The *X Factor(s)*: New antibiotic resistance mechanisms in community-associated methicillin resistant *Staphylococcus aureus*

A thesis submitted to the National University of Ireland, Galway
for the Degree of Doctor of Philosophy
by

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March 2017

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains typically express low-level heterogeneous (HeR) resistance from which high-level homogeneously resistant (HoR) mutants can be isolated following oxacillin exposure. The HoR phenotype typically requires both increased expression of the methicillin resistance gene *mecA*, carried on the *Staphylococcus* cassette chromosome SCC*mec*, and additional mutations elsewhere on the chromosome that have previously been associated with increased c-di-AMP and ppGpp levels. An oxacillin hyper-resistant mutant of the community associated MRSA strain USA300 was isolated from a chemostat culture grown over 13 days in increasing concentrations of oxacillin. This mutant, designated HoR34, was found to contain multiple tandemly amplified copies of SCC*mec* on the chromosome, associated with increased *mecA*-encoded penicillin binding protein 2 a (PBP2A) expression. This is the first report of SCC*mec* amplification as a driver of high level methicillin resistance in MRSA.

A screen of the Nebraska transposon mutant library revealed that mutation of *pgl* was accompanied by increased β-lactam resistance, independent of increased c-di-AMP levels. *pgl* encodes the second enzyme in the pentose phosphate pathway (PPP), 6-phosphogluconolactonase that converts 6-phosphogluconolactone to phosphogluconate. Disruption of the PPP may increase carbon flux through the glycolysis pathway, increasing the intracellular pool of fructose-6-phosphate enabling increased peptidoglycan biosynthesis following exposure to β-lactam antibiotics. Thus, these data suggest that increased β-lactam resistance is not solely reliant on increased c-di-AMP levels but can also be achieved by mutations in metabolic enzymes that enhance peptidoglycan biosynthetic capability.

In contrast to the *pgl* mutation, disruption of the alanine/serine/glycine permease gene, *cycA*, was shown to increase susceptibility to β-lactam antibiotics and the alanine analogue d-cycloserine (DCS). Impaired alanine uptake in chemically defined media (CDM) containing glucose was accompanied by increased β-lactam susceptibility. In contrast, neither alanine uptake nor β-lactam susceptibility was affected in CDM media, indicating an essential role for alanine transport in cell wall integrity and consequently β-lactam resistance. DCS-mediated inhibition of alanine racemase and D-alanine ligase activity in the *cycA* mutant was also shown to

aggravate the impact of impaired alanine uptake on β-lactam susceptibility under *in vitro* and *in vivo* conditions, indicating that DCS/oxacillin combinations offer an exciting new therapeutic option for the treatment of MRSA infections.

Declaration

This thesis is comprised of three manuscripts (in preparation or submitted for publication). I am the first author on all three papers. 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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Laura A. Gallagher

Author Contributions

Chapter 2. Tandem amplification of SCCmec can drive high level methicillin resistance in MRSA

Conceived and designed the experiments: LAG SC TD RF GTF JPOG.

Performed the experiments: LAG (Figure 2.1, Figure 2.2C, Figure 2.3 and Appendix 2), SC (Figure, 2.2, Illumina genome assembly and copy number variation detection using read coverage), NSB (assisted in SNP calling), PL (TEM sample preparation), BW (SCCmec amplification search of 404 S. aureus genomes), MW (MinION genome sequencing).

Analysed the data: LAG SC EMW, JPOG.

Wrote the paper: LAG JPOG.

The chemostat experiment was performed in close collaboration with Dr Gerard Fleming.

Chapter 3. β-Lactam Resistance in Methicillin-Resistant Staphylococcus aureus USA300 Is Increased by Inactivation of pgl gene.

Conceived and designed the experiments: LAG JPOG

Performed the experiments: LAG (all figures)

Analysed the data: LAG JPOG

Wrote the paper: LAG JPOG.

C-di-AMP measurements were performed in the Research Core Unit Metabolomics, Hannover Medical School, Hannover, Germany by Prof. Volkard Kaever.

Chapter 4. Using D-cycloserine and related drugs to overcome resistance to β-lactam antibiotics in MRSA

Conceived and designed the experiments: LAG EMW PDF JPOG

Performed the experiments: LAG (Figure 4.1 and 4.4, Tables 4.1 and 4.2, Appendix 4), EMW (Figure 4.5) CF (Figures 4.2 and 4.5) PDF (4.3 and Appendix 5 and 6)

Analysed the data: LAG EMW CC EON AK PDF JPOG

Wrote the paper: LAG JPOG.

Cell peptidoglycan analysis was performed by DSMZ GmbH (Braunschweig, Germany).

Acknowledgements

I would like to acknowledge and thank all the people that have made this thesis and PhD both possible and overall an enjoyable experience. I am forever grateful to you all.

Firstly I would like to thank my supervisor Prof. James P. O'Gara. The PhD has been a rollercoaster of emotions where your unwavering support and enthusiasm for the research has brought me through the most difficult times. Returning to studies and having the opportunity to work as a member of your research team has been one of my best experiences and I am eternally grateful for the opportunity.

Thank you to Dr. Ger Flemming and Dr. Conor O'Byrne for your valuable input at various GRC meetings. It has been a pleasure working with you both. In addition I would like to thank the Thomas Crawford Hayes Research Fund, for funding our LC-MS/MS and cell wall analysis. I have been fortunate to have collaborated with a number of research groups throughout my PhD and would like to express my gratitude to you all. Especially to Dr. Elaine Waters for our fruitful collaboration as well as your valuable advice and encouraging words throughout the project. To our collaborators Dr. Kirsten Dorries, Prof. Michael Lalk, and Prof. Paul Fey, thanks for your invaluable expertise and assistance with this project.

I can't express enough how grateful I am to the fantastic technical team in the Microbiology department. Thank you to Maurice, Ann, Mike, Katrina, Caroline and Seamus for all the chats, problem solving debates and fixing things over the years! A special thanks to Maurice for putting up with my OCD teaching ways.

I was fortunate to have worked alongside some truly talented individuals briefly in UCD and also in the new and old JOG lab at NUIG. To the YMCA girls in Dublin; Hannah, Elaine, Siobhan and Alyson, you are a great bunch of girls who know how to throw a welcome party. Even though it was a brief visit, you made the start of my PhD journey fun, thank you. To the first JOG lab group in NUIG, Hannah, Justine, Nikki and Colm, thank you for all the help and laughs in the lab. Hannah you have been an incredible support and your constant encouragement over the years has been truly amazing. Your valuable expertise set me off to a good start and I enjoyed working with you. Justine, thank you for your guidance and your experimental expertise during our 3 years working together. It was fun. Nikki we spent most of our time together near the end and thank you for your patience

regarding my OCD over everything. We made a good team and thank you for all the laughs and chats in the lab. I am lucky to call you all my friends and got the opportunity to enjoy many outings together. To the newbies, a big thank you to Andrea, Chris and Claire. Andrea, you are a tonic that has brought fun and laughter to the group. Chris, you have brought an element of calmness to the lab. It has been fun working with you and having the opportunity to share your musical tastes. Claire, I have enjoyed working with you, as you share the same level of detail for everything. Thanks guys for all the laughs, nights out, all the important coffee/tea breaks and help over the last two years. A sincere thank you to Chris and Claire for being my proof readers, an incredible help.

Thanks to Kerrie, Justine, Hannah, Beth, and Tara, 'The Spice Girls'. Thanks girls for being there, for the girlie nights in and out. Your friendship is truly appreciated. A big thank you goes to Kate, you have been a wealth of advice and support over the last year. To all my non-science friends, especially Catherine, Susan, Jessica and Michael, thank you for support, your home cooked dinners and cosy fires.

To my parents, John and Antoinette, thank you for being my pillar of strength. Dad you're never ending support and encouraging words has allowed me to accomplish this and I am eternally grateful. I want to thank you for all your endless hard work, your patience and sacrifices during my early years of college, you made this possible. Mum, your constant love and unyielding support has got me through the most difficult times, I appreciate all you have done. I couldn't have gotten through this project without my family, Denis, Shaun, Caroline, Brian, Declan and Darren, you all have been so patient, understanding and loving. Caroline and Declan, thank you both for lending your ear on many an occasion, you have been an unyielding support.

Finally, thank you to Philip, my rock. You have been a phenomenal support to me through this entire experience. You had to endure many moments of struggles. Your positivity, your encouraging words, your taxi service and most of all, your love has enabled me to achieve this. Thank you for always being there for me and I am so grateful for all your sacrifices you have made for me. This thesis would be impossible without your dedication. I may not say it enough but I am truly grateful.

To my parents John and Antoinette

Chapter 1:
Introduction

1.1. Medical significance

*Staphylococcus aureus*is a Gram-positive coccoid opportunistic pathogen, which is a leading cause of hospital-acquired infections' ranging from superficial skin and soft tissue infections to life threatening systemic infections (1, 2). *S. aureus* is one of the common commensals which colonise the anterior nares and the epithelial and mucosal surfaces of 30% of the population, with a further 60% being transiently colonised which is a known risk factor for infection of open wounds and intravenous catheters (3-5). In particular, immuno-compromised patients are at significantly greater risk of symptomatic secondary infections once the epithelial or mucosal layers are breached (6). The ability of *S. aureus* to cause a wide variety of infections is due to an array of virulence factors that help it evade host immune defences. These include microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (7), exotoxins, enterotoxins (8), leukotoxins, leukocidins and toxic shock syndrome toxin (9-11), as well as other secreted and cell surface-associated factors, which enable biofilm development that inevitably leads to *S. aureus* having a major impact on patient treatment and overall mortality. To further exacerbate the issue, the emergence of multi-drug resistance has added a relatively new complexity to the burden of *S. aureus* infections treatment (12). Since the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in 1961, the development of resistance to additional antibiotics is a growing concern. In many parts of the developed world, 40-60% of *S. aureus* hospital-acquired infection isolates are resistant to oxacillin and the number of vancomycin-resistant isolates are continually increasing (13, 14). As a result, these healthcare-associated infections (HAIs) of *S. aureus* and MRSA pose a major threat to hospital patients (15, 16). Both *S. aureus* and *S. epidermidis* are significant nosocomial pathogens and are frequently associated with infections of indwelling medical devices, by their abilities to form biofilms. The use of medical devices is increasing worldwide, with the advancement in medical treatments resulting in extended life expectancy of over 65's predicted to increase by 57-79% by 2021. Therefore, staphylococcal device-related infections are on the increase due to the increased reliance on indwelling medical devices over the past decade (17). There is growing recognition that device-related infections caused by biofilm-forming pathogens, the majority of which are staphylococcal species, place a major financial burden on the healthcare sector and

are a significant cause of morbidity and mortality in patients (15, 16). Furthermore, this problem has been exacerbated by the emergence of MRSA in the community setting.

1.2. Community-associated MRSA

The incidence of MRSA infections has greatly increased in recent years due to the emergence of community-associated MRSA (CA-MRSA). Unlike HA-MRSA, CA-MRSA typically causes infection among individuals who have no predisposing risk factors or exposure to the health care setting, and is a growing concern. CA-MRSA has been shown to be prevalent among incarcerated populations, underserved urban populations, children and other groups such as athletes. Issues such as overcrowding and confined spaces increase the risk of individuals contacting CA-MRSA (2). CA-MRSA was first reported in United States prisons by the Centers for Disease Control and Prevention (CDC). In 2000, the CA-MRSA isolate USA300 emerged, and since then it has been detected in both community and hospital settings, blurring the epidemiological distinction (18). Although the CDC has reported a steady decline in healthcare-associated MRSA (HA-MRSA), the emergence of CA-MRSA negates this, allowing MRSA to remain on the WHO list of pathogens for which new antibiotics are urgently needed (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/).

It has been noted that CA-MRSA is more easily treated but nevertheless more virulent than HA-MRSA. Recent research has shown genetic differences between CA-MRSA and HA-MRSA (19), yet it does not explain why HA-MRSA is restricted to the healthcare sector while CA-MRSA is not. In the USA, two specific MRSA strains are responsible for the majority of community acquired infections; USA300 and USA400. USA300 is the more virulent and highly invasive of the two (20). It almost always carries the staphylococcal cassette chromosome *mec* (SCC*mec*) type IV, along with the genes for Panton-Valentine leukocidin (PVL). By 2011, it was the predominant CA-MRSA strain type circulating in the USA.

CA-MRSA was first isolated in Denmark in 1993, followed by reports in 1997 in many other European countries including Finland, Greece and France (21-

23). The incidences of infection with ST80—IV CA-MRSA, USA300 CA-MRSA strains, and other lineages has been reported in the hospital and community settings associated with skin and soft tissue infections. However the incidences of MRSA are much less prevalent in Europe with the European ST80-IV CA-MRSA clone being the most common (24-26).

1.3. Peptidoglycan cell wall

Peptidoglycan (PGN), also known as murein, is the main constituent of *S. aureus* cell wall and accounts for approximately 90% of the cell dry weight. The essential role of PGN in maintaining cell shape, structural integrity and turgidity makes it the ideal target for drug design. PGN is found on the outside of the cytoplasmic membrane of almost all bacteria and serves as a platform for anchoring other cell envelope components including surface proteins and teichoic acids. It consists of long glycan chains of two alternating β-1, 4-linked sugars, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The carboxyl group of each MurNAc residue is amidated by the stem peptide L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala, and cross-linked by short flexible interpeptide bridges comprised of five glycine residues (27). *S. aureus* has a considerable degree of crosslinking, more than 93% (28, 29). Crosslinking between side chains extends from the amino group of residues three to the carboxyl groups of D-Ala at position four. The resulting net-like matrix that encases the bacterial cell prevents lysis by turgor pressure. The integrity of the PGN structure is vital for bacterial survival during constant remodelling processes such as degrading, rebuilding during growth and cell division. β-lactam antibiotics are the main drug used for treatment of methicillin-sensitive *Staphylococcus aureus* (MSSA) infections due to their ability to inhibit normal PGN assembly. β-lactams target penicillin-binding proteins (PBPs) which are involved in the final stages of PGN synthesis (30). *S. aureus* possesses four native PBPs, namely PBP1, PBP2, PBP3, and PBP4, which are membrane-bound enzymes catalysing transpeptidase (cross-linking) and transglycosidase reactions (extending the glycan chain) during PGN synthesis.

1.4. Methicillin Resistance

S. aureus is a spectacular microorganism due to its resilient adaptive nature. Since the introduction of penicillin in the early 1940's, *S. aureus* has managed to find means to overcome the bombardment of antibiotics by gene acquisition. In the 1950's, a decade after the introduction of the β-lactam benzylpenicillin, *S. aureus* resistant strains were being isolated from patients. These resistant strains produced an enzyme known as β-lactamase that inactivated the β-lactam rendering the drug ineffective (31-33). This led to the introduction of methicillin in the 1960s, a penicillin derivative that was resistant to β-lactamase hydrolysis. Unfortunately, its effectiveness in clinical use was short lived due to the emergence of MRSA (34). By the 1970s, MRSA had become one of the major public health concerns worldwide resulting in significant morbidity and mortality.

As noted above the four native PBPs are all targets of β-lactam antibiotics (35, 36). The interaction of β-lactams with the PBPs takes place on the external surface of the cytoplasmic membrane and results in a reduction of peptidoglycan cross-linking at the last stage of peptidoglycan synthesis. This leads to a mechanically weakened cell wall, resulting in cytoplasmic seepage and cell death due to normal autolytic enzyme activity during cell division that continues in the presence of β-lactams. However, *S. aureus* has developed a powerful resistance mechanism to methicillin and oxacillin (the clinically used derivative of methicillin). This was achieved by the acquisition of the *mecA* gene which encodes an alternative 76KDa penicillin-binding protein 2A (PBP2a) and it is believed to have been acquired by horizontal transfer from a coagulase negative *Staphylococcus* species (37). This gene is localised on a mobile genetic element, *Staphylococcus* cassette chromosome *mec* (SCC*mec*) and the encoded PBP2a has a low affinity for almost all β-lactam antibiotics (38, 39). Therefore, PBP2a can take over the transpeptidation reactions of the host and facilitate cell wall turnover in the presence of β-lactams (40, 41). However, PBP2a is not able to completely compensate for the other PBPs and requires the transglycosylase activity of the native PBP2 to confer resistance (42). Inactivation of the transglycosylase domain but not the transpeptidase domain of PBP2 was shown to reduce methicillin resistance therefore highlighting the need for cooperation between the native PBP2 and the more recently acquired PBP2a to complete cell wall synthesis in the presence of β-lactams (43, 44).

The synthesis of PBP2a is regulated by two genes adjacent to *mecA*, *mecR1* and *mecI*, which are divergently transcribed. *mecR1* encodes a transmembrane β-lactam sensing protein that acts a signal transducer while *mecI* is bound to the operator region of *mecA* and encodes a transcriptional repressor. Exposure of MRSA to β-lactam antibiotics induces MecR1-mediated cleavage of MecI thus relieving MecI-dependent repression of *mecA* and allowing synthesis of PBP2a (45, 46). However, *mecA* expression can also be controlled by BlaI, the β-lactamase repressor that shows homology to MecI. The BlaR1-BlaI regulatory module is responsible for expression of the β-lactamase-encoding gene *blaZ*. *mecA* expression is under dual control by BlaR1-BlaI and MecR1-MecI (45). Resistance levels to β-lactams are regulated at a different rate depending whether BlaR1-BlaI or MecR1-MecI are involved and are normally kept at low levels. The MecR1-MecI regulatory system has a much slower rate of induction in the presence of β-lactams than BlaR1-BlaI, which responds to most β-lactams. This is due to MecI being a tight regulator of *mecA* transcription, and most β-lactams antibiotics including oxacillin and methicillin do not efficiently activate MecR1. Therefore, strains that possess *mecA* with an intact regulatory system may present as phenotypically susceptible to methicillin. However, selective pressure by antibiotic usage has promoted *S. aureus* strains to acquire mutations or deletions in the regulatory genes that inactivate the repressor and enhance the level of PBP2a expression (47, 48).

The SCC element that carries *mecA* varies in size (20-70kb) and genetic content, and is currently classified into five major groups in *S. aureus* (SCC*mec* type's I-V) (49-51). There are currently eleven identified SCC*mec* types and it is likely that others will be discovered (52). SCC*mec* elements share similar structural homology, however two main features are common among all SCC*mec* elements and are used to define the SCC*mec* type: (1) the *mecA* gene complex and (2) the cassette chromosome recombinase genes *ccrAB* or *ccrC* and associated *ccr* recombination sites (49, 53, 54). These large serine recombinases are responsible for site and orientation-specific excision and integration of this mobile SCC*mec* into a location close to the origin of replication, at the 3'-end of an open reading frame, *orfX* (55). As a result of integration, the SCC*mec* element is flanked by characteristic direct repeats (DR) and they contain integration site sequences (ISS) that are recognised by the *ccr*-complex to mediate correct integration into or precise excision of the

SCC*mec* element from the chromosome as well as integration sites for the next SCC element (55). The remainder of the SCC*mec* element is referred to as the joining regions and consists of pseudogenes, insertion sequences or truncated copies of transposons that have no known function. These regions previously known as junkyard regions are now used for the subtyping of various *SCCmec* types (49, 50, 56).

Since the discovery of MRSA, it initially appeared that these strains were confined to the hospital environment where factors such as broad-spectrum antibiotic exposure, inserted medical devices and immuno-compromised patients lead to increased susceptibility to infection. However, by the 1990's MRSA infections began to occur in the community and have become more prevalent since then. These CA-MRSA infections were identified among people who had no known risk factors and who had no prior exposure to a hospital setting. Genetic analyses of both HA-MRSA and CA-MRSA revealed SCC*mec* type differences between the two groups. HA-MRSA tends to carry the older and larger SCC*mec* types I-III along with other resistant determinants to multiple antibiotics that impose a fitness cost upon the strains. CA-MRSA tends to carry the smaller SCC*mec* types IV and V (55, 57, 58). These new elements typically only carry the *mec*-gene complex and no other resistance genes, rendering less of a fitness burden on CA-MRSA (59, 60). To date no genetic basis has been found to explain the restricted nature of HA-MRSA to the hospital environment.

1.5. Heterogeneous and homogeneous methicillin resistance

An intriguing characteristic of methicillin resistance is its typical heterogeneous expression that consists of different subpopulations (61, 62). The majority of cells grown in the presence of a β-lactam antibiotic are resistant to a level sometimes just above that of a susceptible *S. aureus* strain (1 to 5 μg/ml), with only a small minority of cell population expressing high-level antibiotic resistance (63). This high level of resistance is referred to as homogeneous resistance (HoR) and can be selected for *in vitro* with increasing concentrations of β-lactams (>100 μg/ml). The number and frequency at which highly resistant subpopulations of MRSA occur is reproducible, strain specific and usually stable, with few exceptions (64). This

heterogeneous population that gives rise to a homogenous population upon antibiotic exposure is thought to operate in the clinical setting and provides one reason for β-lactam treatment failure against MRSA. The mechanisms or genetic model governing the transition from a HeR to a HoR phenotype is not yet completely understood, but the abundance of PBP2a does not necessarily correlate with the varied resistance phenotypes (65). Furthermore, it is clear that although *mecA* is a primary genetic determinant for resistance, additional mutations on the chromosome at sites other than *mecA*, *mecR1-mecI* and *blaR1- blaI* mutations account for the strain-specific differences in resistance profiles (65). As well as the genetic differences that affect resistance, the HeR to HoR conversion is strongly dependent on growth condition such as temperature, osmotic stress, and the presence of sucrose in media (64). Due to MRSAs prevalence in hospital and community infections, on-going research is needed to investigate the mechanisms that underpin the formation of these highly resistant sub-clones.

1.6. Chromosomal genes affecting methicillin resistance

To elucidate the causes of altered resistance levels, a number of chromosomal factors were characterized. These included several genetic determinants involved in cell wall biosynthesis and turnover. Inactivation of these genes initially termed *fem* (factor essential for methicillin resistance) and other *aux* (auxiliary) factors lead to reduced resistance levels (40, 66-69).

1.6.1. Contribution of PBP4 to resistance

Methicillin resistance does not depend on PBP2a production alone since several genetic factors have been attributed to altered levels of resistance. Even though PBP2a does not contribute to cell wall synthesis in the absence of methicillin, its involvement in transpeptidase activity under antibiotic pressure is vital for cell wall cross-linking. The mode of action of β-lactam antibiotics is to generally target the transpeptidase domain of PBPs, specifically the high-molecular-weight PBP1, PBP2, and PBP3 which have high affinities for β-lactam antibiotics. The current theory is that PBP2a substitutes for native PBPs that become acylated and inactive

under inhibitory concentrations of β-lactams. However PBP4, a native low-molecular-weight PBP, which has low affinity for most β-lactam antibiotics and possesses transpeptidase and carboxypeptidase activities, appears to be involved in the resistance phenotype, specifically in CA-MRSA. Previous research has shown that PBP4 was linked to low-level methicillin resistance in *mecA* negative strains and its overexpression was noted to result in an increase in β-lactam resistance and more cross-linking in MRSA peptidoglycan. It was also reported that the loss of PBP4 resulted in reduced PBP2 transcription in cells challenged with oxacillin in CA-MRSA strain MW2 but not in HA-MRSA strain COL. Further studies identified that the loss of PBP4 function in CA-MRSA strains MW2 and USA300 led to a 16-fold reduction in oxacillin and nafcillin resistance (70). However this phenotype wasn't seen in HA-MRSA strains COL, N315 and Mu50 following the same deletion of PBP4 (71). Collectively these works indicate that PBP2a is not the sole determinant of methicillin resistance and that PBP4 plays a significant role in CA-MRSA strains.

1.6.2. Fem factors (factor essential for methicillin resistance)

The *fem* genes reside outside, and are independent of the *mecA* locus on the chromosome. They have been shown to play a significant role in peptidoglycan metabolism. The seven *fem* genes, *femA, femB, femC, femD, femE, femF* and *femX* encode enzymes involved in the synthesis of the amino acid side chains of peptidoglycan precursors and are implicated in altered methicillin resistance. The *femABX* operon encodes FemX, FemA, and FemB respectively, required for the formation of the pentaglycine interbridge that crosslinks and stabilizes the peptidoglycan cell wall. FemX encodes a protein factor that causes the first glycine to be added to the ε-amino group of lysine of the stem peptide (72). The *femX* gene is essential in *S. aureus* (72-74). The sequential addition of the remaining glycines is carried out by FemA and FemB, FemA catalyses the addition of the gly2-gly3 and FemB catalyses the addition of the gly4-gly5 (75-77). Mutations in these genes lead to mono, di- and tri-glycine residues in the staphylococcal cross bridges, which results in increased sensitivity and reduced cross linking in the presence of β-lactam antibiotics (69, 75, 78). In addition, a mutation in *femA* leading to complete *femAB* inactivation resulted in increased resistance to lysostaphin, an endopeptidase that

cleaves between the second and third glycine of the pentaglycine cross bridge (79, 80). Disruption of the *femAB* leads to altered resistance levels without affecting PBP2a and PBP expression, therefore confirming the requirement for PBP2a to have proper pentaglycine interbridges to perform cross-linking reactions (75-77, 81, 82).

femC (glnRA) mutants were identified by a Tn 551 insertion into the glutamine synthetase repressor gene *glnR*, which resulted in a polar effect on the downstream glutamine synthetase gene *glnA*. This led to the overall reduction of glutamine in the cell and reduced amidation of the iD-glutamate (83). These peptidoglycan precursors with a non-amidated iD-glutamate residue in the stem peptide take part in peptidoglycan crosslinking less readily, thus resulting in reduced methicillin resistance. However, the addition of glutamine to growth media can restore the amidation of iD-glutamine, leading to normal peptidoglycan composition and the corresponding resistance phenotype. This shows that PBP2a needs a specific peptidoglycan precursor for normal cell wall assembly. However, the *femC* mutants that have reduced methicillin resistance can become highly resistant under methicillin pressure selecting for compensatory mutations that do not restore amidation of the iD-glutamine. These low frequency spontaneous mutations occur in an unknown pathway, enabling the mutants to exhibit homogenous high level resistance.

Inactivation of *femD* (*femR* or *glmM*) leads to inhibition of the first step of peptidoglycan precursor formation (84, 85). The interruption of the *glmM* gene, a phosphoglucosamine mutase that catalyses the interconversion of glucose-6-phosphate into glucosamine-1-phosphate leads to increased susceptibility to β-lactams and a decreased rate of peptidoglycan precursor formation (86). In addition to this, a 10-fold increase in susceptibility to the glycopeptide teicoplanin is also present in a *glmM* mutant. These phenotypes can be fully restored by complementation with the *orf1-orf2-glmM* operon. Furthermore, high level homogenous resistant methicillin suppressor mutants can be selected from *glmM* mutants that maintain hyper susceptibility to teicoplanin (87). This mechanism of resistance is suggested to be due to additional mutations on the chromosome that affects an unknown pathway.

A *femF* (*murE*) mutant results in a heterogeneous resistance phenotype where the incorporation of lysine into the peptidoglycan stem peptide is impaired (78). This leads to an accumulation of precursor dipeptides and reduced pentapeptides in the cell wall. These shortened muropeptides or the abnormal balance of peptidoglycan precursors in the cell wall are believed to be responsible for the reduced resistance (88).

1.6.3. *Fmt* (methicillin resistance factor)

FmtA is suggested to encode a low-affinity penicillin binding protein in *S. aureus* due to its structure, which harbors two of the three conserved motifs found in the serine active site of penicillin binding proteins (PBPs) and β-lactamases, SXXK and SND. It has also been found to interact covalently with β-lactams via a serine residue in the SXXK motif (89). Based on genome studies, *fmtA* is reported to be a part of the core cell wall stimulon. This is due to its increased expression in response to cell wall inhibitors and also when genes involved in peptidoglycan biosynthesis are deleted (90-92). It was found that an insertion into *fmtA* disrupts homogeneity and leads to reduced resistance to methicillin, cefoxitin and imipenem and this is more pronounced when Triton X-100 is present. The inactivation of *fmtA* also led to a slight reduction in the molar concentration of peptidoglycan, suggesting its involvement in *S. aureus* cell wall synthesis (93). More recently the global regulator SarA has been shown to control expression of *fmtA* by binding to the promoter region of *fmtA* following induction by cell wall specific antibiotics (94). In addition, FmtA interacts with teichoic acids by hydrolyzing the ester bond between D-Ala and the back bone of teichoic acids, and the conserved SXXK motif was also shown to be important for the D-amino esterase activity of FmtA (95). Overall, the regulation and function of FmtA appears to be related to cell division, autolysis, and biofilm formation due to its esterase activity and its capacity to modulate teichoic acid charge.

1.6.4. *llm*

The *llm* gene, which encodes a 38 kDa lipophilic membrane protein of unknown function, has also been shown to be involved in the homogeneous resistance phenotype, independently of *mecA* or PBP2a expression. A *llm* mutation results in reduced growth and decreased methicillin resistance, to a lower heterogeneous resistance phenotype (67). An *llm* mutant has also been shown to have altered Triton-X-100-induced autolytic activity. The 3'-terminal sequence of *llm* is not highly conserved among *S. aureus* strains and it has been shown that some Llm activity remains in a mutant which is able to form resistant revertants. This is achieved by IS*256* insertion in an area upstream of the *llm* mutant, thereby creating a new promoter and leading to transcription of the truncated Llm protein, demonstrating its involvement in homogeneous resistance (96).

1.6.5. Lytic Enzymes

S. aureus native hydrolases are important for growth and turnover of the peptidoglycan cell wall and are highly regulated (97). These hydrolases target certain parts of the bacterial cell wall and any errors in their function can lead to cell lysis. A number of autolytic enzymes have been identified in *S. aureus*. However the effect of β-lactam antibiotic exposure on autolytic behaviour is contradictory (98, 99). It has been reported that increased methicillin resistance can be coupled with either reduced or increased autolytic activity highlighting the differences among varied genetic backgrounds. Mutation of the *atl* gene encoding the major autolysin is involved in cell separation and β-lactam antibiotic-induced lysis, leads to only a small decrease in methicillin resistance (100). However, more recent works have shown that the inactivation or deletion of the *lytH* gene, leads to increased methicillin resistance (101, 102).

1.6.6. Secondary messenger: cyclic diadenosine monophosphate

Extensive research into the mechanisms governing the switch from HeR to HoR resistant phenotypes in MRSA have been undertaken. Nonetheless these resistance profiles have yet to be fully elucidated. Recently, a new second messenger was shown to play a role in methicillin resistance in *S. aureus*; cyclic diadenosine

monophosphate (c-di-AMP), which is the second cyclic dinucleotide shown to be produced in bacteria after cyclic di-GMP (103-105). However these second messengers appear to regulate different processes in the bacterial cell. These small molecules, along with other nucleotides, regulate various cellular processes including biofilm formation, virulence and cell cycle. In addition to altered antibiotic resistance, a variety of other phenotypes have been linked to altered c-di-AMP levels such as acid resistance, altered salt tolerance and heat stress (106-109).

C-di-AMP is synthesised from two molecules of ATP by diadenylyl cyclase, DacA, and was first identified in 2008 by Witte et al. in *Bacillus subtilis* (104). The cellular levels of c-di-AMP are further regulated by its degradation to 5'pApA by the phosphodiesterase GdpP (106, 110). Several studies, including those of our research group, have identified non-synonymous single nucleotide polymorphisms (SNPs) in *gdpP*. These *gdpP* mutations, which are predicted to increase intercellular c-di-AMP levels, were found to increase tolerance or/and resistance to β-lactam antibiotics in *S. aureus*, *B. subtilis* and *Listeria monocytogenes* (111). In addition, a SNP in the *dacA* gene was associated with reduced resistance in a MRSA isolate along with an increased growth rate (112). This *dacA* SNP was associated with decreased c-di-AMP levels, reduced autolysis and increased salt tolerance. This suggests that altering c-di-AMP levels is a route to resistance and the HeR to HoR transition in *S. aureus*. The c-di-AMP intracellular concentration is believed to be altered in response to environmental stimuli, which modulates whether c-di-AMP interacts with specific receptors or target proteins to regulate their activity.

Another signalling molecule which has also been linked to increased homogeneous resistance is the alarmone (p)ppGpp. The *relA* gene codes for the synthesis of (p)ppGpp (guanosine penta-phosphate) or ppGpp (guanosine tetra-phosphate), the effector of the stringent response. When *S. aureus* is faced with amino acid deprivation, it produces high levels of (p)ppGpp, which results in the initiation of complex cellular responses to regulate gene expression and slow growth. The *relA* gene encodes a bifunctional RelA/SpoT homologue (RSH) that contains a C-terminal regulatory and an N-terminal enzymatic domain. In total RelA contains four domains, the hydrolysis domain (HD) responsible for degrading (p)ppGpp, the synthetase (SYN) domain that synthesizes (p)ppGpp and the remaining two domains whose functions are not fully understood, TGS and ACT. Research has shown that a

single mutation in the *relA* gene after the SYN domain created a premature stop codon, resulting in increased (p)ppGpp levels and leading to increased resistance levels by inducing the stringent response (113, 114). Further studies showed that mupirocin, which activates the stringent response, also promotes expression of a HoR phenotype (113). *S. aureus* also contains two other genes encoding proteins involved in the synthesis of (p)ppGpp; *relP* and *relQ* which are induced by antibiotic exposure and mediate tolerance (115). More recent findings have demonstrated an interesting connection between the stringent response and the secondary messenger, c-di-AMP. The alarmone (p)ppGpp was shown to be a competitive inhibitor of GdpP activity. Therefore, under stress conditions that trigger the stringent response, the phosphodiesterase activity of GdpP is inhibited and this leads to elevated c-di-AMP levels (116). Besides antibiotic resistance, c-di-AMP has also been implicated in cell wall homeostasis in *S. aureus.* An otherwise lethal lipoteichoic acid defective mutant in *S. aureus* was viable following selection of a compensatory mutation in *gdpP*, which was associated with a 15-fold increase in c-di-AMP levels along with increased peptidoglycan cross-linking by yet an unknown mechanism (106). Cell wall structure changes have been reported in HoR isolates with altered c-di-AMP levels indicating a link between cell wall synthesis, c-di-AMP levels and resistance (106) .

1.6.6.1. C-di-AMP Targets

Identification of c-di-AMP targets is of scientific importance due to its potential exploitation for the development of new therapeutic treatments. Cellular pathways regulated by c-di-AMP levels could offer new approaches to drug development where current treatments are becoming less effective and antibiotic discovery has stalled somewhat.

Several c-di-AMP receptors have recently been identified in *S. aureus.* This was achieved by an unbiased approach that utilised an affinity pull down assay. This analysis identified one major target protein, KtrA, a cytoplasmic potassium transporter-gating component. KtrA is required for growth under potassium limiting conditions. Accumulation of intracellular c-di-AMP levels results in its specific interaction with the C-terminal RCK-C domain of KtrA and inhibition of potassium

uptake (117). This was supported by the observation that *gdpP* mutants along with *ktrA* mutants are more sensitive to salt stress. A second protein, cation proton antiporter A (CpaA) was also shown to bind c-di-AMP. CpaA also contains the RCK-C domain also present in KtrA and is also believed to be involved in the transport of potassium or sodium. The interaction of KtrA homologues and c-di-AMP is observed in other bacterial species including *B. subtilis*, *Corynebacterium glutamicum* and *Streptococcus pneumoniae*. Corrigan *et al*, 2013, also utilised a whole genome sequencing approach to identify two other proteins in *Escherichia coli*, KdpD and PstA that bind c-di-AMP (117). KdpD, a histidine kinase has the potential to also be involved in potassium homeostasis. KdpD is a part of a bi-component regulatory system, where it regulates the second type of potassium uptake system, in conjunction with its cognate response regulator, KdpE. This demonstrates a direct link between c-di-AMP and ion homeostasis, and illuminates the fundamental importance of potassium transport in all living cells to aid adaptation to osmotic stress, pH regulation and maintenance of membrane potential for cell integrity and growth. The final c-di-AMP target identified to date is a PII-like signal transduction protein and it is the most prominent target of c-di-AMP. It is designated PstA in *S. aureus* and *L. monocytogenes* and DarA in *B. subtilis* (117-119). The function of PstA has not been determined in any of the organisms studied and loss of protein does not lead to any apparent phenotypic consequences (118). Similar proteins that contain this structural domain are PII nitrogen regulatory proteins that belong to the GlnB superfamily, but no link has been confirmed. Overall, from the first identified c-di-AMP binding protein, the transcription factor DarR in *Mycobacterium smegmatis* that regulates fatty acid synthesis, to the most recently identified target PstA in *S. aureus*, none of the known c-di-AMP targets are essential. However, the existence of an unidentified target for c-di-AMP that is essential remains entirely possible, and may help explain the connection between increased c-di-AMP levels, altered cell wall structures and antibiotic resistance.

1.7. Biofilm-associated infections caused by staphylococci

S. aureus is an important nosocomial pathogen due to the acquisition of antibiotic resistance determinants along with the ability to form biofilm on implanted medical devices. Biofilms are associated with more than 80% of all microbial infections and certain species of staphylococcal are the most likely candidates to cause such infections as they are frequent commensals on human skin (6, 120, 121). Staphylococci may colonise compromised host tissue such as post-surgical and burn wounds, leading to the establishment of deep-seated skin and soft tissue infections. Further dissemination can occur, causing endocarditis, sepsis and a range of other serious life-threatening illnesses (122-124). The ability of *Staphylococcus* species to colonise biomaterials such as catheters, cerebrospinal fluid shunts, cardiac pacemakers and joint prosthetics that have become common place in modern healthcare treatments has led to increased chronic device related infections (DRIs) involving biofilms (16, 125, 126). The capacity of biofilm to resist therapeutic treatment and evade host immune defences enables *S. aureus* to be a successful pathogen in healthcare and community settings (121, 127).

The ability of bacteria to attach and adhere to surfaces has been documented since the early 1920's among aquatic bacterial populations. However the role of biofilm in the context of human infection has only been recognised in more recent times. Antimicrobial drugs are selected for their ability to inhibit or kill planktonic cells, yet they have limited effectiveness against biofilms, including *S. aureus* biofilms. Biofilms are organised communities of microorganisms encased in a self-produced extracellular matrix which has the ability to protect itself from chemotherapies and host defences, and aids attachment to surfaces, rendering DRIs extremely difficult to treat (121). These are sophisticated bacterial communities that use quorum sensing to communicate and regulate growth and development of the biofilm community (128). Staphylococcal biofilms resist therapeutic treatment in several ways, one of which is the cell's ability to enter into dormancy and reduce there metabolic and growth rates. These dormant or slow growing cells are termed persister cells. They are recognised as the main mode of drug resistance in biofilms and they contribute to the chronic nature of DRIs (129). These persister cells within a biofilm are less susceptible to the action of antibiotics in comparison to the fast growing free-floating planktonic cells (130). The biofilm matrix itself offers

protection by inhibiting diffusion of some drugs across the biofilm barrier and therefore preventing interaction with cells of the biofilm (120). The ability to form biofilms is a key virulence factor, especially in healthcare settings where antibiotic usage is high. Inability to completely disrupt the biofilm at even high doses of antibiotics *in vivo*, results in infected medical devices often requiring surgical removal leading to more discomfort and increased durations in patient care. These DRI's caused by the resilient nature of biofilms expose patients to a higher risk of mortality (6).

Biofilm development has been shown to occur in at least three distinct stages (Fig. 1.1). Initially the bacteria exist in a planktonic state, and gain access to the implanted medical device and undergo initial attachment to a surface (7). This is followed by an accumulation of cells via cell-cell adhesion resulting in a mature multilayer biofilm that can resemble unique pillars or mushroom-shape structures. This biofilm matrix will contain sophisticated channel networks that facilitate the transport of waste and nutrients (131). Varying environmental conditions such as oxygen and nutrient limitations throughout the biofilm promote heterogeneity among the bacterial community (132). This stressed environment produces dormant phenotypes that results in low metabolic levels and reduced cell division, resulting in many slow growing cells that are tolerant to high levels of antibiotics but also contain a small proportion of persister cells. Finally, the seeding, dispersal or cellular detachment and dissemination of individual cells, or clusters of cells from the biofilm matrix to secondary sites within the host encourages bacterial spread (6). This promotes the development of systemic infections such as bacteraemia and sepsis within the host. Biofilm-forming staphylococcal bacteria lead to persistent infections that are resistant to conventional antimicrobial treatment and are regarded as the primary cause of nosocomial bloodstream infections globally (6). A recent review by Moormeier and Bayles suggests that biofilm development occurs through a five–stage process including: 1) attachment, 2) multiplication, 3) exodus, 4) maturation, and 5) dispersal. This model was suggested based on data from new biofilm assays combining microfluidic flow-cell systems and time-lapse microscopy to evaluate biofilm in a near real-time manner. It was suggested that biofilm structures involve metabolic heterogeneity and differential gene expression, which leads to proliferation within a maturing biofilm population, rather than the release of

cell clusters from an unstructured thick biofilm (133). Therefore, a clear understanding of the mechanics of staphylococcal biofilm formation and regulation is vital for the development of new therapeutic strategies, in order to effectively treat these infections.

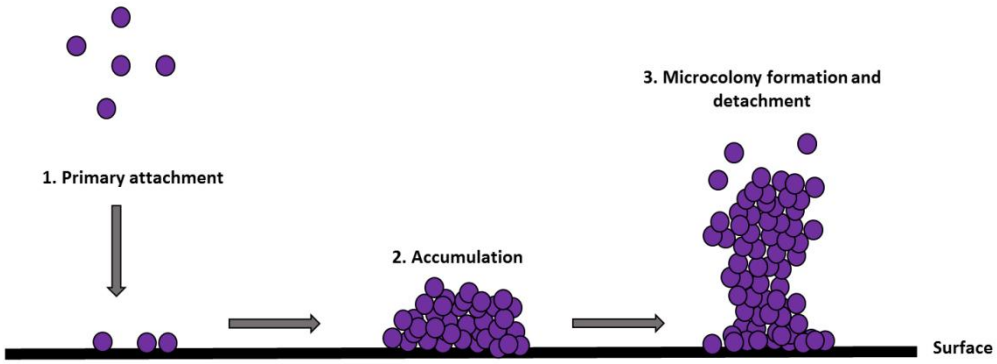

Figure. 1.1. The major stages of biofilm formation by staphylococci. 1) Primary attachment to the surface. 2) Cellular proliferation and accumulation of a multi-layered biofilm separated by fluid filled channels. 3) Mature microcolony develops and individual cells or clusters of cells detach and can disseminate to other parts of the host.

1.8. Biofilm formation in *Staphylococcus aureus*

The *icaADBC* operon-encoded polysaccharide intercellular adhesion (PIA) or poly-N-acetylglucosamine (PNAG) production is the most studied mechanism of *S. aureus* biofilm formation. This is the main biofilm type to be associated with MSSA infections. However, the acquisition of methicillin resistance has impacted biofilm phenotypes through the formation of *ica*-independent biofilms. Numerous studies suggest that MSSA and MRSA isolates utilise different types of biofilms to cause infection relating to implanted medical devices.

1.8.1. Initial Attachment to surfaces

The initial step in biofilm formation is the attachment of staphylococci to biotic or abiotic surfaces and may be mediated by both specific and non-specific interactions (6). Non-specific interactions such as cell surface hydrophobicity,

polarity and van der Waals forces aid in the attachment of cells to abiotic surfaces such as catheters and other uncoated medical devices (134). The presence of cell surface-associated proteins such as the major autolysin can mediate attachment to hydrophilic and hydrophobic polystyrene surfaces (135, 136). Negatively charged teichoic acids have also been shown to aid attachment to polystyrene and glass surfaces (137). Furthermore, after surgical insertion, implanted medical devices become coated with host extracellular matrix proteins (138-140) and interact with microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) that facilitate primary attachment (141). Many of these proteins also play an important part in the accumulation phase of biofilm formation.

1.8.2. The *ica* operon and production of PIA/PNAG

The first mechanism of biofilm development in *S. aureus* is the formation of an exopolysaccharide (EPS) such as polysaccharide intercellular adhesin (PIA) or polymeric N-acetylglucosamine (PNAG) that are produced by the intercellular adhesion (*ica)* operon (142, 143). The *ica* operon consists of four biosynthesis genes, *icaA, icaD, icaB* and *icaC* along with a divergently transcribed repressor, *icaR* (131). Research on the *ica* locus has been conducted in *S. epidermidis*, which has 78% amino acid identity with *S. aureus*, and homologous systems have also been found in a range of other bacteria (142). The production of PIA by the *ica* locus is necessary for biofilm formation and virulence among certain strains of *S. aureus* and *S. epidermidis* (144, 145). This sticky polysaccharide is composed of β-1,6-linked N-acetylglucosamine residues (80-85%) and an anionic fraction with a lower content of non-N-acetylated D-glucosaminyl residues, providing an overall positive net charge which is crucial for resistance of AMPs such as LL-37, HBD3, and dermcidin (146), as well as promoting intercellular aggregation and attachment of cells to inert surfaces (147).

PIA synthesis begins with the up-regulation of the *icaA* gene which encodes a transmembrane enzyme with N-acetylglucosaminyl-transferase activity (148, 149). IcaA synthesises a partially de-acetylated β-1, 6-linked N-acetylglucosamine homopolymer. However optimal IcaA activity needs the expression of the *icaD* gene to produce a N-acetyl–glucosamine polymer (131, 148-150). IcaC is a putative

membrane protein that produces longer chains of N-acetylglucosamine polymers (131, 148). The exact role of IcaC has yet to be determined, but it is believed to be involved in the translocation of the polymeric *N*-acetylglucosamine chain through the cytoplasmic membrane and elongation of the growing polysaccharide (131). IcaB is a surface attached enzyme that facilitates de-acetylation of the *N*-acetylglucosamine residues to the outer surface of the bacteria. This introduces a positive charge to the polymer that is considered important for PIA-mediated intercellular accumulation as well as immune evasion (146). An isogenic *icaB* deletion mutant strain led to impaired biofilm formation and PIA/PNAG production thus demonstrating the importance of polysaccharide de-acetylation in this biofilm phenotype (146). The divergently transcribed regulator gene *icaR* is located upstream of the *ica* operon and encodes a transcriptional repressor that is bound to the *ica* operon promoter region close to the *icaA* start codon (151). IcaR is a member of the TetR family of transcriptional regulators in *S. aureus* and tightly regulates the expression of the *ica* locus in response to environmental stimuli, such as anaerobic growth conditions, extreme temperature, osmolarity, addition of ethanol, and antibiotics, all of which have been shown to influence PIA-dependent biofilm formation (17, 151-153).

This PIA polysaccharide has been shown to be important for both *S. epidermidis* and *S. aureus* virulence *in vivo* and its elaboration seems to be a characteristic of most clinical *S. aureus* strains (144, 145). However, one of the first studies to report PIA-independent biofilm formation was in bovine mastitis isolates of *S. aureus* and it identified the biofilm associated protein (*bap*) gene as contributing to biofilm formation. These *bap*-positive strains contained a mutation in the *ica*-operon but remained able to form biofilms. The significance of Bap in human infections is questionable due to the absence of the *bap* gene in most human isolates (154, 155). A separate study also showed that an *ica* mutation in UAMS-1 *S. aureus* isolate did not impair biofilm development alluding to the possibility of a *icaADBC*-independent biofilm phenotype (156).

1.8.3. *IcaADBC* independent biofilm formation

Carriage of the *ica* locus is common among most clinical isolates, although more recent findings have highlighted the ability of *S. aureus* to form biofilm

independent of PIA production and it appears to be associated mainly with MRSA isolates. PIA/PNAG-independent mechanisms of biofilm formation involve both sortase-anchored and non-covalently anchored surface proteins. In addition to these, eDNA also contributes to primary attachment and intercellular accumulation of the biofilm phenotype.

1.8.3.1.Anchoring of surface proteins to the cell wall by sortase

S. aureus expresses 28 surface proteins of which 21 are covalently attached to cell wall peptidoglycan. Genome sequencing shows that these 21 surface proteins are predicted to contain a Leu-Pro-X-Thr-Gly (LPXTG) binding motif that enables normal display on the cell surface (157). Surface proteins that are transported across the cytoplasmic membrane have an N-terminal signal peptide. The sortase enzyme, encoded by the *srtA* gene is an extracellular transpeptidase that recognizes the C-terminus sorting signal, LPXTG (158) and cleaves the precursor polypeptide at the threonine and glycine residues of the LPXTG motif (159). The carboxyl group of threonine is then amide-linked to the free amino group of the peptidoglycan crossbridge, (which in *S. aureus* is pentaglycine) resulting in these proteins being covalently linked to the cell wall. A *srtA* deletion interferes with normal LPXTG surface protein display, affects *ica*-independent biofilm accumulation and results in reduced virulence in numerous animal models (160-162). To date, a number of LPXTG surface proteins have been linked to biofilm development, namely Bap (Biofilm-associated protein), Aap of *S. epidermidis* (accumulation-associated protein), SasG in *S. aureus* (surface protein G) (163), Spa (protein A) (164), SasC in *S. aureus* (surface protein C) (165), and the fibronectin binding proteins A and B (FnBPA and FnBPB) (166, 167).

1.8.3.2.The major autolysin Atl and extracellular DNA

The major autolysin Atl is a wall-anchored bifunctional peptidoglycan hydrolase that is involved in daughter cell separation, cell wall homeostasis and muropeptide turnover (168, 169). It was first implicated in the primary attachment of biofilm in *S. epidermidis* and has since been shown to have a key role in the early

stages of MRSA *ica*-independent biofilm formation (136, 170). Mutations in *atl* gene results in impaired cell wall turnover and inhibition of biofilm development (171), while an *atl* mutation in an MRSA strain leads to increased sensitivity to penicillin-induced lysis (168). Atl can initiate attachment by several means including hydrophobic interactions of bacteria with uncoated surfaces (37, 38, 47). It can also trigger primary attachment through direct interactions with host extracellular matrix proteins such as fibronectin, fibrinogen or vitronectin (170, 172, 173), and finally, the major autolysins can also mediate primary attachment indirectly through cell lysis and the release of extracellular DNA (eDNA) (174).

Several studies have reported the importance of tightly regulated autolysis and extracellular eDNA in early stages of biofilm development in MRSA that produce a PIA-independent biofilm phenotype (136, 175). The addition of DNaseI or polyanethole sodium sulfanate (PAS) to media inhibits biofilm development in HA-MRSA but has no effect on mature biofilm dispersal (136). Similar mutations that affect eDNA release in *S. aureus* also affect biofilm attachment. In CA-MRSA strain USA 300, mutations in the *nuc* gene which encodes a nuclease that leads to degradation of eDNA (176) or *lrgAB* that encodes an anti-holin that regulates cell lysis, has been shown to positively impact biofilm thickness *in vitro* (177). This further implicates eDNA as a vital component for attachment and/or early stage of biofilm development. However not all these phenotypes have been presented in *in-vivo* work, indicating additional studies are needed to identify the role of these enzymes in MSSA and MRSA biofilm phenotypes.

1.8.3.3.Role of the fibronectin-binding proteins, FnBPA and FnBPB

Previous research by our group has shown that the fibronectin-binding proteins, FnBPA and FnBPB have an important role at the accumulation phase in ica-independent biofilm under static and flow conditions (167). FnBPs were recognised based on their ability to bind fibronectin. In addition to this, they are also capable of binding to both elastin and fibrinogen (178). The FnBPs consists of an N-terminal A domain (comprising the subdomains N1, N2, N3) that binds fibrinogen and elastin, and a C-terminal wall spanning LPXTG-anchoring domain involving tandem repeats (1 to 11) that bind fibronectin. FnBPA and FnBPB are multi-

functional surface proteins that are capable of recovering the biofilm defect of a double *fnbpAB* mutant. The *fnbA* and *fnbB* genes are closely linked but are independently transcribed (179), with the exponential phase of growth presenting maximal expression (180). Furthermore, an *fnbAB* double mutation abolished biofilm and has been shown not to contribute to PIA-mediated biofilm in MSSA (167). Research by Vergara-Irigaray *et al*. (2009) also presented evidence that *fnbAB* genes are important for biofilm development (166). However more recent findings by Geoghegan *et al*. (2013) (181), demonstrated that the expression of FnBPs was constitutive in MRSA producing an FnBP dependent biofilm and restricted to exponential phase of growth in MSSA for PIA-dependent biofilms. Furthermore a strong association between carriage of *fnbps* and invasive staphylococcal diseases has been reported (3, 182). In a mouse foreign body infection model, *fnbAB* mutants had reduced colonisation of implanted catheters versus the parent or *icaADBC* mutant strains, supporting the significance of FnBP-dependent biofilm phenotype in infections (166).

1.9. The staphylococcal accessory gene regulator, Agr

Staphylococci require a sophisticated and intricate regulatory network of virulence regulators to establish infection in a constantly changing environment. However, the production of virulence factors is metabolically expensive for the bacterium and not necessarily required under certain conditions. Therefore the tight regulation of these virulence factors by global regulators is key to bacterial survival in the host.

The accessory gene regulator (Agr) is one of the best characterised quorum sensing systems in staphylococci and has been shown to be pivotal in *S. aureus* virulence (183-185). As illustrated in figure 1.2, the Agr system is composed of two divergently transcribed mRNA transcripts, RNAII and RNAIII which are driven by their own promoters, P2 and P3 respectively (186). RNAII encodes a quorum sensing cassette (AgrBD), a two component system (AgrAC) and RNAIII. The Agr system produces an autoinducing peptide (AgrD), which upon reaching a critical concentration (following bacterial accumulation) binds to AgrC (187), a membrane kinase which results in the activation of AgrA through autophosphorylation. This

leads to the activation of its cognate response regulator, AgrA which binds to and activates the P2 (its own operon) and P3 promoter (188). The P3 promoter transcribes the gene encoding for both RNAIII effector molecule and phenol soluble modulin (PSM) δ-toxin. Furthermore, the Agr system regulates the expression of the *psm* operons by encoding for the expression of PSMα and PSMβ molecules by direct binding of AgrA to the promoters (189). RNAIII proceeds to regulate the expression of target genes, which includes the up-regulation of secreted toxins and degradative enzyme and the down regulation of surface associated factors such as adhesins via indirect and direct mechanisms. Direct targets identified to date include *hla* (α-toxin) (186), *spa* (protein A) (190), *coa* (coagulase) (191), *rot* (repressor of proteins) (192), and *map* (major histocompatibility complex class II analogous protein) (193). In this way, not only can RNAIII control the expression of several hundred target genes, but it can also interfere with the immune system via MAP which is an important mediator in the immune response to infections (193).

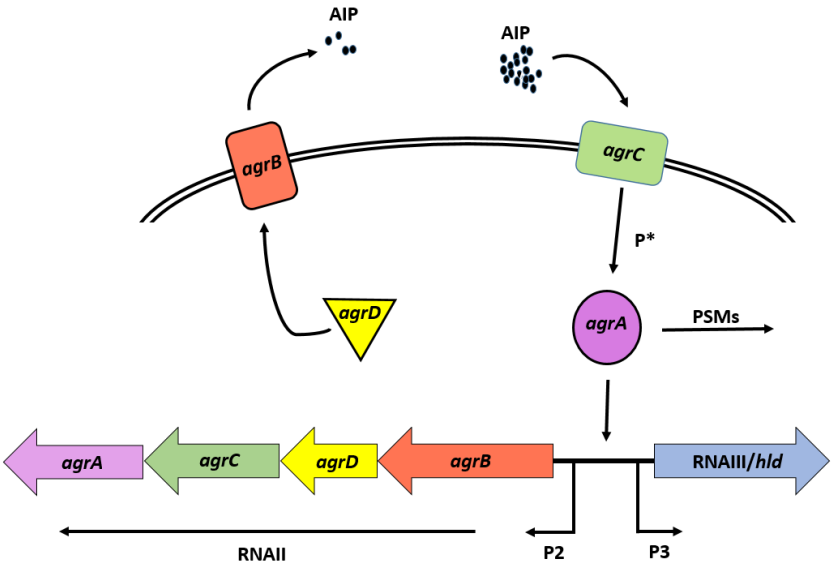

Figure. 1.2. A model of the Agr system showing two divergently transcribed mRNA transcripts, RNAII and RNAIII which are driven by their own promoters, P1 and P2 respectively. RNAIII is represented in blue.

Studies have shown that the Agr system is important for multiple stages of staphylococcal biofilm formation (234). A mutation in the Agr system has been shown to enhance biofilm formation (194) through regulating biofilm detachment.

The addition of exogenous AIP activated the Agr system and increased the production of extracellular proteases resulting in bacterial detachment (128). Subsequently, the addition of glucose was shown to have negative effect by repressing the Agr system, and promoting biofilm (128, 195). This would suggest that the Agr system is important for biofilm formation and dissemination in infections, as well as resisting treatment by antibiotics.

1.10. The Nebraska transposon mutant library

In 2012, the Nebraska Centre for Staphylococcal Research constructed a sequence-defined transposon mutant library of 1,952 strains to enhance the research potential in the *S. aureus* field (196). The library was created in JE2, a derivative of the strain USA300 LAC, a highly characterized epidemic CA-MRSA isolate. Each of the 1,952 non-essential genes contained a single mutation by the introduction of the *mariner*-based transposon *bursa aurealis*, which is known to create random transposon mutations in *S. aureus*. The Nebraska group carried out phenotypic screens on the library mutants to assess its utility for research and to validate the effectiveness of their approach. The group focused on haemolysis activity, protease production, pigmentation and mannitol utilization. As expected, they identified genes already known to be involved in these processes, but also previously undiscovered genes related to these phenotypes. Furthermore, the NTML has been utilised to examine the effects of low-dose amoxicillin on *S. aureus* USA300 biofilms, where sub-MIC levels of β-lactam stimulated the formation of thicker biofilm formation which correlate with the production of eDNA under both static and flow conditions (197). Overall, this library has shown itself to be a valuable tool for research and should prove important in the discovery of genes linked to altered antibiotic sensitivities, thereby also aiding in the development of novel antibacterial drugs. This Nebraska transposon mutant library (NTML) is an invaluable tool for the staphylococcal research community and has been utilised in the research presented in this thesis.

1.11. Continuous culture chemostats

The chemostat was originally introduced in the 1950s by Monad, and Novick & Szilard, as a means of culturing bacteria at a controlled growth rate for an indefinite time period (182, 198). The benefit of chemostat culture was that it allowed researchers to overcome the limitations of batch cultures, such as nutrient depletion, the inability to precisely control the environmental conditions and reproducibility. By the 1960s, chemostats had become the most widely used tool to establish steady state cultures for various applications. They have been widely employed instead of batch cultures in the areas of microbial physiology and biochemistry. The chemostat consists of a culture vessel which permits the continuous addition of fresh medium to replace the spent culture medium being removed. This is carried out at a constant rate to match the rate of cell division. This means that the total number of cells in the chemostat does not change overtime, leading to a steady state culture. Chemostats offered an attractive approach by our group to look at the adaptive nature of bacteria to antibiotic stresses. The chemostat has already proved useful in studying the effect that nutritional status of microorganisms and growth rate has on bacterial susceptibility to antimicrobial agents (199). With continuous cultures it is possible to capitalise on the precise control of cell growth rates and the external environment, making it possible to assess the adaptive evolution of cells growing in increasing concentration of antimicrobial agents and evaluate the contribution of post-antibiotic effects to resistant mechanisms and the dynamics of antibiotic treatment.

1.12. Animal models of sepsis

In 1935, an animal model of sepsis was first utilised in antibiotic research, to demonstrate the efficacy of sulphonamides against *Streptococcus pyogenes* (118). Since then, these models have been used extensively in research to study infections and investigate antimicrobial efficacy. To date, the mouse sepsis model of infection is a standard method of testing antimicrobial compounds *in vivo* before progression to larger animals or humans. The popularity of the sepsis model is due to its relative ease, with small and inexpensive animals, short-duration of experiments with reproducible infections, and also the simple end-points. These experiments are an

essential part of our research to determine the impact of drug combination therapy on the pathogenesis of *S. aureus* infections. This approach provides us with the necessary insights into the physiological relevance of our *in vitro* data and reinforces the commercialisation potential of these discoveries.

Our collaborative group has previously established the experimental parameters for the establishment of a nonlethal model of sepsis in CD1 mice with strain USA300. This mouse model requires the injection of inoculum of 5×10^6 colony forming units (CFUs) to establish bacteraemia infection, which leaves the blood in under 4 hours but continues to persist in the organs. An antibiotic treatment is administered subcutaneously every 12 hours and infection is allowed to proceed for approximately 6 days. During the course of the experiments, mice are routinely scored using the method outlined by Morton (71) to monitor for symptoms of illness which would warrant termination. Homogenates of organs obtained from mice with sepsis are plated onto blood agar. These mouse experiments are approved by the United Kingdom Home Office (Home Office Project License Number 40/3602) and the University of Liverpool Animal Welfare and Ethics Committee.

1.13. Aims of this research

The overarching objective of the research described in this thesis is to advance our understanding of the mechanisms underpinning the modulation of methicillin resistance levels in MRSA. The specific aims of the research were:

1. To characterise the mechanistic basis for expression of methicillin hyper-resistance in USA300 isolates recovered from a chemostat culture in which the antibiotic concentration was increased over 13-days.
2. To explore the role of the *pgl* gene, which encodes the second enzyme in the pentose phosphate pathway, in the control of β-lactam resistance levels in MRSA.
3. To determine how mutation of the *cycA* amino acid permease increases susceptibility to β-lactam antibiotics and the potential role of amino acid analogues to potentiate the activity of oxacillin against MRSA.

Chapter 2

Tandem amplification of SCC*mec* can drive high level methicillin resistance in MRSA

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Running title: Amplification of SCC*mec* is a new mechanism of high level β-lactam resistance in MRSA

Abstract

Hospital-associated methicillin-resistant *Staphylococcus aureus* strains typically express high level, homogenous (HoR) β-lactam resistance, whereas community-associated MRSA (CA-MRSA) more commonly express low level heterogeneous (HeR) resistance. Expression of the HoR phenotype typically requires both increased expression of the *mecA* gene, carried on the *Staphylococcus* cassette chromosome SCC*mec* element, and additional mutational event(s) elsewhere on the chromosome. Here the oxacillin concentration in a chemostat culture of the CA-MRSA strain USA300 was increased from 8 μg/ml to 130 μg/ml over 13 days to isolate highly oxacillin resistant derivatives. A stable, small colony variant, designated HoR34, which had become established in the chemostat culture was found to have acquired mutations in *gdpP*, *clpX*, *guaA* and *camS*. However, complementation of these mutations did not significantly change the hyper-oxacillin-resistant phenotype of HoR34. Closer inspection of the genome sequence data revealed that reads covering SCC*mec* were ~10 times over-represented compared to other parts of the chromosome. qPCR confirmed >10-fold higher levels of *mecA* DNA on the HoR34 chromosome, and MinION genome sequencing verified the presence of 10 tandem repeats of the SCC*mec* element. qPCR further demonstrated that sub-culture of HoR34 in varying concentrations of oxacillin (0–100 mg/ml) was accompanied by accordion-like contraction and amplification of the SCC*mec* element. Although slower growing than USA300, HoR34 out-competed the parent strain in the presence of sub-inhibitory oxacillin. These data identify tandem amplification of the SCC*mec* element as a new mechanism of high-level methicillin resistance in MRSA, which may provide a competitive advantage for MRSA under antibiotic selection.

2.1. Introduction

In recent decades, the overall incidence of methicillin resistant *Staphylococcus* aureus infections has greatly increased due to the emergence of community-associated MRSA (CA-MRSA), which are increasingly displacing hospital associated-MRSA (HA-MRSA) strains in healthcare settings (200). Methicillin resistance is mediated by the *mecA*-encoded low affinity penicillin binding protein 2a carried on the mobile *Staphylococcus* cassette chromosome *mec* element (SCC*mec*). Heterogeneity is a feature of *S. aureus* methicillin resistance (201). In general clinical CA-MRSA isolates exhibit heterogeneous, low level methicillin resistance (HeR) under laboratory growth conditions, whereas HA-MRSA isolates exhibit high level or homogeneous methicillin resistance (HoR). However HeR strains can express a HoR phenotype after selection on elevated concentrations of β-lactam antibiotics, via mechanism(s) involving the stringent response and altered c-di-AMP signalling (106, 201-203).

Despite the resistance of MRSA to methicillin, β-lactam antibiotics retain therapeutic potential in the treatment of MRSA infections. Using detailed *in vitro* and *in vivo* modelling, we recently showed that β-lactams attenuate MRSA, significantly reducing the bacterial burden, preventing dissemination and increasing host survival in invasive pneumonia and sepsis (204). Consistent with this, a randomised controlled trial involving 60 patients showed that flucloxacillin/vancomycin combination therapy reduced the duration of MRSA sepsis from 3 to 1.9 days compared to vancomycin alone (205). However, the long-term implications of using β-lactams as an adjunct to current CA-MRSA treatment regimens is unclear. Thus, while HA-MRSA strains are typically more resistant to antibiotics and less virulent (201), the effect of β-lactam exposure on the other virulence phenotypes remains unknown.

In general, the capacity of pathogens like MRSA to become resistant to new drugs only becomes apparent months or years after their introduction into clinical practice, during which time exposure of the pathogen to new drugs gradually increases, as does the likelihood that endogenous resistance will emerge. This clinical scenario can be mimicked in the laboratory using standard, batch culture techniques to isolate bacterial mutants exhibiting resistance to an antimicrobial drug.

However, such artificial culture conditions can mask the impact of acquired AMR on bacterial fitness (206, 207), a phenomenon that plays a significant role in determining maintenance and spread of the antimicrobial resistance (AMR) genotype in natural bacterial populations, and affects the disease-causing capacity of the pathogen. Here we used a continuous-growth chemostat to address this limitation by creating a more dynamic and competitive environment from which to isolate physiologically-relevant β-lactam resistant mutants. A USA300 culture was exposed to increasing concentrations of oxacillin (8-130 μg/ml) over a thirteen-day period. Among the hyper-resistant mutants isolated was a stable small colony variant in which the tandem amplification of the SCC*mec* element was identified as a new mechanism of high level, β-lactam resistance in MRSA.

2.2. Results and discussion

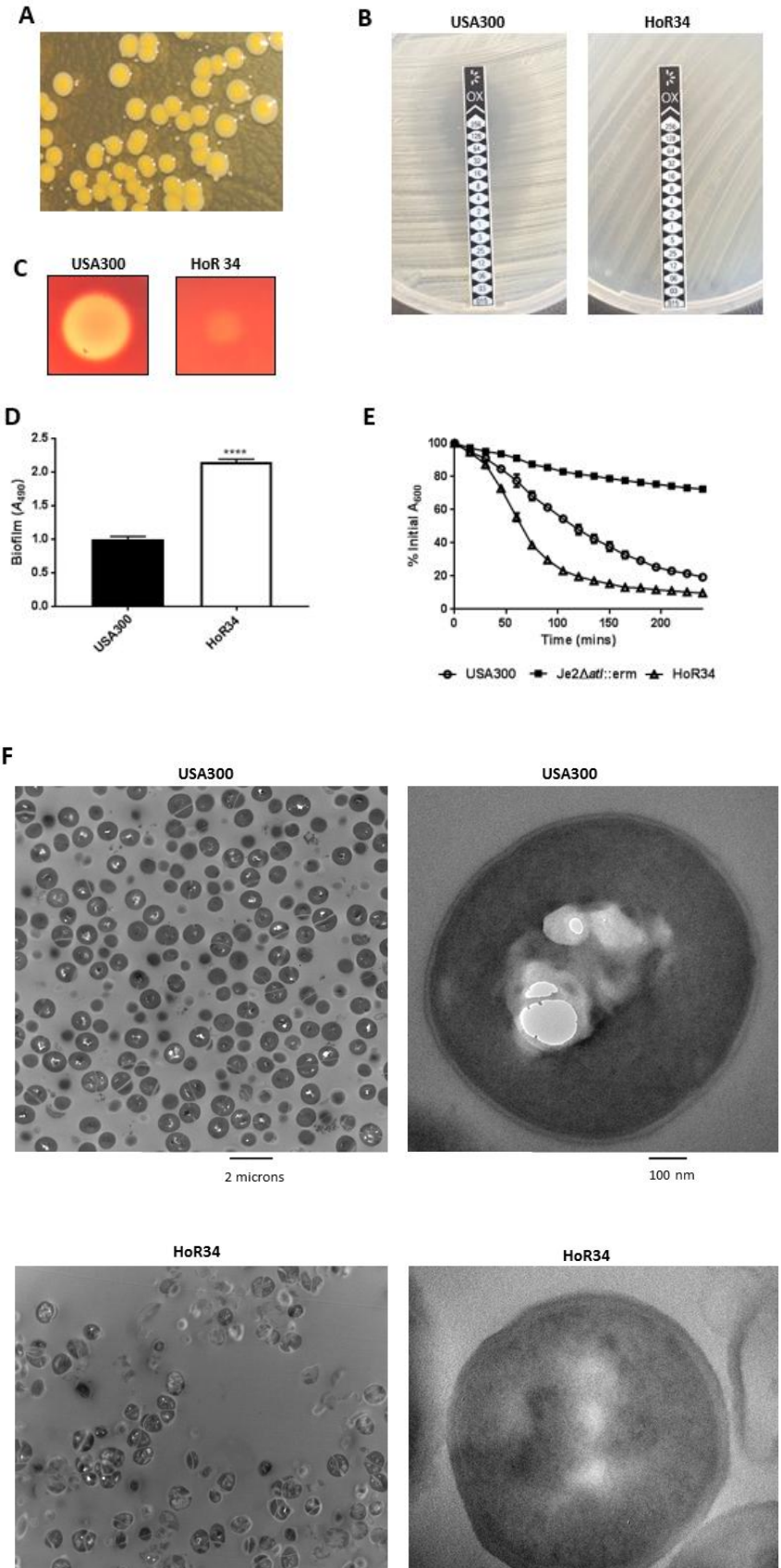
2.2.1. Isolation of USA300 oxacillin hyper-resistant mutants.

A USA300 nutrient broth culture was grown in a chemostat for 13 days. A sub-MIC concentration of oxacillin was used at the start of the chemostat culture and increased on an incremental, daily basis up to 130 μg/ml (equivalent to >800 μg/ml on Mueller Hinton, BHI or nutrient agar), as described in the methods. Isolated hyper resistant mutants were readily differentiated into i) small colony variants with no pigmentation, designated SCV HoR and ii) regular-sized, pigmented colonies, designated HoR (Fig. 2.1A). All HoR mutants selected exhibited oxacillin MICs of 800 μg/ml. Further analysis revealed that the SCV HoR mutants appeared to be clonal, exhibiting similar patterns of biofilm forming capacity and repressed β-haemolysis (data no shown). In contrast the faster growing HoR mutants appeared to be heterogeneous, exhibiting different patterns of biofilm forming capacity and β-haemolytic activity on sheep blood agar (Appendix 1.). Whole genome sequencing further revealed a variety of different mutations in nine of the regular sized HoR mutants recovered from the chemostat (Table 2.1.). These included four mutants with $Ser_{67}Lys$ amino acid substitutions in the diadenylate cyclase DacA, which is responsible for synthesis of c-di-AMP (112), four mutants with five different mutations in genes encoding predicted lipoproteins and one mutant with a $Glu_{227}Gln$ substitution in a predicted ABC transporter designated *abcA* (208) (Table 2.1.). Mutation of the *abcA* gene, located adjacent to the *pbpD* gene, may influence β-lactam resistance by up-regulating expression of *pbpD*, which encodes penicillin binding protein 4 leading to increased peptidoglycan cross linking (208).

Table 2.1. Genetic alterations in USA300 HoR mutants

Isolate	Genome	Nucleotide	Amino acid	Locus tag/gene
HoR20	703854	G-C	$Glu_{227}Gln$	RS03375/*abcA*
HoR18, 21, 27, 36	110748, 110752,111618, 111630, 111648	Multiple	Multiple	RS00520-RS00525
HoR33, 41, 43, 46	2288896	G-A	$Ser_{67}Lys$	RS11640/*dacA*
HoR34	19122	A-C	$Thr_{260}Pro$	*gdpP*
		C-T	$Ala_{314}Val$	*guaA*
	44078	G-T	$Glu_{511}Asp$	*guaA*
	1775825	C-A	$Glu_{37}STOP$	*clpX*
	2046530	G-A	$Gln_{305}STOP$	*camS*

In addition to a small colony size (Fig. 2.1A), impaired growth (data not shown) and a high oxacillin resistance (Fig. 2.1B), a representative SCV HoR, designated HoR34, also exhibited pleiotropic phenotypic changes including reduced β-haemolytic activity (Fig. 2.1C), increased biofilm formation (Fig. 2.1D), increased autolytic activity (Fig. 2.1E), altered cell morphology including defective septa formation (Fig. 2.1F) and an approximately 2-fold increase in cell wall thickness (18.6 ± 1.8 nm in USA300 versus 36.1 ± 4.2 nm in HoR34) (Fig. 2.1F). Genome sequence analysis of HoR34, revealed that a native plasmid pUSA02 (which carries tetracycline resistance) had been lost and identified non-synonymous mutations in the *gdpP*, *guaA*, *clpX* and *camS* genes (Table 2.1). As noted above mutations in *gdpP* have previously been implicated in the HoR phenotype (201, 202) but were not associated with a small colony phenotype, which is clinically important in persistent infections (209). Therefore to determine if the *guaA*, *clpX* or *camS* mutations (alone or in combination) were involved in the small colony size of HoR34, the mutant was subjected to daily subculture in the absence of antibiotic selection for 2 weeks in an effort to isolate fast-growing revertants. However both the SCV and the oxacillin hyper-resistance phenotypes of HoR34 were stable and no fast growing revertants were isolated even after repeated attempts. However the oxacillin MIC of the passaged HoR34 strain was reduced from 800 μg/ml to 300 μg/ml, indicating that although the strain continued to be hyper-resistant, oxacillin resistance levels in this strain can be regulated.


A
B
USA300
HoR34
C
USA300
HoR 34
D
Biofilm (A_{490})
****
USA300
HoR34
E
% Initial A_{600}
Time (mins)
USA300
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USA300
2 microns
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HoR34
HoR34
2 microns
100 nm

Figure 2.1. Phenotypic characteristics of homogeneously oxacillin resistant strains recovered from the chemostat culture. A. Small colony variants and other isolates recovered from the chemostat culture after 13 days at an oxacillin concentration of 130 µg/ml grown on BHI agar for 24 h. **B.** Oxacillin MIC of USA300 and HoR34 determined using Etests. **C.** β-haemolytic activity of USA300 and HoR34 on sheep blood agar. **D**. Biofilm assay of USA300 and HoR34. **E.** Autolytic activity in USA300, HoR34 and a USA300 JE2 *atl* mutant (negative control) were grown to early exponential phase in BHI at 37°C and washed in PBS and adjusted to A_{600} = 1.0 in 0.01% Triton X-100. The A_{600} was measured initially and at 15 min intervals thereafter with shaking incubation at 37°C. Autolytic activity is expressed as a percentage of the initial A_{600}. Results of three independent experiments are shown. **F**. Cell morphology and cell thickness of USA300 and HoR34 determined using transmission electron microscopy at 8000X and 100,000× magnification.

Although mutations in *gdpP* have previously been implicated in the HoR phenotype (106, 201, 204), complementation experiments unexpectedly revealed that the wild type *gdpP* allele carried on plasmid pLI50 had no effect on oxacillin MIC (Appendix 2A) and the small colony phenotypes (data not shown). For control purposes and consistent with previous studies implicating *gdpP* in autolytic activity (106, 201, 204), HoR34 exhibited increased autolytic activity that was successfully complemented with the p*gdpP* plasmid (Appendix 2C). The potential role, if any, of the identified mutations in *guaA, clpX* and *camS* in the HoR phenotype is unknown. However the *clpX* and *camS* genes contain mutations introducing stop codons (Table 2.1) suggesting they are unlikely to encode functional proteins, while predicted loss of function mutations in *guaA* have previously been implicated in the HoR phenotype. Consistent with this, p*guaA*, p*clpX* and *pcamS* plasmids were unable to complement the small colony (data not shown), high oxacillin MIC (Appendix 2A), biofilm (Appendix 2B) or autolytic (Appendix 2C) phenotypes of HoR34. Taken together, these data suggest that the mutations in *guaA, clpX, camS and gdpP*, at least on their own, are not involved in HoR34 hyper-resistance, and raised the possibility that other genomic rearrangements were responsible for this phenotype.

2.2.2. Chromosomal amplification of the SCC*mec* element in HoR34.

A number of recent studies have indicated that large regions of the *S. aureus* chromosome can undergo duplication and amplification events (210, 211). To investigate if such genomic rearrangements had taken place in HoR34, read coverage across the genome was analysed. Illumina sequence reads covering the SCC*mec* element were >10 times over-represented compared to other parts of the chromosome (Fig. 2.2A). LightCycler qPCR confirmed 10-fold higher levels of *mecA* in HoR34 gDNA samples compared to USA300 (data not shown). To determine whether the SCC*mec* element had amplified on the chromosome or excised and re-integrated at multiple sites around the chromosome, we attempted to assemble the Illumina sequence reads corresponding to the SCC*mec* element into contigs. However these efforts were hampered by the short reads. To address this we used MinION genome sequencing, which generates sequence reads of 10Kb. The MinION genome sequence was used as a scaffold onto which the Illumina sequence reads were mapped (Fig. 2.2A). The combined MinION/Illumina sequence data revealed the presence of 10 tandem SCC*mec* element repeats on the HoR34 chromosome (Fig. 2.2B). All 10 copies of SCC*mec* were completely intact and no additional DNA sequences were identified at the join sites. Oligonucleotide primers designed to span the join sites of tandem SCC*mec* elements amplified PCR produced of the predicted size from HoR34 but not USA300, whereas control primers targeting *mecA* amplified PCR products of the predicted size from both HoR34 and USA300 (Fig. 2.2C).

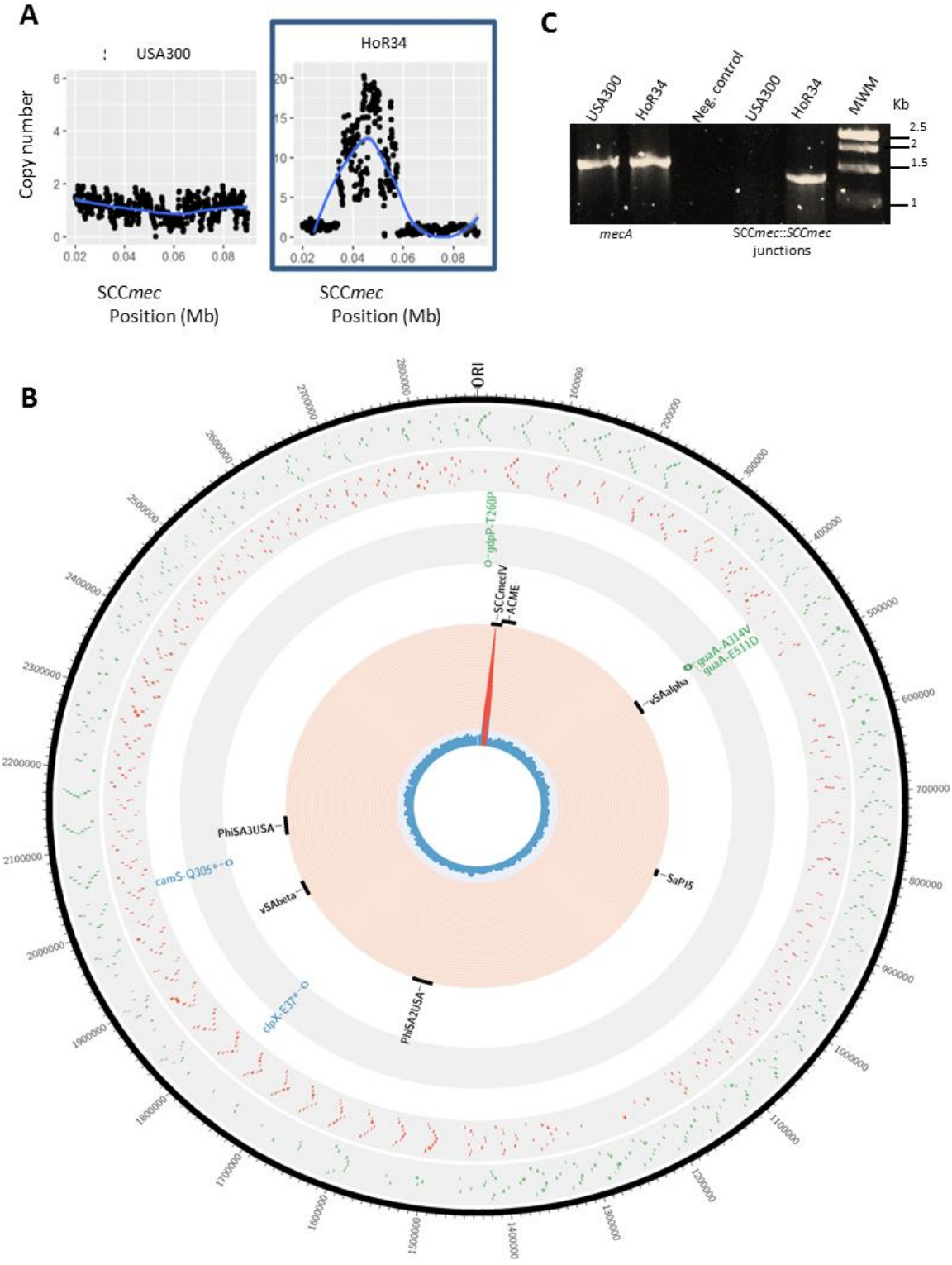

Figure 2.2. Genome sequence read coverage of USA300 and HoR34. **A.** Copy number as determined by Illumina sequence read coverage across SCC*mec* for USA300 (Sample 1A_S1), HoR34 (Sample 8A_S8, highlighted with blue box). **B.** Chromosomal organisation of HoR34 depicting expansion of the SCC*mec* element and locations of *gdpP, clpX, camS* and *guaA* mutations. On the circular map, the inner track shows copy number of 10 kb non-overlapping loci across the genome with loci that had copy number greater than two shown in red and those with copy number

less than two shown in blue. The next track shows black blocks illustrating different regions on the genome e.g. SCC*mec* and ACME. Single nucleotide polymorphisms are shown on the third track. Missense mutations are labelled in green whereas stop gain mutations are labelled in blue. Genes are shown in the outermost tracks. Genes transcribed in the forward (5' > 3') direction are labelled in green and are in the outside track whereas those transcribed in the reverse direction are labelled in red. **C.** PCR amplification across the SCC*mec* junctions in HoR34. Amplification of the *mecA* gene in both USA300 and HoR34 was used as a control.

2.2.3. Stability of the SCC*mec* amplification event.

Although the small colony phenotype of HoR34 was stable in the absence of antibiotic selection, the genomic stability of the SCC*mec* amplification was unknown. Using qPCR the relative abundance of *mecA* was compared in USA300 and HoR34 grown in the presence and absence of oxacillin. Unlike the original chemotat isolate HoR34 in which *mecA* levels were 10-fold higher than USA300, the *mecA* levels in HoR34 passaged strain in antibiotic-free media for 2 weeks, were only 3-fold higher than USA300 (Fig. 2.3A), indicating that up to seven of the amplified SCC*mec* elements were excised/lost from the original chemostat isolate during this time. However further passage of this HoR34 culture in 0.5, 64 and 100 μg/ml oxacillin was accompanied by a significant, concentration-dependent increase in *mecA* levels (up to 17-fold compared to USA300) (Fig. 2.3A). These data suggest that recombination between the tandem SCC*mec* elements in HoR34 facilitates accordion-like contraction and expansion in response to oxacillin exposure. Consistent with these qPCR data, Western blot analysis of HoR34 grown in 0, 0.5, 64 and 100 μg/ml oxacillin also revealed concentration-dependent increases in PBP2a expression (Fig. 2.3B).

To investigate why a small colony variant may have been selected and maintained in the chemostat, we performed competition experiments between the USA300 and HoR34. Predictably the wild type USA300 out-competed the slower-growing HoR34 in the absence of antibiotic selection (Fig. 2.3C). However in the presence of sub-inhibitory oxacillin (0.5 μg/ml), HoR34 exhibited a significant fitness advantageous and strongly outcompeted the wild type (Fig. 2.3D). Collectively these data identify tandem amplification of the SCC*mec* element as a new mechanism of high-level methicillin resistance in MRSA, which may provide a competitive advantage for MRSA under antibiotic selection.

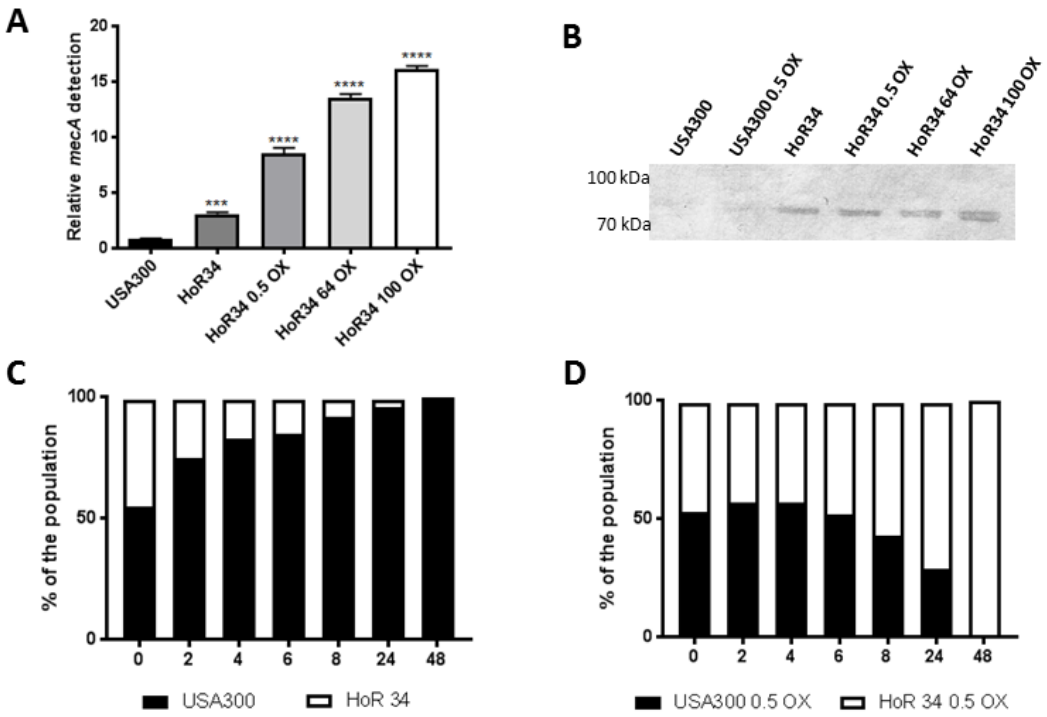

Figure 2.3. Chromosomal amplification of the SCC*mec* element can drive high level oxacillin resistance. A. Comparison of relative *mecA* abundance by LightCycler qPCR in HoR34 grown for 24 h in BHI supplemented with 0, 0.5, 64 or 100 µg/ml oxacillin. B. Comparison of relative PBP2a expression by Western blot analysis in USA300 and HoR34 grown in BHI and BHI supplemented with 0, 0.5, 64 or 100 µg/ml oxacillin. **C.** Competitive growth of USA300 and HoR34 over 48 hours in BHI and BHI supplemented with oxacillin (0.5 µg/ml). The CFU of each strain was enumerated on BHI agar to count all bacteria and BHI oxacillin (30 µg/ml) to count HoR34. The ratio of the two strains in each culture is shown. The data presented are mean and SD of three experiments. Statistical evaluation was performed using a paired two tailed t-test.

2.3. Concluding remarks.

Several genetic mechanisms may have contributed alone or in combination to the SCC*mec* amplification event in HOR34. Expression of the *ccr* recombinase genes which excise SCC*mec* (54, 212) can be increased by β-lactams and vancomycin (213), potentially generating multiple, extra-chromosomal copies of SCC*mec* capable of subsequent reintegration. This possibility is supported by a recent study which identified a replication initiator gene upstream of the *ccr* recombinase genes suggesting that the element may be replicative (214). Other mechanisms that may have contributed to the SCC*mec* amplification, alone or in combination with Ccr-mediated excision, include RecA-dependent non-equal homologous recombination or RecA-independent mechanisms such as recombination between single-stranded repetitive sequence on sister chromatids at the replication fork (215). The absence of repeat sequences flanking the SCC*mec* amplification may also suggest that an initial double-strand break (DSB), followed by RecA-dependent DSB repair during rolling circle replication may drive the production of long tandem arrays in a single generation, which have previously been implicated in fast adaption to drug treatment (216, 217). Following the initial SCC*mec* duplication/amplification, the long stretches of homology are likely to facilitate RecA-mediated expansion and contraction of the element in different concentrations of oxacillin, as recently observed in a *S. lugdenensis* strain carrying an amplified *isd* locus (211).

Recombination events leading to partial deletion of the SCC*mec* locus have been described previously. For instance, increased vancomycin resistance has been linked to site-specific insertion sequence-mediated SCC*mec* excision, including *mecA* (218). Coupled with previous work on intermediate vancomycin-resistant (hVISA) isolates that had a partial deletion of *recA* (210), this suggests that distinct RecA-independent mechanisms may be favoured in high β-lactam or vancomycin environments such that the number of *mecA* copies is optimised to be either high or low (respectively), reflecting the incompatibility of β-lactam and vancomycin resistance (219).

Even though multiple copies of SCC*mec* were maintained by HoR34 following repeated subculture in the absence of oxacillin selection, no evidence for

SCC*mec* amplification was found in a search of 404 *S. aureus* genomes using read coverage of the *mecA* gene normalised with read coverage of three single copy genes (data not shown). On the other hand, our growth competition experiments revealed the increased competitiveness of HoR34 in the presence of oxacillin was balanced by a significant loss of competitiveness in the absence of antibiotic selection, suggesting that MRSA strains carrying multiple SCC*mec* elements are unlikely to be maintained under physiological conditions or in clinical environments where exposure to antibiotics is sporadic. Furthermore, although the *gdpP, camS, clpX and guaA* mutations in HoR34 did not contribute to the high oxacillin MIC, it is possible that one or more of these mutations played a role in the expansion and maintenance of the SCC*mec* element. Taken together our data identify chromosomal amplification of the SCC*mec* element as a new mechanism that may be used by MRSA to adapt to, and be more competitive in, high oxacillin environments.

2.4. Materials and Methods

2.4.1. Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 2.2. *S. aureus* strains were grown overnight at 35°C or 37°C in Brain-Heart Infusion (BHI) media (Oxoid) or Mueller Hinton (MH) (Oxoid) supplemented with 10 μg/ml chloramphenicol (Sigma) or 0.5 - 100 μg/ml oxacillin (Sigma) as indicated. Solid media were further supplemented with 1% agar. *Escherichia coli* strains were grown at 37°C on Luria Bertani (LB) medium supplemented, when required, with ampicillin (100 μg/ml).

Table 2.2. Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant Details	Reference or source
S. aureus		
RN4220	Restriction-deficient laboratory *S. aureus.*	(220)
USA300	CA-MRSA expressing heterogeneous resistance to oxacillin	(221)
HoR34	USA300 derivative expressing high level resistance to oxacillin	This study
ATCC® 29213	MSSA strain for susceptibility testing	(222)
E. coli	*E. coli* Top10	(223)
Plasmids		
pLI50	*E. coli-Staphylococcus* shuttle vector. Ap^r (*E. coli*), Cm^r. (*Staphylococcus*)	(224)
PDrive	*E. coli* cloning vector	(225)

2.4.2. Measurement of oxacillin minimum inhibitory concentration (MIC).

The oxacillin MIC for the *S. aureus* strains used in this study was determined in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines using E-tests strips from Biomerieux on Mueller Hinton agar (Oxoid) containing 2% NaCl.

2.4.3. Isolation of USA300 oxacillin hyper-resistant mutants using a continuous culture chemostat system.

A 580 ml capacity laboratory reactor containing 500 ml of nutrient broth (Oxoid) was used as described previously (226). The CA-MRSA strain USA300 (oxacillin MIC = 32 mg/l on MHA) was inoculated into the chemostat and allowed to grow to stationary phase for 2 days at 37^{o}C in the absence of any antibiotic selection or media replacement. A growth media reservoir containing 20L of nutrient broth was then connected to the chemostat and fed to the chemostat using a peristaltic pump at a flow rate of 100 ml/h, replacing the entire nutrient broth volume of the chemostat every 5 h. After 24 h continuous culture growth in the absence of antibiotic selection, the nutrient broth in the feeding tank was supplemented with oxacillin at a concentration of 8 mg/L. Thereafter the oxacillin concentration in the growth medium reservoir was increased in a step-wise manner every day reaching a final concentration of 130 mg/L on Day 12. Culture samples were collected aseptically from the chemostat after 24 hours culture at each oxacillin concentration before being serially diluted and inoculated onto BHI agar supplemented with oxacillin 100 μg/ml. The minimum inhibitory concentrations (MICs) of colonies recovered from these plates were determined on BHI agar supplemented with oxacillin ranging from 100-1200 μg/ml. All isolates examined were hyper-resistant and capable of robust growth on BHI agar supplemented with 800 μg/ml oxacillin.

2.4.4. Complementation of HoR34 with *gdpP, guaA, camS* and *clpX*.

The *gdpP, guaA, camS* and *clpX* genes were amplified from USA300 genomic DNA by PCR using primers listed in Table 2.3., before being cloned into the cloning vector pDrive (Qiagen) in *Escherichia coli* TOP10. The sequence of inserts in recombinant plasmids was verified by Sanger sequencing (Source Biosciences) before being subcloned on *Eco*RI or *Bam*HI/*Hin*dIII restriction fragments into the *E. coli-Staphylococcus* shuttle plasmid pLI50 (224). The plasmids were transformed by electroporation into the restriction-deficient strain RN4220, and subsequently into HoR34. All plasmid-harbouring strains were cultured in medium supplemented with 100 μg/ml ampicillin (*E. coli*) or 10 μg/ml chloramphenicol (*S. aureus*) to maintain plasmid selection.

2.4.5. Haemolysis, biofilm and autolysis assays.

β-haemolysis was assessed by growth of cultures on BHI agar supplemented with 5% sheep blood. Inoculated sheep blood agar plates were incubated overnight at 37^{o}C and then for a further 24 hours at 4^{o}C. Semi-quantitative measurements of biofilm formation were determined under static conditions using Nunclon Hydrophilic 96 well polystyrene plates that were tissue culture treated (Nunc, Denmark) as described previously (151). A biofilm-positive phenotype was defined as an $\boldsymbol{A}_{490} \geq 0.17$. Triton X-100 induced autolysis assay were performed essentially as described previously (204). Each experiment was repeated at least three times and average data presented.

2.4.6. Transmission Electron Microscopy (TEM).

Overnight BHI cultures were diluted 1:200 in fresh BHI and grown at 37°C to an A_{600} = 1.0. Cell pellets collected from 10 ml culture aliquots were resuspended in fixation solution (2.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.4]), incubated overnight at 4°C before being treated with 2% osmium tetroxide followed by 0.25% uranyl acetate for contrast enhancement. The pellets were then dehydrated in increasing concentrations of ethanol followed by pure propylene oxide, and transferred to a series of resin and propylene oxide mixtures (50:50, 75:25, pure resin) before being embedded in Epon resin. Thin sections were cut on an ultramicrotome. Images were analysed using a Hitachi H7000 instrument. At least 3 to 5 measurements of cell wall thickness were performed on each cell and 88 cells were measured for each sample.

2.4.7. PCR and Quantitative PCR.

Amplification of the *mecA* gene and the SCC*mec* junctions in HoR34 was achieved using the primers in Table 2.3. Quantitative PCR (qPCR) for *mecA* was performed on the Roche LightCycler 480 instrument using the LightCycler 480 Sybr Green Kit (Roche) and the mecA1 primer set in Table 2.3. The *gyrB* gene was used as an internal standard for all reactions using previous described primers (201). For each reaction, the ratio of *mecA* and *gyrB* transcript number was calculated as

follows: $2^{(Ct\ gyrB\ -\ Ct\ mecA)}$. Each qPCR experiment was performed at least three times and average data and standard errors are presented.

Table 2.3. Oligonucleotide primers used in this study

Target Gene	Primer Name	Primer Sequence (5'-3')
GdpP	gdpP_Fwd	GCCGAATGCAGTAACGATTT
	gdpP_Rev	TTGTTGGCGTTCTTGTTTTG
guaA	guaA_Fwd	AGAGGACAAAGCGCCTAAGA
	guaA_Rev	CCTTACCCCTTTTCCGTCCT
clpX	clpX_Fwd	AACGCAAAGTTCGTTGAAGG
	clpX_Rev	TGAGCGTCAACTTTGATTGG
camS	camS_Fwd	GCTGGTGAAGATGCAGGTTC
	camS_Rev	CCTGGTGCATTTGTTGAAACTG
mecA	mecA_Fwd	CATATCGTGAGCAATGAACTGA
	mecA_Rev	CATCGTTACGGATTGCTTCA
SCCmec Junction	SCCmecAJn_Fwd	CTTGCTGGGTGCTATTTGA
	SCCmecAJn_Rev	CGCTGTCTTCCTGTATTTCG
mecA	mecA1_Fwd	TGCTCAATATAAAATTAAAACAAACTACGGTAAC
	mecA1_Rev	GAATAATGACGCTATGATCCCAA
gyrB	gyrB_Fwd	CCAGGTAAATTAGCCGATTGC
	gyrB_Rev	AAATCGCCTGCGTTCTAGAG

2.4.8. Analysis of PBP2a expression

Total cell protein preparations were prepared from overnight cultures grown in 0, 0.5, 64 or 100 μg/ml oxacillin. Cell pellets were re-suspended in distilled water containing 5μg/ml lysostaphin, 10 units of DNase I, and 50:l of 10% SDS before being incubated at 37^{o}C for 30 minutes. Insoluble material was pelleted by centrifugation and the supernatant used for Western blotting as described previously (204).

2.4.9. Growth competition experiments.

Overnight cultures of USA300 and HoR34 cultures were diluted to A_{600} = 0.05 in fresh BHI media and grown for 6h. The cell density of both exponential phase cultures was adjusted to A_{600} = 0.1 in 500 ml flasks containing 50ml BHI or BHI supplemented with 0.5 µg/ml oxacillin and incubated at 37°C with shaking. The number of colony forming units in samples collected at 0, 2, 4, 8, 24 and 48h was determined by plating serial dilutions on BHI agar. Colonies formed by each strain were readily differentiated based on their tetracycline resistance and appearance i.e. the HoR34 colonies were tetracycline sensitive and had a while-coloured SCV phenotype whereas versus USA300 colonies a regular sized, tetracycline resistant and pigmented.

2.4.10. 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 P value <0.05 was deemed significant.

2.4.11. DNA Isolation & Sequencing

Genomic extractions were prepared on overnight cultures of the USA300 HoR mutants grown in BHI media or BHI media supplemented with 100 µg/ml oxacillin using the Wizard Genomic DNA kit (Promega). The optimised technique required the bacterial cells to be pre-treated with 2 µl of a 1mg/ml solution of lysostaphin (Ambi products, New York) in 200 µl 50 mM EDTA to assist lysis for DNA extraction. DNA sequencing was performed using an Illumina MiSeq platform by the Biomedicum Functional Genomics Unit (Finland). Sequencing read length ranged from 36 to over 300 bases.

2.4.12. Quality control of genome sequence data.

Read quality was assessed by screening the read length, nucleotide and quality score distributions using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). The DNA reads were trimmed based on quality scores. Potential adaptor sequence was removed using Trimmomatic v0.32 (227), which scanned reads using a four-base sliding window and trimmed reads where the average Phred base quality of the window was below 30. All ambiguous 'N' bases and reads shorter than 35 bp were removed. The first 20 bases of the DNA reads were removed because they had a nucleotide content that deviated from the expected 25% rate for each base. The DNA reads were corrected using BayesHammer (228) to reduce sequencing errors that can reduce the alignment quality, increase false positive SNP rates and reduce the number of valid SNPs (229). These steps retained 84% of the initial DNA reads among HoR isolates from the chemostat yielding median quality values > 30 across the reads. Insert sizes were an average of 185. Read lengths after trimming and filtering averaged 185 bp and the average coverage per sample on the chromosome, calculated using the Bedtools genomecov function (230) on mapped reads, ranged from 47 to 197.

2.4.13. Genome assembly.

The error-corrected paired and unpaired reads for each DNA sample were assembled using SPAdes v3.1.1 [5] with k-mers 21, 33, 55, 77, 99 and 127 and the 'careful' parameter, which minimized the number of mismatches in the contigs (231). The resulting assemblies were compared to the reference USA300_FPR3757 (PMID:16517273) chromosome using QUAST v2.3 (232). The GC content of each assembly was 32.6%, and there were between 31 and 51 scaffolds per assembly, with N50 values > 200 Kb. One or two short gaps (<500 bp) were found in each assembly that could not be fully closed using Gapfiller (233).

2.4.14. Single nucleotide polymorphism (SNP) calling using assembly and read mapping.

The chromosome and three plasmids (GenBank accessions NC_007790-NC_007793) were indexed with *k*-mer of thirteen and step size of two using SMALT v5.7 (http://www.sanger.ac.uk/science/tools/smalt-0). The error-corrected DNA reads were mapped to the genome with SMALT, which applied a Smith-Waterman sequence alignment algorithm. The SAM (sequence alignment/map) files were converted to BAM (binary alignment/map) files using Samtools v0.1.18 (52). The BAM files were then coordinate-sorted, the paired and unpaired files were merged, and PCR duplicate reads were removed. Candidate SNPs were detected where the base quality (BQ) was >25, the mapping quality (MQ) was >30, and the read depth was <100 using Samtools Mpileup v0.1.18, Bcftools v0.1.17-dev, and the Samtools v0.1.11 vcfutils.pl function. The read depth allele frequency of the non-reference allele (RDAF) and local coverage were estimated using Samtools Pileup v0.1.11.

To call SNPs using an assembly-based approach, the scaffolds produced by SPAdes were aligned to the USA300 reference genome using nucmer in the MUMmer v3.23 package. This was followed by eliminating conflicting repeat copies using the 'delta-filter' command and the 'show-snps' comand to call SNPs and indels. The union of SNPs called by nucmer and SNPs called by Bcftools was used as a candidate SNP set. These sites were queried across all samples using the Samtools Pileup files to find false negative SNPs uncalled by nucmer or Bcftools. The RDAF of the non-reference alleles was reported for each SNP using Samtools Pileup output. Each candidate SNP was assessed using the following additional criteria:

1) SNP Quality (SQ) >30
2) read coverage >5
3) forward-reverse read coverage ratio between 0.1 and 0.9
4) non-reference read allele frequency >0.1
5) 2+ forward reads
6) 2+ reverse reads

Results were converted to variant call format (VCF) and annotated. SNPs were homozygous if the RDAF was ≥ 0.85 and heterozygous if $0.1 < \text{RDAF} < 0.85$. Insufficient read depth coverage was present to predict SNPs with RDAF < 0.1.

2.4.15. Indel calling using split-read mapping.

Deletions and short insertions (indels) were called using the samtopindel script to convert the BAM files, and then with Pindel (234) to only keep indels with at least ten supporting reads. The RDAF of the indels smaller than the read length were calculated using the BAM files in IGV (number of reads with indel at locus / all reads at the locus). For indels greater than one bp in length, the sum of the number of reads with the indel was divided by the sum of the number of reads at each site in the indel. This approach may be limited by uneven coverage at a locus. If the indel was longer than the read length, then a lack of read coverage at the sites predicted to have the mutation was considered evidence of the deletion and the RDAF was set to one.

2.4.16. Variant annotation.

The functional effect of SNPs and indels was estimated by annotation with SnpEff v4.0e (235) using the 'Staphylococcus_aureus_USA300_FPR3757_uid58555' database file from the SnpEff database. Results were manually checked using the reference genome annotation.

2.4.17. Copy number variation detection using read coverage.

Copy number variants (CNVs) were screened using the BAM files containing reads with MQ > 30 to reduce false positive rates [12–14]. Coverage was calculated for every base using genomecov in Bedtools with the '-d' flag (230) so that the median chromosomal coverage could be calculated for each sample. Genome-wide coverage levels were analysed in 10 Kb and 25 Kb windows and plotted as 5 Kb sliding windows with a 2.5 Kb step using the Bedtools makewindows function (230). Coverage for each window was normalised by dividing it by the median coverage of the chromosome to produce a copy number estimate. Windows with copy number ≥ 2 were reported. The copy number of plasmids was determined by dividing the median read coverage of the plasmid by the median read coverage of the chromosome.

2.4.18. MinION long-read genome sequencing

To evaluate the number of SCC*mec* copies and their location contiguous with or excised from the chromosome, genomic DNA from HoR34 was amplified to generate long reads using a Oxford Nanopore Technologies (ONT) MinION. The DNA was sheared with Covaris g-tubes, end-repaired and dA-tailed with NEB Blunt/TA Ligase before adapter ligation, purification using MyOne C1 Streptavidin beads and sequencing using the MinION, which determines the nucleotides at each site based on changes in the ion current flow. ONT Library loading bead and Genomic DNA Sequencing kits were used. The FAST5 file generated was screened with Poretools (236) and the data assembled using an overlap-layout-consensus (OLC) approach applied by the LQS pipeline in Nanopolish (237).

ACKNOWLEDGMENT

We thank Cyril Carroll for assistance with the chemostat experiment.

FUNDING

This study was funded by a grant from the Irish Health Research Board (HRA_POR/2012/51) (to J.P.O'G).

Transparency declarations

The funding agency for this research (Health Research Board) have not played any decision-making role in the research. The authors declare no conflicts of interest relating to this study.

Chapter 3

β-Lactam Resistance in Methicillin-Resistant *Staphylococcus aureus* USA300 Is Increased by Inactivation of *pgl* gene.

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Running title: Mutation of *pgl* facilitates expression of high level methicillin resistance in CA-MRSA

Abstract

Highly virulent community-acquired (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) have recently emerged as a frequent cause of infection in individuals who had no predisposing risk factors or exposure to health care settings. Recognition that these CA-MRSA isolates are responsible for many healthcare associated infections has increased their clinical significance. Previous studies showed that MRSA strains carrying the *mecA* gene which encodes a low affinity penicillin binding protein, PBP2a, express low level, heterogeneous resistance (HeR) to β-lactam antibiotics. Conversion of HeR to high level homogeneous resistance (HoR) was associated with accessory mutations in genes increasing levels of the stringent response alarmone (p)ppGpp and cyclic diadenosine monophosphate (c-di-AMP), and activation of *mecA*. Here using a genetic screen of the Nebraska Transposon Mutant Library, we demonstrated that inactivation of the *pgl* gene which encodes 6-phosphogluconolactonase, resulted in increased resistance to β-lactam antibiotics. The *pgl* mutant had a thicker cell wall and reduced cell size, both of which are consistent with elevated β-lactam resistance. The *pgl* mutant also exhibited reduced haemolysis as well as a growth defect. However, LC/MS-MS metabolite analysis showed that high level resistance in the *pgl* mutant was not associated with elevated c-di-AMP levels. Transduction of the *pgl* mutation into a laboratory MSSA isolate had no effect on oxacillin resistance but was accompanied by increased tolerance to β-lactam antibiotics. We hypothesise that the *pgl* mutant could redirect glucose flow to the glycolysis pathway. This could increase the availability of fructose-6-phosphate and enable sufficient peptidoglycan biosynthesis following exposure to β-lactam-induced cell wall stress.

3.1. Introduction

Staphylococcus aureus is a facultative pathogenic bacterium capable of causing a wide variety of diseases ranging from superficial skin and soft tissue infections to life threatening systemic infections (1, 2). Since the introduction of penicillin in the early 1940's, *S. aureus* has acquired resistance against nearly all antibiotic classes causing limitations in treatment options (12, 31, 32). Most concerning was the identification and rapid spread of methicillin resistant *S. aureus* (MRSA), a leading cause of nosocomial infections worldwide resulting in significant morbidity as well as 20 to 40% mortality (238). However, the incidence of MRSA infections has greatly increased in recent years due to the frequent isolation of community-associated MRSA (CA-MRSA) strains of USA300 type causing infections among individuals who have no traditional healthcare related risk factors (18). In addition, CA-MRSA isolates are now implicated in many healthcare associated infections exacerbating the issue for the healthcare system (239).

Methicillin and other β-lactams inhibit native penicillin binding proteins (PBPs) by binding to the transpeptidase domain which catalyses the cross-linking of the peptidoglycan cell wall. However, *S. aureus* has resistance to methicillin and oxacillin (the clinically used derivative of methicillin) through the acquisition of the *Staphylococcus* cassette chromosome *mec* (SCC*mec*) element harbouring the *mecA* gene, which encodes an alternative transpeptidase penicillin-binding protein 2A (PBP2a) (37). This gene has a low affinity for almost all β-lactam antibiotics, which are the first line treatment for *S. aureus* infections (39).

A major characteristic of methicillin resistance in community acquired-MRSA and to a lesser extent healthcare associated-MRSA is heterogeneity characterised by the majority of cells within a population exhibiting low to moderate levels of resistance (61, 62, 64). However subpopulations (≤0.1%) express high level resistance or a homogeneous methicillin resistance (HoR) phenotype (63). Expression of homogenous high level resistance not only depends on PBP2a production but also on the accumulations of mutations that impact stringent response and/or c-di-AMP signalling (111-115). Despite extensive investigation of this HoR MRSA strains continue to be responsible for therapeutic failures, and MRSA

remains on the WHO list of pathogens for which new antibiotics are urgently needed.

In a recent effort to explore the mechanisms that underpin β-lactam resistance, we described a genetic screen to identify novel mutations that impact the expression of resistance to β-lactam antibiotics in MRSA. Using the Nebraska Transposon Mutant Library, that comprises approximately 2,000 sequence-defined transposon mutants created in JE2, a derivative of the CA-MRSA strain, USA300-LAC (196), we identified mutants with increased resistance to cefoxitin, a β-lactam recommended in tests for *mecA* mediated oxacillin resistance in MRSA isolates by the CLSI standards, 2015. Through this approach, we identified a mutation in the *pgl* gene from the pentose phosphate pathway (PPP) that increased resistance to cefoxitin and other β-lactams. The involvement of Pgl, 6-phosphogluconolactonase which catalyses the hydrolysis of 6-phosphogluconolactone to phosphogluconate and the PPP in β-lactam resistance has not been previously reported. In addition, we concluded that this mechanism of resistance was independent of c-di-AMP levels, and at the same time affected a number of cell wall properties consistent with increased resistance.

3.2. Results

3.2.1. Mutation of *pgl* significantly increases β-lactam resistance in MRSA.

During a screen of the Nebraska Transposon mutant library (NTML) (196) for mutants exhibiting increased resistance to cefoxitin, a β-lactam antibiotic recommended by the Clinical and Laboratory Standards Institute for measuring *mecA*-mediated oxacillin resistance in MRSA strains, we identified a mutant with elevated resistance levels. Previous studies have shown that random mutations or gene disruption by transposon insertion can inactivate genes leading to elevated resistance in MRSA strains including USA300 (106, 201, 240). This screen utilised the NTML comprising approximately 2000 mutants that were created in the laboratory strain JE2, a derivative of USA300 LAC that is cured of plasmids p01 and p03. The parent strain USA300 LAC is the most prevalent strain associated with CA-MRSA infections worldwide (9, 241-243). Previous studies by our group have demonstrated that the transposon mutant NE810 (SAUSA300_1642) exhibited increased sensitivity to β-lactams and d-cycloserine (manuscript in preparation). In addition, the validity of this screen was supported by the identification of altered cefoxitin susceptibility in NE1714 (*relA*) (114, 202) and NE1447 (*ispA*) (244), both of which have previously been implicated in β-lactam resistance.

In a cohort of new mutants identified, NE202 (SAUSA300_1902) exhibited significantly increased resistance to oxacillin (Fig. 3.1A) and cefoxitin (Fig. 3.1B). The disrupted gene in this mutant, SAUSA300_1902 (*pgl*) encodes 6-phosphogluconolactonase, the second enzyme in the pentose phosphate pathway that converts 6-P-gluconololactone to gluconate-6-P as shown in Figure 3.1. A role for Pgl in antibiotic resistance and tolerance has not previously been reported. To investigate this, the NE202 mutant was also tested using disk diffusion assays to assess the susceptibilities to other cell wall active antibiotics including other β-lactams, cloxacillin and imipenim (Appendix 3A.) and vancomycin. We found that while the disruption of *pgl* did not alter susceptibility to vancomycin (data not shown), it was associated with increased resistance to the other β-lactams tested. To confirm the contribution of this transposon insertion to increased oxacillin resistance, the SAUSA300_1902 transposon mutation was transduced using phage 80α back into the parent, JE2 and LAC-13C (a USA300 LAC derivative lacking the 27-kb p03

plasmid carrying resistance to erythromycin). JE2 and 13C transductants harbouring the *pgl* transposon mutation exhibited increased β-lactam resistance (Appendix 3B). In addition, complementation of the *pgl* mutation in NE202 by the expression of the *pgl* gene under the control of its own promoter on plasmid pLI50 restored in wild type, heterogeneous oxacillin (Fig. 3.2A) and cefoxitin (Fig. 3.2B) resistance levels.

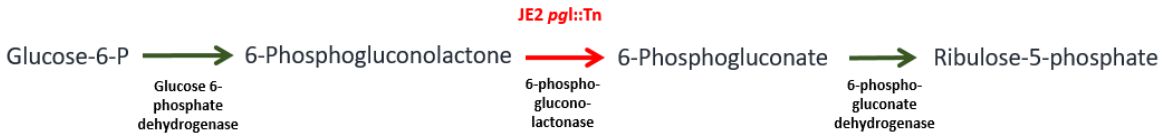

Figure. 3.1. Oxidative phase of the Pentose Phosphate Pathway highlighting the location of the NE202 mutant (JE2 *pgl*::Tn)

To investigate if increased resistance in NE202 was associated with increased expression of *mecA*, RT-pPCR analysis was performed using a LightCycler instrument. These experiments revealed no significant difference in *mecA* expression in NE202 compared to JE2 (Fig. 3.2C). Illumina whole genome sequencing of NE202 demonstrated that the *SCCmec* element was fully intact and that there were no other mutations in the genome that could contribute to the resistance phenotype. Further phenotypic analysis revealed that the NE202 mutant produced colonies that were smaller and more pigmented than wild-type colonies when grown at 37°C (Fig. 3.2E). The complemented NE202 mutant formed wild-type sized colonies indicating that mutation of *pgl* results in a growth defect in *S. aureus*. Growth rate analysis in liquid cultures using optical density (OD) measurements in 96 well plates did not support this (data not shown), and more detailed analysis of growth in NE202 is needed. It was also observed that the *pgl* mutant had no effect on biofilm forming capacity (Appendix 3C), however it resulted in a reduction in β-haemolytic activity when compared to JE2 that was also restored by complementation (Fig. 3.2D). High level oxacillin resistance in clinical isolates has previously been associated with the repression of the Agr system and a reduction of β-haemolytic activity on sheep blood BHI agar (245). Given that the β-haemolysin is encoded by the *hld* gene within the *agr* locus RNAIII transcript, the reduction in haemolytic activity in NE202 suggests that repression of haemolysis may be associated with Agr repression.

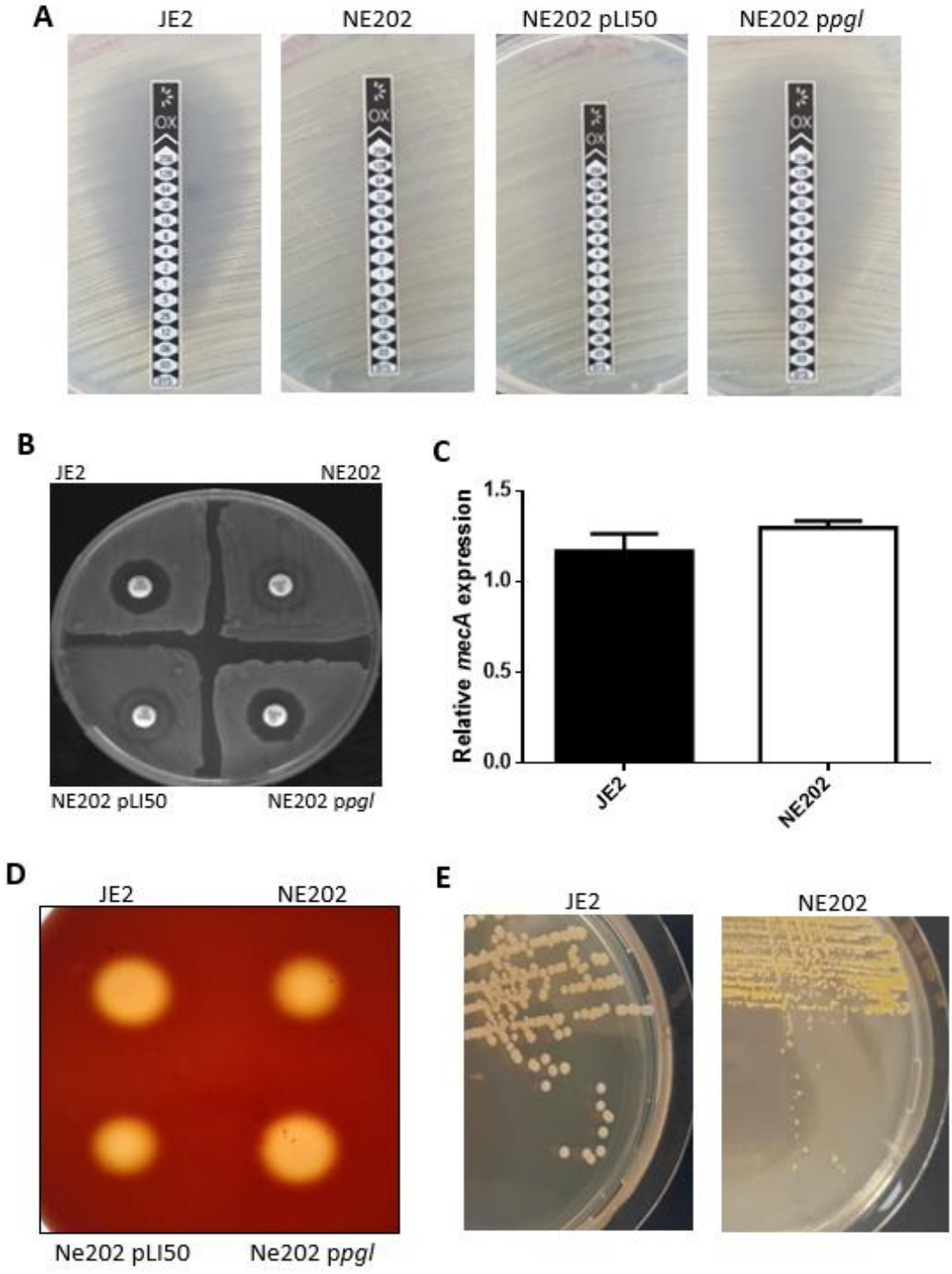

Fig. 3.2. Phenotypic characteristics of NE202. A & B. Oxacillin Etest measurements and cefoxitin disc diffusion of JE2 (wild type), NE202 (*pgl*::Tn), NE202 pLI50 (control) and complemented NE202 p*pgl*. **C.** Comparison of relative *mecA* gene expression by the LightCycler RT-PCR in JE2 and NE202 grown to an A600 =3 in BHI media. A minimum of three biological repeats along with three technical repeats were carried out for each isolate and standard errors are shown. **D.** β-haemolytic activity of JE2, NE202, NE202 pLI50 and NE202 p*pgl* on sheep blood agar. **E.** JE2 and NE202 grown on BHI agar for 24 h.

3.2.2. Mutation of *pgl* impacts cell size and cell wall structure.

The increased resistance of NE202 to β-lactams prompted us to investigate changes in cell size, shape and cell wall thickness. Previous work has shown a link between reduced susceptibility to cell wall active antibiotics and changes in cell envelope structure (116, 240). Scanning and transmission electron microscopy were used to compare whole cell morphology and cell wall thickness in NE202 and JE2. The NE202 cell wall was thicker than that of wild-type cells (Fig. 3.3C and 3.3D) (Table 3.1). Furthermore, quantitative analysis of the cell wall diameter in the SEM images revealed that the NE202 mutant was 12% smaller than the parent JE2 (Fig. 3.3A and 3.3B) (Table 3.1). These data reaffirms reports that mutations leading to increased resistance are accompanied by thicker cell wall architecture. Previous research demonstrated that the disruption of *clpX* or *clpP* led to increases in both resistance and cell wall thickness regardless of strain background (240). In addition, a LAC*Δ*gdpP*::kan mutant that displayed an 8-fold reduction in susceptibility to oxacillin and a 32-fold decrease in susceptibility to penicillin-G also displayed a reduction in cell size by more than 13% with a higher degree of cross-linked muropeptides (trimers and above) in the cell wall (116). Here SEM and TEM analysis showed that the transposon disruption of SAUSA300_1902/*pgl* led to alterations in the cell wall that are consistent with the increased resistance to cell wall targeting antimicrobials. However it remains unclear how reduced cell size impacts resistance levels. As mentioned above, the LAC*Δ*gdpP*::kan mutant that had reduced cell size also possessed increased levels of c-di-AMP (116). In addition, mutations that affect the pool of c-di-AMP within the cell have been shown to express altered resistance levels (112, 116). Therefore, increased c-di-AMP levels may play a role in controlling *S. aureus* cell size, which in turn impacts susceptibility to cell wall active antibiotics.

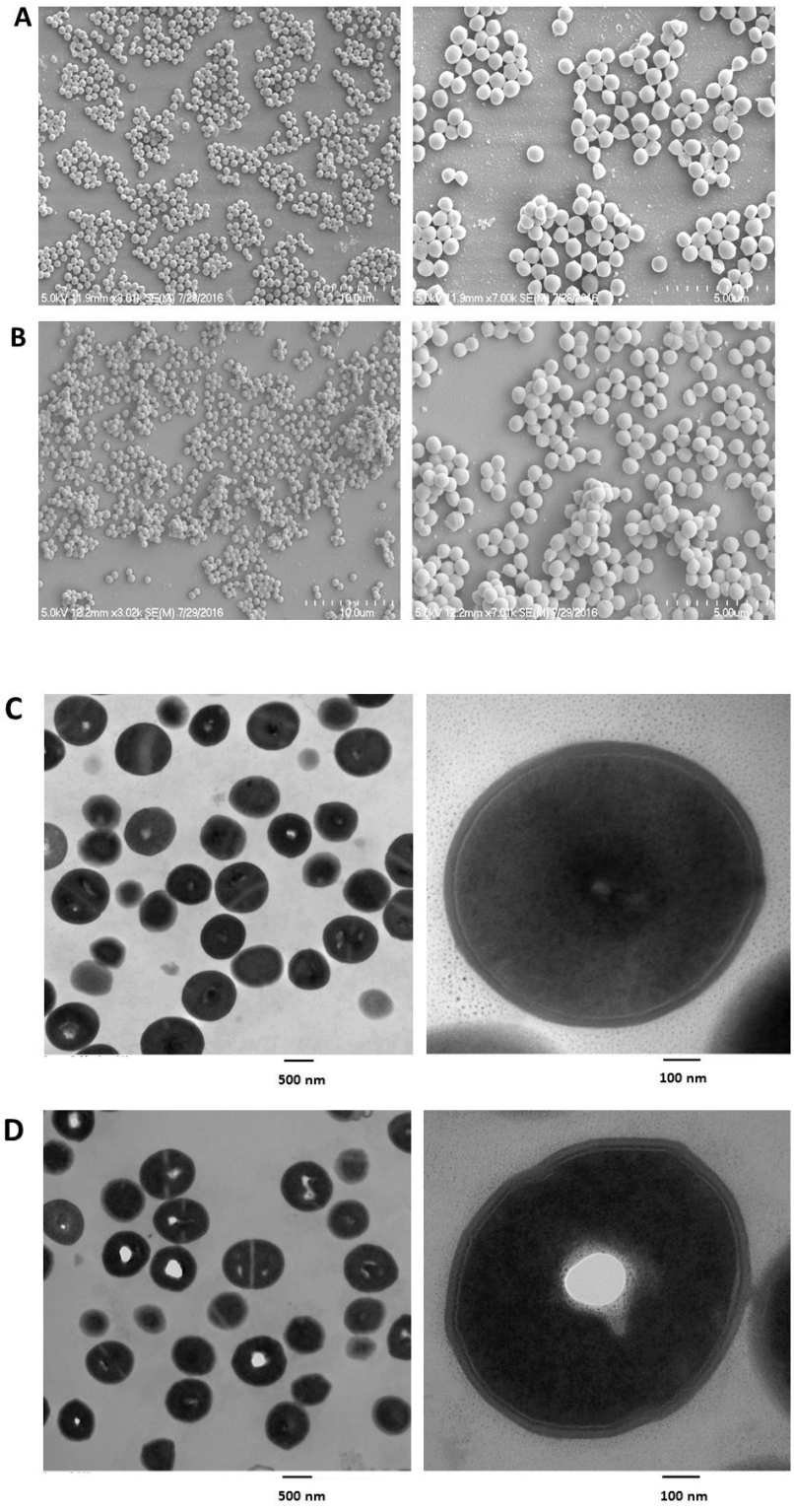

Figure 3.3. Mutation in *pgl* reduces cell size and increases cell wall thickness in MRSA. A and B. Comparisons of JE2 (A) and NE202 (B) cell morphology by scanning electron microscopy (SEM). **C and D.** Comparison of JE2 (C) and NE202 (D) whole cell morphology and cell wall thickness by transmission electron microscopy (TEM). TEM magnifications are ×15,000 (left) and ×70,000 (right). Cell size and cell wall thickness measurements are provided in Table 3.1.

Table. 3.1. Comparison of cell diameter and cell wall thickness in the NE202 *pgl* mutant and wild type JE2

	Mean cell diameter (μm) ± SD	Mean cell wall Thickness (nm) ± SD
JE2	0.896 ± 0.075	25.92787 ± 1.791
NE202	0.790222 ± 0.031	30.33578 ± 2.293

3.2.3. Role of c-di-AMP in the *pgl* mutant resistance phenotype

Previous studies revealed salt hypersensitivity in a spontaneous *gdpP* mutant that exhibited increased oxacillin resistance and a 15-fold increase in c-di-AMP levels (106, 117). Although *S. aureus* is intrinsically salt tolerant (246), mutations in genes associated with controlling the pool of c-di-AMP has been linked to salt hypersensitivity (112). Corrigan *et al.* 2013 identified c-di-AMP targets, more specially KtrA, a potassium transporter that contributes to *S. aureus* growth under osmotic stress (117). Intracellular accumulation of c-di-AMP and binding to KtrA is proposed to inhibit potassium uptake causing growth defects under osmotic stress. We assessed salt sensitivity of the NE202 mutant to ascertain if the *pgl* mutation may be impacting on c-di-AMP levels. As shown in Fig. 3.4A, the NE202 mutant exhibited increased salt sensitivity which was partially reversed in the complemented strain, suggesting that c-di-AMP levels may be elevated in NE202.

Cell extracts were sent to Prof. Kaever in the Hannover Medical School for LC-MS/MS analysis to compare c-di-AMP levels in JE2, NE202 and NE202 p*pgl*. The c-di-AMP concentration was reduced in NE202 and restored in the complimented mutant (Fig. 3.4B). Given that GdpP encodes a c-di-AMP phosphodiesterase and previous reports demonstrating that c-di-AMP levels are increased 15-fold in a *gdpP* mutant, this result was unexpected and suggest that the change in c-di-AMP levels in NE202 do not significantly contribute to increased oxacillin resistance in this strain. It remains unclear why c-di-AMP levels are reduced in the *pgl* mutant. A recent study revealed that inactivation of *clpX*, which has previously been implicated in homogeneous resistance, had no effect on the intracellular levels of c-di-AMP (247). Furthermore, inactivation of either *clpX* or *gdpP* alone compensates for the absence of lipoteichoic acid (LTA) (), and mutations in either gene are associated with decreased cell size, a small increase in

peptidoglycan cross-linking and smaller colonies (106). The data indicates that mutations in either *gdpP* or *clpX* can compensate for impaired LTA biosynthesis and β-lactam susceptibility via c-di-AMP-dependent and c-di-AMP-independent pathways, respectively. Our data suggest that, like *clpX* mutations, inactivation of *pgl* may also impact β-lactam resistance in a c-di-AMP-independent manner.

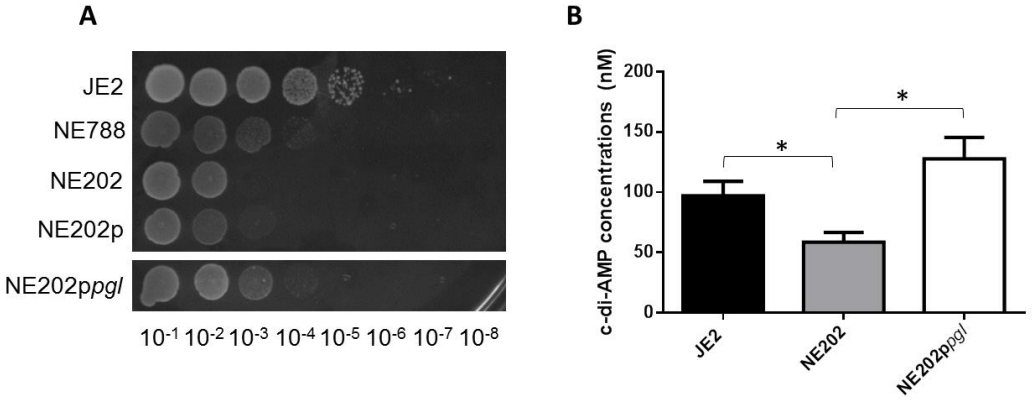

Figure 3.4. Mutation of *pgl* is associated with increased NaCl susceptibility and decreased c-di-AMP. A. NaCl tolerance of JE2, NE788 (*ktr*::Tn), NE202 (*pgl*::Tn), NE202 pLI50 (control) and complemented NE202 p*pgl* grown on BHI agar supplemented with 2.2 M NaCl. Representative results from three independent experiments are shown. **B.** Intracellular levels of c-di-AMP quantified by HPLC-MS/MS analysis. Average values from four biological replicates with standard error are shown. Asterisks indicate a significant difference using Student's *t*-test ($p<0.05$).

3.2.4. Inactivation of *pgl* increased β-lactam tolerance in MSSA.

Because increased oxacillin resistance in NE202 was not associated with increased *mecA* expression (Fig. 3.2C), we hypothesised that mutation of *pgl* may increase β-lactam resistance by increasing antibiotic tolerance levels. To investigate this, we transduced the *pgl* transposon mutation into the laboratory MSSA strain, 8325-4 using phage 80α. Erythromycin resistant candidate transductants were verified by colony PCR of the *pgl* locus (data not shown). Tolerant cells exhibit a bacteriostatic response to challenge with an antimicrobial drug that is bactericidal for the majority of a population. In these experiments, tolerance was defined as a ≤90% drop in viability after 6 hours challenge with 12.5µg/ml of oxacillin (111, 248). An

8325-4 *pgl*::Tn mutant exhibited tolerance (Fig. 3.5) without the concomitant increase in oxacillin resistance when compared to the parent 8325-4 (data not shown). Importantly, increased tolerance to β-lactams in the 8325-4 lineage of strains and *S. aureus* Newman has been linked to the inactivation of *gdpP* (111). Mutation of *gdpP* has also been linked to acid tolerance in *S. aureus, Bacillus subtilis* and *Lactococcus lactis* (249-251). These data demonstrate that mutation of *pgl* increases oxacillin tolerance, which appears to contribute to expression of high level β-lactam resistance in MRSA.

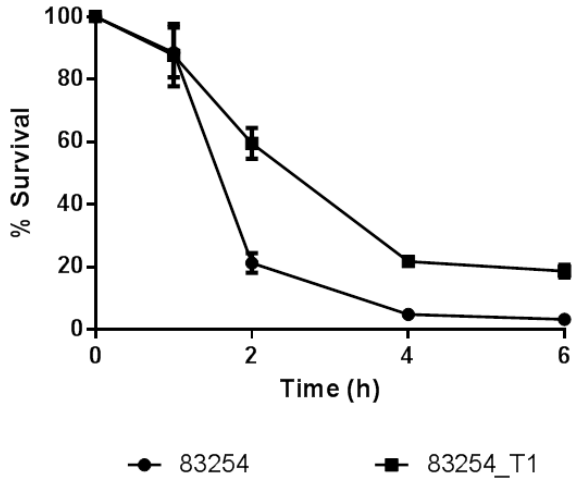

Figure 3.5. Mutation of *pgl* is associated with increased oxacillin tolerance. A. Oxacillin tolerance of *S. aureus* 8325-4 and 8325-4_T1 (*pgl*::Tn) following challenge with 12.5 µg oxacillin/ml for up to 6 hours. Data presented is the average of two independent experiments and standard error is shown.

3.3. Discussion

In a screen of the NTM library for mutants expressing high levels of resistance to cefoxitin, a *pgl* mutant was identified. Pgl, 6-phosphogluconolactonase, catalyses the hydrolysis of 6-phosphogluconolactone to phosphogluconate in the pentose phosphate pathway (PPP). The PPP is important to maintain carbon homoeostasis and to provide precursors for nucleotide and amino acid biosynthesis. The *pgl* mutant exhibited a number of phenotypic hallmarks associated with increased β-lactam resistance including reduced cell size and a thicker cell wall. The cell wall and cell surface appearance of the *pgl* mutant were similar to wild type, but the mutant formed smaller colonies than wild type, indicative of a growth defect. Previous reports have indicated that *pgl* deletions in *E. coli* and *L. monocytogenes* also impacted growth but these defects appeared to dependent on glucose availability in the media (252, 253). To date, a number of other genes have been reported to increase β-lactam resistance when inactivated. Most notably, the disruption of genes associated with controlling the pool of cyclic diadenylate monophosphate (c-di-AMP), a recently discovered essential secondary messenger in *S. aureus* (111-115). Mutation of *gdpP* in USA300 LAC which encodes a phosphodiesterase that specifically cleaves c-di-AMP, led to substantially increased resistance to penicillin, increased peptidoglycan cross-linking and decreased cell size (106). Another nucleotide messenger, (p)ppGpp, the effector molecule of the stringent response, has also been associated with increased resistance to β-lactams (113, 114). The stringent response alarmone enables cells to adapt to starvation conditions such as amino acid depletion, by down regulating pathways that are involved in active growth and up-regulating genes involved in stress adaptation. Recent research has shown that (p)ppGpp is capable of binding to GdpP, inhibiting its phosphodiesterase activity resulting in a concurrent increase in c-di-AMP (116, 254). Thus increased levels of c-di-AMP and (p)ppGpp are implicated in the switch from expression of heterogeneous/low level to high-level resistance.

LC/MS-MS analysis of JE2 and *pgl* mutant cell extracts revealed that c-di-AMP levels appeared to be reduced in the *pgl* mutant, an observation that is inconsistent with a role for this signalling nucleotide in increased β-lactam resistance. Because c-di-AMP levels normally peak in late growth phase (116), it seems possible that c-di-AMP levels in the *pgl* mutant may reflect the growth defect

in this mutant. A separate project in our laboratory identified a mutation in hexose phosphate transporter, *hpt* gene associated with expression of high level resistance in a clinical isolate, and which was also not associated with increased c-di-AMP levels (Nikki Black, personal communication). Furthermore, this is not the first study reporting a lack of correlation between increased β-lactam resistance and elevated c-di-AMP levels. Recent findings by Baek, *et al*. (2014), identified *clpX and clpP* mutants implicated in increased β-lactam resistance in USA300 including a 32-fold increase in imipenem resistance (240). Significant changes in cell wall peptidoglycan in these mutants were independent of altered c-di-AMP levels and PBP2a expression, leading the investigators to speculate that processing of the auxillary factors GlmS, MurI, FemA, FemB, MurC, MurE and PBP2a by the ClpXP proteases may impact β-lactam resistance levels. In mutants lacking ClpXP, higher concentrations of these auxiliary factor proteins that are involved in cell wall biosynthesis may be consistent with the higher MIC of *clpXP* mutants to β-lactam antibiotics.

Previous studies with *gdpP* and *rel* mutants showed that a shift to homogeneous, high level resistance was associated with an increase in PBP2a expression (201, 202, 255). However, neither *mecA* transcription nor PBP2a expression were significantly increased in the *pgl* mutant. Of note *mecA* expression in the *pgl* mutant was not carried out in the presence of sub-inhibitory oxacillin, which is known to activate *mecA*, this was to verify that the *pgl* mutant did not directly impact *mecA* expression. Future experiments to assess the potential involvement of other PBPs, including PBP2 and PBP4, in the *pgl* phenotype are needed, given that these proteins are believed to contribute in part to resistance in CA-MRSA (70).

To investigate the potential impact of the *pgl* mutation on antibiotic tolerance, the mutation was transduced into the laboratory MSSA strain, 8325-4. Tolerance can be defined as the ability of a small minority of bacterial cells in a population to withstand transient exposure to high concentrations of antibiotic without a simultaneous change in MIC (256). This is often achieved by down regulation of essential bacterial processes including growth (257). The 8325-4 *pgl*::Tn mutant, which significantly formed smaller colonies than the wild type parent, was more tolerant to oxacillin. Because *gdpP* mutation has also been linked

to increased tolerance to β-lactam and glycopeptide antibiotics (111), these data suggest that that antibiotic tolerance in *S. aureus* can be achieved by c-di-AMP-dependent and c-di-AMP-independent mechanisms.

In conclusion, we hypothesise that mutation of *pgl* may be accompanied by an increase in the oxacillin MIC by causing a general lowering of metabolic activity, which is evidenced by a growth defect and leads to increased antibiotic tolerance. Increased tolerance may increase the oxacillin MIC by enabling the *pgl* mutant to produce enough PBP2a to resist higher concentrations of β-lactams. During an activated stringent response transcription and translation of *mecA* are increased while growth rate and metabolism are slowed down, demonstrating that *mecA* is exempt from stringent control (255). We further hypothesise that c-di-AMP may not be directly involved in increased β-lactam resistance associated with the *pgl* and other mutations, but rather may control growth and cell wall metabolism in response to cell wall stresses (108, 249, 251). It is clear that c-di-AMP homeostasis is important for bacterial survival; too much or too little c-di-AMP can inhibit growth (108, 116). Also, targets of c-di-AMP have not been directly linked to cell wall assembly but to metabolism and potassium transport when under osmotic cell stress (117).

Alternatively, the disruption of the PPP in the *pgl* mutant could redirect glucose flow to the glycolysis pathway as illustratyed in figure 3.6. This could increase the availability of fructose-6-phosphate and enable sufficient peptidoglycan biosynthesis following exposure to β-lactam-induced cell wall stress. A recent study in *Listeria monocytogenes* has shown that c-di-AMP can inhibit pyruvate kinase (Pyk) (258), which may also increase the intracellular pool of fructose-6-P for cell wall biosynthesis under antibiotic stress. This data highlights the link between the c-di-AMP signalling, central metabolism and cell wall biosynthesis.

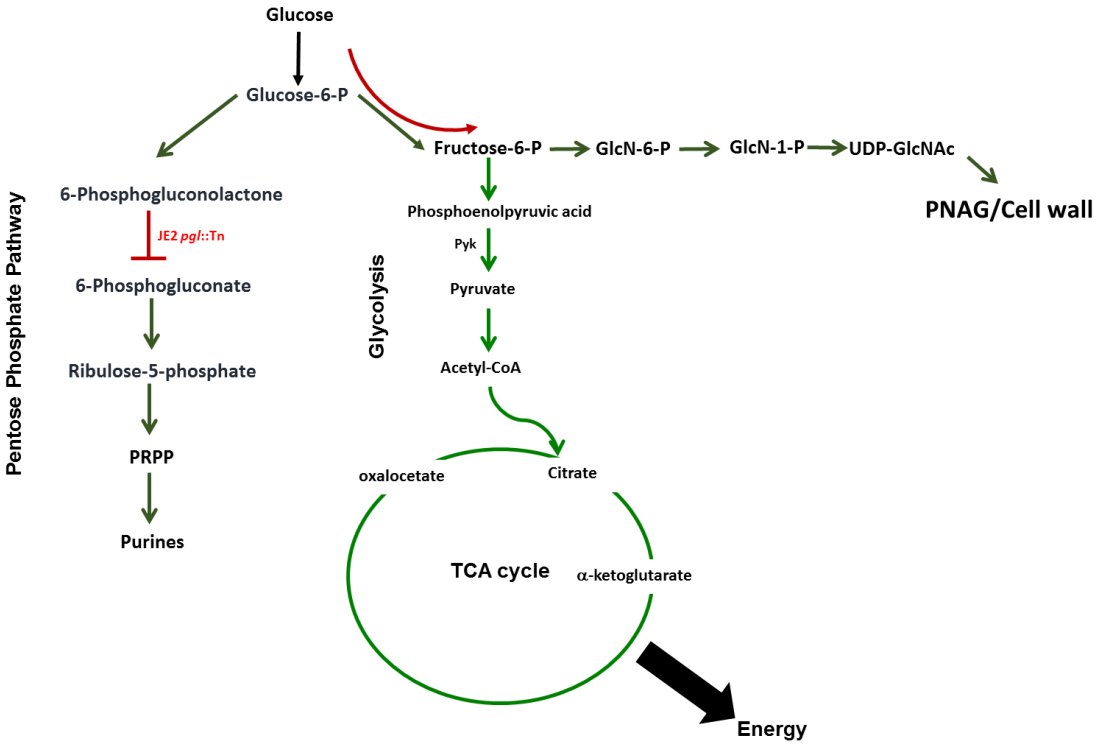

Figure 3.6. An outline of the pentose phosphate pathway and glycolysis. Illustration showing the flow of glucose through the glycolysis pathway leading to an increase in the availability of fructose-6-phosphate for peptidoglycan biosynthesis.

Research in our laboratory is now underway to further examine the effect that the *pgl* mutation has on the PPP, growth and metabolism. Furthermore, assessing the impact of PBP2a and other PBP's under β-lactam exposure will also help explain their contribution to the increased oxacillin MIC of the *pgl* mutant. Taken together, these data suggest that mutations in key metabolic enzymes in *S. aureus* mutants that can contribute to increased antibiotic tolerance and cell wall biosynthesis is manifested as an increase in MIC.

3.4. Materials and Method

3.4.1. Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 3.2. *S. aureus* strains were grown overnight at 35°C or 37°C in Brain-Heart Infusion (BHI) media (Oxoid) or Mueller Hinton (MH) (Oxoid) supplemented with 10 mg/ml chloramphenicol (Sigma) or 10 mg/ml erythromycin (Sigma) as indicated. Solid media were further supplemented with 1% agar. *Escherichia coli* strains were grown at 37°C on Luria Bertani (LB) medium supplemented, when required, with ampicillin (100 µg/ml). β-haemolytic activity was visualized on sheep blood BHI agar by hot-cold lysis.

Table 3.2. Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant Details	Reference or source
S. aureus		
8325-4	8325 derivative cured of known prophages with 11-bp deletion in *rsbU*. MSSA.	(259)
RN4220	Restriction-deficient laboratory *S. aureus.*	(220)
JE2	USA300 LAC derivative cured of plasmids p01 and p03. CA-MRSA, SCC*mec* type IV. Parent strain of NTML.	(196)
13C	USA300 LAC derivative cured of plasmids p03. CA-MRSA, SCC*mec* type IV.	(196)
NE202	JE2 derivative carrying *pgl*::Tn mutation. Erm^r.	(196)
NE788	JE2 derivative carrying *ktrA*::Tn mutation. Erm^r.	(196)
NE1868	JE2 derivative carrying *mecA*::Tn mutation. Erm^r. Ox^s	(196)
ATCC® 29213	MSSA strain for susceptibility testing	(222)
ATCC® 25923	MSSA strain for susceptibility testing	(222)
E. coli	*E. coli* TOP10	(223)
Plasmids		
pLI50	*E. coli-Staphylococcus* shuttle vector. Ap^r (*E. coli*), Cm^r. (*Staphylococcus*)	(224)
PDrive	*E. coli* cloning vector	(225)

3.4.2. Screening of the Nebraska Transposon Mutant Library for altered cefoxitin susceptibility.

The Nebraska Transposon Mutant Library was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) (http://www.narsa.net). This library was created at UNMC in the USA300-derivative strain JE2 (USA300 cured of both plasmids p01 and p03) and contains a collection of 1,952 mutants of non-essential genes in *S. aureus* (196). The mariner-based transposon (Tn) *bursa aurealis* was used to generate random Tn insertion mutations in *S. aureus* as described by Fey *et al*., 2013 (196). The wild-type strain JE2 was used as a positive control for resistance, and NE1868 (*mecA*::Tn) and ATCC® 25923 served as a negative controls for resistance. The entire strain collection was screened to identify mutated genes that lead to altered cefoxitin resistance. This was carried out through the application of antimicrobial susceptibility testing using the disk diffusion method in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines using cefoxitin (30µg) diffusion disks (Oxoid) on Mueller Hinton agar (Oxoid). Briefly, each library mutant was streaked onto BHI agar supplemented with 10µg/ml of erythromycin and grown overnight for 18 h at 37°C. A suspension of cells was prepared by suspending 5-6 isolated colonies from a plate into 5mls of sterile saline and cell density adjusted to the 0.5 McFarland standard. Each bacterial suspension was inoculated onto the surface of a MH agar plate using a swab before the application of the cefoxitin disc. Plates were incubated at 35°C for 18 h. The screen of the library was performed in duplicate initially, with a further two repeats to confirm the phenotypes of mutants exhibiting altered cefoxitin susceptibility. PCR amplification of the loci carrying transposon insertions was used to confirm the locations of transposon insertion in mutants of interest. Phage 80α was then used to transduce transposon mutations from library mutants back into JE2 and 13C (a USA300 derivative lacking the erythromycin resistant plasmid) to verify that secondary mutations were not involved in the observed cefoxitin susceptibility phenotype (as described in detail below). To further ascertain that no secondary mutations were present, the genomes of JE2 and NE202 were determined by Illumina HiSeq sequencing (MicrobesNG, UK) and aligned to the publically available USA300_FPR3757 genome.

3.4.3. Antibiotic profiling

Measurement of oxacillin minimum inhibitory concentration (MIC) and disk diffusion (DD) assays for the *S. aureus* strains in this study were determined in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines using E-tests strips from Biomerieux or antibiotic diffusion disks (Oxoid). ATCC® 25923 and ATCC® 29213 strains were used as controls for susceptibility testing respectively.

3.4.4. Genetic Techniques

Genomic and plasmid extractions were prepared using Wizard Genomic DNA and Plasmid Purification kits (Promega). Prior to DNA extraction, cells were pre-treated with 2 µl of 1 mg/ml lysostaphin (Ambi Products, New York) to facilitate subsequent lysis. Restriction enzyme, *EcoR1* (Roche, UK and New England Biolabs, MA) was used according to the manufacturers' instructions accompanied with the appropriate 10X buffer. Restriction digests were performed at a volume ranging from 20 µl – 50 µl.

3.4.5. Transduction of transposon mutations from NMTL mutants into other *S. aureus* strains.

The selected *bursa aurealis* transposon mutant strain NE202 (JE2 *pgl*::Tn) that exhibited increased resistance to β-lactam antibiotics was backcrossed into JE2 and 13C (USA300 cured of plasmid p03) to ensure that secondary mutations were not involved in increased cefoxitin resistance. The bacteriophage 80α was propagated on NE202 and the resulting lysate used to transduce the mutation into JE2, 13C and 8325-4, a laboratory *S. aureus* strain. Transductants were selected on BHI agar containing erythromycin 10µg/ml and colony PCR was used to amplify the *pgl* locus with the primers NE202_Fwd and NE202_Rev (Table 3.3).

Table 3.3. Oligonucleotide primers used in this study

Target Gene	Primer Name	Primer Sequence (5'-3')
Pgl	NE202_Fwd	TCATCCTTAATTCACCCCAATC
	NE202_Rev	CAGGTGTCCATTTACCACCA
MecA	mecA1_Fwd	TGCTCAATATAAAATTAAAACAAACTACGGTAAC
	mecA1_Rev	GAATAATGACGCTATGATCCCAA
gyrB	gyrB_Fwd	CCAGGTAAATTAGCCGATTGC
	gyrB_Rev	AAATCGCCTGCGTTCTAGAG

3.4.6. Complementation of NE202 with *pgl*.

The *pgl* gene was amplified from JE2 genomic DNA by PCR using primers listed in Table 3.3., before being cloned into the cloning vector pDrive (Qiagen) in *Escherichia coli* TOP10. The sequence of the insert in the recombinant plasmid was verified by Sanger sequencing (Source Biosciences) before being sub-cloned on an *EcoRI* restriction fragment into the *E. coli-Staphylococcus* shuttle plasmid pLI50 (224). The plasmid was transformed by electroporation into the restriction-deficient strain RN4220, and subsequently into NE202. All plasmid-harbouring strains were cultured in medium supplemented with 100 µg/ml ampicillin (*E. coli*) or 10 µg/ml chloramphenicol (*S. aureus*) to maintain plasmid selection.

3.4.7. Static biofilm assays.

Semi-quantitative measurements of biofilm formation were determined under static conditions using Nunclon Hydrophilic 96 well polystyrene plates that were tissue culture treated (Nunc, Denmark) as described previously (151). Briefly, a dilution of 1:200 bacteria to media were grown in individual wells of the 96-well plates containing BHI medium or BHI supplemented with 1% glucose at 37°C for 24h. Subsequently, the plates were gently rinsed three times with distilled water and left to dry at 60°C for 1h. Each well was then stained for 5 minutes using 4% crystal violet and the adhered biofilm was then solubilised using 5% acetic acid and the absorbance of the stained, suspended biofilm was measured at 490nm using a

microtitre plate reader. Each strain was tested at least three times, and average results are presented. A biofilm-positive phenotype was defined as an $\boldsymbol{A}_{490} \geq 0.17$.

3.4.8. Extraction of total RNA.

Strains JE2 and NE202 were grown to an OD600nm = 3.0, washed in RNA Later (Ambion), pelleted and immediately stored at -20°C to ensure maintenance of RNA integrity prior to purification. RNA extraction was carried out using the RNeasy Mini Kit (Qiagen) by following the manufacturer's instructions with the following adaptions. Cells in RNA Later were pelleted, washed in Tris buffer at pH 8.5 to inhibit RNA activity and resuspended in 50 mM EDTA with 4 µl of 1 mg/ml lysostaphin to lyse the cells. Purified RNA was eluted in 50 µl of RNAsecure (Ambion). Residual DNA present in the RNA preparations was removed using Ambion recombinant Turbo DNase. Purified RNA was eluted and stored in RNAsecure resuspension solution (Ambion) and the integrity of the RNA confirmed by agarose gel electrophoresis. RNA concentration was determined using a Qubit Fluorometric quantitation system. Purified RNA was converted to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer's instructions.

3.4.9. Reverse Transcription PCR (RT-PCR)

RT-PCR analysis of *mecA* mRNA was performed using the Roche LightCycler 480 instrument with the LightCycler 480 Sybr Green Kit (Roche) and the primers mecA1 and mecA2 (Table 3.3.). Cycling conditions were 95°C for 5 minutes and followed by 45 cycles of 95°C for 10 seconds, 58°C for 20 seconds and 72°C for 20 seconds. Melt curve analysis was performed at 95°C for 5 seconds followed by 65°C for one minute up to 97°C at a ramp rate of 0.11c/sec with five readings taken for every degree of temperature increase. The *gyrB* gene was used as an internal standard for all reactions using primers gyrB_Fwd and gyrB_Rev (Table 3.3.). For each reaction, the ratio of *mecA* and *gyrB* transcript number was calculated. Each RT-PCR experiment was performed three times and presented as average data with standard errors.

3.4.10. Scanning Electron Microscopy (SEM).

Biofilm imaging, cell shape and cell size analysis were carried out using SEM imaging. JE2 and NE202 biofilms were grown on 4-well tissue culture treated polystyrene slides (Nunc, Thermo-scientific). Overnight BHI cultures were diluted 1:200 in BHI supplemented with 1% glucose and incubated at 37^{o}C for 24h. Subsequently, the slides were gently rinsed three times with distilled water, dried at 60^{o}C 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.

3.4.11. Transmission Electron Microscopy (TEM).

Overnight BHI cultures of JE2 and NE202 were diluted 1:200 in fresh BHI and grown at 37°C to an A_{600} = 1.0. 10 ml culture aliquots were subjected to centrifugation at 8,000 × ***g***, and the cell pellets were resuspended in fixation solution (2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and incubated overnight at 4°C. The fixed cells were further treated with 2% osmium tetroxide, followed by 0.25% uranyl acetate for contrast enhancement. The pellets were then dehydrated in increasing concentrations of ethanol as described above for the SEM cell preparation, followed by pure propylene oxide, and transferred to a series of resin and propylene oxide mixtures (50:50, 75:25, pure resin) before being embedded in Epon resin. Thin sections were cut on an ultramicrotome. Images were analysed using a Hitachi H7000 instrument. At least 3 to 5 measurements of cell wall thickness were performed on each cell and an average or 50 cells were measured for each sample.

3.4.12. Salt tolerance assays.

Overnight cultures were grown in BHI broth at 37°C and cell density standardised to OD_{600}= 1.0. Tenfold serial dilutions down to 10^{-8} were made in sterile BHI and 4 µl of each dilution was inoculated onto BHI agar containing 2.2 M NaCl. Plates were incubated at 37°C for 24 h and photographed using standard methods.

3.4.13. Preparation of cell lysates for c-di-AMP quantification.

Overnight cultures were diluted to OD_{600} = 0.05 and grown to OD_{600} = 2.5. At late exponential phase, 15 X OD units (equivalent to 2.4 mg cell dry weight) of bacterial culture was harvested by filtration on 0.45 µm Whatman filter membranes (Sigma) by using a vacuum dependent fast-filtration approach adapted from the method of Meyer *et al* (260). The filter membranes were washed once with 50ml ice cold isotonic NaCl solution and immediately transferred to a tube filled with 5ml pre-cooled extraction solution (60% ethanol) before it was snap frozen by liquid nitrogen. For intracellular extraction, mechanical cell disruption was carried out. The bacterial cells were washed off the filter by using the extraction solution and vortexing. The resuspended cells where transferred into tubes (2ml or 50ml) containing 0.10-0.11mm diameter glass beads (Sigma). Mechanical cell disruption was carried out by using the FastPrep-24 instrument (MP Biomedicals, LLC) twice for 40 S at 6 m/s and cell extracts were transferred to fresh 15 ml tubes. The remaining beads and cell debris were washed with ultrapure water as a second extraction step, and combined with the ethanolic cell extract. Following this, the cell extract was centrifuged for 5mins at 4°C at 10,000X g, the supernatant was collected and stored at -80°C for freeze drying. c-di-AMP was detected and quantified by LC-MS/MS using a protocol published previously for the detection of c-di-GMP (261) and c-di-AMP (106).

3.4.14. Oxacillin tolerance assay.

Overnights cultures of 8325-4 and 8325-4 *pgl*::Tn were grown in BHI at 37°C, adjusted to OD_{600} = 0.05 in fresh BHI media and grown for approximately 3 h.

The number of colony forming units in the cultures was determined by plating serial dilutions of the cultures on BHI agar before they were supplemented with 12.5µg/ml of oxacillin. At indicated times, an aliquot of cells was removed, serially diluted and plated on BHI agar to enumerate survivors. Tolerance was defined as a ≤90% drop in viability after a 6-h challenge with 12.5 µg/ml of oxacillin (248, 261, 262).

3.4.15. Statistical analysis

Two-tailed, two-sample equal variance Student's t-Tests were used to determine statistically significant differences in assays performed during this study. A *P* value <0.05 was deemed significant.

ACKNOWLEDGMENT

We thank Volkhard Kaever for assistance with c-di-AMP measurements.

FUNDING

This study was funded by a grant from the Irish Health Research Board (HRA_POR/2012/51) (to J.P.O'G). C-di-AMP measurements were funded in part by the Thomas Crawford Hayes Research Fund (to LAG).

Chapter 4

Using D-cycloserine and related drugs to overcome resistance to β-lactam antibiotics in MRSA

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Running title: Using amino acid analogues to overcome resistance to β-lactam antibiotics in MRSA

Abstract

Healthcare-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) is one of the most significant multidrug resistant pathogens impacting patient treatment worldwide. However resistance incurs a fitness cost which initially confined MRSA to hospital settings. The emergence of community-associated MRSA (CA-MRSA) as etiological agents in MRSA infections in otherwise healthy individuals has exacerbated the problem with these pathogens. Furthermore CA-MRSA have increasingly displaced HA-MRSA in healthcare settings and MRSA remains on the WHO list of pathogens for which new antibiotics are urgently needed. Identifying genes that affect resistance in MRSA could inform the design of new drugs and lead to more-effective antimicrobial strategies. Here we screened the Nebraska Transposon Mutant Library (NTML) and identified a mutation in an amino acid permease, CycA, which rendered MRSA more susceptible to β-lactam antibiotics in brain heart infusion (BHI) medium. Furthermore the *cycA* mutant also exhibited hyper-susceptibility to the alanine analogue d-cycloserine (DCS), that inhibits alanine racemase and D-alanine ligase activity which are both required for cell wall cross-linking. Amino acid uptake studies revealed that alanine uptake in the *cycA* mutant was impaired in chemically defined medium (CDM) supplemented with glucose, which correlated with increased susceptibility to oxacillin. However in CDM without glucose, the *cycA* had no impact on alanine uptake or oxacillin susceptibility, suggesting that an alternative alanine permease can compensate for CycA under these growth conditions. Substantial differences in whole cell morphology and cell wall thickness in the *cycA* mutant correlated with reduced incorporation of glycine and alanine into the peptidoglycan cell wall. These data indicate that alanine transport via CycA is important for cell wall integrity and consequently resistance to β-lactam antibiotics in media supplemented with glucose. Furthermore, DCS-mediated inhibition of alanine racemase and D-alanine ligase activity in the *cycA* mutant exacerbates the impact of impaired alanine uptake on cell wall architecture, offering a plausible explanation for both DCS and oxacillin hypersusceptibility. Building on these findings we also report the capacity of DCS to potentiate the activity of β-lactam antibiotics against MRSA *in vitro* and in a mouse model of sepsis which offers a new therapeutic option for the treatment of MRSA infections.

4.1. Introduction

Up to 30% of the human population are permanently colonised with *Staphylococcus aureus* and a further 60% transiently colonised, continuing to present a significant risk of infection (4, 5). Treatment options were complicated by the discovery of methicillin resistant *S. aureus* (MRSA) in 1961 (263), further compounded by reports since then of resistance to all licenced anti-staphylococcal agents including vancomycin (12). Although the Centres for Disease Control has reported a steady decline in healthcare-associated MRSA (HA-MRSA) infections in the United States since 2008, the epidemic of community-associated MRSA (CA-MRSA) infections in individuals with no predisposing risk factors or exposure to a health care setting has been alarming (18). Furthermore CA-MRSA isolates are now implicated in many healthcare associated infections blurring the epidemiological distinction between HA- and CA-MRSA.

Resistance to methicillin in *S. aureus* is mediated by acquisition of *mecA* on the staphylococcal cassette chromosome *mec* (SCC*mec*), which encodes an alternative penicillin binding protein, PBP2a, which can continue to cross-link the cell wall in the presence of β-lactam antibiotics (40, 54). However, resistance to methicillin and related antibiotics is not uniform. Populations of MRSA cells are heterogeneous, with a sub-population (≤0.1%) expressing high level β-lactam resistance (64, 264, 265). Exposure of this heterogeneous population (HeR) to β-lactam antibiotics selects for the acquisition of accessory mutations, including mutations that affect the stringent response and c-di-AMP signalling, that facilitate expression of homogeneous, high-level resistance (HoR) (111-115).

Here we performed a genetic screen for new mutations that impact resistance to β-lactam antibiotics in MRSA. Using the Nebraska Transposon Mutant Library, which comprises 1,952 sequence-defined transposon mutants constructed in a derivative of the CA-MRSA strain, USA300-LAC (196), we identified a mutation in an amino acid permease that resulted in increased sensitivity to cefoxitin. Building on these findings we report the capacity of d-cycloserine to potentiate the activity of β-lactam antibiotics against MRSA *in vitro* and in a mouse model of sepsis.

4.2. Results

4.2.1. Mutation of *cycA* significantly reduces β-lactam resistance in MRSA.

An unbiased screen of the Nebraska Transposon Mutant Library was performed using a disk diffusion assay to identify mutants with increased susceptibility to cefoxitin, the β-lactam drug recommended by the Clinical and Laboratory Standards Institute for measuring *mecA*-mediated oxacillin resistance in MRSA isolates. The parent strain of the library JE2, a derivative of USA300 LAC cured of plasmids p01 and p03 (196), was used as a positive control. Phage 80α-mediated transduction of candidate transposon mutations back into the JE2, together with genetic complementation were used to confirm the role of putative genes involved in cefoxitin resistance. Only mutants verified by both transduction and complementation were examined further. To support our screen, 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 (114, 197, 202). 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 (244).

Among the transposon mutants identified was NE810 (SAUSA300_1642), which exhibited significantly increased susceptibility to oxacillin (Fig. 4.1A) and cefoxitin (Appendix 4A). Additional susceptibility tests were also carried out with other cell wall active antibiotics including other β-lactams (cloxacillin (Appendix 4B) and imipenim) and vancomycin. We found that while the disruption of *cycA* did not alter susceptibility to vancomycin (data not shown), it was associated with increased sensitivity to all β-lactams tested. Consistent with this, NE810 was recently reported to be more sensitive to amoxacillin (197). Lightcycler RT-qPCR analysis revealed that expression of *mecA* was not significantly affected in NE810

compared to JE2 (Fig. 4.1B). Illumina whole genome sequencing of NE810 further revealed that the SCC*mec* element was fully intact in this mutant with no additional mutations (data not shown). In addition to JE2, transduction of the SAUSA300_1642 transposon mutation into LAC-13C (a USA300 LAC derivative lacking the 27-kb p03 plasmid carrying resistance to erythromycin (196) and MRSA strain COL was also accompanied by a significant increase in oxacillin susceptibility (Fig. 4.1C). Complementation of the *cycA* mutation in NE810 by the expression of the *cycA* gene under the control of its own promoter on plasmid pLI50 restored in wild type, heterogeneous oxacillin resistance levels (Fig. 4.1A) and resistance to all β-lactams tested (Appendix 4B). Further phenotypic analysis revealed that NE810 growth rate in BHI media (data not shown) and biofilm forming capacity in BHI and BHI glucose were unaffected (Appendix 4C), although interestingly β-haemolytic activity was elevated in NE810 compared to JE2 (Appendix 4D).

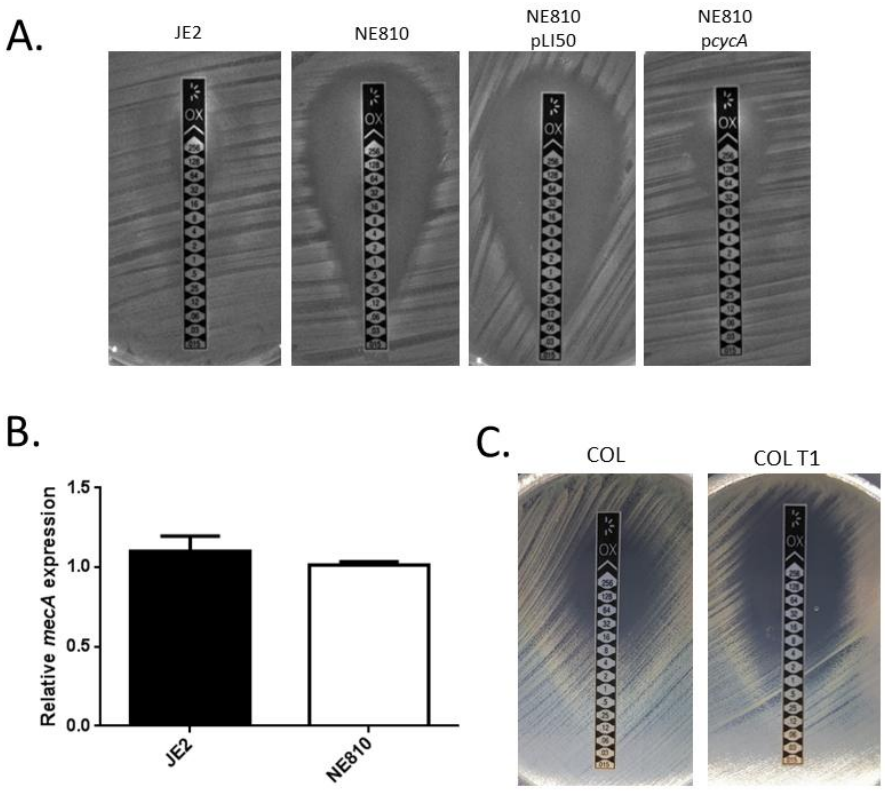

Figure 4.1. Mutation of *cycA* increases oxacillin susceptibility in MRSA. A. Etest measurement of oxacillin minimum inhibitory concentrations (MICs) in JE2 (wild type), NE810 (*cycA*::Tn), NE810 carrying pLI50 (empty plasmid as a control) and NE810 p*cycA*. **B.** Comparison of relative *mecA* gene expression by the LightCycler RT-PCR in JE2 and NE810 grown to A_{600}=3 in BHI media. The experiment was repeated three time and standard deviations are shown. **C.** Etest measurement of oxacillin MICs in MRSA strain COL and COL *cycA*::Tn.

4.2.2. Mutation of *cycA* increases susceptibility to d-cycloserine.

SAUSA300_1642 is a putative D-serine/L- and D-alanine/glycine transporter with homology to CycA in *Mycobacterium tuberculosis* (266, 267). A *cycA* point mutation contributes, in part, to increased d-cycloserine (DCS) resistance in *M. bovis* (266, 267). In contrast our data showed that the NE810 *cycA* mutant was significantly more sensitive to DCS than the wild type JE2 (Fig. 4.2). NE810 also showed increased sensitivity to β-Chloro-d-alanine (βCDA) (data not shown), an alanine analogue that inhibits d-glutamate-d-alanine transaminase and alanine racemase (268, 269). DCS (a cyclic analogue of alanine and serine) is a broad-spectrum antibiotic produced by several *Streptomyces* species used as a second line drug in the treatment of TB infections in humans. This drug blocks the alanine racemase enzyme that converts L-alanine to D-alanine and the ligase enzyme that links two D-alanine amino acids together (4). These are important enzymes in both peptidoglycan (cell wall cross-linking) and teichoic acid biosynthesis in Gram-positive bacteria. A mutant in the predicted D-alanine: D-alanine ligase (*ddl*, SAUSA300_2039) is not available in the NTML library suggesting that this gene may be essential. USA300 is predicted to have two alanine racemase enzymes, SAUSA300_2027 (*alr*) and SAUSA300_1292 (*alr2*). In several Gram-negative bacteria, a second L- and D-alanine-regulated alanine racemase, designated *dadX*, is involved in the use of alanine as a carbon and energy source, rather than the generation of D-alanine for cell wall biosynthesis (270). The NE799 mutant carrying an *alr2* transposon mutation exhibited no change in cefoxitin susceptibility (data not shown), whereas the NE1713 *alr* mutant was significantly more susceptible to cefoxitin (Appendix 4A) and oxacillin (Data not shown). These data suggest that, as in several other bacteria, Alr and Alr2, which share only 29% identity and 48% similarity (data not shown), may also have different functions in *S. aureus* and different affinities for DCS. Nevertheless, the increased susceptibility of the *alr* mutant to cefoxitin and oxacillin is consistent with an important role for D-alanine in *S. aureus* peptidoglycan biosynthesis and β-lactam resistance.

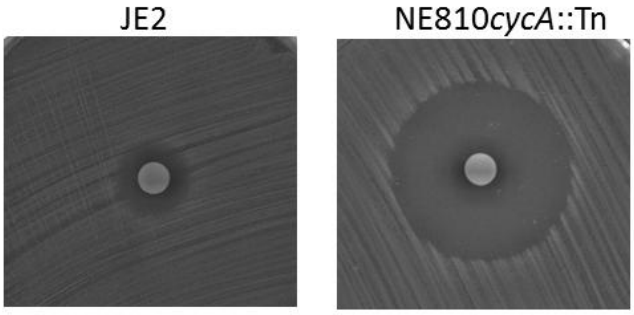

Figure 4.2. Mutation of cycA increases susceptibility to D-cycloserine. A Comparison of zones of inhibition around d-cycloserine 30µg disks on lawns of JE2 and NE810 *(cycA*::Tn) grown on Mueller Hinton (MH).

4.2.3. Mutation of *cycA* impairs alanine uptake.

To investigate the role of CycA as a permease, JE2 and NE810 were grown in CDM and CDMG media for 12 h and amino acid uptake in spent media was measured. These experiments demonstrated significantly reduced alanine uptake by NE810 compared to JE2 in CDMG (Fig. 4.3A). In contrast in CDM media, alanine uptake by NE810 and JE2 was similar (Fig. 4.3B). Consistent with these data, growth of both JE2 and NE810 is significantly impaired in CDM lacking alanine when compared to CDM revealing a critical role for alanine uptake in CDM (Fig. 4.3C and 4.3D). Furthermore, analysis of oxacillin susceptibility in CDM media revealed no difference between NE810 and JE2 (Fig. 4.3E), whereas in CDMG NE810 exhibited significant susceptibility to oxacillin (Fig. 4.3F). These data indicate that under growth conditions where CycA plays no role in alanine uptake, mutation of *cycA* has no impact on oxacillin susceptibility. Utilisation of other amino acids by NE810 and JE2, including serine and glycine, were not significantly different in CDMG but showed a slightly slower uptake rate (Appendix 5) or CDM media (Appendix 6). These data indicate that *cycA* is a major alanine transporter when grown in media containing glucose but not in CDM media.

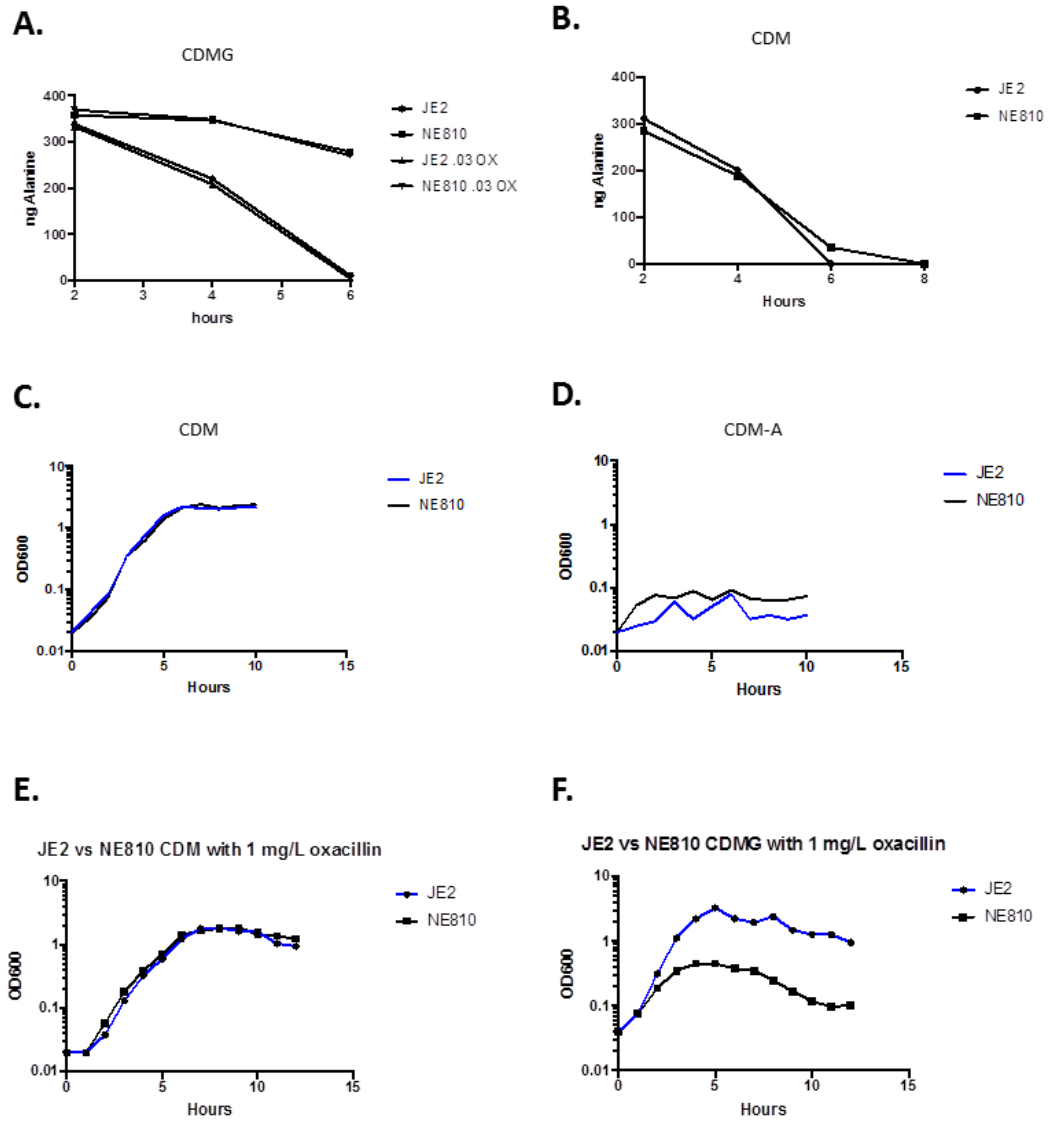

Figure 4.3. Mutation of *cycA* impairs alanine uptake in *S. aureus*. A. and B. Amino acid consumption by JE2 and NE810 grown aerobically in chemically defined media containing 14mM of glucose (CDMG) (A) or CDM (B). Residual alanine was measured in spent media after 2, 4 and 8 h growth. **C and D.** Cell densities of JE2 and NE810 cultures grown in CDM (C) and CDM lacking alanine (D). **E.** Comparison of JE2 and NE810 grown aerobically in CDM supplemented with 1 mg/L oxacillin. **F.** Comparison of JE2 and NE810 grown aerobically in CDMG supplemented with 1 mg/L oxacillin.

4.2.4. Mutation of *cycA* impacts cell wall structure.

The impaired uptake of alanine and increased susceptibility of NE810 to β-lactams and DCS prompted us to compare whole cell morphology and cell wall thickness in NE810 and JE2. Scanning and transmission electron microscopy revealed gross differences in whole cell morphology and cell wall thickness in NE810 compared to JE2. NE810 exhibited a highly irregular cell shape when compared to JE2, and many cells appeared collapsed (Fig. 4.4A and 4.4B). TEM imaging revealed that NE810 cells had a wrinkled cell surface and a thinner cell wall compared to JE2 (Fig. 4.4C and 4.4D), although cell size (mean cell diameter) was unaffected (Table 4.1). Given that alanine uptake, as well as alanine racemase and D-alanine ligase activity, are important for alanine incorporation into peptidoglycan, cell wall extracts of JE2 and NE810 were shipped to DSMZ GmbH (Braunschweig, Germany) for muropeptide analysis to compare the molar ratios of muramic acid, lysine, alanine, glycine and glutamic acid. The overall muropeptide profile of the two strains was similar, however the data revealed that the peptidoglycan interbridge of the wild type JE2 may contain 5-6 glycine residues whereas the NE810 interbridge contains approximately 4 glycine residues. In addition to this, the overall incorporation of alanine was also reduced in the NE810 mutant compared to the parent. Given that the amino acid uptake experiments revealed similar glycine uptake in NE810 and JE2, it remains unclear why the glycine interbridge in NE810 is affected and more detailed cell wall analysis is needed. However the reduced length of the cross-bridge and resulting thinner cell wall are consistent with increased susceptibility to β-lactams.

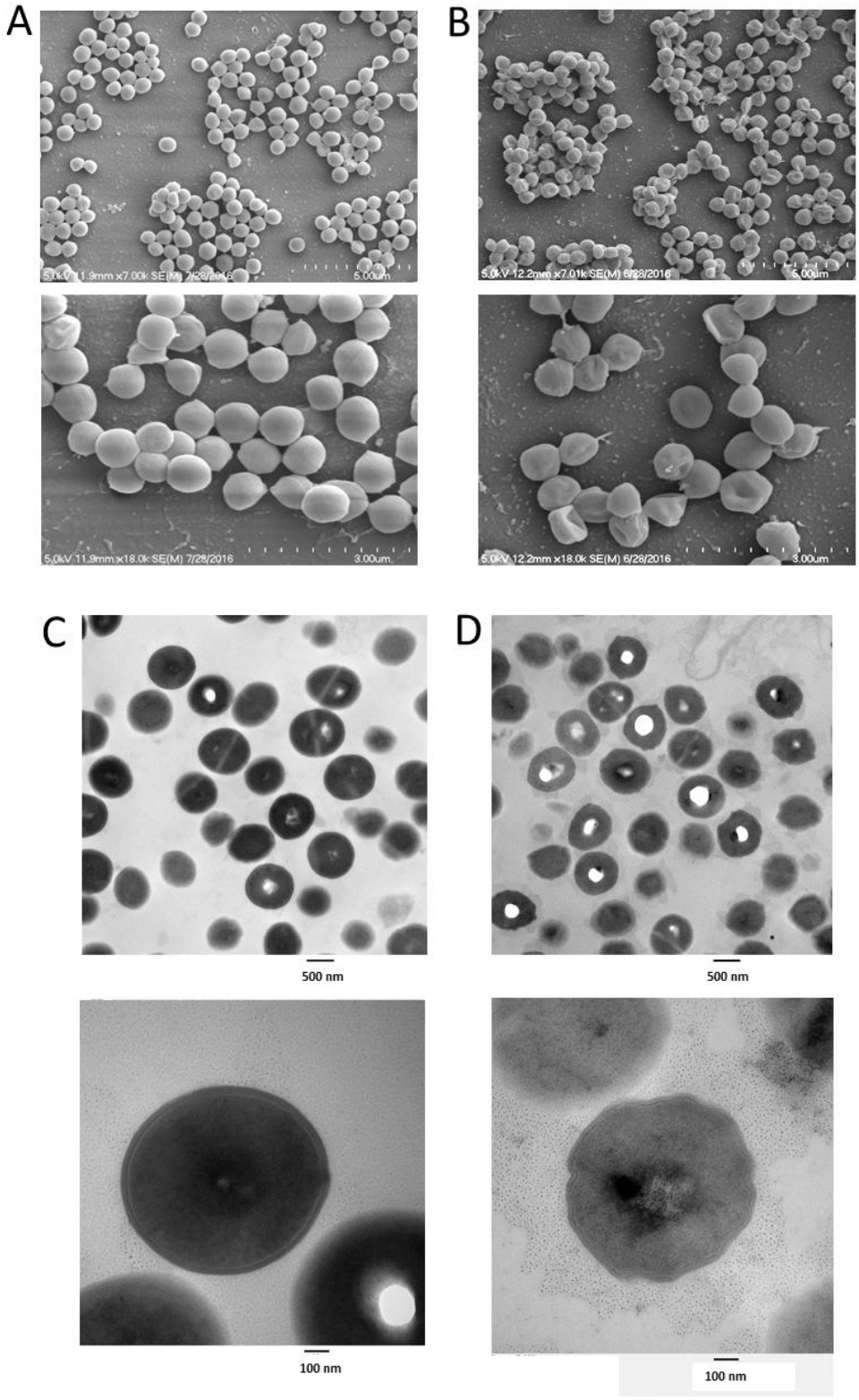

Figure 4.4. Mutation of *cycA* reduces cell wall thickness in MRSA. A and B. Comparison of JE2 (A) and NE810 (B) cell morphology by scanning electron microscopy (SEM). **C and D.** Comparison of JE2 (C) and NE810 (D) cell wall thickness by transmission electron microscopy (TEM). TEM magnifications are ×15,000 (top) and ×70,000 (bottom) and cell wall thickness measurements are provided in Table 4.1.

Table 4.1. Comparison of cell diameter and cell wall thickness in the NE810 *cycA* mutant and wild type JE2.

	Mean cell diameter (μm) ± SD	Mean cell wall Thickness (nm) ± SD
JE2	0.896 ± 0.075	25.92787 ± 1.791
NE810	0.886167 ± 0.050	18.9555556 ± 2.544

4.2.5. D-cycloserine and oxacillin act synergistically against MRSA.

As noted above, mutation of *cycA* increased susceptibility of NE810 to DCS. Working on the premise that impaired alanine uptake in the *cycA* mutant or DCS-mediated inhibition of alanine racemase and D-alanine ligase may both render the cell wall more susceptible to β-lactams, we investigated DCS/oxacillin synergism against MRSA. DCS potentiated the activity of oxacillin against wild type JE2 and the NE810 *cycA* mutant (Fig. 4.5A and 4.5B). Using the checkerboard microdilution method, exposure of USA300 to sub-inhibitory concentrations of DCS resulted in a ~4-fold reduction in the oxacillin MIC from 32μg/ml to 8μg/ml (Table 4.2), suggesting that reduced uptake of alanine in the *cycA* mutant is associated with increased susceptibility to DCS. Consistent with these data, DCS and βCDA have both been shown to act synergistically to inhibit bacterial growth in the presence of β-lactam antibiotics (271, 272). Thus, the reduced uptake of alanine in the *cycA* mutant appears to impair normal cell wall biosynthesis thereby increasing susceptibility to β-lactam antibiotics. Impaired cell wall synthesis is compounded by the addition of DCS which further inhibits alanine racemase and D-alanine ligase, resulting in a concomitant increase in susceptibility. These data reveal a correlation between susceptibility to β-lactam antibiotics and drugs based on serine/alanine/glycine analogues raising the possibility that drugs based on amino acid analogues may be used in combination with β-lactams to overcome antibiotic resistance in the treatment of MRSA infections.

However, the increased susceptibility of the *cycA* mutant to DCS indicates that this amino acid analogue is still being transported into the cell in NE810 and suggest the involvement of a second permease. On-going work in our laboratory is underway to screen the NTML library for a mutant that have increased resistance to DCS, on the assumption that such a mutant(s) will have impaired DCS uptake

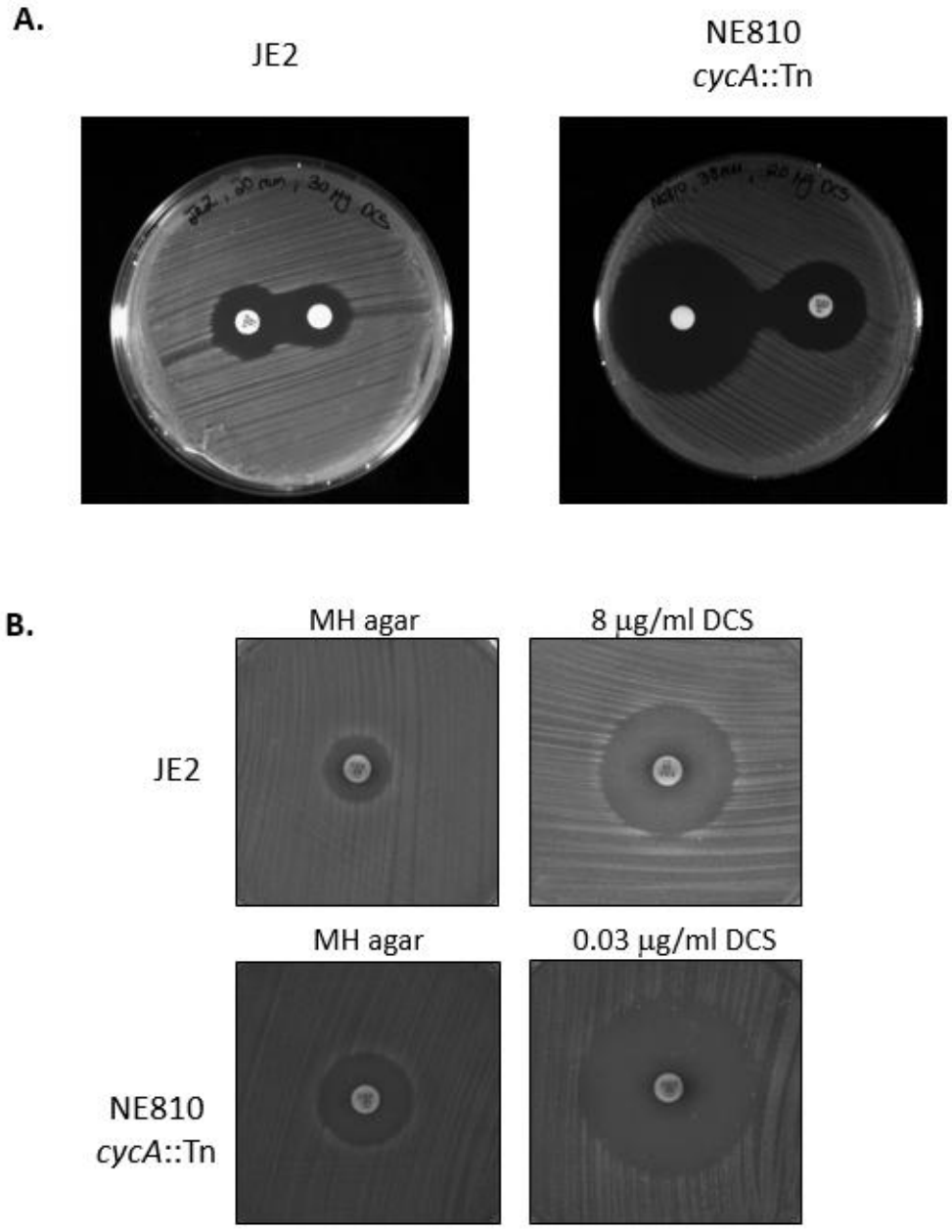

Figure 4.5. D-cycloserine potentiates the activity of β-lactam antibiotics against MRSA. A Synergism between cefoxitin (30µg) and DCS (30µg for JE2 and 20 µg for NE810)) against lawns of JE2 (left) and NE810 (right) grown on Mueller Hinton (MH) agar. **B** Comparison of zones of inhibition around cefoxitin 30µg disks on lawns of JE2 and NE810 *(cycA*::Tn) grown on Mueller Hinton (MH) agar or MH agar supplemented with d-cycloserine (DCS) (8 µgml for JE2 or 0.03 µg/ml for NE810).

Table 4.2 Minimum inhibitory concentrations of JE2 and NE810 to oxacillin and D-cycloserine alone or in combination.

	MIC (μg/ml)		Combination treatment MIC (μg/ml)	
Strain	Oxacillin	D-cycloserine	Oxacillin	D-cycloserine
JE2	32-64	32	6	8
NE810	8	8	1	1

4.2.6. Combination therapy with d-cycloserine and oxacillin significantly reduces the bacterial burden in the spleen and kidneys of mice infected with MRSA.

The therapeutic potential of oxacillin in combination with DCS in the treatment of MRSA infections was assessed in mice. Firstly, sepsis was established in sets of 5 mice via tail vein injection with 5 x10^6 CFU of JE2 or NE810 strain, and the infection established for 16 hours. Mice were treated with 75 mg of oxacillin/kg, 10mg of DCS/kg, a combination of both oxacillin and DCS or left untreated (control). The antibiotic treatment was administered subcutaneously every 12 hours and the infection was allowed to proceed for 7 days. Treatment with oxacillin and DCS in combination significantly reduced the bacterial burden in the kidneys and spleen of infected animals after 7 days when compared to treatment with oxacillin or DCS alone (Fig. 4.6). Treatment with oxacillin and DCS completely cleared the infection from the spleen and also significantly reduced the number of CFUs in the kidneys (Fig. 4.6). Furthermore, consistent with the hypersensitivity of NE810 to β-lactams in the laboratory, the *in vivo* results showed that infections caused by NE810 were completely cleared from the spleen and significantly reduced in the kidney following treatment with oxacillin alone. These data demonstrate that d-cycloserine potentiates the activity of β-lactam antibiotics against MRSA *in vivo*, which has important therapeutic implications.

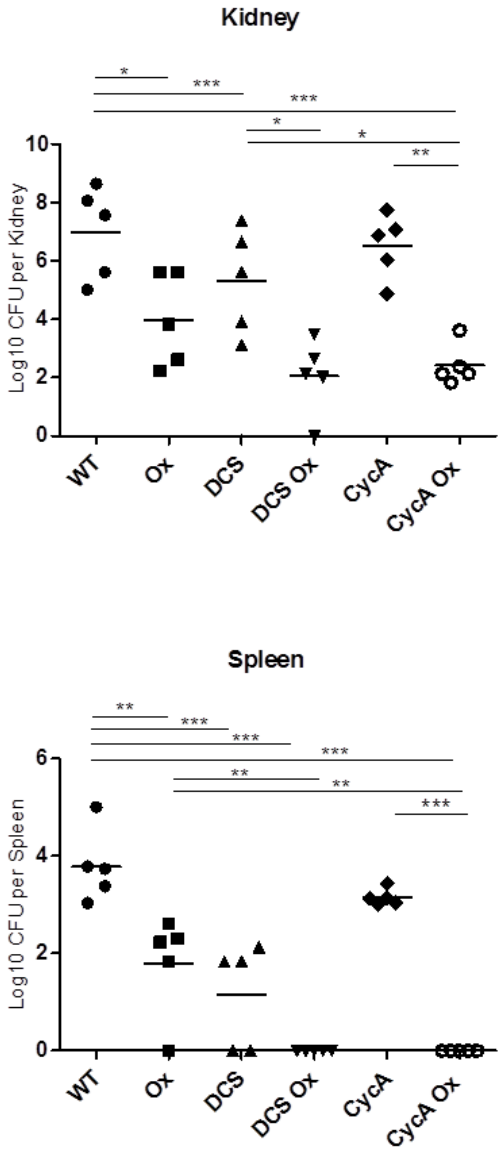

Figure 4.6. Combination therapy with d-cycloserine and oxacillin significantly reduces the bacterial burden in the spleen and kidneys of mice infected with MRSA. The number of colony-forming units (CFU) recovered from the kidney and spleen of mice infected by tail vein injection with 5×10^6 JE2 or NE810 (*cycA*::Tn) and left untreated (control) or treated with 75 mg of oxacillin(ox) /kg subcutaneously, 10mg of DCS/kg subcutaneously or a combination of both oxacillin and DCS every 12 hours for 7 days . (The first antibiotic dose was given 16 hours after infection) before being euthanized on Day 7. Significant differences determined using a one-way analysis of variance and Turkey's multiple comparison test as post hoc analysis are denoted using asterisks (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$).

4.3. Discussion

These data demonstrate that mutation of the *S. aureus cycA* gene increases susceptibility to the β-lactam antibiotics cefoxitin and oxacillin, as well as d-cycloserine (DCS), an amino acid analogue that inhibits alanine racemase and D-alanine ligase activity. CycA is annotated as a D-serine/L- and D-alanine/glycine transporter, with 46% identity to CycA in *M. tuberculosis* and 53% identity with *E. coli* CycA. In contrast to our data, a *cycA* point mutation in *M. bovis* contributes, in part, to increased DCS resistance (267). In *E. coli*, a *cycA* mutation can also result in increased DCS resistance, but only in minimal media and not in complex media, indicating that uptake of the toxic amino acid analogue by *E. coli* is significantly dependent on growth media (273-276). The reason for the opposite effects of *cycA* mutation on DCS susceptibility in *S. aureus* and *E. coli* are unclear. Presumably DCS uptake is not significantly blocked by the *cycA* mutation in *S. aureus*, raising the question of what permease/transport system is involved in uptake of this amino acid analogue. Although the identity of the permease involved in DCS uptake remains unknown, ongoing work in our laboratory is seeking to identify if any transposon mutants in the NTM library are more resistant to DCS, which may be indicative of impaired uptake. In contrast, uptake of alanine (and to a much lesser extent serine and glycine) are impaired in the NE810 *cycA* mutant in chemically defined medium supplemented with glucose (CDMG) as evidenced by amino acid consumption studies in this mutant. Impaired uptake of alanine in CDMG correlated with increased susceptibility to oxacillin, suggesting that alanine utilisation via CycA is important for cell wall integrity and consequently resistance to β-lactam antibiotics. However, this is not the case in CDM media lacking glucose, in which alanine, serine and glycine uptake were not affected and oxacillin resistance levels were similar to wild type. The different susceptibilities of the cycA mutant to oxacillin in CDM and CDMG appears to be the result of altered alanine uptake. Peptidoglycan analysis identified a shortened glycine interbridge in the *cycA* mutant, which is likely to negatively impact the cell wall. In this context DCS-mediated inhibition of alanine racemase and D-alanine ligase activity in the *cycA* mutant is likely to exacerbate the impact of impaired alanine uptake on the cell wall architecture, offering a plausible explanation for both DSC and oxacillin hyper-susceptibility in NE810.

Our working hypothesis proposes that the *cycA* mutation does not increase *S. aureus* susceptibility to oxacillin in CDM media because a second active permease must substitute for CycA to transport alanine into the cell (thus satisfying the requirement for L- and D-alanine incorporation into peptidoglycan). Given that CycA cannot be involved in the transport of DCS, we further hypothesise that this second alanine permease is also involved in the transport of DCS into the cell, as illustrated in figure 4.7. It remains unclear how or why the addition of glucose to CDM impacts the activity of two alanine/DCS permeases. The enhanced metabolic activity and protein synthesis in cells grown in media supplemented with glucose may increase the requirements for all amino acids. Under such conditions, the second putative permease implicated in alanine and DCS uptake may have a higher affinity for another substrate(s), effectively blocking alanine and DCS transport.

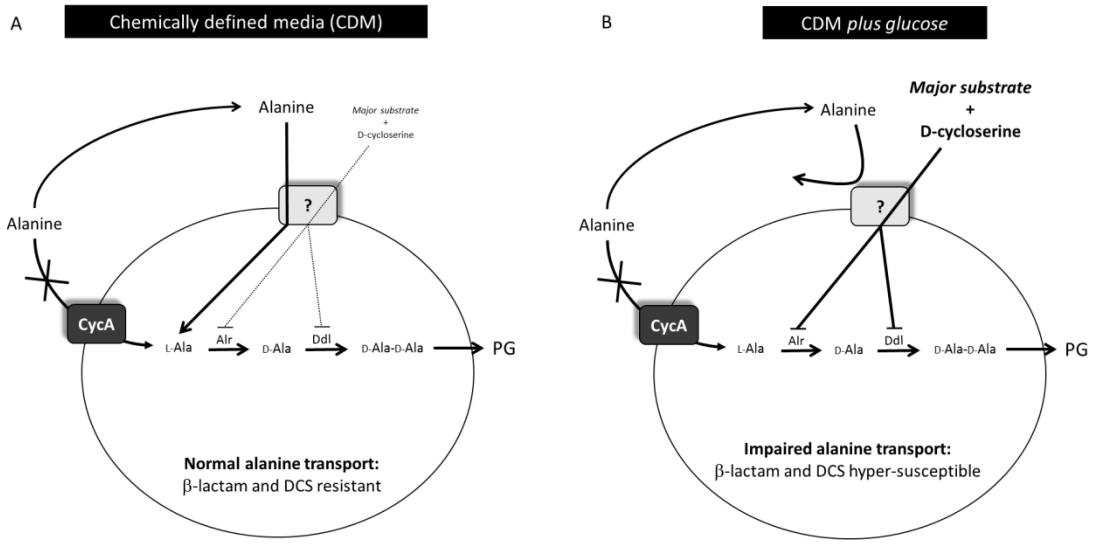

Figure 4.7. Proposed model for CycA-mediated transport of alanine and d-cycloserine in chemically defined medium (CDM) and CDM plus glucose. A. In CDM alanine is transported into the cell by CycA, but in the absence of *cycA* can also be transported by an alternative permease whose major substrate is not required for growth under these conditions. This alternative alanine permease transports d-cycloserine at a relatively low efficiency in CDM. **B.** In CDMG, the major substrate of the alternative permease is required for growth and transported into the cell at high efficiency, competitively excluding transport of alanine. Thus in a *cycA* mutant, alanine transport is significantly impaired. D-cycloserine is transported into the cell via the alternative alanine permease at relatively high efficiency in CDMG.

Halsey *et al.* (2017) recently characterised amino acid catabolism in *S. aureus* grown in CDM and CDMG (277). In media lacking glucose, alternative carbon sources such as amino acids become critical for growth (277). Specifically, growth of *S. aureus* in CDM lacking glucose was reliant on glutamate and amino acids generating glutamate, particularly proline (278). However, the growth requirement for other amino acids was increased in CDMG, supplemented with glucose, particularly lysine which was rapidly consumed in CDMG but only slowly consumed in CDM (277). Consistent with this, our studies also demonstrated that lysine was completely catabolised when *S. aureus* was grown in CDMG (Appendix 5) but not in CDM (Appendix 6). However, it is unclear how and why lysine is rapidly utilised in CDMG, given that analysis of the *S. aureus* genome does not support the existence of a metabolic pathway to ferment lysine to acetate (278) (a secondary carbon source) (277). It is tempting to speculate that the putative second alanine/DCS permease is a high affinity lysine permease that fulfils the cell's requirement for this amino acid in media supplemented with glucose. Thus, in CDMG media continuous transport of lysine through this permease would preclude alanine and DCS transport, with knock-on effects on cell wall architecture. In CDM media, the requirement for lysine is negated, allowing uptake of alanine, normal cell wall biosynthesis and wild type levels of β-lactam susceptibility.

Regardless of the identify and biology of the second, putative alanine/DCS permease, our data show the therapeutic potential of β-lactam / DCS combinations in the treatment of MRSA infections and have begun to elucidate the mechanisms underpinning this therapeutic effect. New therapeutic regimes along these lines have assumed increased strategic importance given that *S. aureus* isolates resistant to all licensed anti-staphylococcal drugs have been reported and the regulatory barriers to the introduction of new antimicrobial drugs. The excellent safety profile of β-lactam antibiotics makes these drugs particularly attractive as components of combination antimicrobial therapies. Currently, DCS is a second line drug in the treatment of tuberculosis and has known neurological side effects when administered at the required dose for treatment. Nonetheless physicians are turning to DCS for treatment of multi-drug resistant infections where alternatives are limited. Our data with MRSA demonstrated that the MICs for DCS and oxacillin were reduced when the two drugs were used in combination, opening up the possibility of using DCS in

the treatment of MRSA at concentrations that minimise the possibility of neurological side effects. Furthermore, DCS may serve as a scaffold drug from which it may be possible to synthesis more active and less toxic derivatives. Because oxacillin resistance in *S. aureus* is linked mainly to changes in the cell wall and because almost all synthesis of D-alanine is controlled by alanine racemase, a target for DCS, we propose that the combination of DCS and oxacillin represents an exciting new therapeutic option for the treatment of MRSA infections.

4.4. Materials and Method

4.4.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 4.3. Strains were grown overnight at 37°C in LB (Sigma), BHI broth (Oxoid), Mueller Hinton (Oxoid) or nutrient (Oxoid) media supplemented with ampicillin, chloramphenicol, erythromycin, oxacillin or d-cycloserine as indicated. Solid media were further supplemented with 1% agar. These antibiotics were sourced from Sigma and added to media at the specified concentrations.

Table 4.3. Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant Details	Reference
S. aureus		
RN4220	Restriction-deficient laboratory *S. aureus.*	(220)
JE2	USA300 LAC derivative cured of plasmids p01 and p03. CA-MRSA, SCC*mec* type IV. Parent strain of NTML.	(196)
13C	USA300 LAC derivative cured of plasmids p03. CA-MRSA, SCC*mec* type IV.	(196)
NE810	JE2 carrying *bursa aurealis cycA* mutation. Erm^r. CA-MRSA isogenic USA300 JE2 Δ*cycA*::φNΣ	(196)
NE1868	JE2 carrying *bursa aurealis mecA* mutation. Erm^r. CA-MRSA isogenic USA300 JE2Δ*mecA*::φNΣ	(196)
COL	MRSA Clinical Isolate	(263)
ATCC® 29213	MSSA strain for susceptibility testing	(222)
ATCC® 25923	MSSA strain for susceptibility testing	(222)
E. coli	*E. coli* HST08	(279)
Plasmids		
pLI50	*E. coli-Staphylococcus* shuttle vector. Ap^r (*E. coli*), Cm^r. (*Staphylococcus*)	(224)

4.4.2. Phenotypic library screen

The Nebraska transposon library was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) (196). This library was created in the USA300 genetic background, an epidemic CA-MRSA isolate, and consists of approximately 2,000 *S. aureus* derived strains that contain a single random transposon mutation within each of the non-essential genes of Je2, the CA-MRSA isolate USA300 cured of both plasmids p01 and p03. The overall aim of this screen is to identify genes which as a result of being disrupted by transposon insertion would lead to altered cefoxitin resistance levels. This was carried out through the application of Antimicrobial susceptibility testing by the application of disk diffusion method in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines using cefoxitin (30 µg) diffusion disks (Oxoid) on Mueller Hinton agar (Oxoid). Briefly, each library candidate was freshly streaked onto BHI agar supplemented with 10ug/ml of erythromycin and grown overnight for 18 h at 37°C. The wild-type strain JE2 was used as a positive control for resistance and NE1868 (JE2Δ*mecA*) was used along with ATCC® 25923 as controls for susceptibility. A bacterial suspension was prepared and standardised to 0.5 McFarland. Each bacterial suspension was used to inoculate the surface of a MH agar before the addition of the cefoxitin disc. Plates were then incubated at 35°C for 18 h. The entire library screen was carried out in duplicate initially with a further two repeats to confirm phenotypes. PCR was also carried out to confirm the transposon insertion in the allocated gene according to NARSA. Screening the entire library identified numerous library candidates that presented with either increased susceptibility or reduced susceptibility to cefoxitin, other β-lactams and antibacterial agents.

4.4.3. Antibiotic susceptibility testing

Measurement of oxacillin minimum inhibitory concentration (MIC) and disk diffusion tests (DD) for the *S. aureus* strains in this study were performed in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines using E-tests strips from Biomerieux on Mueller Hinton agar containing 2% NaCl or antibiotic diffusion disks (Oxoid) on Mueller Hinton agar.

4.4.4. Transduction of NE810 transposon mutation into other *S. aureus* strains.

The selected transposon mutant, NE810 that exhibited increased sensitivity to β-lactams and DCS was back crossed into JE2 background to ensure that secondary mutations were not involved in increased cefoxitin susceptibility. The bacteriophage 80α was propagated on NE810 and the resulting lysate used to transduce the mutation into JE2, 13C and COL, a MRSA clinical isolate. Transductants were selected on BHI agar containing erythromycin 10ug/ml and colonies screened by PCR using the primers NE810_Fwd and NE810_Rev (Table 4.4.) to confirm the presence of the transposon insertion in the *cycA* gene. To further ascertain that no secondary mutations were present, the genomes of JE2 and NE810 were determined by Illumina HiSeq sequencing (MicrobesNG, UK) and aligned to the publically available USA300_FPR3757 genome.

Table 4.4. Oligonucleotide primers used in this study

Target Gene	Primer Name	Primer Sequence (5'-3')
CycA	NE810_Fwd	ACAGAATAGCCACAAATAGCACC
	NE810_Rev	ACAGAATAGCCACAAATAGCACC
cycA	NE810F1_Fwd	GTCTTCAAGAATTCGGCCACAAATAGCACCATTAA
	NE810F1_Rev	CGACTCTAGAGGATCATGTCCCAAGCCCTAAAAC
mecA	mecA1_Fwd	TGCTCAATATAAAATTAAAACAAACTACGGTAAC
	mecA1_Rev	GAATAATGACGCTATGATCCCAA
gyrB	gyrB_Fwd	CCAGGTAAATTAGCCGATTGC
	gyrB_Rev	AAATCGCCTGCGTTCTAGAG

4.4.5. Complementation of NE810 with the *cycA* gene.

Genetic complementation of NE810 was achieved by cloning the *cycA* gene (SAUSA300_1642) into *Escherichia coli-Staphylococcus* shuttle plasmid pLI50 using the In-fusion HD cloning kit by Clontech. First, the *cycA* gene from JE2 was amplified by PCR using CloneAmp DNA polymerase with the primers

NE810F1_Fwd and NE810F1_Rev (Table 4.4.). The primers were designed using the CloneAmp In-fusion tool for the amplification of *cycA* with 15bp extensions that were complementary to the ends of the linearized vector. The amplified 1608 bp PCR product and restricted plasmid pLI50 were ligated using the In-Fusion enzyme which recognised the 15bp overlaps at their ends. The recombinant plasmid generated was transformed into *E. coli* HST08 and verified by Sanger sequencing (Source Biosciences). The plasmid was transformed by electroporation into NE810 via the restriction-deficient laboratory strain RN4220. All plasmid-harbouring strains were cultured in medium supplemented with 100 µg/ml ampicillin (*E. coli*) or 10 µg/ml chloramphenicol (*S. aureus*) to maintain plasmid selection. Sigma-Aldrich supplied oligonucleotide primers used for PCR and RT-PCR.

4.4.6. Growth measurements of JE2 and NE810.

Overnight cultures of JE2 and NE810 grown at 37°C in TSB were washed in phosphate-buffered saline (PBS) and inoculated at a starting cell density of A_{600} = 0.05 into chemically defined medium (CDM), CDM lacking alanine or CDM supplemented with 14mM glucose (CDMG) (280). Where indicated the media was supplemented with 0.125 mg/L oxacillin. Cell density was determined by measuring the A_{600} every hour for 12 hours.

4.4.7. Static biofilm assays.

Semi-quantitative measurements of biofilm formation were determined under static conditions using Nunclon Hydrophilic 96 well tissue culture treated polystyrene plates (Nunc, Denmark) as described previously (151). Each strain was tested a minimum of three times, and average results are presented. A biofilm-positive phenotype was defined as an $\boldsymbol{A}_{490} \geq 0.17$.

4.4.8. Reverse Transcription-PCR (RT-PCR).

RT-PCR for *mecA* was performed on the Roche LightCycler 480 instrument using the LightCycler 480 Sybr Green Kit (Roche) with the *mecA* primer set. The following programme was used: denaturation conditions were 95 ^{o}C for 5 minutes and followed by 45 cycles of 95 ^{o}C for 10 seconds, 58 ^{o}C for 20 seconds and 72 ^{o}C for 20 seconds at which point readings were taken. Melt curve analysis was performed at 95 ^{o}C for 5 seconds followed by 65 ^{o}C for one minute up to 97 ^{o}C at a ramp rate of 0.11c/sec with five readings taken for every degree of temperature increase. The *gyrB* gene served as an internal standard for all reactions. For each reaction, the ratio of *mecA* and *gyrB* transcript number was calculated. Each RT-PCR experiment was performed three times and presented as average data with standard errors. The RT-PCR primers used in this study are listed in Table 4.4.

4.4.9. Scanning Electron Microscopy (SEM).

Biofilm imaging, cell shape and cell size analysis were carried out using SEM imaging. JE2 and NE810 biofilms were grown on tissue culture treated polystyrene slides with detachable wells (Nunc, Thermo-scientific). Overnight BHI cultures were diluted 1:200 in BHI broth supplemented with 1% glucose and incubated for 24h at 37^{0}C. Subsequently, the slides were rinsed three times with distilled water, dried at 60^{o}C 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.

4.4.10. Transmission Electron Microscopy (TEM)

Overnight BHI cultures of JE2 and NE810 were diluted 1:200 in fresh BHI and grown at 37°C to an A_{600} = 1.0. 10 ml culture aliquots were subjected to centrifugation at 8,000 × ***g***, and the cell pellets were resuspended in fixation solution

(2.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.4]) and incubated overnight at 4°C. The fixed cells were further treated with 2% osmium tetroxide, followed by 0.25% uranyl acetate for contrast enhancement. The pellets were then dehydrated in increasing concentrations of ethanol as described above for the SEM cell preparation, followed by pure propylene oxide, and transferred to a series of resin and propylene oxide mixtures (50:50, 75:25, pure resin) before being embedded in Epon resin. Thin sections were cut on an ultramicrotome. Images were analysed using a Hitachi H7000 instrument. At least 3 to 5 measurements of cell wall thickness were performed on each cell and approximately 30 cells were measured for each sample.

4.4.11. Preparation of cells for peptidoglycan analysis.

Overnight cultures of JE2 and NE810 were used to inoculate fresh BHI in flasks at A_{600} = 0.05. Cultures were grown to mid-log (A_{600}=1.5), cooled on ice and 3-4 g wet weight of cells collected by pelleting before being washed once in ice cold sterile molecular grade water. Cell samples were sent for peptidoglycan analyses by DSMZ GmbH (Braunschweig, Germany) according to published protocols (281).

4.4.12. Antibiotic synergy analysis using microdilution checkerboard assay.

The synergistic activity of DCS and oxacillin against JE2 and NE810 was measured using the checkerboard microdilution method in 96-well plates. The final inoculum in each well was 5×10^5 CFU/ml, and the results were read after an 18/24-h incubation at 37°C. Negative controls were MH broth and the DCS-antibiotic combination while positive controls were MH broth and bacterial suspension. The fractional inhibitory concentration (FIC) index was calculated for each drug combination. An FIC index of ≤ 0.5 was considered synergistic, one of >0.5 to <2 was considered indifferent, and one of >2 was considered antagonistic. All experiments were performed in triplicate.

4.4.13. Antibiotic synergy analysis using antibiotic impregnated disks on agar plates.

Suspensions of JE2 and NE810 cells adjusted to the 0.5 McFarland standard and inoculated onto the surface of a MH agar plate with or without DCS as indicated, before the addition of a cefoxitin disk or both cefoxitin and DCS disks. Plates were incubated at 35°C for 18 h.

4.4.14. Amino acid uptake analysis.

Overnight cultures of JE2 and NE810 grown at 37°C in TSB were washed in phosphate-buffered saline [PBS] and inoculated at a starting cell density of A_{600} = 0.05 into CDM or CDMG with or without 0.03 µg/ml of oxacillin and grown for 12 h. 1 ml culture aliquots were collected every 4 h, cells pelleted for 3 min at 14,000 rpm and the supernatants filtered through Amicon Ultra centrifugal filters (Millipore) (3,000 molecular weight cutoff [MWCO]) according to the manufacturer's protocol. Amino acid analysis was performed by the Protein Structure Core Facility, University of Nebraska Medical Center (UNMC), using a Hitachi L-8800 amino acid analyser.

4.4.15. *In vivo* mouse model of MRSA infection

Age-matched, 6–8-week-old, outbred CD1 female mice (Charles River, United Kingdom) were used in a nonlethal model of sepsis. JE2 and NE810 cultures were grown to an A_{600} of 1 in BHI broth for the sepsis model, washed in PBS, and adjusted to appropriate cell densities. For bacteremia, 5×10^6 CFU USA300 (5 mice/group) were injected into the tail vein and left untreated (PBS control), treated with 75 mg oxacillin/kg/12 hours, DCS at 10mg/kg (made fresh each time), or in combination with oxacillin and DCS (the first antibiotic dose was administered 16 hours after infection), before being euthanized on 7 days. Homogenates of organs obtained from mice with sepsis were plated on blood agar. Statistical significance was assessed using the 2-tailed Student *t* test. Mouse experiments were approved by the United Kingdom Home Office (Home Office Project License Number 40/3602) and the University of Liverpool Animal Welfare and Ethics Committee.

4.4.16. Statistical analysis

Two-tailed Student's t-Tests were used to determine statistically significant differences in assays performed during this study. A *P* value <0.05 was deemed significant.

ACKNOWLEDGMENT

We thank Pierce Laylor for assistance with TEM cell wall analysis.

FUNDING

This study was funded by a grant from the Irish Health Research Board (HRA_POR/2012/51) (to J.P.O'G). Peptidoglycan cell wall analysis was funded by the Thomas Crawford Hayes Research Fund (to LAG).

Chapter 5

Conclusions and future directions

The opportunistic pathogen *S. aureus* has become a major burden on the healthcare sector due to its persistent and virulent mechanisms which has compromised both patient care and caused infections among healthy individuals in the community who have had no pre-exposure to the hospital setting. Recognition that these CA-MRSA isolates are also responsible for many healthcare associated infections has increased their significance. A contributing factor in therapeutic failures of *S. aureus* infections is the ability of this pathogen to accumulate mutations during treatment which promote survival and proliferation. Since the 1960's, following the initial isolation of MRSA from hospital environments, multidrug resistant isolates have emerged to all classes of clinically-used antibiotics. These resistance mechanisms provide a clear advantage for bacterial survival in response to treatment strategies. However survival comes at a fitness cost for the bacterium. Antimicrobial resistance has a strong influence on therapeutic options worldwide and advances in our understanding of mechanisms underpinning resistance will assist efforts to develop improved treatment regimens for patient care.

Our research group has previously reported that a mutation in GdpP, a c-di-AMP phosphodiesterase, leads to high-level resistance to methicillin, attenuated virulence and an altered biofilm phenotype in MRSA. Furthermore, the disruption of the *S. aureus dacA* gene, which encodes a c-di-AMP synthase was also shown to play a role in the switch from expression of high-level, homogeneous resistance to low-level heterogeneous resistance. Our group and others have also implicated another nucleotide messenger, (p)ppGpp, the effector molecule of the stringent response, in the expression of increased resistance to β-lactam antibiotics. Evidence suggests that GdpP is tightly regulated by ppGpp, implicating the stringent response in controlling c-di-AMP signalling and modulating resistance to β-lactam antibiotics, biofilm and virulence. Furthermore, altered c-di-AMP levels have also been linked to controlling cell size and envelope stress response, antibiotic tolerance and altered resistance in other bacterial species including *B. subtilis, S. pneumonia* and *L. monocytogenes*. We hypothesised that the increased β-lactam resistance was reliant on increased c-di-AMP levels in HoR mutants and that therapeutics designed to disrupt ppGpp and c-di-AMP signalling could reduce resistance, offering a novel approach in the management of MRSA infections.

In this thesis, two scientific approaches were utilised to better understand secondary mechanisms associated with high level resistance in the epidemic USA300 clone LAC. First, using a chemostat the adaptation of USA300 to increasing concentrations of oxacillin was characterised in a continuous culture. Secondly, the NTML was screened for mutations impacting levels of resistance to the β-lactam cefoxitin. Since exposure of HeR MRSA to sub-inhibitory concentrations of β-lactams can select for highly resistant mutants, we believe that both of these approaches are clinically relevant.

5.1. Amplification of SCC*mec* is a new mechanism of high level β-lactam resistance in MRSA

Acquired secondary mutations outside SCC*mec* have long been implicated in high level, homogeneous methicillin resistance, augmenting expression of *mecA*/PBP2a. However, our research has revealed tandem amplification of SCC*mec* on the chromosome as a novel route to increased resistance. The increasing levels of *mecA*-encoded PBP2a in this mutant, designated HoR34, is responsible for the corresponding increase in oxacillin resistance levels from 300 µg/ml to 800 µg/ml. The significant loss of competitiveness in the absence of antibiotic selection suggests that MRSA mutants carrying multiple SCC*mec* elements are unlikely to be maintained under physiological conditions, perhaps explaining why tandem duplication and amplification of SCC*mec* has not been previously identified in clinical isolates. However despite the fitness burden, the USA300 mutant carrying multiple SCC*mec* elements was more competitive than wild type USA300 in the presence of oxacillin indicating that this resistance mechanism may be clinically important under appropriate growth conditions.

A number of genetic mechanisms may have contributed to SCC*mec* amplification. The role of the *ccr* recombinases, which are responsible for site and orientation-specific excision and integration of SCC*mec*, are likely to be involved in the initial duplication event, particularly given that these genes are activated by β-lactams. Following the initial duplication event, it seems likely that amplification of SCC*mec* is RecA-mediated. RecA activity is also likely to explain why the number of copies of SCC*mec* can be expanded and contracted as oxacillin concentrations are

increased and decreased, respectively. Future work to mutate *recA* in HoR34 will determine if RecA is required for expansion and contraction of the amplified SCC*mec* element.

5.2. Mutation of *pgl* facilitates expression of high level methicillin resistance in CA-MRSA

Recently, research into *clpX* or *clpP* mutations associated with increased resistance to β-lactams found no association with increased c-di-AMP levels, indicating that alternative routes to β-lactam resistance independent of c-di-AMP are possible. Furthermore, the reported essentiality of this nucleotide in mollicutes lacking a cell wall points to a broader role for c-di-AMP signalling in cellular physiology (282-284).

In this research, we identified a *pgl* mutant expressing high levels of resistance to β-lactams, independent of increased c-di-AMP levels. 6-phosphogluconolactonase, encoded by *pgl*, is the second enzyme in the pentose phosphate pathway (PPP) and converts 6-phosphogluconolactone to phosphogluconate. This mutant exhibited significant changes in cell wall structure which have been previously associated with β-lactam cell wall stress and resistance. Furthermore, this mutant appeared to have growth defects which has also been reported for *pgl* mutants in *L. moncytogenes* and *E.coli* in glucose limiting media (252, 253) .

We hypothesise that this disruption in the PPP could increase glucose flow through the glycolysis pathway, which would leave the *pgl* mutant primed to upregulate peptidoglycan biosynthesis following exposure to β-lactam-induced cell wall stress. In this scenario, the increased demand for cell wall precursors by PBP2a and other native PBPs would be met, enabling increased cell wall biosynthesis and consequently increased resistance to β-lactams. Metabolomic studies by Dörries *et al.,* 2014, on the impact of antibiotics on *S. aureus* metabolome revealed increased glucose uptake and a switch from the PPP to the glycolysis and TCA pathway 60 mins after β-lactam exposure, which is consistent with the demand for both increased cell wall biosynthesis and ATP generation via the TCA cycle (285). Increased carbon flux through glycolysis and the TCA cycle in a *pgl* mutant is also consistent

with our data showing increased β-lactam tolerance in an MSSA *pgl* mutant, in which we speculate that native PBPs can quickly avail of the increased supply of cell wall precursors thus directing carbon flux to cell wall biosynthesis.

A recent study in *L. monocytogenes* showed that c-di-AMP inhibits pyruvate kinase activity causing a TCA cycle dysfunction that could increase the availability of fructose-6-P for cell wall synthesis under antibiotic stress (258). Consistent with this a study in *S. epidermidis* indicated that TCA cycle dysfunction was associated with increased β-lactam resistance (286). Indeed this data may also suggest that elevated c-di-AMP levels contribute to increased β-lactam resistance by inhibiting the pyruvate kinase to increase carbon flux to cell wall biosynthesis. Overall these data highlight the complex interplay between the c-di-AMP signalling, central metabolism and cell wall biosynthesis.

Consistent with data from *L. monocytogenes* (253) and a previous study in *S. aureus* (287), the absence of a glucose-6-phosphate dehydrogenase *zwf* mutant in the NTML library suggests that this gene is essential. This enzyme catalyses the conversion of glucose-6-phosphate to 6-phosphogluconolactone, which is the first step of the PPP. A *zwf* mutation apparently blocks the oxidative branch of the PPP. It is unclear why the *pgl* mutant is viable, which may suggest that this mutation does not completely inhibit the PPP. Research is currently underway to further examine the effect of the *pgl* mutation on the PPP, glycolysis, the TCA cycle and cell wall biosynthesis. Collaborations are underway to utilise LC/MS-MS to assess the entire metabolome of the *pgl* mutant. Measuring the expression of PBP2a and PBPs levels through western blots and *mecA* transcription by RT-PCR will also be performed to reveal their contribution to the increased oxacillin MIC of the *pgl* mutant. Our working hypothesis proposes that mutations in key metabolic enzymes contributes to increased antibiotic tolerance in both MSSA and MRSA, driven in part by increased cell wall biosynthesis, leading to an overall increase in resistance levels in MRSA.

5.3. Using amino acid analogues to overcome resistance to β-lactam antibiotics in MRSA

The data presented in this thesis demonstrates that mutation of the *S. aureus cycA* gene increases susceptibility to the β-lactam antibiotics cefoxitin and oxacillin, as well as d-cycloserine (DCS), in CDMG, BHI media, and under *in vivo* conditions in mice. Our collaborator Prof Paul Fey demonstrated that alanine uptake is impaired in the *cycA* mutant indicating that alanine is important for cell wall integrity and consequently resistance to β-lactam antibiotics. In this context DCS-mediated inhibition of alanine racemase and D-alanine ligase activity in the *cycA* mutant was shown to aggravate the impact of impaired alanine uptake on β-lactam susceptibility. As oxacillin resistance in *S. aureus* is linked mainly to changes in the cell wall and the correct incorporation of alanine into the cell wall is vital, we propose using oxacillin in combination with DCS to potentiate the activity of β-lactams against MRSA as an exciting new therapeutic option for the treatment of MRSA infections.

A comparison of alanine uptake in CDMG and CDM suggested that a second active permease must compensate for alanine transport in the *cycA* mutant in CDM resulting in no change in oxacillin susceptibility. Furthermore this data indicates that CycA cannot be involved in the transport of DCS and that DCS transport may also be facilitated, at least in part, by the proposed second alanine permease. It remains unclear how or why the addition of glucose to CDM has such an impact on the activity of the second alanine/DCS permease. We have speculated that the second putative permease implicated in alanine and DCS uptake may have a higher affinity for another substrate(s), effectively blocking alanine and DCS transport when in media lacking glucose, however this remains to be verified. Currently, our research group is screening the NTML library for a mutant(s) exhibiting increased resistance to DCS, based on impaired DCS uptake. Amino acid uptake will be measured in CDM and CDMG to identify if the transport of other substrates is also impacted in DCS resistant mutant(s). Furthermore, if possible a double mutant lacking *cycA* and the gene(s) involved in DCS resistance will be constructed to assess the impact on alanine transport, β-lactam and DCS susceptibility.

Antibiotic resistance continues to be a major threat to our healthcare systems particularly given the limited number of new antimicrobial drugs that are in clinical

trials and the focus on new therapeutic approaches that currently rely solely upon a single bactericidal mechanism. MRSA remains on the WHO list of pathogens for which new antibiotics are urgently needed. Multidrug resistance in *S. aureus* clinical isolates including resistance to "new" clinical antibiotics such as daptomycin highlights the continued threat from this pathogen. However the one drug - one target model has limitations, and combination therapy offers an alternative approach to MRSA treatment. Combination therapies could present several advantages over the development of new antibiotics, including the decreased likelihood of resistance development and the capacity to repurpose antibiotics for which resistance is prevalent. Based on the research presented in this thesis, increased β-lactam resistance is not solely reliant on increased c-di-AMP levels but also on an enhanced capacity for cell wall biosynthesis. Implicating glycolysis, the TCA cycle and cell wall biosynthetic pathways in high levels resistance may expand the range of potential targets for combination therapies. Impeding the cells ability to adapt to antimicrobials could reduce our need for new antibiotics in the future. Targeting signalling systems in bacterial pathogens has the potential to reduce antibiotic resistance thus offering new therapeutic potential for β-lactam drugs alone or in combination with existing antimicrobials.

5.4. Final thoughts

This body of research has highlighted the potential of the cell wall biosynthesis pathways to be a key antibiotic target for the treatment of MRSA infections. These results postulate that β-lactam resistance is associated with an increase in cell wall biosynthesis leading to an increased demand on cell wall biosynthetic components. The acquisition of the multiple copies of SCC*mecA* would allow for a greater degree of transpeptidation impacting peptidoglycan crosslinking in the HoR 34 mutant. The differences that may be created by increased transpeptidation in the overall peptidoglycan structure could possibly alter cell morphology, drug permeability, and sensitivity to antimicrobial agents that target peptidoglycan biosynthesis. Redirecting the glucose flow through the glycolysis pathway, the *pgl* mutant may allow for optimal glycolysis coverage with increased accessibility to fructose 6-P and acetyl-CoA that are major components for the

synthesis of the peptidoglycan cell wall precursors. A plentiful supply of these precursors are necessary to address the over production of PBP2a proteins and other PBPs when the bacterium is subjected to β-lactam exposure. However, the chemical structure of the peptidoglycan that has been gathered over a number of decades is still very limited. More research is needed regarding the finer structures and residue arrangements to address particular types of cross linking that impact resistance.

As oxacillin resistance in *S. aureus* is linked mainly to changes in the cell wall and the correct incorporation of amino acids and cell wall precursors into the cell wall, we propose using oxacillin in combination with drugs which target and inhibit proteins that have key roles in cell wall synthesis along with limiting there availability. The *S. aureus* peptidoglycan cell wall uniquely contains a 5 residue stem-peptide along with a penta-glycine interbridge, which are of major structural importance. Thus the reduced uptake of alanine and glycine in the *cycA* mutant impairs normal cell wall biosynthesis and increases susceptibility to β-lactam antibiotics.

These data prompt a re-evaluation of drug combination therapies using β-lactams and other cell wall active antibiotics including drugs based on serine/alanine/glycine analogues to identify new treatments that suppress resistance development and promote synergism. Impeding the cells ability to adapt to antimicrobials could reduce our need for new antibiotics in the future. Importantly, our research offers a promising alternative to the treatment of multidrug resistant infections in humans and animals. Apart from the enormous benefits that would be experience by patients, the overall economic savings in healthcare and veterinary care as a result of effective treatment would be enormous.

Importantly, this work could be expanded to address other ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter*) which could determine the overall therapeutic impact that DCS in combination with β-lactams in overcoming resistance. If DCS and other amino acid analogues potentiate the activity of β-lactam antibiotics in other AMR pathogens, the impact of this work could have major therapeutic implication.

Chapter 6

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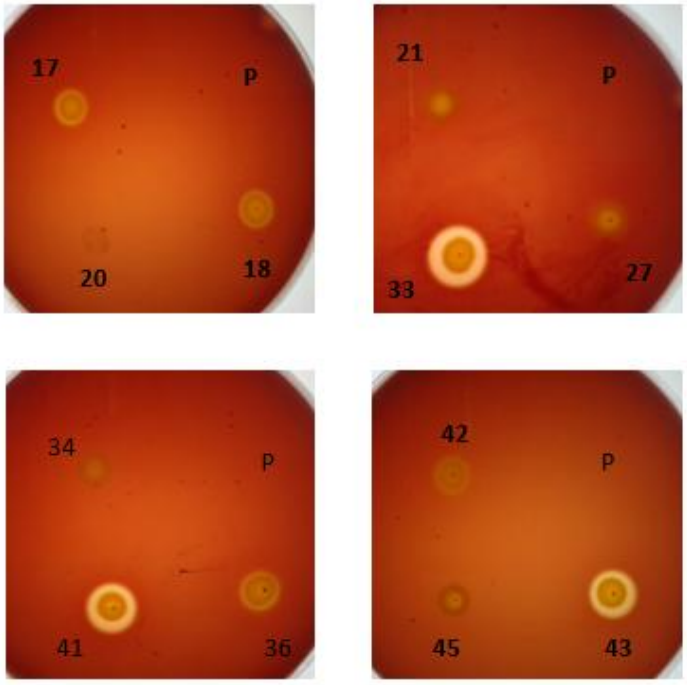
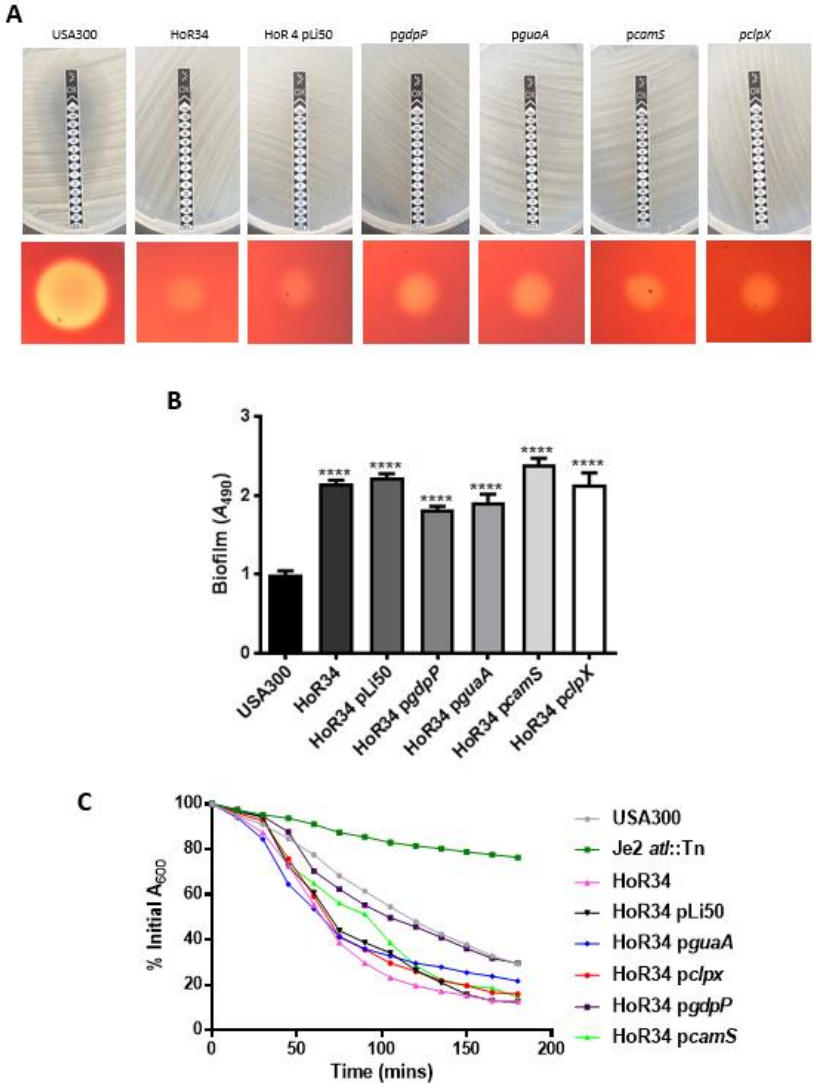
Appendices

Appendix 1



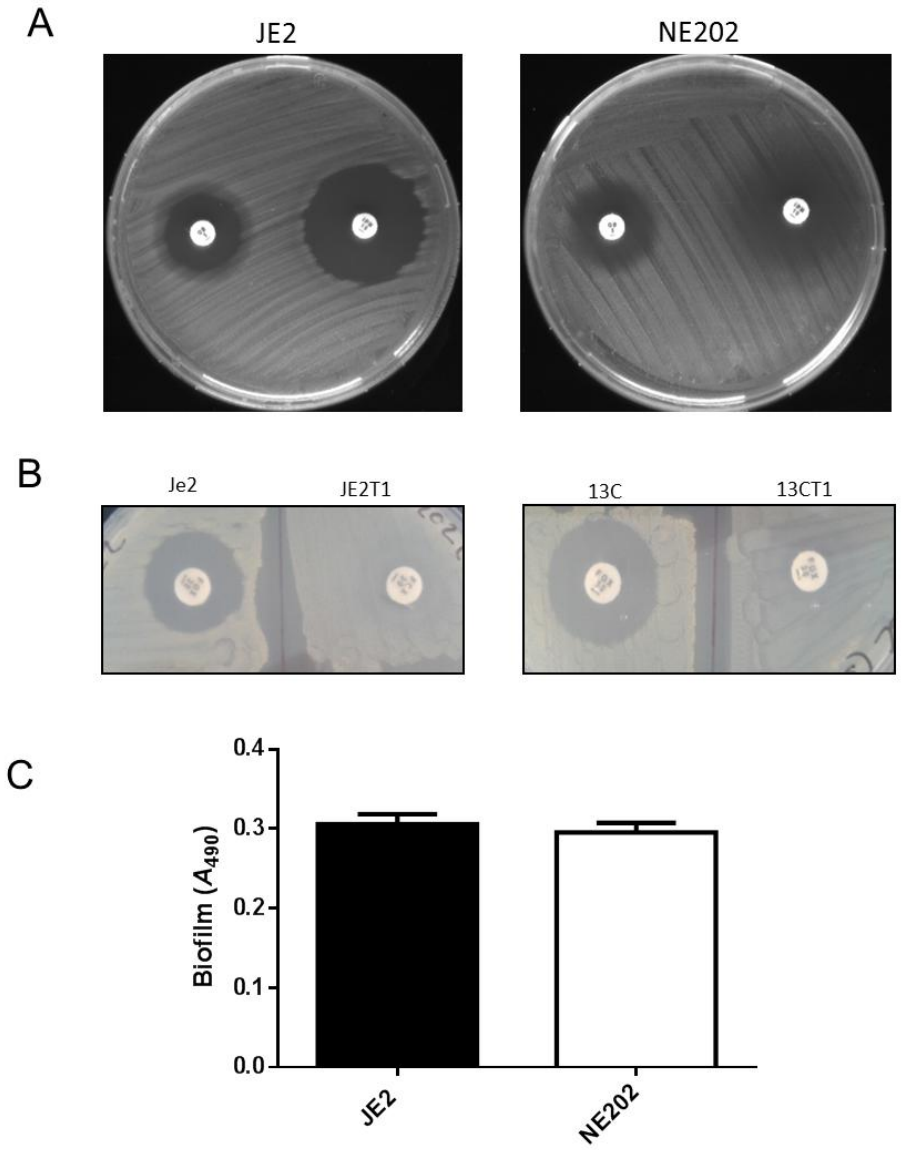
Appendix 1. β-haemolytic activity of USA300 HoR mutants isolated from the chemostat. Overnight cultures standardised to an OD 1.0 were plated onto BHI 5% sheep agar containing 100 μ/ml oxacillin and incubated for 24h. β-haemolytic activity was assessed based on lysis of cells. P indicates parent that hasn't grown in the presence of oxacillin.

Appendix 2



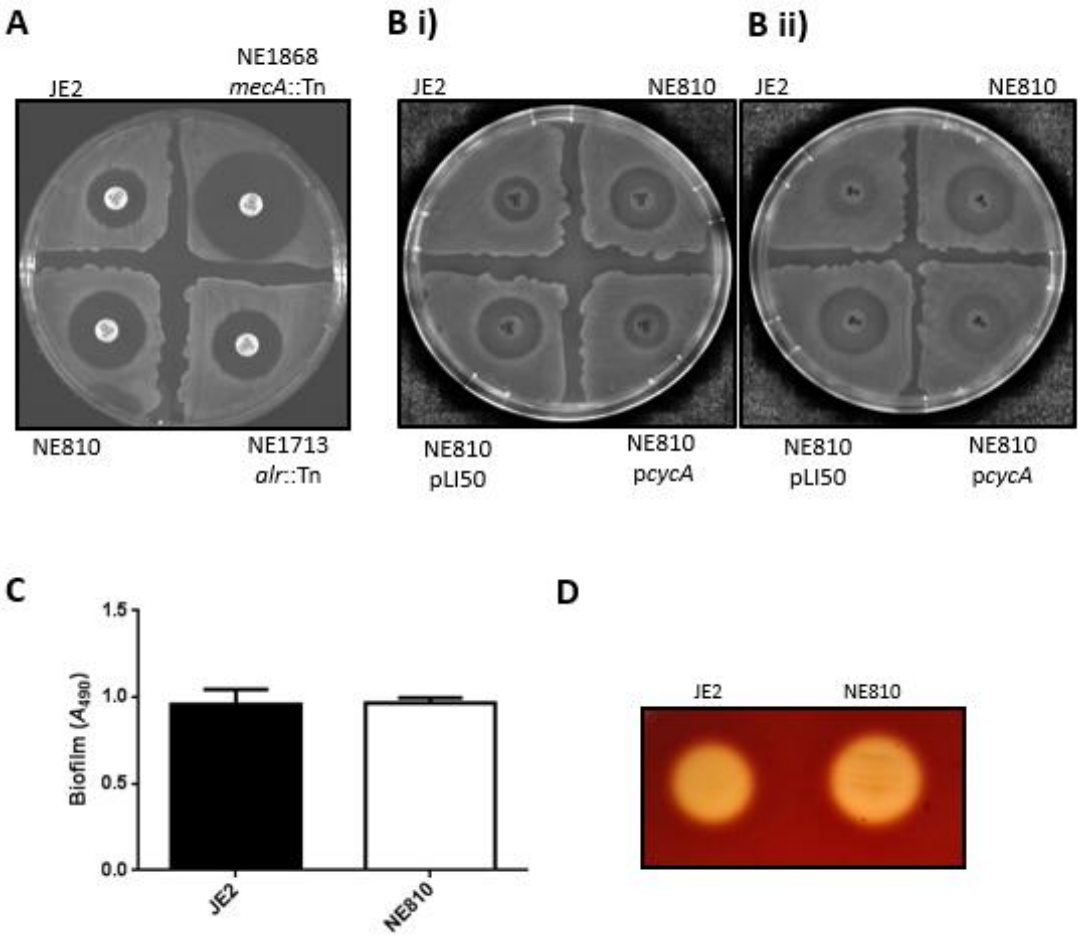
Appendix 2. Complementation of HoR34 phenotypes with *gdpP, guaA, clpX* and *camS*. A. Oxacillin MIC of USA300, HoR34 and HoR34 carrying plasmids pLI50 (control), p*gdpP*, p*guaA*, p*clpX* and p*camS* determined using Etests. β-haemolytic activity of USA300, HoR34 and HoR34 carrying plasmids pLI50 (control), p*gdpP*, p*guaA*, p*clpX* and p*camS* on sheep blood agar. **B.** Comparative biofilm formation by USA300, HoR34, and HoR34 carrying plasmids pLI50 (control), p*gdpP*, p*guaA*, p*clpX* and p*camS*, 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 deviation. Significant differences are indicated (Student's two-tailed t-test, ***P < 0.0001). **C.** Autolytic activity in USA300 and HoR34. USA300, HoR34, HoR34 carrying plasmids pLI50 (control), p*gdpP*, p*guaA*, p*clpX* and p*camS*, and a USA300 JE2 *atl* mutant (negative control) were grown to early exponential phase in BHI at 37°C and washed in PBS and adjusted to A_{600} = 1.0 in 0.01% Triton X-100. The A_{600} was measured initially and at 15 min intervals thereafter with shaking incubation at 37°C. Autolytic activity is expressed as a percentage of the initial A_{600}.

Appendix 3



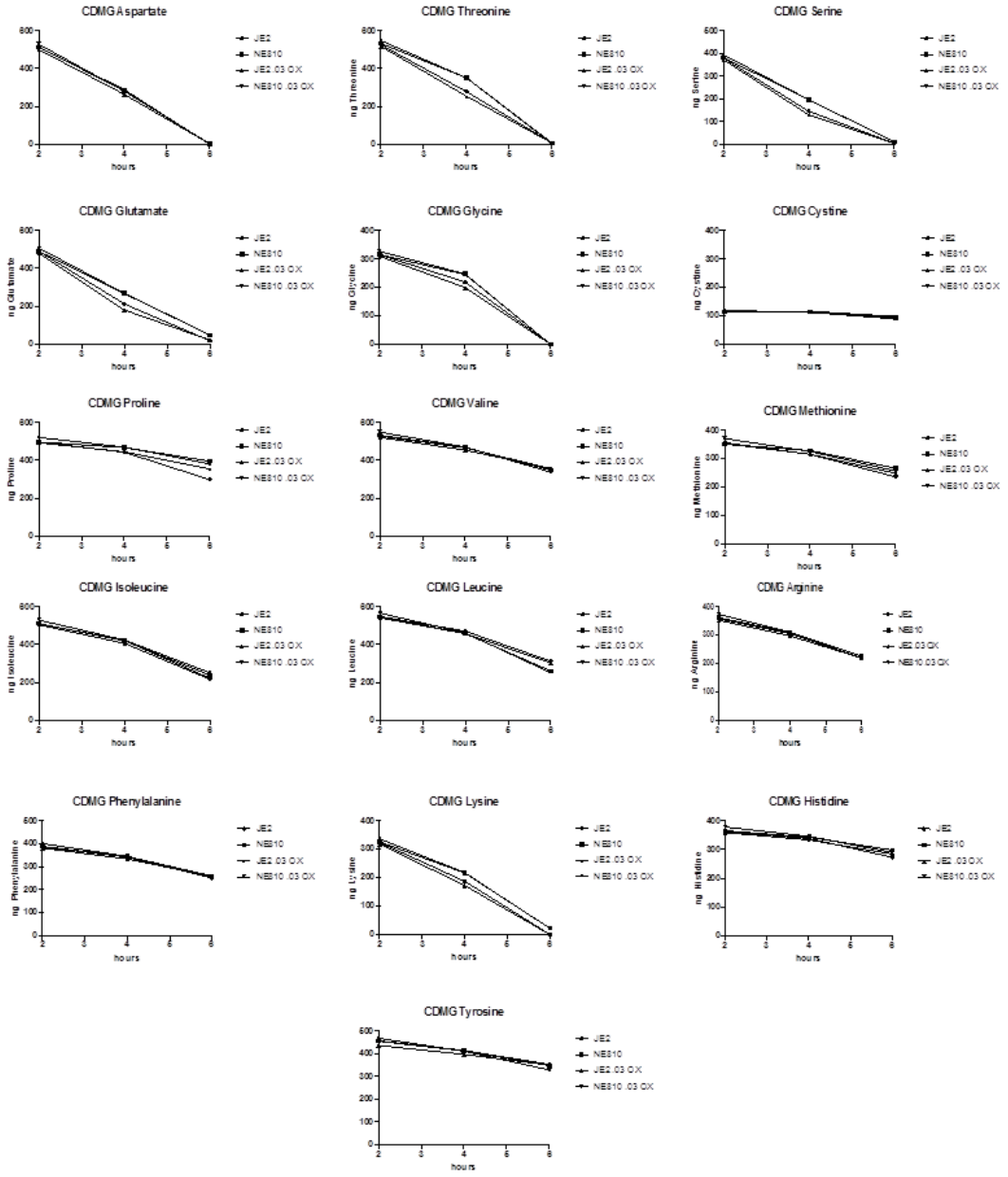
Appendix 3. A. Susceptibility of JE2 and NE202 to cloxacillin and ipipenim determined using disk diffusion assays. **B.** Disk diffusion assay to measure cefoxitin sensitivity of JE2, 13C and their corresponding *pgl*::Tn mutants JE2T1 and 13CT1. **C.** Comparative biofilm formation of JE2 and NE202 (*pgl*::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.

Appendix 4



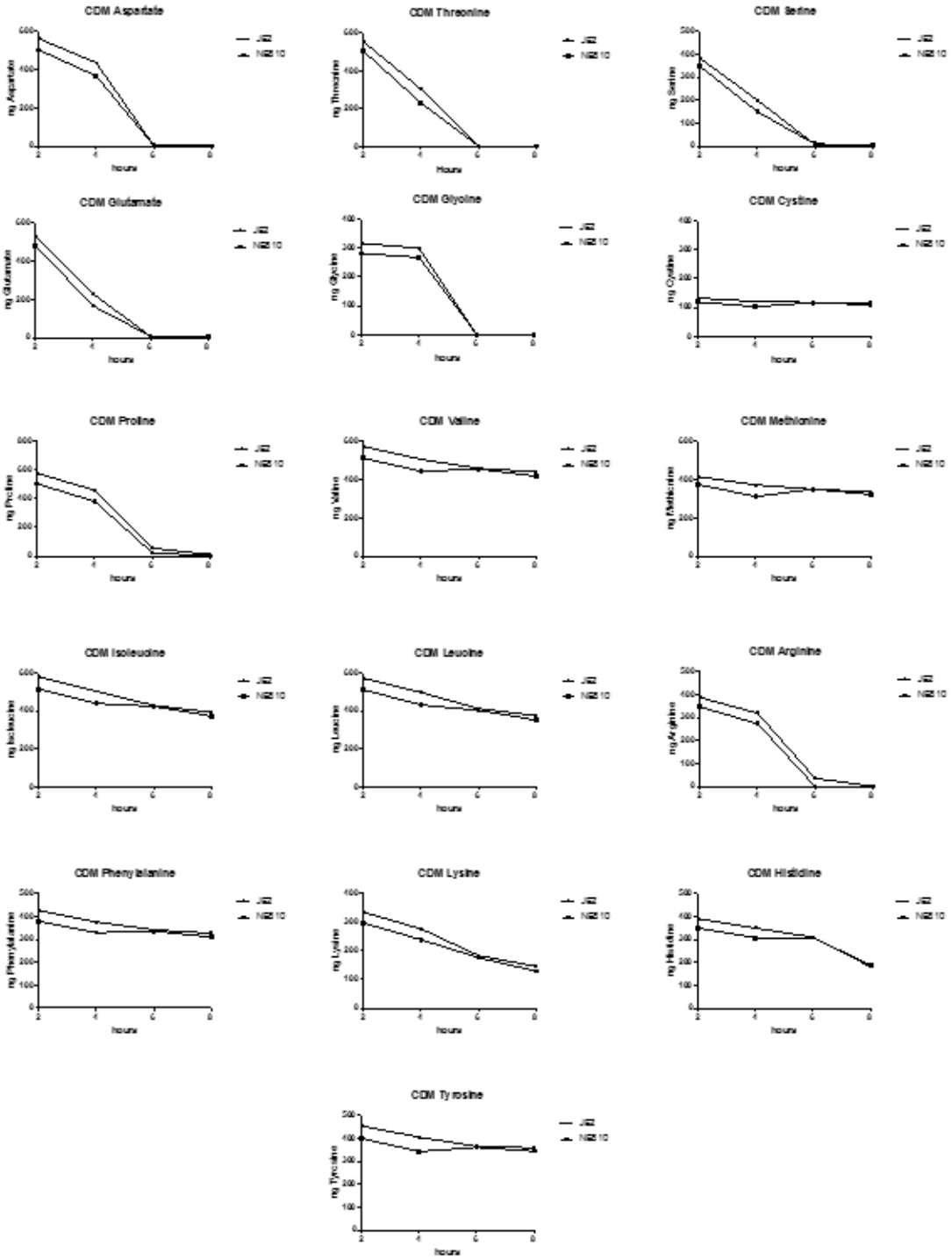
Appendix 4. A Cefoxitin susceptibility profiling of JE2, NE1868 (*mecA*::Tn), NE810 and NE1713 (*alr*::Tn). **B** B i) Cefoxitin and B ii) cloxacillin susceptibility profiling of JE2, NE810, NE810 carrying plasmids pLI50 (control) and NE810 complemented with p*cycA* determined using disk diffusion assays. **C** Comparative biofilm formation of JE2 and NE810 grown for 24 h at 37^{o}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 deviation. **D** Overnight cultures standardised to an OD 1.0 were plated onto BHI 5% sheep agar and incubated for 24h. β-haemolytic activity was assessed based on lysis of cells.

Appendix 5



Appendix 5. Amino acid consumption of JE2 and NE810 following growth in CDM containing 14mM of Glucose (CDMG). JE2 and NE810 were grown aerobically in CDMG, and amino acid concentrations (mM) were measured from the spent medium at 2, 4 and 8 h of growth

Appendix 6



Appendix 6. Amino acid consumption of JE2 and NE810 following growth in CDM. JE2 and NE810 were grown aerobically in CDM, and amino acid concentrations (mM) were measured from the spent medium at 2, 4, 6 and 8 h of growth.