and 72ºC for 20 seconds. Melt curve analysis was performed at 95ºC for 5 seconds followed by 65ºC for one minute up to 97ºC at a ramp rate of 0.11c/sec with five readings taken for every degree of temperature increase. The *gyrB* gene amplified with primers gyrB_Fwd and gyrB_Rev (Table 4.2) served as an internal standard for all reactions. For each reaction, the ratio of *mecA* and *gyrB* transcript number was calculated as follows: $2^{(Ct\ gyrB\ -\ Ct\ mecA)}$. Each RT-qPCR experiment was performed three times and presented as average data with standard errors.

4.3.6 Growth assays. For growth assays, cultures were grown in 50ml of BHI broth in a 250ml flask, with a starting A_{600}=0.01. Culture aliquots were collected at 1 hour intervals and cell density measured at A_{600}. Growth curves in CDM were performed in 96-well plates, using a Tecan Sunrise plate reader, measuring A_{600} every 30mins. Growth experiments were repeated at least 3 times and standard error of the mean calculated.

4.3.7 Population analysis profile. Population analysis profiles (PAPs) were carried out as previously described [278]. Briefly, overnight cultures were standardised to an A_{600}=1, before being serially diluted and the number of CFUs enumerated on BHI agar plates supplemented with a range of oxacillin concentrations, from 0.25µg/ml to 100µg/ml. The average data from at least three independent experiments are presented with error bars representing the standard error of the mean.

4.3.8 Oxacillin tolerance assay. Tolerance assays were performed as described by Griffiths *et al.*[311]. Briefly, overnight cultures were sub-inoculated to A_{600}=0.01 in 10ml BHI broth. Cells were grown to mid-exponential phase; aliquots were removed before the addition of oxacillin 12.5µg/ml and every hour thereafter for 6 hours for CFU enumeration. Survival is expressed as a percentage of viable cells compared to pre-treatment.

4.3.9 Salt tolerance assay. Overnight cultures were grown in BHI broth at 37ºC and cell density standardised to A_{600}= 1.0. Tenfold serial dilutions down to 10^{-7} were made in sterile BHI and 4µl of each dilution was spotted onto BHI agar containing 2.2 M NaCl. Plates were incubated at 37ºC for 24h and imaged.

4.3.10 Scanning electron microscopy. Biofilm imaging, cell shape and cell size analysis were carried out using SEM imaging. JE2, NE650 and NE1419 biofilms were grown on tissue culture treated polystyrene slides with detachable wells (Nunc, Thermo-scientific). Overnight cultures were diluted 1:200 in BHI broth and incubated for 24h at 37ºC. Subsequently, the slides were rinsed three times with distilled water, dried at 60ºC for 1h, before being rinsed with 0.1M phosphate buffer, fixed in 2.5% glutaraldehyde for 2h, rinsed again with 0.1M phosphate buffer, dehydrated in ethanol (20%, 30%, and 50% for 10 min each step; 70% ethanol with 0.5% uranyl acetate for 30 min; and then 90%, 96%, and 100% ethanol), soaked in hexamethyldisilazane (HMDS) for 30 minutes and finally dried overnight. The slides were fixed to metal stubs before being coated in gold and imaged by SEM using a Hitachi S2600N Variable Pressure Scanning Electron Microscope.

4.3.11 Nucleotide quantification using UPLC-MRM/MS. Overnight cultures were standardised to A_{600} in 50ml BHI broth and grown to mid-exponential phase. Cells were washed three times in cold PBS and flash frozen in liquid nitrogen for analysis by MtoZ Biolabs (Boston) using the following protocol.

80% methanol was added to each sample to a total volume of 200µL. Cells were lysed on a MM400 mill mixer at 25Hz for 1min x 2 below 0ºC, with the aid of two 4mm stainless steel balls, followed by sonication for 3 min in an ice-water bath. The tubes were centrifuged at 15,000 rpm in an Eppendorf 5420 R centrifuge and at 4ºC for 15min. Clear supernatants were collected for assay of nucleotides using the following procedures.

50µL of each supernatant was mixed with 50µL of a 13C10-labled GTP (internal standard) solution, and 50µL of chloroform. The tubes were vortex-mixed for 30s, followed by centrifugal clarification. The organic phase was removed and discarded. The aqueous phase was dried in a nitrogen evaporator at 30°C under a gentle nitrogen gas flow. The residues were reconstituted in 50µL of water. In addition, a standard mix containing all the targeted phosphate-containing metabolites and nucleotides was dissolved in a solution containing the same internal standard to prepare standard solution S1. This solution was serially diluted at a ratio of 1 to 4 (v/v) to have standard solutions of S2 to S9. 10µL of each sample solution or each standard solution was injected into a C18 UPLC column (2.1 x 150mm, 1.7µm) for gradient elution with tributylamine acetate-containing mobile phase A and acetonitrile-mobile phase B. The efficient gradient was from 15%B to 50%B in 12min, with a flow rate of 250µL/min and a column temperature of 40°C. The metabolites were detected by UPLC-MRM/MS with (-) ion detection.

For all the above quantitation, concentrations of the detected metabolites were calculated from their linear-regression calibration curves with internal calibration. All the concentrations were normalized with the protein content in each sample.

4.4 Results

4.4.1 Mutation of the purine nucleoside phosphorylase, *deoD*, in MRSA increases β-lactam resistance in MRSA. A screen of the NTML, using the cefoxitin disk-diffusion assay according to CLSI guidelines[281] was conducted, in order to identify novel genetic loci that may contribute to β-lactam resistance. Library mutants were screened at least twice with mutants showing altered resistance profiles compared to the parent, JE2, being repeated a minimum of 5 more times. NE650 exhibited increased resistance on a cefoxitin disk-diffusion assay (Fig. 4.1A). JE2 has a zone diameter of 16mm while NE650 has a diameter of 10mm. Furthermore, oxacillin E-test strips and agar dilution assays confirmed that the NE650 mutant had an increased MIC, from 32μg/ml to 128μg/ml (Fig. 4.1B). NE650 contains a transposon insertion in *deoD*, a purine nucleoside phosphorylase. The increased resistance phenotype could be complemented by cloning a 1387bp fragment, containing a wild type *deoD* gene and its upstream promoter region, on the *E. coli-Staphylococcus* shuttle vector, pLI50, which was then electroporated into NE650 (Fig. 4.1A & B). Insertion of an empty pLI50 plasmid had no effect on cefoxitin susceptibility (Fig. 4.1A) or oxacillin resistance (data not shown). NE650 contains a transposon insertion in the purine-nucleoside phosphorylase, *deoD* (Fig. 4.1C). Interestingly, *deoD* is located downstream of *deoA* (NE424) and *deoC* (NE913), both of which are nucleoside phosphorylases. However, *deoA* is a pyrimidine-nucleoside phosphorylase, and *deoC* is a deoxyribose-5-phosphate aldolase[312], and neither is reported to function in purine metabolism.

To ensure that background mutations in NE650 were not responsible for the resistance phenotype, phage 80α was used to backcross the NE650 transposon into the parent strain, JE2, as well as the USA400 background, MW2 (Table 4.3). PCR was used to confirm the transposon insertion at the correct position on the genome. In both strains the oxacillin MIC and the cefoxitin resistance was increased when *deoD* was disrupted via transposon insertion.

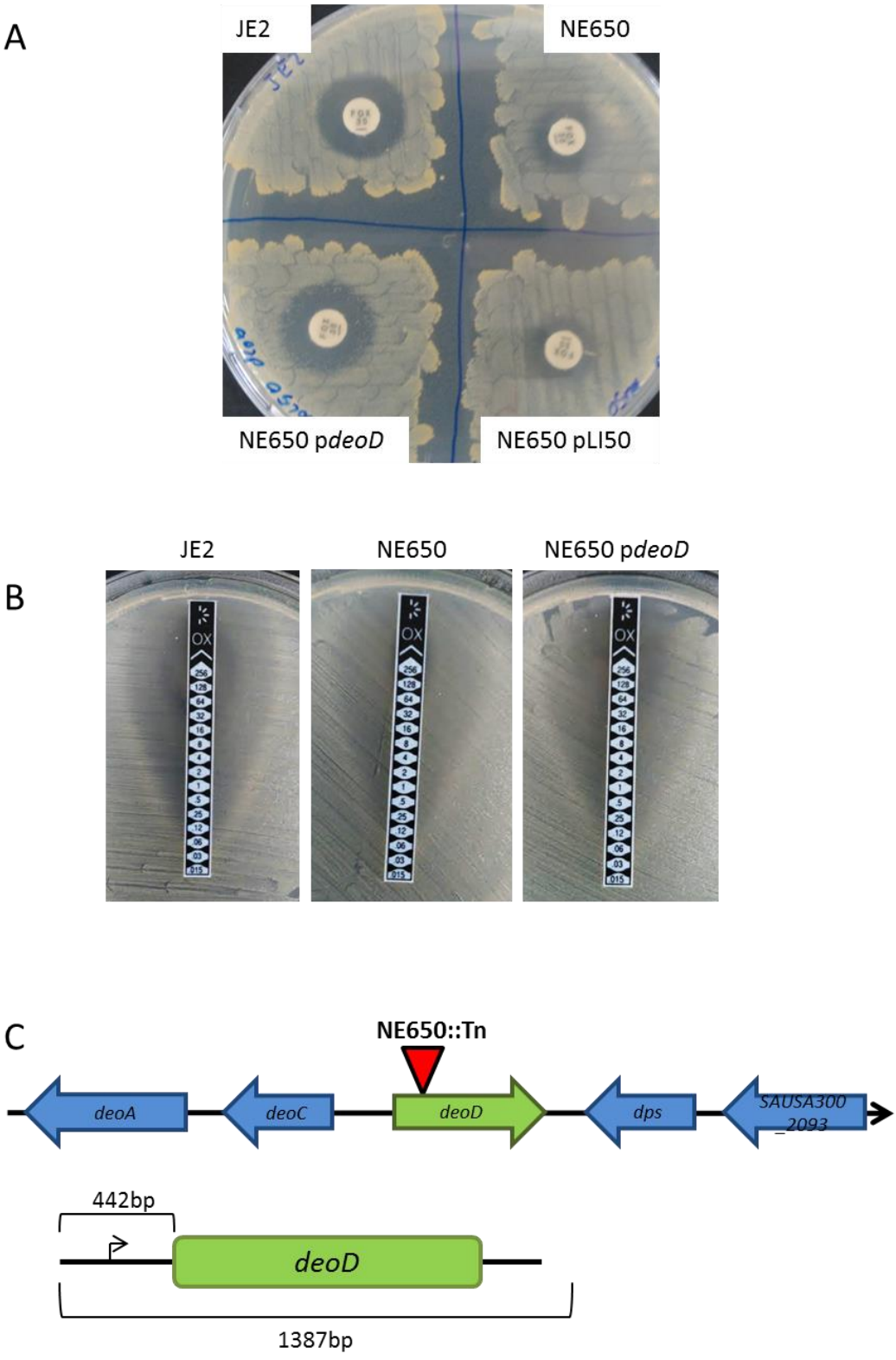

Figure 4.1. Mutation of *deoD* increases β-lactam resistance in MRSA. A. Susceptibility of JE2, NE650 (*deoD*), NE650 pLI50 and NE650 p*deoD* grown on MH agar to cefoxitin (FOX, 30µg disks). **B.** Etest measurement of oxacillin MIC (µg/ml) in JE2, NE650 and NE650 p*deoD*. **C.** Structure of the *deoD* chromosomal neighbourhood, indicating the location of the transposon insertion in NE650. The 1,387bp region encompassing the *deoD* gene on the complementation plasmid p*deoD* is indicated.

A second mutant present in the library, with a transposon insertion in a gene annotated *deoD* (SAUSA300_0138), did not display any variation in either cefoxitin sensitivity or oxacillin resistance (Table 4.4). The two *deoD* genes are not located together on the chromosome. However, they share 73% and 67% identity at the nucleotide and amino acid level, respectively. There are two annotated purine nucleoside phosphorylases (PNPs) in *B. subtilis*[92], [313], however Blast sequence alignment did not identify any sequence similarity between either gene between the two bacteria. Purine nucleoside phosphorylases typically occur as trimeric or hexameric polypeptide structures[110], with the trimer structure common to mammals[314] and the hexameric form in prokaryotes[110]. Bacteria containing both trimer and hexamer PNPs have been identified[315]–[317]. The oligomerization status of *S. aureus* DeoD, alone or potentially in combination with SAUSA300_0138, is unknown and may be important for enzyme activity.

Table 4.3. Increased cefoxitin (Fox) and oxacillin (Ox) resistance phenotypes could be transduced into an MW2 background.

Strain	Fox (mm)*	Ox MIC (μg/ml)**
JE2	15	32
NE650	10	128
NE1419	7	256
MW2	16	32
MW2 *deoD*::Tn	10	128
MW2 *nupG*::Tn	7	128

* Cefoxitin (FOX) susceptibility was determined by disk diffusion assays on MH agar and expressed as zone diameters (mm) around FOX (30 μg/ml disks).
** Oxacillin (Ox) minimum inhibitory concentration (μg/ml) was determined by agar dilution.

Although *deoD* (SAUSA300_2091) has not previously been implicated in high-level β-lactam resistance, several other purine metabolism genes have been implicated in this phenotype. Mutations in IMP dehydrogenase (*guaB*), GMP synthase (*guaA*), ribose-phosphate pyrophosphate (*prsA*), hypoxanthine phosphoribosyltransferase (*hpt*), guanylate kinase (*gmk*), adenylosuccinate lyase (*purB*) and GTP pyrophosphokinase (*relA*), whose functions are involved in both the *de novo* and salvage pathways of purine metabolism, have been

identified in highly resistant HoR strains [114], [144], [318], [319]. These acquired mutations provide compelling evidence to suggest that altered purine metabolism can contribute to expression of high-level resistance to β-lactams.

4.4.2 NE1419 displays increased β-lactam resistance and contains a transposon mutation in *nupG*, a putative purine transporter. To further investigate the role of purine metabolic genes in β-lactam resistance, the susceptibility of NTML strains with transposon insertions in purine metabolism genes to both cefoxitin and oxacillin was measured (Table 4.4). Mutations in the *guaB*, *guaA*, *prsA*, *hpt*, *gmk* genes were absent from the library, suggesting that their functions may be essential. Among the mutants examined, NE1419 (SAUSA300_0631), which encodes a putative nucleoside transporter, was found to exhibit a similar increase in cefoxitin (Fig. 4.2A) and oxacillin resistance (Fig. 4.2B) to that observed in NE650. The increased resistance phenotype of NE1419 was successfully transduced into JE2 and MW2, and complemented by the wild type plasmid-borne gene (Table 4.3, Fig. 4.2B). The SAUSA300_0631 predicted amino acid sequence is 85-86% identical to the *nupG* homologues in *Mycobacteroides abscessus* and *Streptococcus pneumonia.* In *Bacillus subtilis* the *nupG* gene encodes a guanosine transporter, with an upstream 5'UTR guanine riboswitch known as a G-box [99], [108]. The G-box is a guanine binding motif, found in several organisms[99], and allows cells to down-regulate the expression of purine salvage genes when nucleotides are abundant. Other purine metabolism genes with the G-box motif include *xpt*, *pbuX, pbuG* and *guaA*[108], [320], [321].

Interestingly, the *nupG* gene lies between two other hypothetical genes also predicted to encode membrane transporters (Fig. 4.2C). The gene encoding penicillin-binding protein 4, PBP4, is also in this chromosomal neighbourhood (Fig. 4.2C). PBP4 has been implicated in high-level resistance in CA-MRSA, in concert with PBP2a[322]. Furthermore, overexpression of *pbp4* has been reported to confer low-level resistance to β-lactams in *mecA*-negative *S. aureus*[323], [324].

To confirm the absence of background mutations that may contribute to increased resistance in NE650 and NE1419, whole genome sequencing (MicrobesNG, Birmingham) was employed. In NE650 a synonymous base pair change (C→T) was found at position

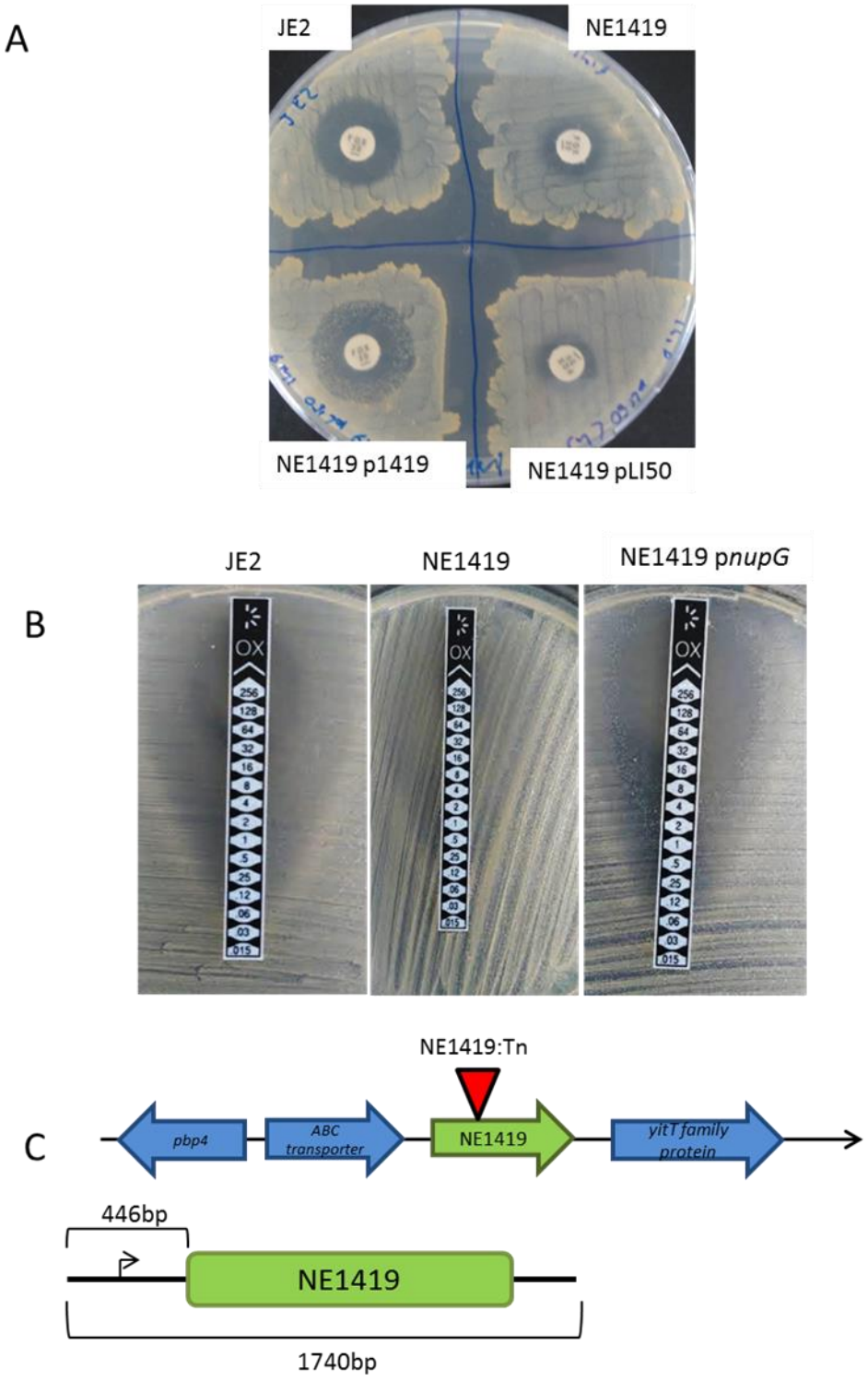

Figure 4.2. Mutation of NE1419 (SAUSA300_0631) increases β-lactam resistance in MRSA. A. Susceptibility of JE2, NE1419, NE1419 pLI50 and NE1419 p*nupG* strains grown on MH agar to cefoxitin (FOX, 30μg disks). **B.** Etest measurement of oxacillin minimum inhibitory concentrations (MICs; μg/ml) in JE2, NE1419 and NE1419 p*nupG*. **C.** Structure of the *nupG* chromosomal neighbourhood, indicating the location of the transposon insertion in NE1419. The 1,740bp region encompassing the *nupG* gene on the complementation plasmid p*nupG* is indicated.

1,899,146 of SAUSA300_1716, a pyrimidine nucleotide-disulfide oxidoreductase. Two additional intergenic mutations were also found in NE650; a 69bp duplication at position 2,699,017, and an A to T substitution at position 1,844,130 which also present in NE1419. It is difficult to envisage how these mutations could impact resistance, and when combined with our complementation data would appear not to be relevant to our observed phenotypes.

4.4.3 NE650 and NE1419 continue to express heterogeneous resistance to β-lactams. NE650 and NE1419 carry mutations in the purine salvage pathway. Nucleoside transporters allow the bacteria to utilise exogenous purines to by-pass the energy expensive process of *de novo* synthesis, and nucleoside phosphorylases carry out the first processing step for imported purines, as well as recycling internal nucleosides back into the general nucleotide pool. Growth curves performed on both mutants demonstrated that the transposon insertions in the purine salvage pathway did not significantly affect growth (Fig. 4.3A).

Mutations identified through whole genome sequencing of heterogeneously resistant MRSA strains that underwent selection for homogenous resistance, have been found in both the *de novo* and salvage pathway [114], [144]. In these homogenously resistant (HoR) strains, the entire population displays resistance to β-lactams, unlike the typically heterogeneously resistant (HeR) strains where the majority of individuals are killed off upon antibiotic exposure. To characterise the resistance phenotypes of NE650 and NE1419, population analysis profiles were carried out (Fig. 4.3B).

These experiments revealed that although the both mutants are more resistant to β-lactams, they retain the heterogeneous susceptibility population profile of the wild type parent. The healthcare-associated MRSA strain BH1CC was used as a control for homogenous resistance (Fig. 4.3B). Thus, these data show that the increased β-lactam resistance in the *deoD* and *nupG* mutants can be considered a different phenotype to the homogeneous resistance associated with accessory mutations in other purine metabolism genes, such as *guaB*, *guaA*, *prsA*, *hpt*, *gmk*, *purB* and *relA*.

Table 4.4. Susceptibility of purine metabolism mutants to cefoxitin (FOX) & oxacillin (Ox)

Strain	Mutation	Fox (mm)*	Ox MIC (µg/ml)**
JE2	Wild type	10	32
NE650	*deoD*	6	128
NE1419	*nupG*	6	256
NE280	*pbuX*	10	32
NE283	*pbuG*	10	32
NE353	*purH*	13	32
NE424	*pdp*	10	32
NE477	*deoD*	10	32
NE494	*purQ*	11	11
NE522	*purB*	11	11
NE544	*nupC*	10	32
NE581	*purF*	11	64
NE622	putative *nupC*	10	32
NE744	*purK*	11	64
NE750	*purD*	11	64
NE932	nucleosidase	10	32
NE1101	*purM*	11	64
NE1134	*purS*	12	64
NE1237	*purR*	10	32
NE1331	*ndk*	9	32
NE1407	*pyk*	10	32
NE1464	*purL*	10	64
NE1583	nucleosidase	10	32
NE1637	hydrolase	9	32
NE1785	*purN*	10	64

* Cefoxitin (FOX) susceptibility was determined by disk diffusion assays on MH agar and expressed as zone diameters (mm) around FOX (30 µg/ml disks).
** Oxacillin (Ox) minimum inhibitory concentration (µg/ml) was determined by agar dilution.

To further investigate the role of *mecA* in *deoD* and *nupG*-mediated β-lactam resistance, phage 80α was used to transduce the transposon mutations into the laboratory MSSA strain, 8325-4. PCR was used to confirm transduction of the *deoD*::Tn and *nupG*::Tn alleles into 8325-4. Mutations in *gdpP* have been shown to increase MSSA tolerance towards oxacillin[311]. Wild-type 8325-4 was highly susceptible to cefoxitin and the *deoD* or *nupG*

mutations had no effect on this susceptibility as evidenced by measurement of zone diameters around cefoxitin (30µg) disks (Supp. Fig. 6.3). These data indicate that disruption of *deoD* or *nupG* is insufficient to increase β-lactam resistance, independent of *mecA.* Additionally, tolerance to oxacillin challenge (defined as a ≤90% drop in viability after a 6hr challenge with oxacillin 12.5 µg/ml[15],[35]) was also unaffected in 8325-4 and its *deoD* and *nupG* mutants (Fig 4.3C). Apparently, disruptions in purine metabolism homeostasis, and in particular purine salvage, are accompanied by increased β-lactam resistance in *mecA*-positive *S. aureus* backgrounds only. However, it is noteworthy that 8325-4 is RsbU-deficient, which may affect the cells response to environmental stresses such as antibiotic exposure. Further work is required to characterise the effects of *deoD* and *nupG* mutations in clinical MSSA isolates. RT-qPCR revealed that the transcriptional activity of *mecA* was also similar in NE650 or NE1419 compared to JE2 (Fig. 4.3D), suggesting that the increased resistance to β-lactams exhibited by strains with mutations in purine metabolism genes was not associated with increased *mecA* expression. These data indicate that although *mecA* is required for the high-level resistance expressed by NE650 and NE1419, the mutations themselves do not affect expression of *mecA*.

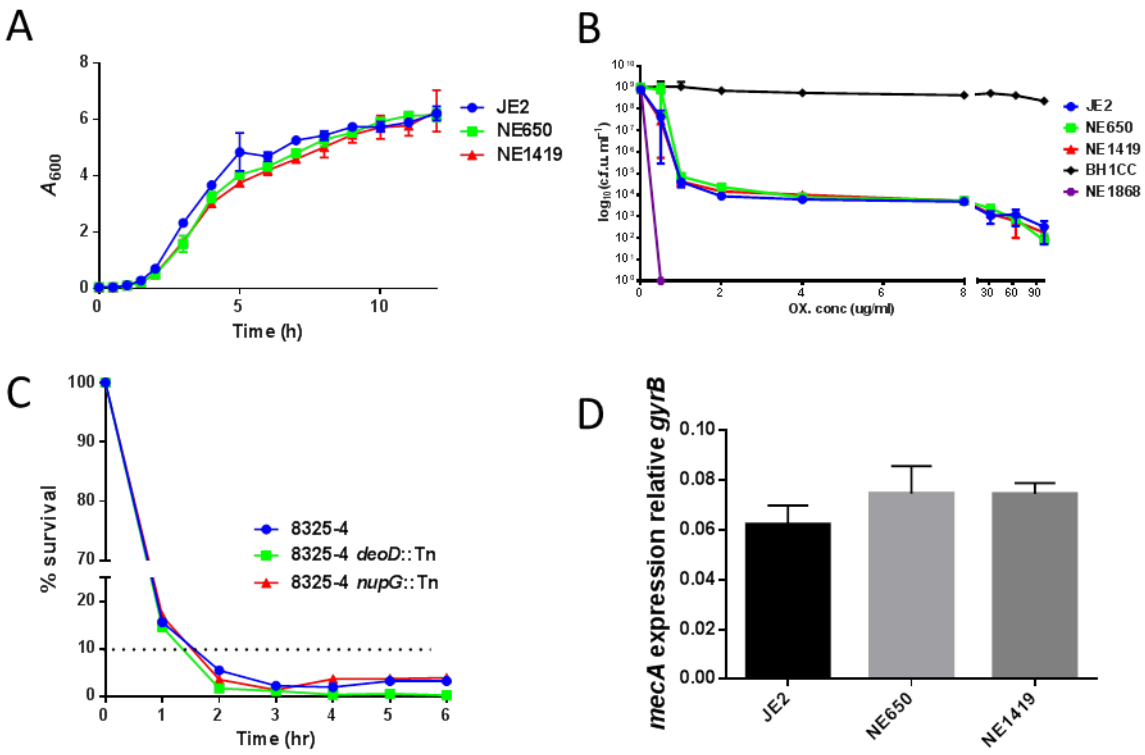

Figure 4.3. Increased β-lactam resistance in NE650 and NE1419 does not affect growth and is *mecA*-independent. A. Growth of JE2, NE650 and NE1419 in BHI at 37°C. Culture aliquots were collected every hour for 12 hours and cell density measured at A_{600}. Experiments were repeated three times and standard error of the mean is shown. **B.** Population analysis profile of JE2, NE650 and NE1419 grown in BHI media to exponential phase. Cultures were standardised and serially diluted before plating onto BHI agar supplemented with increasing concentrations of oxacillin for enumeration of survivors. Experiments were repeated three times and error bars denote the standard error of the mean. Homogenously resistant strain BH1CC and NE1868 (*mecA*::Tn) were included as positive and negative controls, respectively. **C.** 8325-4, 8325-4 *deoD*::Tn and 8325-4 *nupG*::Tn were grown to mid-exponentail phase in BHI broth and treated with oxacillin 12.5µg/ml. Survival was assessed by CFU enumeration every hour thereafter, and percentage survival of CFUs before treatment shown. Dotted line indicates tolerance threshold. Data representitive of three independent experiments. **D.** Comparison of *mecA* transcription by LightCycler RT-qPCR in JE2, NE650 and NE1419 grown to mid-exponential phase in BHI broth. Experiments were repeated at least three times and standard error of the mean shown.

4.4.4 Increased β-lactam resistance in *deoD* and *nupG* mutants appears to be independent of c-di-AMP. Mutations in the c-di-AMP phosphodiesterase gene *gdpP,* which result in the accumulation of c-di-AMP, have been associated with high-level β-lactam resistance in MRSA[111], [326]. Interestingly c-di-AMP is linked to purine metabolism because it is synthesised by the condensation of two molecules of either ADP or ATP[327]. Given that c-

di-AMP is synthesised from products of the purine metabolic pathway, we investigated whether increased levels of c-di-AMP might correlate with increased resistance in NE650 and NE1419.

To do this, we took advantage of several known phenotypes associated with altered c-di-AMP levels and used these as a surrogate for biochemical quantification of the metabolite itself. Corrigan *et al.* reported a 13% decrease in cell size in a *gdpP* mutant compared to its parent[111]. Using scanning electron microscopy, cells from JE2, N650 and NE1419 cultures grown in BHI or BHI supplemented with 0.5µg/ml oxacillin were not shown to be significantly different (Fig. 4.4A & B). Furthermore, increased c-di-AMP levels have been associated with decreased tolerance to osmotic stress in *S. aureus*, and other bacteria including *B. subtilis* and *Lactococcus lactis*[124], [328]. C-di-AMP binds to the potassium ion transporter, KtrA, inducing hypersensitivity to osmotic stress[124], [326]. Salt tolerance assays performed on JE2, NE650 and NE1419 revealed no differences in tolerance, whereas the *ktrA* (NE788) mutant, which was used as a control, exhibited significantly decreased salt tolerance (Fig. 4.4C). These data suggest that c-di-AMP homeostasis is being maintained in the *nupG* and *deoD* mutants and that increased β-lactam resistance is not dependent on this secondary messenger (Fig. 4.4C). However quantitative measurements of c-di-AMP in the mutants are required to confirm this conclusion.

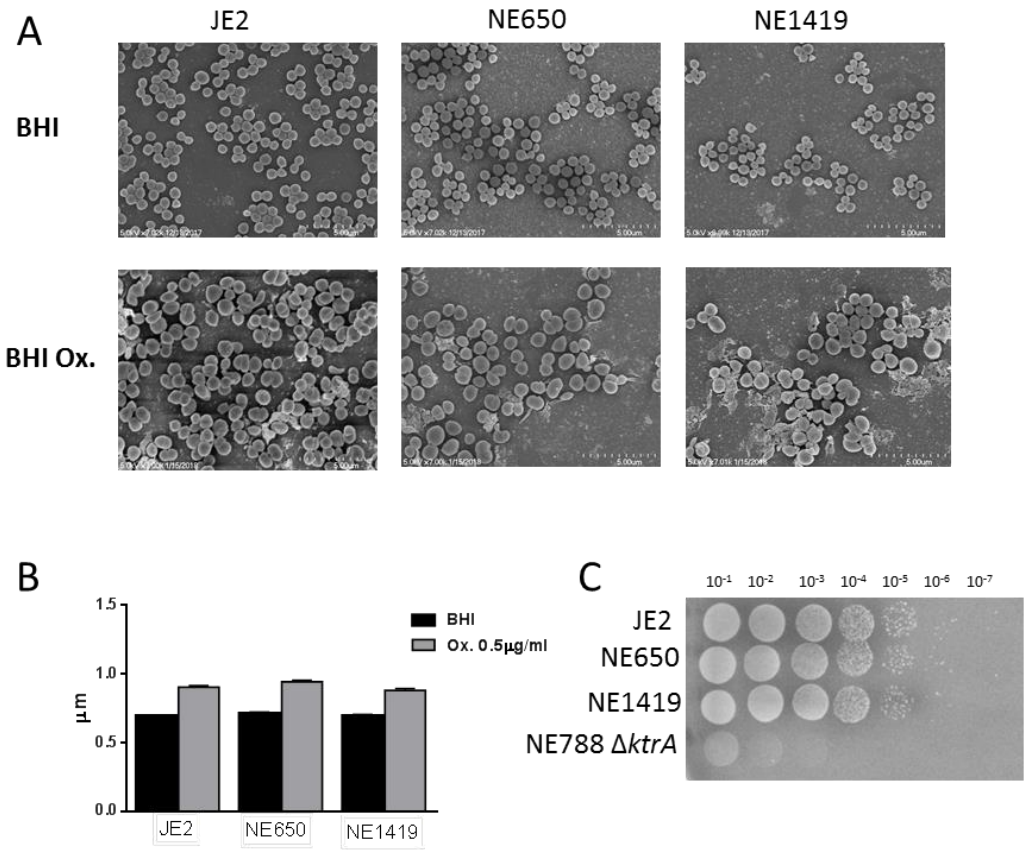

Figure 4.4. Salt tolerance and SEM imaging indicates that increased β-lactam resistance in NE650 and NE1419 is independent of c-di-AMP levels. A. Representative images of JE2, NE650 and NE1419 using SEM magnified X7,000. **B.** Cell sizes of JE2, NE650 and NE1419 grown for 24 hours in BHI and BHI supplemented with oxacillin 0.5µg/ml. Cell size measured using ImageJ software. Error bars indicate standard deviation of 50 independent cell measurements. **C.** NaCl tolerance of JE2, NE650, NE1419 and NE788 (*ktr*::Tn) grown on BHI agar supplemented with 2.2M NaCl. Representative results from three independent experiments are shown.

4.4.5 Guanine depletion in chemically defined media increases oxacillin resistance. In the purine salvage pathway, exogenous guanosine is converted to its nucleotide base, guanine, by DeoD, and then ribosylated and phosphorylated by nucleotide phosphoribosyltransferases to generate guanosine monophosphate (GMP). The increased oxacillin resistance of the *deoD* mutant suggests that disrupted nucleoside/nucleotide conversion increases β-lactam resistance. We reasoned that exogenous guanine in the media may be transported into the cell and bypass the enzymatic step absent in the *deoD* mutant. To investigate this possibility, oxacillin MICs were measured in chemically defined media (CDM), or CDM lacking guanine (Table 4.5).

The oxacillin MIC in CDM for JE2 and NE650 was 0.25μg/ml, NE1419 displayed increased resistance at 4μg/ml (Table 4.5). This would indicate that a *deoD* mutation does not increase β-lactam resistance under these conditions, unlike in MH media. Surprisingly, when guanine is removed from the CDM media the MIC of all three strains was increased (Table 4.5). JE2 has an MIC of 32μg/ml, while NE1419 displays an increase from 4μg/ml to 64μg/ml (Table 4.5). NE650 had only a small increase to 1μg/ml. These data would suggest that removing guanine from the CDM is increasing resistance to oxacillin, under these conditions.

The initial aim of this experiment was to by-pass the metabolic blockage caused by *deoD* disruption and restore wild type resistance levels. However, in CDM NE650 appears to be to be as susceptible to oxacillin as JE2. NE1419 maintained its increased-resistance phenotype compared to JE2. In this preliminary data, it appears that the presence of guanine, in CDM, is attenuating β-lactam resistance in JE2.

Table 4.5. Oxacillin MIC of JE2, NE650 and NE1419 in CDM with and without guanine

	CDM*	-Gua*	Fold increase
JE2	0.25	32	8
NE650	0.25	1	2
NE1419	4	64	4

*Oxacillin MIC (μg/ml) was determined using broth microdilution in chemically defined media (CDM) and CDM lacking guanine (-Gua).

To investigate if these changes in susceptibility were related to changes in growth, JE2, NE650 and NE1419 were grown in CDM and CDM-Gua and cell density measured over 12 hours. Comparatively, growth in CDM and CDM-Gua was similar (Fig. 4.5), with all strains reaching a slightly higher cell density in CDM-Gua compared to CDM (Fig. 4.5) indicating that guanine has a small but measurable effect on growth.

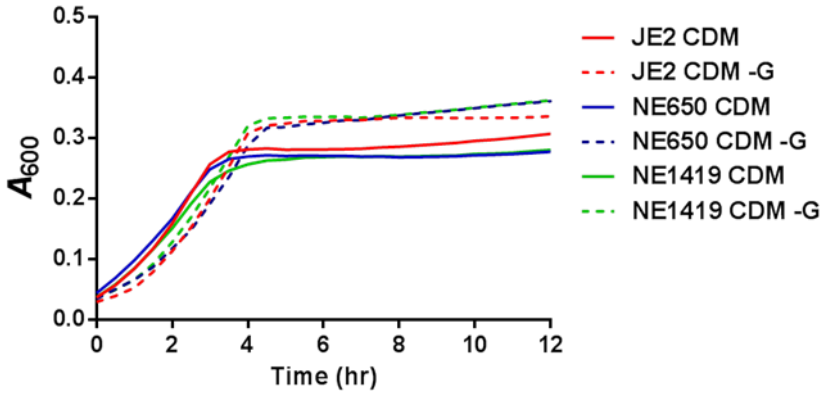

Figure 4.5. Growth of JE2, NE650 and NE1419 in CDM was not affected by the absence of guanine. Overnight cultures were used to inoculate CDM and CDM lacking guanine (-G) in a 96-well plate and cell density was measured every 30mins for 12 hours, at A_{600}. Growth curves are representative of 3 independent experiments.

These preliminary data suggest that removing guanine from the growth media reduces β-lactam resistance. This conclusion appears to be consistent with previous studies showing that mutations in the purine metabolic pathway also increase resistance to β-lactams in MRSA [114], [144] and suggests that disruption of purine biosynthesis or guanine depletion in growth media have similar effects, leading to increased β-lactam resistance. The NE1419 MIC of 4µg/ml in CDM compared to JE2 MIC of 0.25µg/ml in CDM (Table 4.5) supports the idea that NupG is not the only purine transporter in *S. aureus* but that *nupG* is the primary guanine importer. Additional experiments with a mutant with all purine transporters disrupted may further increase the MIC in these conditions. The decreased MIC observed in NE650 in CDM lacking guanine is difficult to explain. Data published from our group revealed that the *pur* biosynthetic operon is strongly repressed by oxacillin exposure[329], suggesting that nucleotide biosynthesis may be impaired in the *deoD* mutant grown in CDM lacking guanine when also exposed to oxacillin. In contrast other permeases may substitute for the absence of *nupG*, to transport other exogenous purines such as adenine. Regardless, guanine appears to increase MRSA susceptibility to oxacillin when grown in CDM. Future experiments to measure the effect of guanine on oxacillin susceptibility in Mueller Hinton media remains to be determined. However, this preliminary data showing that guanine can sensitive MRSA to oxacillin warrants further research. If manipulating the purine

biosynthetic pathway can result in cells being unable to express high-level resistance, this could be a novel treatment option for MRSA infections.

4.4.6 Mutations in *nupG* and *deoD* are associated with reduced intracellular nucleotide concentrations. Ultra-performance liquid-chromatography-multiple reaction monitoring mass spectronomy (UPLC-MRM/MS) performed by MtoZ Biolabs, Boston, was used to compare the intracellular concentrations of phosphorylated nucleotides in NE650, NE1419 and JE2. Cells were grown to mid-exponential phase in BHI broth, before being washed three times and flash frozen in liquid nitrogen. Metabolite concentrations were standardised against total protein concentrations in each cell sample. All phosphorylated nucleotide derivatives were significantly reduced in NE650 compared to JE2 (Fig. 4.6). Nucleotide concentrations were also reduced in NE1419, but these reductions only reached significance in the cases of ADP, GDP, GTP and XMP (Fig. 4.6).

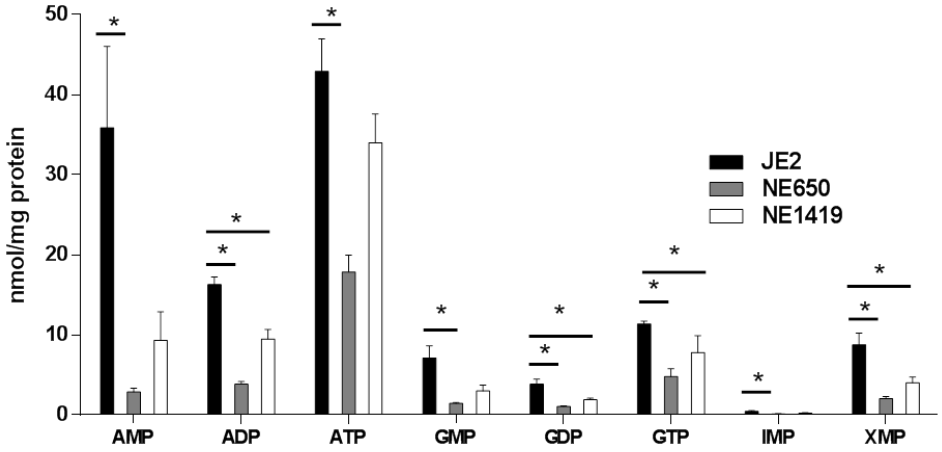

Figure 4.6. UPLC-MRM/MS quantification of nucleotide derivatives in JE2, NE650 and NE1419. Overnight cultures of JE2, NE650 and NE1419 were subcultured to A_{600} 0.05 in 50ml BHI broth and grown to mid-exponential phase. Cells were washed 3 times in cold PBS and flash frozen in liquid nitrogen for analysis. Standard error of the mean represents 3 independent experiments. Asterisks indicate statistical significance (p <0.05).

The significant reduction in AMP and GMP levels in NE650 compared to JE2 suggests that the purine salvage pathway is being disrupted in the *deoD* mutant and that this enzyme is important in maintaining purine nucleotide homeostasis. Furthermore decreased GTP levels in NE650 may indicate that CodY is activated in exponential phase; CodY-mediated repression is normally maintained until stationary phase when GTP concentrations are

reduced[330]. The overall reduction in intracellular nucleotides in NE1419 supports the predicted function of NupG as a purine transporter. Interestingly, *S. aureus* is predicted to possess two additional purine transporters, PbuG and PbuX. In *B. subtilis*, PbuG and PbuX are specifically involved in the transport of hypoxanthine/guanine and xanthine, respectively[42],[43], which may be able to compensate, at least in part, for the loss of NupG in NE1419, as has been proposed in *B. subtilis*[109]. However, mutations in the *pbuG* or *pbuX* genes had no impact on susceptibility to oxacillin (Table 4.4) suggesting that NupG is the primary nucleoside transporter in *S. aureus* or that specific purine nucleoside(s) transported by NupG (and not PbuG or PbuX) are particularly important in the β-lactam resistance phenotype. In any event, these data suggest that disrupted purine transport in the *nupG* mutant or impaired nucleoside phosphorylation in the absence of DeoD causes a decrease in the intracellular purine pool, which triggers expression of high-level oxacillin resistance. More specifically, reduced intracellular GTP may drive a CodY-mediated change in gene expression similar to that observed during the stringent response (Fig. 4.7), which is known to increase resistance to β-lactams. The stringent response results in the accumulation of ppGpp in a RelA-mediated reaction for which GTP is a substrate. RelA-catalysed ppGpp synthesis consumes GTP, which links it to GTP-controlled activity of CodY (Fig. 4.7). Mutations in *deoD* and *nupG* may mimic this process, by-passing ppGpp synthesis, and reducing GTP levels by inhibiting the purine salvage pathway (Fig. 4.7). Future experiments should characterise CodY activity in NE650 and NE1419. In general terms, these data are consistent with the existing literature linking purine metabolism, the stringent response, and a reduction in intracellular GTP with increased β-lactam resistance [114], [135], [143], [144].

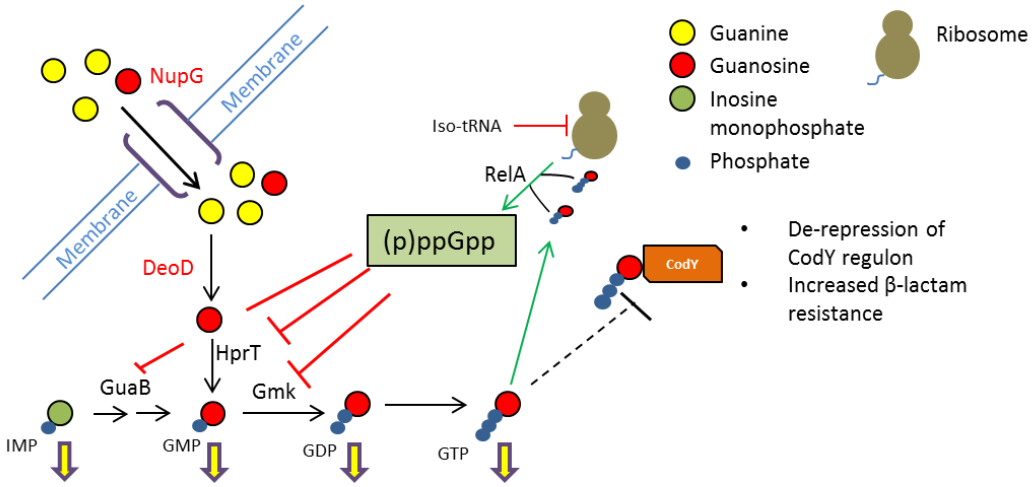

Figure 4.7. The stringent response and purine metabolism are intricately linked via intracellular levels of GTP. The stringent response is triggered by RelA sensing a lack of charged, branched-chain amino acid-tRNA at the ribosome. RelA rapidly synthesises the alarmone (p)ppGpp, depleting intracellular GTP. (p)ppGpp further reduces GTP levels by inhibiting GuaB, Gmk and HprT (Hpt), attenuating purine *de novo* synthesis and salvage pathways. A decrease in intracellular GTP reduces GTP-dependent CodY repression of stringent response genes, resulting in increased β-lactam resistance. Disruption of *nupG* and *deoD* causes a decrease in GTP levels which may cause a similar derepressing effect on the CodY regulon, impacting β-lactam resistance.

4.5 Discussion

The purine metabolic pathway is a vital source of nucleotides for all organisms. GTP and ATP generated from *de novo* synthesis or via the salvage pathway supplies the cell with precursors for DNA and RNA synthesis and maintenance. Furthermore, a rising area of interest is the fate and targets of nucleotide secondary messengers such as c-di-AMP and (p)ppGpp. Through a rigorous screen of the Nebraska Transposon Mutant Library, two mutants with increased resistance to β-lactams were identified. NE650 contains a transposon insertion in *deoD*, which encodes a nucleoside phosphorylase in the purine salvage pathway. DeoD converts nucleosides to nucleotides, which are then converted to their respective monophosphate nucleotides via phosphoribosyltransferases. NE650 exhibits increased resistance to cefoxitin and a 2-fold increase in the oxacillin MIC (128μg/ml) compared 32μg/ml parent strain of the NTML, JE2 (Fig. 4.1A&B). This phenotype was successfully complemented with a plasmid-borne *deoD*, and transferred by transduction with the *deoD*::Tn allele into the USA400 strain MW2.

Extension of our analysis to other purine metabolism genes potentially involved in β-lactam resistance identified NE1419, in which the oxacillin MIC was also significantly increased to >256μg/ml (Fig. 4.2A). NE1419 contains a transposon insertion in SAUSA300_0631, which encodes a predicted protein with significant homology to NupG, a guanosine transporter in *B. subtilis*. Wild type resistance levels were restored to NE1419 by complementation with a functional *nupG* gene, and the increased resistance phenotype was transduced with the *nupG*::Tn allele to MW2.

NE650 and NE1419 did not display an altered growth pattern compared to JE2 in BHI media (Fig. 4.3A), indicating that under the growth conditions tested, the salvage pathway is not essential for normal growth. Mutations in the purine metabolic pathway have been previously linked with a homogenous β-lactam resistance towards β-lactams in MRSA[114], [144]; however population profiling showed that this was not the case in NE650 and NE1419 (Fig. 4.3C). Although both mutants exhibit increased MICs towards oxacillin, they retain a heterogeneously resistant population.

mecA-independent oxacillin resistance has previously been reported in a COL *mecA* mutant background by Banerjee *et al.* [323]. More recently, clinical isolates lacking *mecA,* that express phenotypic resistance to β-lactams, have also been reported[332]. Interestingly some of these *mec*-negative β-lactam resistant clinical isolate contain frameshift mutations

in *gdpP*. To investigate the impact of *deoD* and *nupG* mutations on β-lactam susceptibility in a *mecA*-negative background, the alleles were transduced into the MSSA strain 8325-4, but no change oxacillin tolerance (Fig. 4.3C) or cefoxitin resistance (Supp. Fig. 6.3) was observed. Despite the requirement of *mecA* for expression of elevated β-lactam resistance in NE650 and NE1419, real-time RT-qPCR of *mecA* transcription revealed no differences between the mutants and JE2, suggesting that increased expression of *mecA* was not responsible for the phenotype (Fig 4.3D).

The secondary messenger c-di-AMP is synthesised via the condensation of ADP or ATP by the diadenylate cyclase DacA[327]. Mutations in *gdpP*, a phosphodiesterase responsible for degrading c-di-AMP have been associated with increased β-lactam resistance, decreased cell size, and reduced NaCl tolerance[12],[40]. The impact of *deoD* or *nupG* mutations on NaCl tolerance and cell size, as indicators for increased levels of c-di-AMP, was assessed. Using scanning electron microscopy, no change in cell size was observed in NE650 and NE1419 grown in either BHI or BHI supplemented with oxacillin 0.5µg/ml, when compared to JE2 (Fig. 4.4A & B). Similarly, NaCl tolerance was unaffected in both mutants compared to JE2 (Fig. 4.4C). These data suggest that the increased oxacillin MIC of the mutants is independent of c-di-AMP levels. To confirm this hypothesis, quantitative measurements of c-di-AMP are needed in the future.

Using CDM, we attempted to by-pass the *deoD* mutation in NE650 to restore wild type levels of oxacillin resistance. In doing so we observed an overall increase in oxacillin MIC in JE2, NE650 and NE1419, in CDM lacking guanine compared to CDM (Table 4.5). This preliminary data would suggest that exposing cells to exogenous guanine can sensitise MRSA to oxacillin. This data appears to conform to earlier research showing that an overall reduction in guanine metabolism is associated with increased β-lactam resistance[114], [144]. The reduction in oxacillin MIC in NE1419 from 64µg/ml in CDM to 4µg/ml in CDM-Gua suggests that NupG is a primary purine transporter in *S. aureus*, and that impaired guanine transport in the *nupG* mutant may protect against guanine-induced sensitization to oxacillin. Further studies are needed to more fully understand the effects of CDM media on purine metabolism and the therapeutic potential of guanine to sensitise MRSA to β-lactam antibiotics.

The relationship between purine metabolism and β-lactam resistance was also evident in our previous report that the *pur* biosynthetic operon was the most down-regulated gene cluster in USA300 under stress induced by sub-inhibitory oxacillin [329]. This observation suggested that exposure of MRSA to oxacillin resulted in repression of *de novo* purine biosynthesis, which in turn is also likely to reduce intracellular concentrations of purines and increase the importance of the purine salvage pathway for DNA and RNA synthesis. UPLC-MRM/MS revealed a large reduction in nucleotide concentrations in both the *deoD,* and to a lesser extent, *nupG* mutants (Fig. 4.6). The less significant effect of the *nupG* mutation on intracellular nucleotides suggests that alternative permeases PbuG and PbuX can still transport exogenous purines into the cell. In *B. subtilis* NupG imports guanosine but an alternative purine transporter, NupN, is also present [109]. There is no NupN homologue in *S. aureus*, which may suggest a more important role for NupG for guanosine uptake in this bacterium. Our observation that β-lactam susceptibility is unaffected in the NE280 (*pbuG*) and NE283 (*pbuX*) mutants may also support the idea that NupG is the primary purine transporter and explain why the *nupG* mutation leads to a downstream reduction in GTP levels (Fig 4.7). Further experimental evidence is required to support this model.

Purine metabolism has become an area of considerable interest in the study of β-lactam resistance in MRSA. Several genes within both the salvage and *de novo* purine pathway have been implicated in high-level and homogenous β-lactam resistance[114], [132], [133], [143], [144], [329]. The data presented here is the first report implicating *deoD* and *nupG* in this phenotype. A common finding in the data presented here and in previous studies is the decreased GTP production, either through mutations in biosynthetic genes or via activation of the stringent response which consumes GTP for synthesis of ppGpp (Fig 4.7). In *B. subtilis* the stringent response and the metabolic repressor CodY constitute two overlapping regulatory systems, regulated by the presence of either GTP or branched-chain amino acids[306]. In *S. aureus,* CodY has been shown to modulate the expression of over 200 genes during exponential growth, primarily acting as a repressor[73]. In *S. aureus*, activation of the stringent response leads to increased expression of *mecA*[45],[49], via a mechanism that remains unclear. Several factors influencing purine metabolism have been implicated, along with a decrease in intracellular GTP levels. Here we have provided further evidence that perturbations in this pathway increase oxacillin resistance in MRSA. Further work is required

to fully understand how *deoD* and *nupG* mutations impact intracellular nucleotide pools, and how these changes are influencing the β-lactam resistance.

Chapter 5

5.1 Overall Conclusions

Staphylococcus aureus represents a real and ever-present threat in both clinical and community settings. The commensal nature of this opportunistic pathogen, and *S. epidermidis* means that the risk of human infection is constantly present, particularly for vulnerable individuals in healthcare settings. The rise and spread of antibiotic resistance has led to complications when treating infections that were otherwise treatable in the past. With this in mind, the need to understand the mechanisms by which infections are caused is more important than ever, so that new treatment strategies can be developed.

5.1.1 The Rbf-SarR regulatory pathway is a novel mechanism of biofilm regulation.

For *S. epidermidis,* the primary virulence factor is the capacity to colonise indwelling-medical devices via the production of biofilm. Once cells are encased within a mature biofilm antibiotic treatment is no longer effective, frequently necessitating removal of infected medical devices. Biofilm formation on artificial heart valves and prosthetic joint implants typically requires surgical intervention[333], increasing treatment costs and risks to patients. *In vitro,* biofilm formation can be induced by a number of environmental stresses such as pH, osmolarity and ethanol. Here we have described a novel regulatory pathway induced by high levels of NaCl.

The *ica* operon encodes the machinery that synthesises polysaccharide intercellular adhesion (PIA) / poly-N-acetylglucosamine (PNAG), a major component of the *S. epidermidis* biofilm matrix[178]. The divergently transcribed *icaR* gene encodes the preeminent repressor, which in *S. aureus* is repressed by the transcriptional regulator, SarX[233]. *S. aureus* SarX is activated by a second transcription factor, Rbf[233]. In *S. epidermidis* an *rbf* mutant exhibits an impaired biofilm-forming phenotype. However, Rbf itself does not bind to the upstream promoter region of *sarX* or the *ica* operon. Electrophoretic mobility shift assays (EMSAs) revealed that recombinant Rbf did bind to the upstream promoter region of another member of the Sar family, *sarR*. Furthermore, purified SarR was shown to bind to the promoter region of the *ica* operon.

Our evidence indicates that Rbf regulates *ica* expression via *sarR*. To confirm this hypothesis, a *sarR* mutant was generated and found to have increased biofilm forming capacity compared to the wild type, suggesting that SarR is a negative regulator of biofilm.

Transcription analysis showed that *icaADBC* expression was negatively regulated by SarR, which itself was repressed by Rbf. These data identify Rbf as a positive regulator of the *ica* operon, which represses transcription of the negative regulator, *sarR*.

Several transcription factors are known to regulate biofilm under varying environmental conditions in *S. epidermidis*, including *icaR, sigB* and *sarA*. The regulatory pathway described here relies on SigB-dependent repression of *icaR* caused by osmotic stress in the media. This may suggest that the Rbf/SarR regulatory pathway is utilised for fine-turning *ica* expression in relatively high-salt environments such as human skin which is a natural habitat for these bacteria. Although speculative, it is possible that dry, high-salt environments such as the human epidermis promote *ica*-dependent biofilm formation in *S. epidermidis* via SigB repression of *icaR*. Rbf-mediated repression of *sarR* then increases *ica* expression resulting in the synthesis of PIA. The polysaccharide matrix may serve to sequester water in an otherwise dry environment, aiding in the survival of *S. epidermidis*.

Several questions remain with regards this hypothesis. It is unclear what factors promote the expression of *rbf* and what other genes are under its control in *S. epidermidis.* Furthermore, in *S. aureus* an *rbf* mutant had reduced survival in a murine catheter infection model[241]. The role of *rbf in vivo* has not yet been examined in *S. epidermidis*. Future work should focus on understanding what signal activates *rbf* expression. If this regulator can be targeted and switched off, then it may be possible to attenuate *S. epidermidis* biofilm formation, reducing risk to vulnerable patients requiring the insertion of medical devices.

Succinyl-CoA synthetase plays an important role in promoting β-lactam resistance in MRSA.

β-lactam resistance in *S. aureus* clinical isolates emerged shortly after the introduction of penicillin in the 1940s[334]. Since then the introduction of all new anti-staphylococcal drugs has been accompanied by the co-evolution of resistance. Understanding how antibiotic resistance occurs at a molecular level allows the development of more strategic treatment options to improve patient outcomes and mitigates against the emergence of new resistance mechanisms.

The TCA cycle functions to produce biosynthetic precursors, reducing potential and energy. Through rigorous screening of the NTML we identified two mutants, NE1770 and NE569

containing a transposon insertion in both succinyl-CoA synthetase subunit α and β, respectively. Both mutants exhibited increased sensitivity to β-lactams. Succinyl-CoA synthetase converts succinyl-CoA to succinate. No other TCA cycle mutant exhibited increased sensitivity to oxacillin, indicating that there was a unique property associated with this TCA cycle enzyme.

One possibility that may link the TCA cycle and oxacillin resistance is that the accumulation of succinyl-CoA which may be promoting the uncontrolled succinylation of enzymes involved in cell wall biosynthesis. Succinylation as a form of post-translational modification (PTM) has only recently been described and has been found to occur abundantly in other pathogenic organisms[300], [303], [335]. Work is on-going to probe if a causal link exists between the *sucC* mutant and increased protein succinylation, and whether this succinylation may be affecting peptidoglycan biosynthesis. This exciting prospect opens the door to sensitizing MRSA to β-lactam antibiotics using compounds that target succinyl-CoA synthetase or succinylation generally within the cell. Cell wall composition analysis and proteomics targeting succinylated-peptide fragments will yield further mechanistic insights in the future.

Purine metabolism continues to be implicated in high-level, β-lactam resistance in MRSA.

In our NTML screen, we also identified two purine salvage pathway mutants that exhibit increased β-lactam resistance in JE2. *deoD* and *nupG* encode a purine nucleoside phosphorylase and nucleoside permease, respectively. Mutation of either gene significantly increased oxacillin resistance. Activation of the stringent response pathway and increased c-di-AMP levels in MRSA exposed to β-lactams has previously been implicated in the expression of high-level, homogenous resistance[111], [114], [121], [318]. However, mutations that increase oxacillin resistance prior to any antibiotic exposure have not been reported. Here we report that phosphorylated nucleotide levels are reduced overall, in both mutants, and that the increased resistance phenotype appears to be independent of both the stringent response and c-di-AMP levels.

It is not yet fully understood how β-lactam resistance is increased in strains with mutations impacting purine homeostasis. The alarmone ppGpp induces the stringent response by inhibiting specific purine biosynthetic enzymes such HprT and Gmk[132]. The reduction in

intracellular GTP reduces the activity of the repressor, CodY, thereby activating expression of many amino acid biosynthetic and transport genes. Thus, the reduction of GTP levels in NE650 and NE1419 may initiate the same response, independent of ppGpp levels. Furthermore, the oxacillin MIC of JE2 appears to increase when guanine is removed from chemically defined media, suggesting that reducing exogenous guanine availability similarly impacts GTP concentrations and β-lactam susceptibility. Future work will be needed to more fully understand the relationship between exogenous purines and β-lactam resistance. The observation that NE650 does not maintain an increased resistance phenotype compared to JE2 in CDM media highlights how purine metabolism as a whole is important to cell homeostasis.

This work raises several questions concerning purine metabolism and oxacillin resistance in MRSA. *deoD* appears to play an important role in β-lactam resistance in MH media but not CDM, without affecting overall growth. Furthermore, our preliminary data suggests that exogenous guanine may have the capacity to modulate β-lactam resistance. Data presented here, and from other groups[113], [114], [144], have shown that a reduction in purine metabolism facilitates high-level oxacillin resistance. Inferring from our data showing that removing exogenous guanine also promotes resistance in CDM, it may be possible to sensitise MRSA by increasing levels of exogenous guanine. A greater understanding of the purine metabolic pathway and the cross-talk between purine secondary messengers is required to explore this possibility.

Final remarks

Antibiotic resistance will continue to be a major treatment burden unless novel methods of targeting pathogenic bacteria become available. In order to achieve this, a full and comprehensive knowledge of resistance mechanisms is required. This would allow accessory factors contributing to resistance to be targeted, to induce sensitivity to existing or novel therapeutic agents. Understanding the mechanisms of β-lactam resistance in MRSA can be difficult due to its tendency to appear sensitive initially, and upon β-lactam exposure, acquire accessory mutations that facilitate high-level antibiotic resistance.

The resistance element, *mecA*, is mechanistically yet to be fully understood. β-lactam resistance via *mecA* is unusual compared to other resistance mechanisms. The presence of

mecA and its expression is not necessarily accompanied by high-levels of resistance. Instead *S. aureus* relies on accessory factors to work alongside PBP2a to confer increased resistance. The reasons why upregulating *mecA* expression in itself does not confer high-level resistance to oxacillin remains unknown. Unpublished data from our group has revealed that over-expression of *mecA* on a high-copy plasmid is lethal to cells; suggesting that high concentrations of PBP2a may be toxic, possibly by interfering with the activity of other penicillin-binding proteins during peptidoglycan biosynthesis. The SCC*mec* element is a foreign piece of DNA, thought to originate in *Staphylococcus sciuri*[336]. This raises the possibility that expression of this foreign element imposes a fitness cost *S. aureus* that in turn necessitates the acquisition of compensatory mutations to maintain cellular homeostasis.

Identifying accessory factors that contribute to *mecA*-dependent β-lactam resistance remains the most promising route to uncovering the mechanism, or mechanisms, underpinning β-lactam resistance in MRSA. We have contributed to this area of research by showing that purine metabolism and the TCA cycle can influence oxacillin resistance. Whether or not MRSA utilises multiple independent mechanism that can promote resistance remains, or whether there is a unifying element in these mechanisms remains to be uncovered.

The cell utilises intracellular GTP levels as a monitoring system for nutrient scarcity. A reduction in GTP can be caused by an increased in RelA-catalysed biosynthesis of ppGpp, which in turn, inhibits various enzymes in the GTP synthesis pathway. GTP concentrations can also be reduced by point mutations in purine metabolic pathway genes such as *gmk*, *hprT* and *guaB*[337]. When the stringent response is activated, reduced intracellular GTP levels serve to decouple CodY from its DNA binding sites, derepressing genes that cope with nutrient stress[73]. Perhaps one or more factors in the CodY regulon contribute to increased β-lactam resistance in concert with PBP2a. Functionally linking the CodY regulon and β-lactam resistance will require more extensive research.

Other research groups have shown that an increase in TCA cycle activity is required for the selection of homogenously resistant MRSA[79], [279]. However, the stringent response to amino acid limitation down-regulates central metabolism and cell division, making it difficult

to reconcile these two lines of research. It is possible that mutations induced by ROS in an upregulated TCA cycle occur in genes involved in GTP synthesis which in turn down regulates purine metabolism, resulting in increased resistance.

Ultimately, we do not know whether MRSA uses a single accessory factor, reached by multiple pathways to promote β-lactam resistance, or whether many independent pathways can lead to resistance. The data presented in this thesis have provided new insights on the mechanisms underpinning β-lactam resistance and the importance of continuing to investigate this phenotype in order to develop new therapeutic approaches for the treatment of infections caused by MRSA and other pathogens resistant to beta-lactams antibiotics.

Chapter 6

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Supplementary data

The TCA cycle enzyme succinyl-CoA synthetase influences resistance to β-lactam antibiotics in MRSA

Table 6.1. JE2 and NE569 MIC in trimethoprim/sulfamethoxazole (TMP/SMX) and ciprofloxacin (Cip).

	TMP/SMX*	Cip*
JE2	0.125/2.375	32
NE569	0.125/2.375	32

*****TMP/SMX and Cip minimum inhibitory (μg/ml) concentration was determined using broth microdilution assay.

Table 6.2. Cefoxitin (Fox) sensitivity and oxacillin (Ox) MIC of JE2, NE569, MW2 and MW2 *sucC*::Tn.

	Fox (mm)*	Ox. MIC (μg/ml)**
JE2	10	32
NE569	24	0.5
MW2	10	32
MW2 *sucC*::Tn	15	0.5

* Cefoxitin (FOX) susceptibility was determined by disk diffusion assays on MH agar and expressed as zone diameters (mm) around FOX (30 μg/ml) disks.
** Oxacillin (OX) minimum inhibitory concentration (μg/ml) was determined by agar dilution assay.

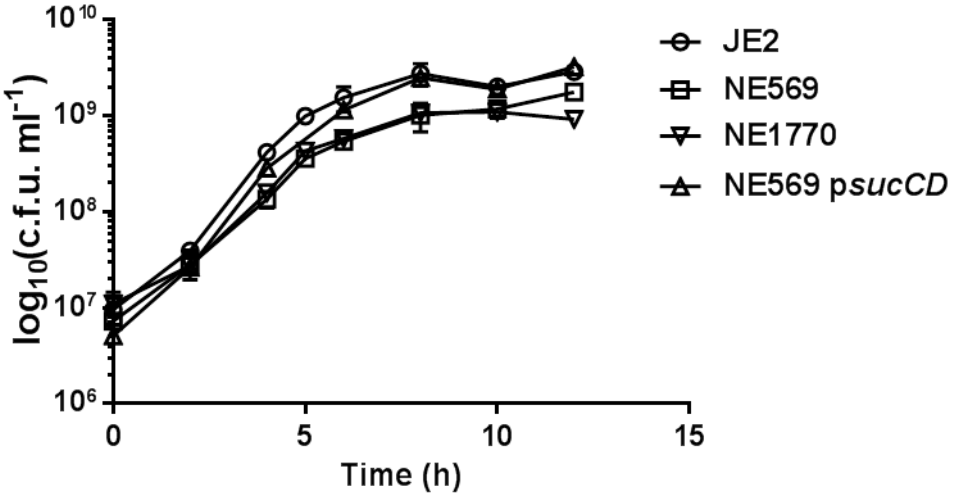

Figure 6.1. Growth of succinyl-CoA synthetase mutants is impaired in MH media. Growth of JE2, NE569 (*sucC*), NE569 p*sucCD* and NE1770 (*sucD*) in MH broth. Culture aliquots were collected every hour for 12 hours and CFUs enumerated. Growth experiments were repeated at least three times and standard error of the mean shown.

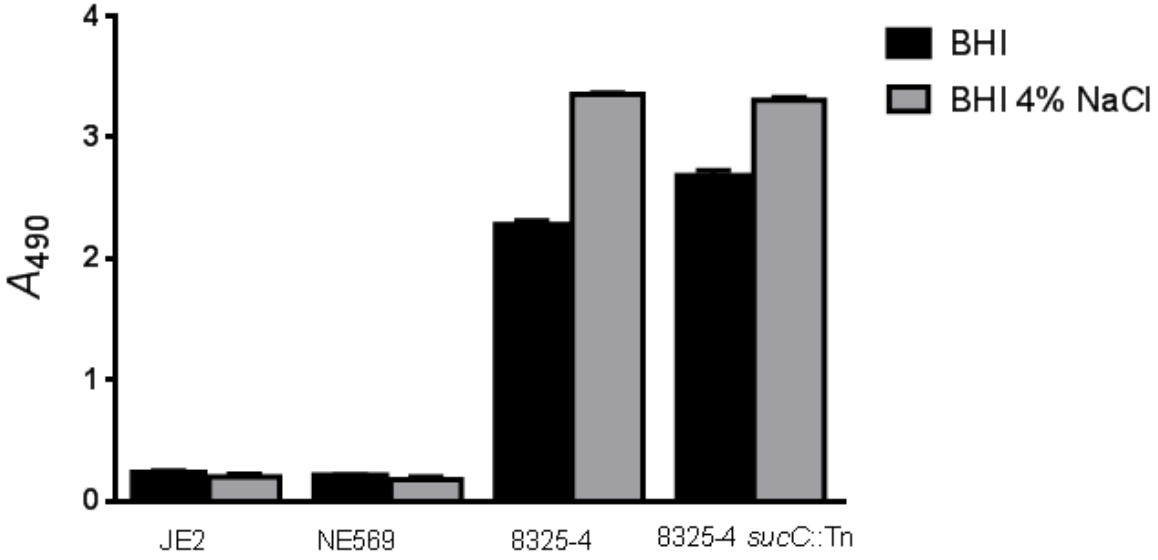

Figure 6.2. Mutation of *sucC* in JE2 or the MSSA strain 8325-4 does not impact biofilm formation. Comparative biofilm formation of JE2, NE569, 8325-4 and 8325-4 *sucC*::Tn grown for 24 h at 37°C in BHI in hydrophilic 96-well polystyrene plates. The density of crystal violet-stained biofilms was measured at A_{490}. Data presented are the means of three independent experiments ± standard error.

Table 6.3. Susceptibility of JE2, NE569 (*sucC*), NE547 (*sucA*), NE626 (*sdhA*) and the double mutants *sucC/sucA* and *sucC/sdhA* to cefoxitin (FOX) and oxacillin (OX)

	Fox (mm)*	Ox. MIC (µg/ml)**
JE2	11	32
NE569 (*sucC*)	24	0.5
NE547 (*sucA*)	10	32
NE626 (*sdhA*)	10	32
sucC/*sucA*	11	32
sucC/*sdhA*	24	4

* Cefoxitin (FOX) susceptibility was determined by disk diffusion assays on MH agar and expressed as zone diameters (mm) around FOX (30 µg/ml) disks.
** Oxacillin (OX) minimum inhibitory concentration (µg/ml) was determined by agar dilution assay.

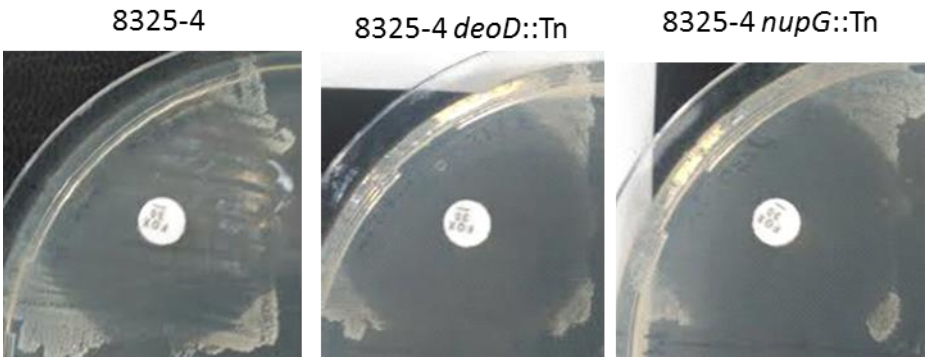

Figure 6.3. Transposon insertion into *deoD* and *nupG* in MSSA strain, 8325-4, does not increase cefoxitin tolerance. Susceptibility of 8325-4, 8325-4 *deoD*::Tn and 8325-4 *nupG*::Tn grown on MH agar to cefoxitin (FOX, 30µg disks).

Special dedication

To the hundreds-of-billions of bacteria, who selflessly sacrificed themselves to make this work possible.