The role of penicillin binding protein 4 in β-lactam resistance in Staphylococcus aureus

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Ahang H. Mawlood

Abstract

Staphylococcus aureus possesses four native penicillin binding proteins PBPs (PBP1-4). Because of the structural resemblance between their natural substrate, the D-Ala-D-Ala end of the stem pentapeptide precursor, and penicillin-type antibiotics, the late stage peptidoglycan synthesizing enzymes are sensitive to penicillin. Resistance to penicillin-type antibiotics in *S. aureus* arises due to acquisition of an alternative PBP (PBP2A, encoded by *mecA*), expression of β-lactamase, or point mutations in genes encoding endogenous penicillin binding proteins, which reduces penicillin binding to the enzyme active site. The reduced sensitivity to penicillin G and cefotaxime *in vitro* selected mutant RS1/19 cells is likely related to PBP4 overproduction, because antibiotics that selectively inhibit PBP4 activity cause the greatest reduction in penicillin G MIC (minimum inhibitory concentration), when used in combination.

To test whether PBP4 overproduction is sufficient and necessary for the penicillin resistance observed in RS1/19, the *pbp*4 gene was cloned into a high copy number plasmid and introduced into different genetic backgrounds of *S. aureus*. Antibiotic sensitivity testing showed that there was no difference in the minimum inhibitory concentration (MIC) of penicillin G or oxacillin, this means that increased PBP4 activity is not sufficient for resistance. Allelic replacement using double cross-over event was used to delete *pbp4* in the background of RS1/19 strain. RS1/19Δ *pbp4* tested in this study have a moderately increased sensitivity to cefoxitin and cefsoludin, but a marked increase in sensitivity to cefotaxim, oxacillin, and penicillin G compared to RS1/19. These data lead to the conclusion that PBP4 is necessary for the penicillin and methicillin resistance phenotype in RS1/19.

In addition, PBP4 must work with a network of genes that collectively associates with the resistance observed in RS1/19 strain. The data presented here clearly shows that PBP4 is not the only factor involved in resistance in RS1/19, but that it is

necessary for the full expression of resistance. It has been concluded that deletion of monofunctional trasglycosylase *mgt* is not essential for viability and expression of resistance in both wild-type and RS1/19strains. Future studies of RS1/19 are likely to uncover other factors that are essential for full expression of methicillin resistance in *mecA* negative strains of *S*. *aureus*.

The role of penicillin binding protein 4 in β-lactam resistance in *Staphylococcus aureus*

Thesis submitted for the degree of Doctor of Philosophy

at the University of Durham

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Declaration

I declare that the work within this thesis, submitted for the degree of Doctor of Philosophy, is my own original work and has not been submitted for a degree at this or any other University.

Signed ……..

Date ……..

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My heartiest thanks to my loving husband Salah for sharing all good and bad times with me over many years away from home and my family.

DEDICATION

This Thesis is Dedicated to My Husband Salah, My Mum and Dad. It is Also Dedicated Proudly to My Sisters and Brothers and All Loved Ones.

CHAPTER ONE

General Introduction

1. 1. General Characteristics of *Staphylococcus aureus*

S. *aureus* was first isolated and characterized by Alexander Ogston, in 1880, in pus of surgical absce, who reported that it was different in appearance to the previously characterized *Streptococcus* genus (Ogston, 1882). The word *Staphylococcus* is derived from the Greek word staphylos, meaning a "bunch of grapes", and indicates that *S*. *aureus* divides in more than one plane, forming clusters looking like grape bunches under the light microscope (Kloos et al., 1998).

Historically, scientists faced difficulties in differentiating staphylococci from other cocci shaped bacteria, especially those forming clusters, like the genus *Micrococcus*. Later, it was discovered that micrococci were different from staphylococci as they show a consistently high resistance to novobiocin, the presence of multiple menaquinones in the membrane, and lack the ability to ferment glucose anaerobically (Cohen. 1972). Additionally, the G+C content in staphylococcal DNA was found to be much less than that of micrococci (Silvestri, L and Hill., 1965). As new technologies became available in the 1990s which allowed more detailed biochemical, morphological, and physiological insights into bacterial phylogeny, the staphylococcal species became easier to distinguish in the laboratory and clinic from closely related bacteria (Schleifer and Kroppenst., 1990; Crossley and Archer., 1997).

There are 32 species and eight sub-species in the genus *Staphylococcus*, many of which preferentially colonise the human body (Kloos and Bannerman, 1994). *S*. *aureus* has long been recognised as one of the major human pathogens and is by far one of the most common nosocomial organisms, being responsible for most post-surgical infections. It is an opportunistic bacterium frequently part of human micro flora, causing disease when the immune system becomes compromised (de Sousa and de Lencastre, 2004).

S. *aureus* is non-motile, non-spore forming Gram-positive cocci with a diameter of approximately 1μm (Kloos et al., 1998). Colony morphology appears as raised, smooth, and glistening with complete margins (Schleifer and Bell, 2009). *S*. *aureus* can be distinguished from other staphylococcal species on the basis of gold

pigmentation of colonies due to the production of staphyloxanthin, a membrane-localized carotenoid pigment that gives *S*. *aureus* its characteristic golden colour. Nevertheless, some strains make little to no pigment and appear white or grey in colour (Pelz et al., 2005). Staphylococci are tolerant to high concentrations of salt (Wilkinson, 1997) and show resistance to heat (Kloos and Lambe 1991). They are usually facultative anaerobes, growing better under aerobic than anaerobic conditions, capable of both respiration and fermentation. The genus is susceptible to lysis by lysostaphin, which cleaves the interpeptide glycine bridge of its peptidoglycan, and is relatively resistant to lysozyme (Kloos et al., 1998).

S. *aureus* is a one of the leading causes of healthcare-associated infections and is responsible for a substantial burden of disease in hospitalized patients. Despite increasingly severe infection control guidelines, the prevalence and corresponding negative impact of *S*. *aureus* infections remain considerable (Anderson et al., 2012).
One of the most important risk factors associated with increased occurrence of surgical site infection (SSI) is nasal colonization with either *S*. *aureus* or methicillin resistant *S*. *aureus* (MRSA). In addition there is an economic cost related to SSI following orthopaedic surgery, due to longer hospital stays and increased hospital costs (Goyal et al., 2013). The Department of Health, England (DoH) reported that about 9% of patients in hospitals in the UK acquire health care associated infections (HAIs), including those caused by MRSA costing the National Health Service (NHS) about £1 billion per annum (Collins et al., 2011). Furthermore, approximately 19, 000 patient die annually from infections caused by MRSA in the USA alone, resulting in approximately $3–4 billion of additional health care costs per year. In 2005, the U.S. The press have described MRSA as a "superbug" that killed more people than AIDS. Moreover, MRSA has been identified as one of most out-of-control antimicrobial-resistant pathogens in hospitals. Therefore, the rapid and reliable detection of MRSA strains is crucial not only for infection control, but also for effective patient treatment at early stages of infection (Chen et al., 2013).

1. 2. Treatment of *S. aureus* Infections and Antibiotic Resistance

Morbidity and mortality attributed with staphylococcal infections is connected to a combination of two important factors; the aggressive virulence potential of the

pathogen and its remarkable ability to become resistant to antibiotic treatments (Otto M., 2010). To date *S*. *aureus* has acquired resistance to all clinically relevant classes of antibiotics by means of both horizontal DNA transfer such as transduction, conjugation or transformation, and antibiotic-driven chromosomal mutation (Berger-Bächi, 2002 ; Chambers and Deleo, 2009). *S*. *aureus* is associated with a wide variety of infections, from minor skin infections and chronic bone infections to devastating septicaemia and endocarditis (Howden et al., 2010). More than 60% of nosocomial *S*. *aureus* isolates are now resistant to methicillin, and some strains have developed resistance to more than 20 different antimicrobial agents in some countries (Liu et al., 2011). The acquisition of antimicrobial resistance and changing patterns of staphylococcal disease has been common themes in the staphylococcal literature over the past 50 years (Howden et al., 2010). Therefore, the need to develop novel antibiotics to treat these organisms is a big challenge.

The cell wall biosynthesis pathway is one of the most important pathways to be exploited for antibiotic treatment because the target molecules are localized on the outside of the bacterial cytoplasmic membrane, so that the antibiotics do not have to cross the membrane to reach the target. The importance of this feature for antibiotic development was recently highlighted when potent inhibitors of enzymes, identified by genomic strategies as potential antibiotic targets, proved inactive due to their inability to reach the cytoplasmic target (Schneider and Sahl, 2010).

To reduce the emergence of resistant bacteria developing during selective pressure, antimicrobial agents must be used carefully. Therefore using an appropriate drug at the appropriate dosage and for appropriate duration is an important clinical consideration (Tenover et al., 2006). Multidrug resistance is now the norm among many pathogens and *S*. *aureus* is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life-threatening infections, and its adaptability. All *S*. *aureus* strains were susceptible to penicillin G when the latter was initially introduced in the early 1940s, but by 1942 the first report of penicillin-resistant *S*. *aureus* had already appeared (Rammelkamp and Keefer, 1942), and today virtually all strains of *S*. *aureus* are resistant to natural

penicillins, aminopenicillins, and antipseudomonal penicillins (Rice, 2006). Resistance to these drugs occurred because of the acquisition of genes that encode drug-inactivating enzymes (Figure 1. 1), initially known as penicillinases and now called β-lactamases (Rice, 2007). *blaZ* is the gene that encodes β-lactamase, which is an extracellular enzyme, synthesized when staphylococci are exposed to β-lactam antibiotics; it hydrolyses the β-lactam ring, and renders the β-lactam inactive. *blaZ* is under the control of two adjacent regulatory genes, the anti-repressor *blaR1* and the repressor *blaI*. The signalling pathway responsible for β-lactamase synthesis requires sequential cleavage of the regulatory protein *blaR1* and *blaI* (Lowy, 2003).

Due to the rapid increase of the emergence of penicillin resistant *S*. *aureus*, the semi-synthetic penicillin "methicillin" was introduced in 1959 as a therapeutic agent active against infections caused by penicillin-resistant *S*. *aureus*. Less than two years later there were reports, from the UK, of *S*. *aureus* isolates that had acquired resistance to methicillin (Jevons, 1961). In MRSA, resistance is mediated by the *mecA* gene encodes a penicillin binding protein, PBP2a, which reacts with β-lactams extremely slowly. The acylation efficiency of PBP2a by penicillin G, characterized by the second-order rate constant $k2/Kd$=15 M^{-1} s^{-1}, is one to three orders of magnitude slower than that of the four native PBPs from *S*. *aureus*. The acylation rate of PBP2a at therapeutic concentrations of *β*-lactams is thus negligible compared with the bacterial generation time (*t½* for acylation > 1 h with 10 μM of penicillin) (Zapun et al., 2008). Therefore, the PBP encoded by *mecA* can compensate for reduced muropeptide cross-linking and cell wall destruction due to PBPs inhibition (Deurenberg et al., 2007). The genes, *mecI* and *mecR1*, regulate the *mecA* response to β-lactam antibiotics in a pattern similar to that of the regulation of *blaZ,* by the genes *blaR1* and *blaI* following exposure to penicillin (Lowy F., 2003).

Vancomycin is a glycopeptide antibiotic that has as its primary targets the D-Ala-D-Ala subunits of the Gram positive cell wall, which causes cell death by inhibiting cell wall cross-linking. The first MRSA strains that became resistant to vancomycin occurred in Japan in 1996 (Hiramatsu et al, 1997) and subsequently several vancomycin intermediate *S*. *aureus* (VISA) strains developed in other countries (Hiramatsu, 2001). VISA is not mediated by a simple mechanism (e.g. target site modification, production of antibiotic-modifying enzyme, drug efflux or

impermeability) (Mainardi et al., 2008). Rather, it is characterized by the presence of a thickened cell wall, reduced degree of cross-linking in the peptidoglycan, and the abundance of the D-Ala-D-Ala termini on the pentapeptide of the glycan chain. In addition to reduced or non-existence of *pbp4* expression, which has both transpeptidase and DD,carboxypeptidse activity and is require to catalyse highly cross-linked peptidoglycan. Change in the expression of *pbp4* may be associated with altered and thickening of the cell wall and these strains usually exhibit a VISA phenotype (Wootton et al., 2005).

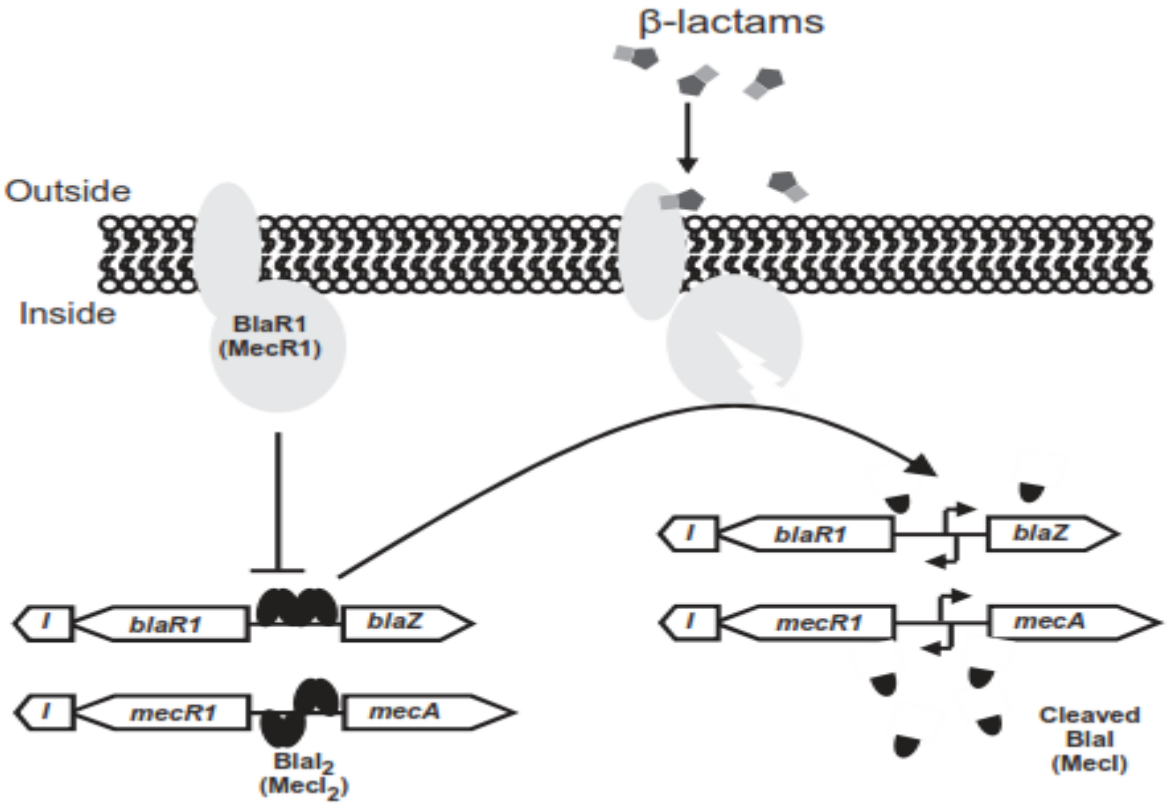


Figure 1. 1. Induction of Beta-lactamase andPBP2a synthesis in the presence of the β-lactam antibiotics. Mechanism of *S*. *aureus* resistance to penicillin and methicillin proceeds in a similar fashion. Binding of β-lactam antibiotics to the transmembrane sensor-transducer BlaR1*and* MecRI results in cleavage of the BlaI/MecRI repressors and transcription of *blaZ* (β-lactamase) and *mec*A (PBP2a). The extracellular enzymes hydrolyse the β-lactam ring of the antibiotics, thereby rendering β-lactams inactive. Images taken from McCallum et al., (2010).

1. 3. The Staphylococcal Cell Wall

The staphylococcal cell is surrounded by a net like structure of about 20-40nm thick, called peptidoglycan (PG) or murein that is composed of a series of short glycan chains of approximately 20 alternating N-acetylmuramic acid and β-(1,4)-N-acetylglucosamine residues (Figure 1. 2) Attached to each N-acetylmuramic acid residue a stem peptide. Some glycan chains in peptidoglycan are linked together via the last glycine residue of the pentaglycine cross bridge attached to the L-lysine residue (position 3) on one stem peptide and the D-alanine residue (position 4) on another (Stapleton and Taylor, 2002). The main function of this cell-sized macromolecule is to protect cell integrity by withstanding turgor (Sauvage et al., 2008). This strong, yet flexible structure provides the framework of the bacterial cell wall, withstanding the high osmotic pressure and maintaining cell shape, (Pereira et al., 2009) and serves as a platform for anchoring other cell envelope components such as proteins and teichoic acids. It is intimately involved in the processes of cell growth and cell division (Vollmer et al., 2008).

Teichoic acids (TA) are composed of repetitive polyol phosphate subunits such as ribitol phosphate (Rbo-P) or glycerol phosphate (Gro-P). *S*. *aureus* wall teichoic acid (WTA) is covalently linked to the 6-OH of N-acetyl muramic acid (MurNAc) via a disaccharide composed of N-acetylglucosamine (GlcNAc)-1-P and N-acetylmannosamine (ManNAc), which is followed by two units of Gro-P (Brown et al., 2008). Lipoteichoic acid (LTA) polymers are attached to the cytoplasmic membrane via a glycolipid anchor, which is a diglucosyl diacylglycerol in *S*. *aureus* and most other staphylococcal species (Dreisbach et al., 2011). WTA has an important role in processes such biofilm formation, phage infectivity, and pathogenesis (Brown et al., 2012). Furthermore, enzymes that participate in wall teichoic acid biosynthesis are localized at the septum (Atilano et al., 2010). They act as regulators for penicillin binding protein 4 (PBP4) localization that is required for the production of highly cross-linked peptidoglycan (Figure 1. 3; Farha et al., 2013). Besides, wall teichoic acid mediates the localization of the major autolysin, AtlA, in the division septum. Therefore, wall teichoic acid plays an important role in regulation of both biosynthesis and degradation of nascent peptidoglycan in the septum (Atilano et al., 2010).

Additionally surface proteins of *S. aureus* are linked to the cell wall by sortase, a transpeptidase enzyme that cleaves polypeptidesn between the Gly and Thr of the LPXTG (Leu-Pro-any-Thr-Gly) and catalyses the formation of an amid bond between the carboxyl-group of threonine and the amino-group of the cell wall peptidoglycan (Maresso and Schneewind, 2008).

1. 4. Peptidoglycan Biosynthesis

Peptidoglycan biosynthesis is a complex process involving the coordination of both biosynthetic and degradation pathways. Peptidoglycan synthesis occurred through several steps which are: nucleotide-linked precursors, lipid II, and polymerization and cross-linking, and peptidoglycan manufacture (Stapleton et al., 2007).

1. 4. 1. Nucleotide Precursor Synthesis

The biosynthesis of peptidoglycan is a complex process involving approximately 20 enzyme reactions, which likely work in a coordinative, dynamic but, as yet, largely uncharacterized association (Lovering et al., 2012). Synthesis of the nucleotide precursors takes place in the cytoplasm, while synthesis of lipid linked intermediates and polymerization reactions takes place at the inner and outer surfaces of the cytoplasmic membranes respectively (van Heijenoort J., 2001; Figure 1. 4). Cytoplasmic steps can be divided into four main sets of reactions:

1. Formation of UDP-Glc NAc from fructose-6-phosphate.
2. Formation of UDP-Mur-NAc from UDP-Glc NAc.
3. Assembly of the peptide stem leading to UDP-Mur NAc-pentapeptide.
4. Side pathways of synthesis of D-glutamic acid and the dipeptide D-alanyl-D-alanine. The end product of these reactions is the synthesis of phosphor-MurNAc-pentapeptide (Barreteau et al., 2008).

1. 4. 2. Assembly of Lipid II on the Inner Cytoplasmic Membrane

The first membrane- associated step begin with the transfer of the phosphor-MurNAc-pentapeptide from the cytoplasmic precursor to the membrane acceptor undecaprenyl phosphate (C55-P), a reaction catalyzed by the integral membrane protein MraY, yielding undecaprenyl-pyrophosphate-Mur-NAc- (pentapeptide) or called lipid I.

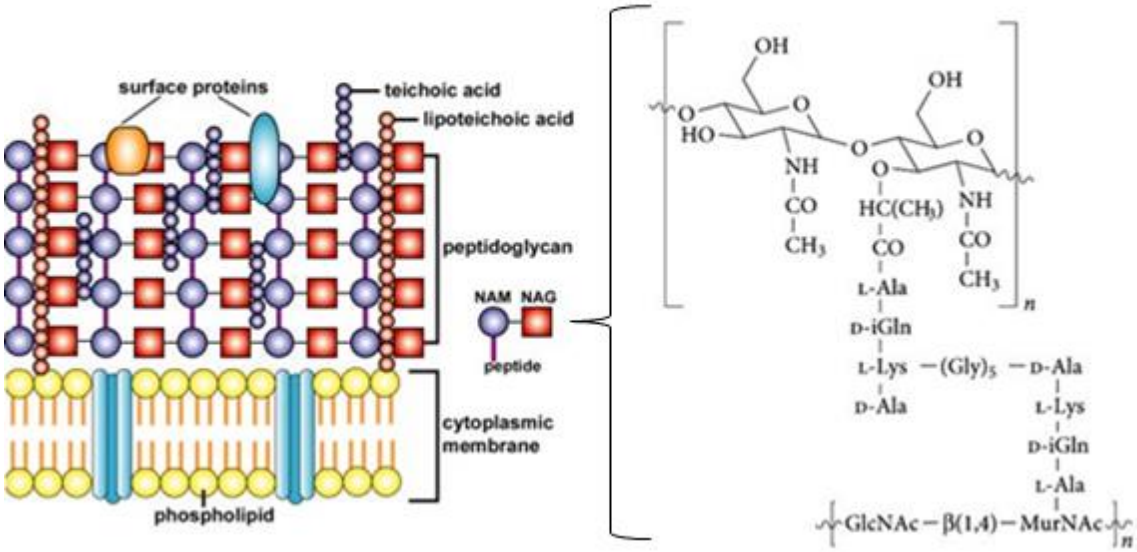


Figure 1. 2. Schematic diagram of the cell wall architecture of *S. aureus*. The cell wall is composed of a thick layer of peptidoglycan which comprises approximately 90% of the cell wall structure as well as lipoteichoic acid and wall teichoic acid. Peptidoglycan is a Polymer of β-(1-4)-N-Acetyl-D-glucosamine units. Alternating residues are modified to form N-acetylmuramic acid with the addition of lactate which is covalently linked to a tetrapeptide. The tetrapeptides of adjacent polymers are linked by penta-glycine bridges. The cross-linked peptidoglycan polymers form a mesh-like network over a phospholipid bilayer plasma membrane. Image taken from Niwa et al., 2005).

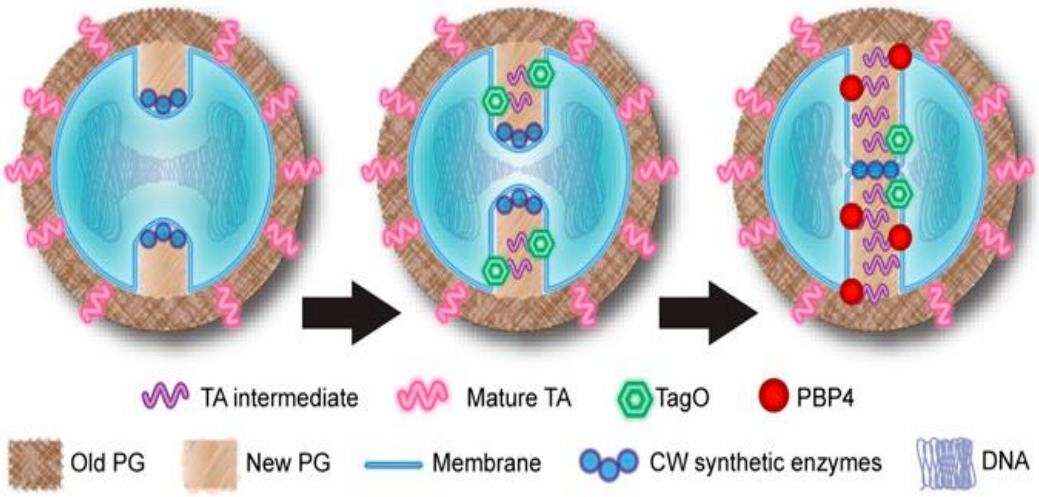


Figure 1. 3. Role of teichoic acids (TA) synthesis in PBP4 localization to the mid-cell. The early cell-wall synthetic machinery assembles at the division site, leading to the synthesis of new peptidoglycan, with low levels of crosslinking (Left). TagO, which is the first enzyme in the teichoic acid biosynthetic pathway, together with other WTA synthetic enzymes is localized to the septum by an unknown mechanism, leading to the synthesis of intermediate molecules in TA biosynthesis (Center). These intermediates (or another cellular component dependent on TA biosynthesis) function as a temporal and spatial cue for PBP4 recruitment to the division septum, allowing the synthesis of highly cross-linked Peptidoglycan to occur in a regulated manner (Right). Image taken from Atilano et al., (2010).

Subsequently, beta-N- acetylglucosaminyltransferase (*murG*) adds GlcNAc from UDP-N-acetylglucosamine to lipid I to form undecaprenyl-pyrophosphate-Mur-NAc- (pentapeptide)-GlcNAc or called lipid II (Bouhss et al., 2008).

The formation of the pentaglycine cross-link in *S. aureus* requires glycyl tRNA and lipid II. The *femABX* genes encode proteins involved in the addition of the pentaglycine bridge; FemX is responsible for addition of the first glycine, FemA the next two glycines; and FemB the final two glycines (Bouhss et al., 2007). The final step in the inner cytoplasmic membrane for peptidoglycan synthesis is to flip the lipidII building block to the outer side of the cytoplasmic membrane (Lovering et al., 2012). Recently a role for an essential division protein has been implicated in lipid II transport; in *Escherichia coli* model membrane vesicles and trans-bi-layer movement of lipid II requires the presence of FtsW. However, the mechanism underlying this function and governing molecular feature in cell wall biosynthesis remain unknown (Mohammadi et al., 2011).

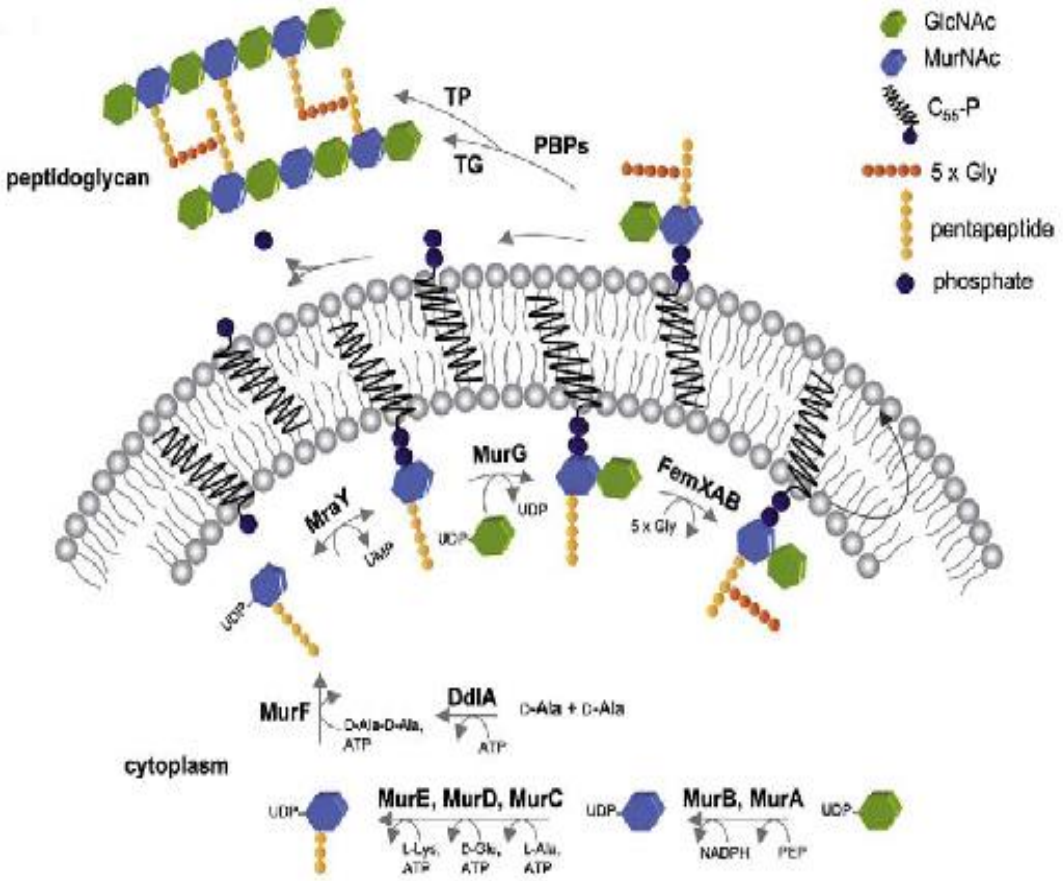


Figure 1. 4. Steps of peptidoglycan biosynthesis in *S. aureus*. UDP-MurNAc-stem peptide synthesized in the cytoplasm, then transferred to a lipid carrier in the membrane to form lipid I which modified to generate Lipid II. Lipid II is flipped to the inner face of the cell wall. The peptidoglycan monomers are then cross-linked by the action of PBPs, through glycosylation in which MurNAc of the nascent PG are covalently linked to the GlcNAc of lipid II precursor. A sub-set of PBPs can also cross-link the lipid II pentapetide to a pentaglycine cross-bridge via transpeptidation reaction. Images taken from (Schneider and Sahl, 2010)

1. 4. 3. Polymerization and Cross-linking

After the completion of the cellular stages of peptidoglycan synthesis, the subsequent extracellular steps require the energy of bonds present in lipid II monomer to drive reactions forward (Lovering et al., 2012). This occurs through two main reactions; first polymerization involves the covalent cross-linking of nascent lipid II subunits to the growing peptidoglycan strand on the outer face of the cell membrane. Two glutamate residues in the (glycosylation) GT domain active site of membrane bound monofunctional and bifunctional proteins are responsible for the formation of covalent β-1, 4 linkages between the MurNAc precursor of the nascent PG and the GlcNAc precursor of the lipidII (Goffin and Ghuysen, 1998 ; Sauvage et al., 2008). Cross-linking of peptidoglycan occurs via transpeptidation (TP) reaction of peptide units in adjacent glycan strands. The cross-linkage extends from the amino group of the side-chain of the residue at position 3 of one peptide subunit (acyl acceptor) to the carboxyl group of D-Ala at position 4 of another (acyl –donor) (3-4 cross-linkage), through an interpeptide bridge (Magnet et al., 2007). There is a considerable variation in the degree of cross-linkage, for example in *S*. *aureus*, the percentage of monomers is low (10%), most peptidoglycan units being present as oligomers (Vollmer et al., 2008).

1. 4. 4. Peptidoglycan Manufacture

The growth model of peptidoglycan in Gram positive bacteria is “inside-to-outside” growth, which means that new glycan strands are inserted on the inner face of the wall, where the PBPs are localised (Reith and Mayer, 2011). According to the surface stress theory, newly synthesized cell wall material is deposited to the inner face of the cell wall in a relaxed conformation. As peptidoglycan polymerization progresses, the wall moves outwards and become stretched bearing the stress due to hydrostatic pressure. According to the inside-to-outside model of growth, the cell wall is never weakened since new covalently layers of the murein are formed before the older and outer ones are breached (Scheffers and Pinho, 2005).

1. 4. 5. Cell Division and Separation of Daughter Cells in *S. aureus*

S. *aureus* is a spherical shaped bacterium that appears to have only one mode of cell wall synthesis at the mid-cell (Scheffers and Pinho, 2005 ; Strauss et al., 2012) and cell division planes are built perpendicular to previous plane (Tzagoloff and Novick, 1977 ; Periera et al., 2009). A division plane has to be tightly coordinated with chromosome segregation to avoid bisection of the nucleoid by the septum (Veiga et al., 2011).

The divisome is a macro-molecular complex that is formed during cell division, it consists of two major components including positive regulators and negative regulators that regulate the construction of an essential cytosekeleton protein Fts Z into a ring-like structure called the Z-ring (Figure 1. 5; Son and Lee, 2013). Proteins that are known or are thought to participate in divisome formation in *S*. *aureus* are FtsZ, FtsA, EzrA, GpsB, SepF, PBP1, PBP2, PBP3, DivIB, DivIC, FtsL, FtsW (Steele et al., 2011). Following the Z-ring formation the cells synthesize a nascent layer of peptidoglycan at the mid-cell in a form that is surrounded by membrane invaginations, this layer of peptidoglycan is called the cross-wall. It has been shown that deletion of FtsZ leads to delocalization of the peptidoglycan biosynthesis results in cell enlargement and lysis (Pinho et al., 2003). It has been reported that *S*. *aureus* at the septum has five zones of alternating densities between the membrane of the two daughter cells, which consist of two high density zones appearing to be sandwiched by three low density zones (figure 1. 6) (Matias and Beveridge, 2006).

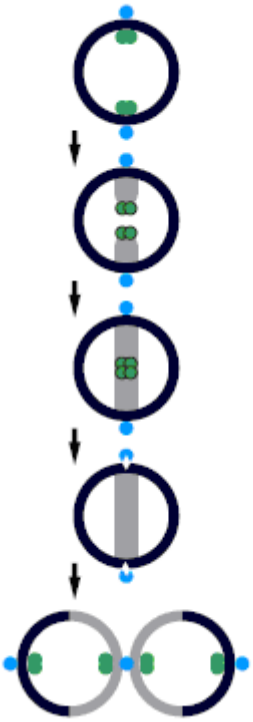


Figure 1. 5. Proposed models for cell wall assembly in spherical cocci *S.aureus.* The parental cell wall is in black, the new cell wall of septal origin is in light gray, septal peptidoglycan synthesis machinery is in green, and the splitting machinery is in cyan, with a jaw to indicate activity. This diagram shows that in staphylococci it synthesizes the cell wall at the division site using one septal machinery. Image taken from Zapun et al., (2008).

The most distinctive feature in the centre of the nascent cross wall was a thin electron-dense layer, called the splitting system of the cross wall, is 7 to 10 nm wide and has shown to consist of a concentrically arranged system of about 14 to 18 ring-shaped tubuli, each 7 to 10 nm in diameter. It has been assumed that teichoic acid-like material and lipoteichoic acid is the chemical composition of the splitting system (Giesbrecht et al., 1998; Scheffers and Pinho, 2005). Additionally, minute vesicular structures 50 to 60 nm in diameter, that creates a row of holes in the peripheral cell wall above closed cross walls, possessing a high autolytic activity capable of hydrolysing adjacent peptidoglycan and lead to the splitting of daughter cells. It is suggested that these murosomes are formed due to the autolytic activity of Atl, which forms around 30 holes around the bacterial circumference (Touhami et al., 2004).

After completing the synthesis of the cross-wall, the cells must cleave the nascent septa in a highly regulated manner to form two daughter cells; this is mediated by autolysins, which are peptidoglycan hydrolases that are active during cell growth, cell turnover, and cell division and separation (Singh et al., 2010). There are five types of peptidoglycan hydrolases which are: N-acetyl muramidase, N-acetyl glucosaminidase, N-acetylmuramyl-L-alanine amidase, endopeptidase and transglycosylases (Figure 1. 7) (Liu et al., 2011). Lytic transglycosylases are a further class of autolysins which cleave the β-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine residues of peptidoglycan, with concomitant formation of 1,6-anhydromuramic acid residues (Stapleton et al., 2007). AtlA which is the major extracellular autolysin that is encoded by the *atl* gene. This autolysin undergoes cleavage to generate amidase and glucosaminidase. Three peptidoglycan hydrolases AtlA, LytN and Slel are directly involved in splitting of daughter cells, however, cells remain connected by a small fragment of peptidoglycan (Frankel et al., 2011). Furthermore, sister cells usually change location according to each other while still remaining connected. These factors are apparently responsible for this characteristic formation of irregular cell groups (Tzagoloff and Novick, 1977).

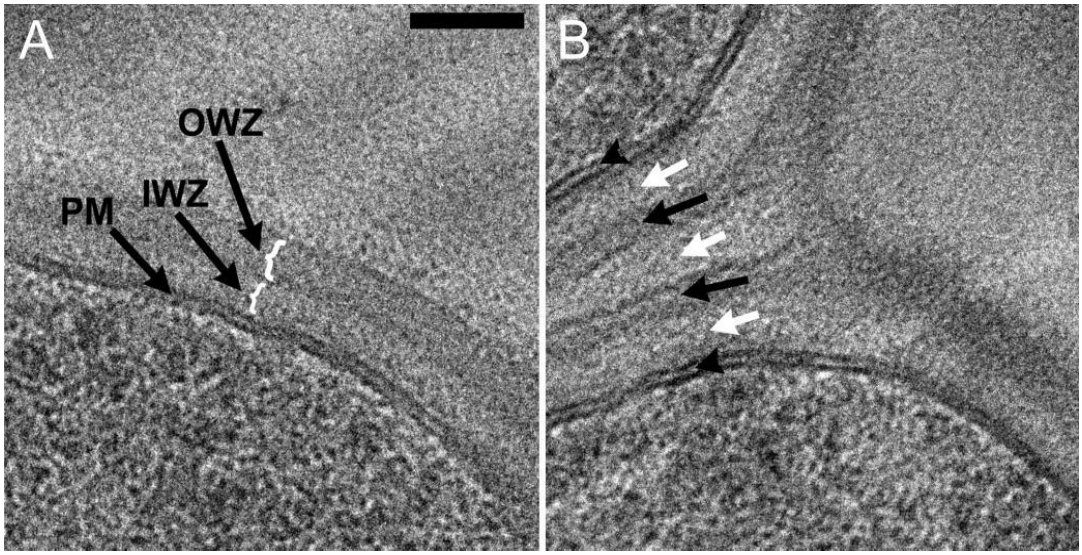


Figure 1. 6. Frozen-hydrated section at high magnification showing the *S. aureus* cell envelope. At non-septal regions, the plasma membrane (PM) is bound by a bipartite wall; a low-density inner wall zone (IWZ) precedes a high-density outer wall zone (OWZ). (B) At the septum, five different zones of alternating low (white arrows) and high (black arrows) densities are distinguished between the two membranes of the septum (arrowheads). Both images are shown at the same magnification. Bar 50 nm. Image taken from Matias,et al., (2006).

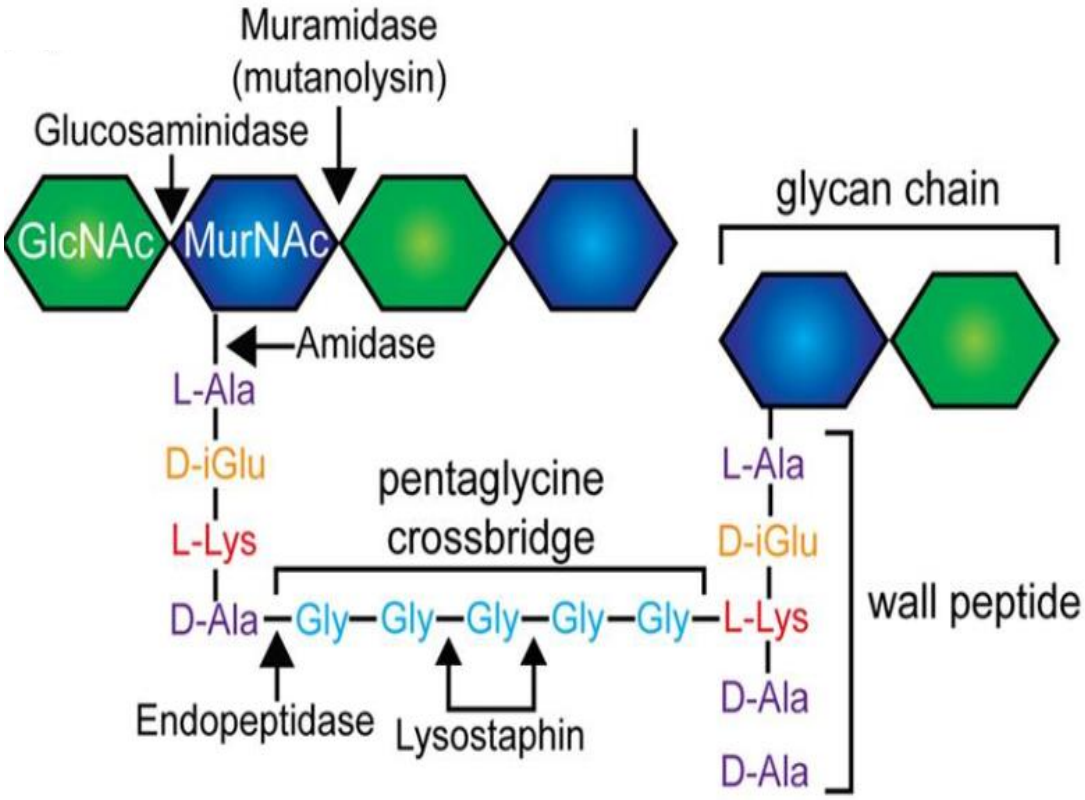


Figure 1. 7. Site of cleavage of peptidoglycan hydrolases. Schematic illustration of cell wall peptidoglycan with selected enzymatic cleavage site. Image taken from Frankel et al., (2011).

The key regulator of peptidoglycan degradation is the two component system *walK/walR*. The phosphorylated response of walR activates the expression of genes that encode for peptidoglycan hydrolases and supress the genes that inhibit hydrolysis (Uehara and Bernhardt, 2011). Hence, a proper balance is required between synthesis and degradation of peptidoglycan to maintain this network (Biswas et al., 2006). During peptidoglycan degradation the stem peptide is hydrolysed by the activity of the amidases and carboxypeptidase, amino sugars and peptides are released during activity of these enzymes in the cell wall compartment. Subsequently, uptake of the cell wall amino sugars such as GlcNAc and MurNAc by the phosphotransferase system (PTS) transporters NagE and MurP, respectively and cell wall-derived peptides such as muropeptides by mpp/opp-like ABC transporters, both well conserved in *S. aureus,* form the major pathway for recycling of cell wall components (Reith and Mayer, 2011).

1. 5. Penicillin Binding Proteins (PBPs)

Bacterial cells; except mycloplamas species; possess a variable number of penicillin binding proteins, which act as cell wall biosynthetic enzymes (Macheboeuf et al., 2006). PBPs that catalyse the polymerization of the glycan strand called transglycosylation (TG) and the cross-linking between glycan chains called transpeptidation (TP). Some PBPs can hydrolyze the last D-ala of stem pentapeptides called DD-carboxypeptidation (CP), or hydrolyze the peptide bond connecting two glycan strands called endopeptidation (EP) (Sauvage et al., 2008).

Penicillin binding proteins are classified according to their molecular weight as low molecular mass (LMM) or high molecular mass (HMM) PBPs (Figure 1. 8). Moreover, depending on the architecture and the catalytic activity of their N-terminal domain, HMM enzymes are subdivided to either class A (bifunctional, having both TG and TP activity) or class B (monofunctional) PBPs having only TP activity. In class B enzymes, the N-terminal domain is believed to play a role in cell morphogenesis by interacting with other proteins involved in the cell cycle (Sauvage et al., 2008). The C-terminal penicillin binding (PB) domain of both classes has a transpeptidase activity, (Sauvage et al., 2008 ; Terrak and Nguyen- Distěche, 2006). LMM PBPs are commonly DD- carboxypeptidase or endopeptidases, and therefore

create their own subfamily (Goffin and Ghuysen, 2002). Antibiotics that target TPase or TGase activity lead to cell wall weakness, inhibit cell division, and subsequently cause cell lysis. Moenomycin is the only known natural product that directly inhibits TGase activity. Unfortunately, this antibiotic cannot be used for therapeutic property because of it is toxicity to human (Walsh, 2003).

The numbering system used for PBP naming is based on the membrane protein profile seen when PBP proteins are labelled with either radioactive or fluorescent penicillin analogues subjected to SDS-PAGE separation. The highest molecular weight PBP is designated as number 1 and the remaining of the PBPs are numbered sequentially according to reducing size (Spratt and Cromie, 1988). Thus, PBP numbering is completely species-specific, which means that the number does not indicate what structure or function that an enzyme has; for example. *E*. *coli* PBP2 is most similar to *S.aureus* PBP3 (Kong et al., 2010).

Class A PBPs can be found in two main types, the majority display GT and TP domains that are interconnected by a linker of different length, followed by a short C-terminal sequence (Macheboeuf et al., 2006). When other protein families, such as a fibronectin-binding domain, follow the C-terminal domain-binding domain it forms the second type of arrangement (Ghuysen, J., 1994).

Class B PBPs can be divided into four main groups, but the most frequent one has the simplest structure. It consists of an N-terminal secretion signal followed by the TP domain. Two groups are characterized by the presence of an additional region of unknown function positioned after the TP domain. Interestingly, this region contains three motifs with homology to the inter-domain linker in class A PBPs, and folds into a very similar structure. It most likely provides support between the N-terminal domain and the TP domain. This region is sometimes called PASTA (PBP and Ser/Thr kinase attached) domains and it has been suggested that PASTA binds unlinked peptidoglycan and also acting as a peptidoglycan-sensing molecule (Yeats et al., 2002). The final group of class B enzymes carries an NTF2 (nuclear transport factor 2)-like domain positioned before the N-terminal end. NTF2 has been found in staphylococcal PBP2a, but the function of this domain is unclear (Lim and Strynadka, 2002 ; Macheboeuf et al., 2006).

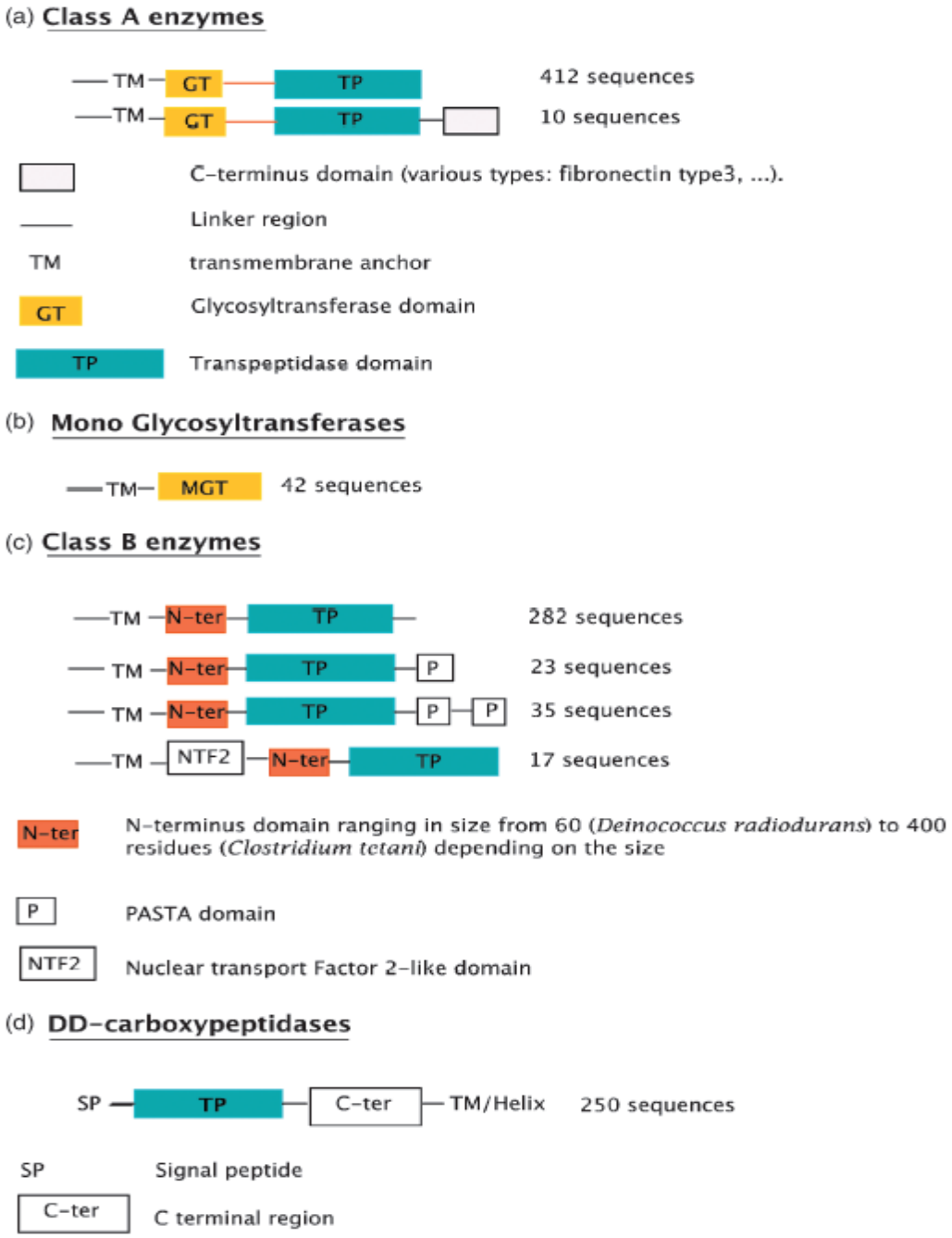


Figure 1. 8. Architecture of penicillin binding proteins (PBPs). There are at least 10 types of arrangement of PBPs in 213 eubacterial genomes identified. All PBPs are connected to the membrane by means of a transmembrane helix (TM) or an amphipathic helix, in the case of LMM PBPs. Images taken from Macheboeuf et al., (2006).

LMM PBPs are unusual because the TP domain is encoded after a signal peptide and their membrane association is accomplished by either a transmembrane or amphipathic helix, both located at the C-terminal end. (Macheboeuf et al., 2006).

1. 5. 1. The Penicillin Binding Proteins in *S. aureus*

S. *aureus* possess four types of PBPs, namely PBP1-4, that differ in their cell wall biosynthesis function and affinity to β-lactam antibiotics (Chambers et al., 1994). In addition, this species also contains two monofunctional transglycosylases: MGT and SgtA (Reed et al., 2011). Methicillin resistant *S*. *aureus* (MRSA) possess a 78 kDa PBP2 variant, which has a low affinity for penicillin. This protein is called PBP2a and is encoded by the *mecA* gene (de Lencastre et al., 2007) that is carried by a mobile genetic element, *SCCmec*. The *mecA* gene has been acquired from commensal staphylococcal species, such as *S sciuri* (Couto et al., 2003) or *S epidermidis* (Basset et al., 2009) upon horizontal transfer (Hiramatsu et al., 2001). The *mecA* gene complex is widely distributed among *S*. *aureus* strains as well as among other staphylococcal species collectively called coagulase negative staphylococci. Therefore, *mecA* may be freely transmissible among staphylococcal species, crossing the staphylococcal species barrier (Ito et al., 1999).

PBP1 is the largest PBP in *S*. *aureus*, (85 kDa) and is a class B, monofunctional, transpeptidase. The function of PBP1 is mainly correlated to cell division. It possesses an N-terminal domain, followed by the TPase domain and two PASTA domains terminated by the C-terminal region (Pereira et al., 2009). PBP1 does not play a major role in the cross-linking of *S*. *aureus* peptidoglycan, and the main phenotypic consequences of PBP1 depletion in *S*. *aureus* occurs at the level of septum formation, a dramatic increase in cell mass, and a complete loss of cell division resulting in cell lysis. This finding together with the localization of PBP1 in the septum, strongly suggests that the essential role of PBP1 in *S*. *aureus* is linked to some specific function of this protein in cell division (Pereira et al., 2007). Interestingly, a point mutation made in the TP domain active site, which inhibits the activity of the enzyme and binding to penicillin, led to abnormality in growth and peptidoglycan composition but did not cause complete inhibition of septum formation or cell lysis. Therefore, it has been suggested that PBP1 has two important

roles in the cell cycle of *S. aureus*, *as* a protein required for septation and also as a transpeptidase that generates a critical signal for cell separation at the end of cytokinesis (Pereira et al., 2009).

PBP2 is the only bifunctional HMM class A PBP (81 kDa); it has two protein domains, an N-terminal TGase and a C-terminal TPase domain (Figure 1. 9). This HMM PBP is essential for growth and viability of the bacteria. During the first steps of cell division, PBP2 is positioned at the septum to form a ring around the future division plane (Pinho and Errington, 2005). Acylation of the TPase domain of PBP2 with oxacillin leads PBP2 to move from the septum to the entirety of the cell wall (Pinho and Errington, 2003). *S. aureus* is distinguished from other bacterial species that it has only one class A PBP, but it has been shown that its TGase activity in *S. aureus* is six times higher than that of class A PBPs of other bacterial species (Barrett et al., 2005). The essentiality of TGase activity of PBP2 in *S. aureus* appears to depend on the strain and the presence of PBP2a. It has been reported that in the MRSA strain COL, mutations in the active site of the TPase domain had no effect on the viability of the cells and did not alter their sensitivity to methicillin, due to the presence of PBP2a which also has a transpeptidase activity. In contrast, a point mutation made to the active site of the TGase domain in the same strain caused a dramatic increase in methicillin sensitivity as well as the presence of very short peptidoglycan chain (Pinho et al., 2001), demonstrating that PBP2 TGase activity is crucial for appropriate peptidoglycan biosynthesis.

Insertional inactivation of PBP2 causes a reduced MIC in MRSA strain COL, suggesting that even in the presence of the active TPase of PBP2a the TGase activity of PBP2 is crucial for appropriate cell wall synthesis (Pinho et al., 1997). Nevertheless, in MSSA mutations made in the active site of the TGase domain did not affect the viability as do strains with inactivated PBP2, suggesting that TPase activity of PBP2 may be also essential in these cells (Pinho et al., 2001). Afterwards it was found that the monofunctional transglycosylase activity of MGT, can replace the TGase activity in both COL Δ*pbp2* and in MSSA TGase domain mutants. MGT activity is only essential in the absence of PBP2 (Reed et al., 2011).

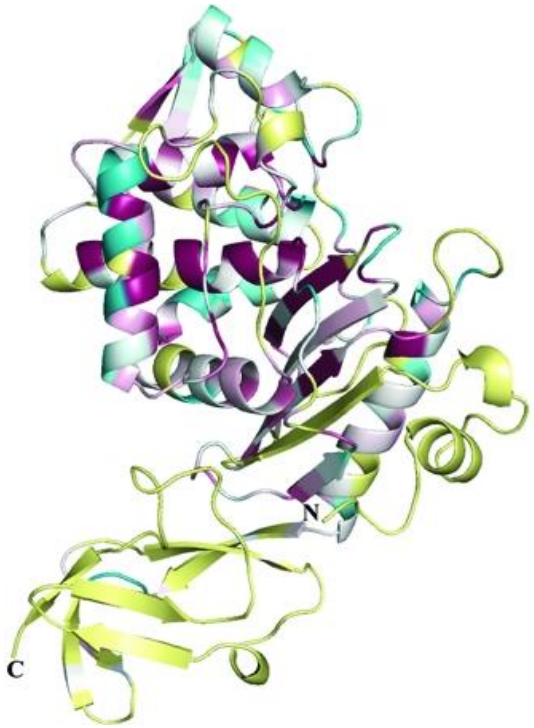


Figure 1. 9. Structure of class A PBP2 in *Staphylococcus aureus*. Overall view of the structure of *Staphylococcus aureus* PBP2. The PB/transpeptidase domain is coloured by secondary structures, the linker region is coloured green, and the big and small lobes of the transglycosylase domain are coloured yellow and salmon respectively. Ct, C-terminus; Nt, N-terminus. Images taken from Sauvage et al., 2008).

Similar to PBP1, PBP3 is a HMM class B protein, (75 kDa), and has monofunctional transpeptidase activity, but PBP3 does not possess PASTA domains at its C-terminal region (Macheboeuf et al., 2006). PBP3 may be involved with septum formation and cell separation but PBP3 mutant cells grow normally. In the presence of a sub-MIC levels of methicillin cells have an abnormal size and shape and have disoriented septa. These data are consistent with idea that the TPase activity of PBP3 may be taken over by other PBPs (PBP1 and PBP2) when PBP3 is not available or is under antibiotic pressure (Pinho et al., 2000 ; Georgopadakou et al., 1982).

PBP4 is the only LMM PBP that belongs to class B protein in *S. aureus* (45 kDa), and is differs from other proteins in this class because it is bifunctional, catalysing both DD-carboxypeptidase and transpeptidase activity (Figure 1. 10) (Navratna et al., 2010). PBP4 is not essential for survival, because cells with depleted or mutated PBP4 continue to grow normally (Leski and Tomasz, 2005 ; Navratna et al., 2010). In most species, the LMM TPases have a low level of carboxypeptidase activity, but PBP4 in *S. aureus* is efficient at both reactions (Kozarich, and Strominger, 1978). A recent report indicates that the biochemical characteristics and conformational features of the active site of PBP4 suggest that this protein also has a β-lactamase activity. This conclusion was supported by several lines of evidence, including mass spectra showing hydrolysed β-lactam antibiotics, electron density at the active site of PBP4, and a nitrocefin-based assay for β-lactamase activity. This implies that the active site of this enzyme can remain free even in the presence of β-lactam antibiotics (Navratna et al., 2010)

TP is the main function of PBP4 *in vitro,* because the level of unlinked peptidoglycan resulting from CP is low approximately (10-15%) (Navratna et al., 2010). Additionally, recent studies have suggested that wall teichoic acid has an important role in localization of PBP4 in the division septum (Figure 1. 2). Usually, the correlation between WTA and peptidoglycan prevents carboxypeptidation occurring at the division septum (Atilano et al., 2010 ; Farha et al., 2013).

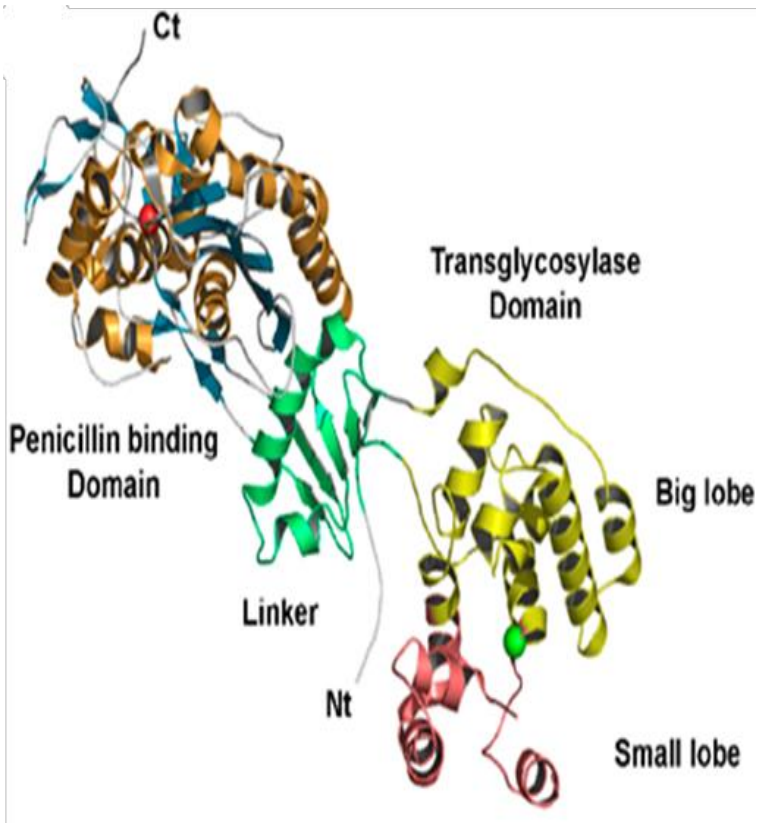


Figure 1. 10. Structural features of *S. aureus* PBP4. (A) Sequence conservation of PBP4 is shown in the context of the crystal structure. Of the two domains in *S. aureus* PBP4, the β-lactamase domain at the N terminus is very well conserved, whereas the all-β domain at the C terminus is not. Images taken from (navartna et al., 2010).

There is a very little information available regarding the regulation of PBP production and the activity of their coding genes, along with other non-*pbp*-related genes, which are indicated to be associated with intrinsic β-lactam resistance. It was shown that a 90 bp deletion near to *abcA* region correlates with an increase to β-lactam resistance as a result of overproduction of PBP4, wherease the level of transcription of *abcA* was only slightly higher than that of its parent. It was concluded that 90 bp deletion of the promoter region, even though it is closer to the abcA ORF, mainly affected *pbp4* transcription (Schrader-Fischer and Berger-Bächi, 2001). Interestingly, an increased level of PBP4 transcripts was noticed in an *abcA* knockout mutant, therefore it has been suggested that *S*. *aureus abcA* regulates PBP4 production (Domanski et al., 1997). Recently it was suggested that AbcA is one of the efflux pumps family which are capable of extruding antibiotics, leading to multidrug resistance (Qin et al., 2013). AbcA overexpression associated with fourfold increase in resistance to methicillin, cefotaxime, penicillin, and nafcillin (Truong-Bolduc and Hooper, 2007).

On the other hand, it was reported that *abcA* and *pbp4* are regulated independently due to their different expression patterns during growth; transcription of *pbp4* later in the exponential growth phase. Furthermore, the increased *abcA* transcription during exposure to methicillin suggested that AbcA has a protective role against methicillin (Schrader-Fischer and Berger-Bächi, 2001).

The global regulatory systems *agr* and *sar* are involved in regulation of virulence determinants in *S*. *aureus* (Novick et al., 2003). While *agr* is known to control toxin and exoprotein production, the *sar* locus was suggested to be essential for the optimal expression of *agr*. Although, *abcA* expression is under the control of the global regulator *agr*, neither *agr* nor *sar* had any effect on *pbp4* expression nor an effect on the PBP4 protein level. Also methicillin induced *abcA* expression but had no effect on *pbp4* expression (Schrader-Fischer and Berger-Bächi, 2001).

Methicillin resistant strains lacking the *mecA* gene have previously been generated, *in vitro* by three research groups and in each case the resistance mechanism is different, indicating that the genome is able to respond in several ways to subtly different selection pressures. The group of Brigitte Berger-Bächi reported that high-

level expression of *pbp*4, caused by a deletion in the region upstream of this gene, resulted in an increase in PBP4 protein in cell membranes, an increase in the thickness of the cell wall and translated to low-level PBP4-linked intrinsic β-lactam resistance in MSSA (Henze and Berger-Bächi, 1996). Recently, Banerjee at al., reported that methicillin resistance in an *in vitro* generated strain is due in part to an amino acid change at the entrance to the binding pocket of PBP4 (Banerjee et al., 2010). CWD *S*. *aureus* were previously generated in our lab on a medium with an increased osmolality and in the presence of sub-lethal levels of penicillin G; on reversion to cell wall competent (CWC) form, the cells were remained resistant. Membrane preparations showed a high level of PBP4 in the CWC strains, indicating that the cells have undergone stable genotypic changes that allow them to avoid the action of β-lactam antibiotics (Fuller et al., 2005).

Furthermore, inactivation of PBP4 caused a marked decrease in highly cross-linked muropeptide components of the cell wall of wild-type and mutants of *S*. *aureus* strains. (Sieradzki et al., 1999). Deletion of PBP4 in hospital acquired methicillin resistant *S*. *aureus* (HA-MRSA) strains did not alter the MIC of β-lactam antibiotics (Katamaya et al., 2003). However, PBP4 has an important role in mediating β-lactam and methicillin resistant in community acquired methicillin resistant *S*. *aureus* (CA-MRSA), PBP4 inactivation in MRSA strains USA300 and MW2 is sufficient to cause a 16-fold reduction in oxacillin and nafcillin resistance. Therefore PBP2a, is not the only determinant of methicillin resistance in CA-MRSA and it has been clearly demonstrated that PBP4 is a key element in β- lactam resistance in CA-MRSA strains (Memmi et al., 2008).

In clinical isolates of vancomycin intermediate *S*. *aureus* (VISA), PBP4 inactivation was found to increase vancomycin resistance, due to an altered peptidoglycan composition, a thickened cell wall, and decreased autolytic activity (Sieradzki and Tomazs, 1997; Howden et al., 2010). This is in agreement with the function of PBP4 that mediates secondary transpeptidation necessary for highly cross-linked peptidoglycan (Sieradzki and Tomazs, 2003). In addition to clinical isolates of VISA, *in vitro* selection for resistance to glycopeptide antibiotics (vancomycin and teicoplanin) produces altered peptidoglycan. Membrane protein profile of VISA strains shows no detectable PBP4 and reduced level of cross-linking. HPLC analysis

showed increased glycan chain lengths and monomer-rich peptidoglycan. The monomer precursors in the peptidoglycan component of the cell wall are non-lethal target for glycopeptide antibiotics (vancomycin), therefore, binding to these targets prevents access of vancomycin molecules to the lethal target adjacent to the plasma membrane (Sieradzki et al., 1999). Although these reports show that low level or inactivation of PBP4 correlates with increased vancomycin resistance not all strains of clinical VISA have altered PBP4 production and it might be strain specific. This means that PBP4 may be associated with vancomycin resistance in only some strains of *S*. *aureus* (Woottoon, M. 2005).

Some species of bacteria possess membrane bound proteins that are capable of catalysing glycan chain polymerization from lipid II called monofunctional glycosyltransferases MGTs; (Terrak and Nguyen-Disteche, 2006). Using genome sequences to search for GTase domains in *S*. *aureus*, only three genes were suggested to have TGase activity PBP2, MGT, and SgtA (Wang et al., 2001). Both the GT domain of class A PBPs and MGTs show high sequence similarity and belong to the GT51 family, which is characterized by five conserved motifs and uses lipid II as substrate (Spratt, et al., 1996). In addition MGT is enzymatically active, because purified MGT protein, isolated from *S*. *aureus*, has the ability to catalyse polymerisation of un-cross-linked glycan chains (Wang et al., 2001).

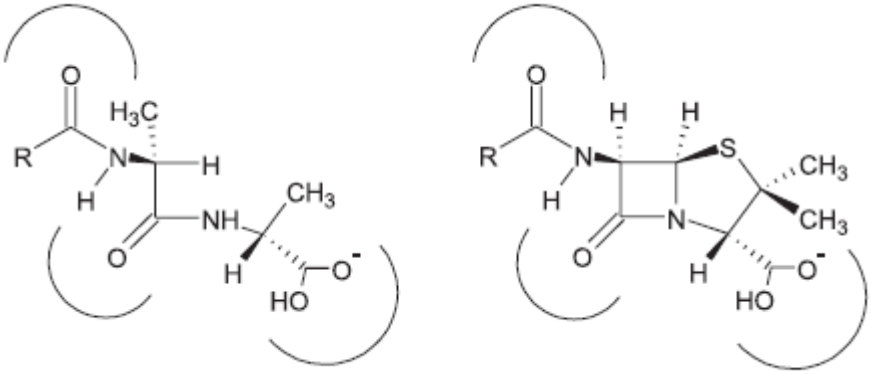
Classification of SgtA as a transglycosylase was previously based only upon sequence homology to other known glycosyltransferase activity (Kuroda et al., 2001). *SgtA* has been shown to be non-essential in the background of the MSSA strain NCTC8325-4, even in the absence of the TGase activity of PBP2; the functioning of SgtA as a transglycosylase in *S*. *aureus* has remained largely uncharacterized (Reed et al., 2011).

1. 5. 2. Affinity of PBPs to β-lactam Antibiotics

All PBPs contain a domain which uses D-Ala-D-Ala dipeptide on the C-terminal end of the lipid II pentapeptide as a substrate for peptidation reactions. In PBPs mediated peptidation the peptidase domain forms a complex with the D-Ala D-Ala, followed by acylation of the C-terminal alanine residue by serine residue in the peptidase

domain active site. This forms a covalent acyl-enzyme complex, which is resolved by a rate limiting deacylation reaction in the final enzymatic step. In transpeptidases, amino group on the pentaglycine bridge serves as the proton acceptor of the reaction, resulting in cross-linking of two peptidoglycan strands. In DD-carboxypeptidase, the acyl-enzyme intermediate is hydrolysed; which results in perminantly shortend stem peptide that is unable to be cross-linked. The proton is then back-donated to the active site, breaking the acyl-enzyme bond and recharching the PBP for another reaction (Zapun et al., 2008).

β-lactam antibiotics mimic the D-Ala-D-Ala dipeptide in an elongated conformation, particularly regarding the distribution of three electrostatic negative wells (Figure 1. 11), forming a stable inhibition complex. The half-life of the covalent acyl-enzyme complex, which is formed between PBP and the penicillin analogue, is much longer than that of the PBP and its natural substrate (Chambers et al., 1994). This is because the acylation of the active site serine generate a massive leaving group that remains part of the acyl-enzyme, limiting access of the acceptor group. As a result, only water has access to the active site and can attach the acyl-enzyme (Ghuysen, 1991). The active sites of PBPs have varying affinity for *β*-lactams depending upon the particular PBP, the type of *β*-lactam used, and kinetics of the deacylation reaction. For example, in case of penicillin K_m values for interactions with staphylococcal PBPs ranges from 0.01-5μM and PBP-pencilloylation ranges from 10-90% depending on the PBP. This wide variation in binding kinetics explains the observation that different *β*-lactam antibiotics have different concentration ranges at which they can damage bacterial cells, even though they have the same target (Tomasz, 1979). The inhibitory efficiency of a *β*-lactam for a PBP is given by the C_{50}, which is the antibiotic concentration needed to inhibit half of the PBP molecule at a steady state (when the acylation and deacylation reactions proceed at the same rate) (Zapun et al., 2008).



N-Acyl-D-Alanyl-D-Alanine peptide Penicillin backbone

Figure 1. 11. Shows the resemblance between *β*-lactams and the D-Ala-D-Ala dipeptide in the lipid II, particularly regarding the distribution of three electrostatic-negative wells. The regions of negative electrostatic potential are indicated by arcs. Images taken from Zapun et al., (2008).

1. 6. Cell Wall Defective Bacteria

S. *aureus* is characterized by the formation of variants both *in vivo* and *in vitro* as a result of stress response adaptation. The mechanisms contributing to developing this population heterogeneity are poorly understood (Marcova et al., 2008). Long term incubation of bacteria in the presence of cell wall inhibiting antibiotic can lead to the formation of abnormal "fried egg shape" colonies; these adapted cells are called L-forms, stain Gram negative and are osmosensitive variants (Tsai et al., 2011).

Isolation of L-form *in vitro* requires high osmotic pressure; therefore these cells provided with certain feature that supports growth and survival in the cells (Morikawa et al., 2010). The division of the L-form in the apparent absence of peptidoglycan is paradoxical (Joseleau-Petit et al., 2007).Several different methods for replication by L-form bacteria have been reported including budding, filamentous growth and binary fission, (Onwuamaegbu et al., 2005). Interestingly, propagation of L-form in *Bacillus subtilus* does not require the normal FtsZ-dependant division machine but occurs by a remarkable extrusion (Leaver et al., 2009). However, it has been observed that peptidoglycan polymer is required for L-forms survival (Yong, 2007). Similarly, it has been reported that the propagation of these L-form bacteria requires D-glutamate, DAP, and Mur activity, all specific to peptidoglycan biosynthesis. L-form bacteria contain approximately 7% of peptidoglycan component in the cells (Joseleau-Petit et al., 2007).

It has been shown that the first essential step in l-form generation involves escape of the cell protoplast from its enveloping cell wall. Therefore mutations in two different genes *walR* and *sepF* contribute to this feature. *walR* is a transcriotional regulator involved in cell wall homeostasis, and sepF, required for accurate and efficient cell division. The *walR* mutation renders the activity of the protein partially constitutive, inappropriately upregulating the acivity of autolytic enzymes that weaken the cell wall. The *sepF* mutation probably works by perturbing the formation of a properly constructed division septum, generating a mechanical breach in the cell wall (Dominguez-Cuevas et al., 2011). Recently it has been reported that l-form does not depend on the well-characterized cytoskeletal systems that are pivotal for cell shape, elongation, and division of walled cells, but that it does depend on having a

particular state of membrane fluidity. This provides biological support for the "membrane only" model and highlights the likely importance of the dynamic biological properties of membranes in cell reproduction (Mercier et al., 2012). Impairing branched chain fatty acid synthesis, which is important for providing a particular state of membrane fluidity, leaded the l-form proliferation to be virtually abolished but growth of walled cells were unaffected. This indicated that mutant cells with reduced membrane fluidity were able to grow in size, but they did not undertake the final membrane scission step needed to generate separate progeny cells (Errington, 2013).

In vivo, L-form bacteria have been reported to persist within cells because they can inhibit phagocytosis (Schmitt-Slomska et al., 1973), however others have suggested that L-form persist in cells as an adaptation response to antibiotic pressure (Onwuamaegbu et al., 2005). Interestingly, an established *E. coli* L-form line (LW1655F1), isolated nearly 40 years ago has acquired mutations in several genes required for peptidoglycan synthesis, the analysis revealed mutations in *ftsQ* and *mraY* genes in this strain, which are responsible for cell division and peptidoglycan biosynthesis respectively in all bacteria with a peptidoglycan-based cell wall (Siddiqui et al., 2006). Studies have shown that subtle changes in the primary structure of cell division proteins can render other division functions dispensable which were believed to be essential {e.g., FtsA can compensate for ZipA (Geissler et al., 2003)}.

A number of bacterial species have been reported to be able to grow and replicate as L-form variants. Cell wall deficient CWD forms of *S. aureus* have been identified clinically in patients with recurrent skin lesions, meningitis, endocarditis, arthritis and other diseases (Stoitsova et al., 2000). When L-form of *S. aureus* was injected to rats the inflammatory response was less intense and the infection appeared later and continued for a long period, compared to the wild strain. This is because these cells did not have cell wall envelope and antigen signals in the protoplast, leading to unsuccessful phagocytosis; as a result they survive and persist for a long time in the host cells or lead to failure of antibiosis therapy and consequential chronic infection (Michailova et al., 2007).

CHAPTER TWO

Material and Methods

2. 1. Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in (Table 2. 1 & Table 2. 2).

Table 2. 1. Bacterial strains used in this study.

***S. aureus* strains name**	**Genotype**	**Refrence**
Oxford ATCC 9144	wild-type reference strain	Kearns et al., 2006
RS1/19	revertant strain originated from Oxford strain.	Fuller et al., 2005.
T12	revertant strain originated from Oxford	This study
RN4220	restriction deficient derivative of Oxford strain	Kreiswirth et al., 1983.
8325-4	Wild type 8325 cured of prophages	Novick R.P., 1967
SH1000	NCTC8325-4 derivative; *rsbU*$^+$ *agr*$^+$	Horsburgh et al., 2002
RN4220(pGL485-*pbp4*)	*pbp4* overexpression transductant	This study
Oxford(pGL485-*pbp4*)	*pbp4* overexpression transductant	This study
8325-4(pGL485-*pbp4*)	*pbp4* overexpression transductant	This study
SH1000(pGL485-*pbp4*)	*pbp4* overexpression transductant	This study
RN4220(pMAD-Δ*pbp4*)	*pbp4* single deletion mutant in vector	This study
RN4220(pMAD-Δ*mgt)*	Δ*mgt* single deletion mutant in vector	This study
RS1/19(Δ*pbp4*)	*pbp4* single deletion mutant	This study
RN4220(Δ*mgt*)	Δ*mgt* single deletion mutant	This study
Oxford(Δ*mgt*)	Δ*mgt* single deletion mutant.	This study
RS1/19(Δmgt)	Δmgt single deletion mutant	This study

E. coli XL-1 Blue	General laboratory cloning strain	Sambrook et al., 1989

Table 2. 2. Plasmids used in this study.

Name	**Genotype**	**Refrence**
pGL485	Expression shuttle vector	Cooper et al., 2009
pGL485(*pbp4*)	Structural gene of *pbp4*	This study
PCR-Blunt II-TOPO	TOPO cloning vector *kanR bla*	Invitrogen
pGEM-3Zf(+)	Standard cloning vector	Promega
(pGEM-3Zf (+) (*5 'pbp4*))	*pbp4* upstream region	This study
pGEM-3Zf + (*5 'pbp4+spc*)	*pbp4* upstream region and spectionmycin *spc* resistance cassette gene	This study
pGEM-3zf (+) (Δ*pbp4)*	*pbp4* disrupted by *spc* gene	This study
pMAD	Vector for allelic replacement in low GC Gram positive bacteria *ermC bla*	Arnaud et al., (2004).
pMAD Δ*pbp4*	*pbp4* upstream and downstream region ligated through *spc* gene	This study.
pBluescript SK(+)	High copy cloning vector	Fermentas.
pBlue(*5 'mgt*)	*mgt* upstream region	This study.
pBlue(*5 'mgt+cm*)	*mgt* upstream region with *cm*	This study.
pBlue(Δ*mgt*)	*mgt* upstream and downstream region disrupted by *Cm.*	This study.

pMAD Δ*mgt*	*mgt* upstream and downstream region disrupted by *Cm*.	This study
pBlue(*pbp1*)	*pbp1* region with upstream and downstream region.	This study.
pBlue(*pbp2*)	*pbp2* region with upstream and downstream region	This study
pBlue(*pbp3*)	*pbp3* region with upstream and downstream region	This study
pBlue(Δ*pbp1*)	*pbp1* gene disrupted by *spc*	This study
pBlue(Δ*pbp2*)	*pbp2* gene disrupted by *spc*	This study
pBlue(Δ*pbp3*)	*pbp3* gene disrupted by *spc*	This study
pMAD(Δ*pbp1*)	*pbp1* gene disrupted by *spc*	This study
pMAD(Δ*pbp2*)	*pbp2* gene disrupted by *spc*.	This study
pMAD(Δ*pbp3*)	*pbp3* gene disrupted by *spc*.	This study
pBlue(5'*SgtA)*	*SgtA* upstream region .	This study
pBlue(5'*SgtA+spc)*	*SgtA* upstream region with *spc* cassette gene	This study
pBlue(Δ*SgtA)*	*SgtA* upstream and downstream region with *spc* cassette gene.	This study
pMAD(Δ*SgtA*)	*SgtA* gene disrupted by *spc*	This study

2. 2. Growth Media and Antibiotics

All growth media was sterilised by autoclaving at 121°C, 20 min at 15 p.s.i. unless otherwise stated.

2. 2. 1. Brain Heart Infusion Broth (BHI)

BHI broth was prepared as 37 g/L of brain heart infusion medium (Oxoid, UK). BHI agar was prepared by the addition of 15 g/L No. 1 Bacteriological Agar (Oxoid) to BHI broth.

2. 2. 2. Cell Wall Deficient Media (CWD)

CWD media consists of BHI medium supplemented with 5% (w/v) sucrose, 0.5 % (w/v) yeast extract and 0.2% (w/v) $MgSO_4$.7H2O as established in previous work (Fuller et al., 2005). After autoclaving and once the mixture had cooled to around 50 ºC, 10 %(v/v) sterile horse serum was added aseptically.

2. 2. 3. Luria Bertani Medium (LB)

Luria Bertani (LB) broth was prepared as described by Sambrock et al (1989) and consisted of 10 g/L tryptone (Oxoid) 5 g/L yeast extract (Berton, Dikinson and company) and 10 g/L NaCl (Fisher Scientific). LB agar was prepared by the addition of 15 g/L No. 1 Bacteriological Agar (Oxoid) to LB broth.

2. 2. 4. Iso-Sensitest Media (ISS)

ISS broth was prepared as 31.4 g/L of ISS medium (Oxoid, UK). ISS agar was prepared by the addition of 15 g/L No. 1 Bacteriological Agar (Oxoid) to ISS broth.

2. 2. 5. B2 Medium

B2 broth consisted of 10 g/L Bacto Casamino Acids (Becton, Dickinson and company) 25 g/L Yeast Extract (Becton, Dickinson and company) 1 g/L K_2HPO_4 (Sigma) 25 g/L NaCl (Fisher Scientific). After autoclaving the sterilised medium was supplemented with 25 ml/L of filter sterilised 20% glucose (Sigma).

2. 2. 6. LK Medium

LK broth consists of 10 g/L tryptone (Oxoid) 5 g/L Yeast extract (Becton, Dickinson and company) and 7 g/L KCl (sigma). LK agar was prepared by addition of 15 g/L No. 1 Bacteriological Agar (Oxoid) to LK broth.

2. 2. 7. SMMP

SMMP medium was prepared as 55ml 2×SMM, 40 ML 4×Penassay broth, and 5 ml 10 %(w/v) bovine albumin; adjust pH the to 7.0; then filter sterilized.

2. 2. 8. 2×SMM

25 ml 0.2 M sodium hydrogen maleate, 40 ml 0.1N NaOH; adjust the pH to 6.5. Add 5 ml 1 M MgCl2, 42.7 g sucrose; dissolve and bring volume to 125 ml. filter sterilize.

2. 2. 9. 4×Penassay Broth

17.5 g Antibiotic medium 3 (Becton Dickinson) dissolved in 250 ml water.

2. 2. 10. 0.2 M Sodium Hydrogen Maleate:

13.7 g maleic acid, 4 g NaOH; dissolve in 500 ml water.

2. 2. 11. Phage Agar (1L)

Consists of 3 g casamino acids, 3 g yeast extract 5.9 g NaCl then add 10 g No. 1 bacteriological agar (for Bottom agar) or 3.3 g agar (for Top agar).

2. 2. 12. Phage Buffer (1L)

1 ml of 1M MgSO4, 4 ml of 1M CaCl2, 50 ml of 1M Tris-HCl ph 7.8, 5.9 g of NaCl, and 1g of gelatin. Adjust pH to 7.8.

2. 2. 13. Antibiotics

Growth media for *E. coli* was supplemented as required with: ampicillin (Amp) at 100 µg/ml, spectinomycin (spc) at 100µg/ml, or kanamycin (Kan) at 50 µg/ml. Growth media for *S. aureus* was supplemented as required with the following: Erythromycin (Ery) 5 µg/ml, chloramphenicol (Cm) at 10 µg/ml or spectinomycin (spc) at 100 µg/ml.

2. 3. Microbial Growth and Storage Conditions

E. *coli* and *S*. *aureus* were grown at 37°C unless otherwise stated. *E*. *coli* and *S*. *aureus* liquid cultures were grown in broth with agitation at 200 rpm unless otherwise stated. For short term storage agar slopes were used by inoculation isolated colonies from the agar plate into deep agar slant and incubated overnight at 37°C, and then stored at 4°C. For long term storage, single colonies were grown in BHI broth media and suspended in BHI liquid medium plus 20% glycerol (v/ v) fast frozen in liquid nitrogen and stored as aliquots in sterile plastic vials at -80°C. Phage stocks were stored at 4°C.

2. 4. Staphytect Test

The latex slide agglutination test staphytect plus (Oxoid, Basingstoke, United Kingdom) was used to identify coagulase-positive *S*. *aureus*. The staphytect test identifies whether an organism is *S*. *aureus* or not by testing for the presence of clumping factor , protein A or certain capsular polysaccharides , if one or more of these are present agglutination occurs and the test is positive. Staphytect plus uses blue latex coated with porcine fibrinogen and rabbit immunoglobulin G including specific polyclonal antibodies raised against capsular polysaccharides of *S*. *aureus*. When the test reagents mixed on a card at room temperature with colonies of *S*. *aureus* , rapid agglutination occur through the reaction between fibrinogen and clumping factor, FC portion of IgG and protein A, or specific IgG and capsular polysaccharide. This is compared to control reagent and a positive result being the presence of agglutination within 20 seconds indicating *S*. *aureus*.

2. 5. Gram Staining

Gram staining uses dyes to stain a bacterial cell so it stands out from its background. It differentiates between Gram-positive and Gram-negative bacteria due to their differing cell wall structure. The specimen is mounted and fixed on a slide before it is stained. The reagents used are crystal violet, the primary stain; Lugol iodine, the mordant; ethanol, the decolouriser and safranin, the counter stain. Gram-positive bacteria incorporated little or no counter stain and will remain blue-violent in colour from the crystal violet whilst Gram-negative bacteria take on the pink colour of the counter stain. The staining procedure from agar plates has been performed by mixing a couple of colonies with a few drops of MQ (deionised and distilled water) on a

glass slide. The smear was allowed to air dry and then the organisms heat-fixed by passing it through a flame several times. Crystal violet was added to the dried smear for 1 min, washed off with MQ and Lugol iodine added for 1 min, which was blotted off. A solution of 95% ethanol was agitated over the stain for 30s and washed off with MQ, and finally a counter stain of Safranin was added for 5s and again washed off with MQ. A mixture of air-drying and blotting dried the Gram stain. While from liquid media, it was carried out by spinning a 500µl aliquot of the culture at 13,000rpm for 5 min and washed in 500µl MQ once, to remove the media allowing a clearer Gram stain. The pallet was re-suspended fully in 100µl MQ. Of this solution 10µl was added to 10µl MQ and mixed on a slide. The Gram stain then proceeded as above.

2. 6. Antibiotic Sensitivity Testing

2. 6.1. MIC Determination

Macro broth dilution determination of MIC was carried out using CWD or ISS broth; the inoculum suspension density was adjusted to be equal that of 0.5 McFarland standards (bioMerieux, Basingstoke, UK) (Andrew JM, 2001). This suspension of bacteria was equivalent to an absorbance at 600 nm of 0.025-0.035. Briefly, liquid media (10ml) was added to 14 universal tubes making the range of antibiotic concentrations of 0.032 to 128.0mg/L. One ml of media containing the antibiotic dilutions was transferred to test tubes with one ml of bacterial suspension. The inoculum suspension was further diluted to give an inoculum size of 10^5 CFU/ ml in the same media of antibiotics. Tubes were mixed thoroughly and incubated for 18-20 h at 37ºC in air and then checked for visible growth. The MIC was determined as the lowest concentration of antibiotic at which there was no visible growth.

2. 6. 2. E-Tests

E-tests were performed following the British Society of Antimicrobial Chemotherapy (BSAC) method (Andrews, 2001) with modifications. Penicillin E-test strips (AB Biodisk, Sweden) were used with ISS or CWD solid medium. The overnight culture of the strain to be tested was diluted with sterile MQ to 0.5 McFarland standards and an aliquot of 100µl spread three ways on the plate with a

sterile cotton wool bud. The plate was then air dried for 30 min, the strip added and the plate incubated at 37 ºC with the results read at 48h.

2. 7. Selection for Penicillin Resistance by Successive Cycling Using Liquid Media

Selection for penicillin-resistant mutants was performed in broth cultures by exposing bacteria to gradual increasing concentrations of penicillin G. Initially, a series of broths containing different concentrations of antibiotic were inoculated; following incubation, the broth culture with the highest concentration of penicillin G that could support growth was used to sub-culture into CWD liquid media containing further incremental increases in the antibiotic concentration. The whole cycling experiment was followed over a minimum of 10 cycles and repeated with 2 independent isolates of *S*. *aureus* Oxford. Following each cycle, Gram staining was carried out for the cells growing in highest level of penicillin G. The percentage of Gram negative cells were calculated from the average of Gram negative cells in 5 different microscopic fields.

2. 8. Growth Curve

A representative growth curve was established to determine the change in the absorbance readings over time for 3 strains of *S*. *aureus* (Wild type, RS1/19, T12). A single colony was inoculated into 5ml of the BHI broth media and incubated overnight at 37ºC; an aliquot of 1.0 ml was then transferred to a 100 ml of fresh liquid media of the same composition, to give a 1:100 dilution. The suspension was diluted so that the turbidity was similar to that of 0.5 McFarlands. Growth was monitored for each of three replicates for about 10h. One ml of the liquid culture was aseptically taken to read the absorbance at 600nm. Absorbance readings were determined every 30 min and the data then plotted to obtain a growth curve. The culture incubated overnight and the last reading was measured after 24h.

2. 9. Analysis of Penicillin Binding Protein (PBPs) Profile

2. 9. 1. Membrane Protein Preparation

Membrane proteins were prepared according to the method of (Sieradzki et al., 1999) with some modifications. Cells grown to the late exponential stage in 1L of BHI

medium were collected by centrifugation at 12,000 × g for 10 min at 4˚C, washed twice in 50mM Tris. HCl, 150mM NaCl, 5mM MgCl2 buffer, (pH 7.5), resuspended in the same buffer, supplemented with 10µl/ml bacterial Protease Arrest™ (Biosciences) (GE Health Care Life Sciences), and 10mM β-mercaptoethanol. Lysostaphin, DNase, and RNase were added to the final concentration of 100, 20, 10 µg/µl, respectively. The suspensions were incubated on ice for 30 min, followed by sonication for a total of 5 min, in 1 minute bursts with 2 min intervals on ice water batch after each 1 min sonication cycle. The suspension were then centrifuged at (5000rpm) for 10 min at 4˚C to pellet the hole cells. Partially lysed /broken cells were harvested by ultracentrifugation at 110,000 ×g (50000rpm) for 45 min at 4ºC, washed twice in 50mM phosphate buffer (pH 7.0), and membranes were solubilized by 2% (v/v)Triton X-100, snap frozen and stored in freezer at -80ºC.

2. 9. 2. Estimation of Protein Concentration

The Bradford assay was used for estimation of protein concentration, which is a dye-binding assay first developed by Bradford (Bradford 1976). It is based on the principal that, upon binding to protein, the maximum absorbance of an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm. The concentration of unknown samples can be estimated by comparison of their absorbance values to the absorbance values of a series of known protein concentrations.

2. 9. 3. Bradford Assay

Estimation of protein concentration was performed using Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Ltd.). A series of BSA standards ranging from 0-40µg was prepared from a stock solution of 2mg/ml BSA in 2%Triton X-100 (2% Triton X-100 in phosphate buffer pH 7.0) and each made up to a final volume of 25µl in 2% (v/v) Triton X-100. Aliquots of samples of unknown concentration were also prepared in a final volume of 25µl of 2% (v/v) Triton X-100. To each of these samples, 75µl ddH_2O was then added. Finally, 900µl of 25% (v/v) Protein Assay Dye Reagent Concentrate was added to each solution, mixed and incubated at room temperature for 10 minutes. Absorbance was measured at 595nm and the protein concentration of each sample calculated from the plotted BSA standard data.

2. 9. 4. Mini-format 1-Dimensional SDS-PAGE

SDS-PAGE analysis of protein samples was performed according to the discontinuous Trisglycine Tris-Glycine buffer system of Laemmli (Laemmli 1970), using Bio-Rad's Mini Protean II vertical gel apparatus (Bio-Rad Laboratories Ltd.).

2. 9. 4. 1. Gel Casting

SDS-PAGE mini gels were performed using 0.75mm thick gels composed of a 12% acrylamide resolving gel and a 5% acrylamide stacking gel. These were cast using the Mini-Protean II Cell (Bio-Rad Laboratories Ltd.) and clean glass casting plates. Resolving gel solutions were prepared containing 12% (w/v) acrylamide (acrylamide:bis-acrylamide 37: 5: 1) (Bio-Rad Laboratories Ltd.), 375mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate (Bio-Rad Laboratories Ltd.), and 0.2% (v/v) TEMED (Bio-Rad Laboratories Ltd.). Stacking gels were similarly prepared containing 5% (w/v) acrylamide and 125mM Tris-HCl pH 6.8 as the buffer. The resolving gel was cast first and overlaid with water-saturated butan-1-ol. Once this had set, the butan-1-ol was rinsed out and excess water removed by blotting. The stacking gel was then overlaid onto the level surface of the resolving gel and individual sample loading wells created by insertion of a Teflon comb. Wells were then rinsed free of any unpolymerised acrylamide.

2. 9. 4. 2. Sample Preparation and Gel Loading

Bocillin FL (Invitrogen, Molecular Probes) a derivative of penicillin V, was used to detect the penicillin binding proteins in membrane preparations. The method of Zhao et al., (1999) was followed. Protein samples 10-15µg/µl in 2% Triton X-100 were solubilised in 5x SDS sample loading buffer (12% (w/v) SDS, 5% (w/v) DTT (Melford Laboratories Ltd., Ipswich, UK), 0.05% (w/v) bromophenol blue, 0.312 M Tris-HCl pH 6.8, 50% (v/v) glycerol) to give a 1x concentration in a volume of up to 16.7µl. To this protein mixture 3.3µl of Bocillin FL 10mM (final concentration) was added to give a final volume of 20µl. The reaction mixture was incubated at 37ºC for 30 min., then denatured at 100˚C for 3 min and centrifuged for 1 min to pellet cell debris and insoluble material. Amersham ECL plex fluorescent Rainbow marker (225, 150, 102, 76, 52, 38, 31, 24, 17, 12 kDa) was used to determine the protein

sizes, 2µl Rainbow markers mixed with 2µl 5X SDS and 6µl of water and the mixture loaded in the gel without heating this used along with labeled protein samples that have been prepared previously.10µl of each reaction mixture (approx. 7.5-15ug of protein was subjected to SDS-PAGE analysis (12% polyacrylamide gel, Bio-Rad laboratories). The protein gels were rinsed with water immediately after electrophoresis, to visualize the labeled PBPs. The gels were directly scanned with The Typhoon 9400 Variable Mode Imager (Amersham Biosciences) was used in fluorescence acquisition mode at normal sensitivity, three channels were used (Cy2 channel excitation at 488nm and emission at 530 nm, Cy3 channel excitation at 532nm and emission at 580nm, and Cy5 channel excitation at 633nm and emission at 670nm.), then the images were overlaid to detect rainbow marker and PBPs together (Zhao et al., 1999).

2. 9. 4. 3. Electrophoresis

Electrophoresis through the stacking gel was conducted at 100V, after which the voltage was increased to 200V until the dye front reached the bottom of the resolving gel. Gels were then carefully detached from the apparatus and proteins visualized immediately by Typhoon scanner.

2. 10. Lysostaphin Susceptibility

The method of Sieradzki et al. (1999) was used for lysostaphin susceptibility test. The overnight culture was used to inoculate 50 ml of liquid media at a 1 in 100 dilution and further incubated until the exponential phase was reached, as judged by an absorbance reading and the previously established growth curves of the relevant strain. The culture was centrifuged at 13,000 rpm for 5 min and washed three times in 20 mM KPO_4 buffer (pH 7.5), and after the final wash re-suspended in the same buffer. The absorbance reading at wavelength 620 nm, using disposable cuvettes and a visible spectrophotometer, was adjusted to approximately 0.25. The spectrophotometer was temperature controlled at 37 °C. One unit of lysostaphin was then added, the cuvette shaken and returned to the spectrophotometer for continuous reading. The data was plotted as a percentage of the initial wavelength 620 nm.

2. 11. Autolysis with Triton X-100

Autolysis with Triton X-100 followed the method by Fournier and Hooper (2000) with minor modifications. Cells were grown in CWD liquid media in triplicate to the exponential phase, OD_{600} of 0.6 to 0.8, using the same overnight culture as the inoculum. The cells were pelleted by centrifugation and resuspended in the same volume containing 50mM Tris (hydroxymethyl) aminomethane hydrochloride Tris-HCl (pH 7.5) and 0.1% Triton X-100. The cells were then incubated at 30°C with shaking, and the changes in OD_{580} were measured.

2. 12. Quantitative Autolysis Assay Using Extracellular Autolysin

Cell wall hydrolysis assays were examined as described by (Zhu et al., 2010). Extracellular peptidoglycan hydrolases of bacteria were isolated from 15 ml of a 16 h culture by centrifugation at 6,000 x g for 15 min at 4°C. The supernatant was filter-sterilized and concentrated 100-fold using Vivaspin 6 Centrifugal Filter units (Milipore, 5 kDa) (Sartorius stedium biotech). The concentration of total proteins in each preparation was determined using the Bradford assay. Briefly, 100 µg of enzyme extract was added to a suspension of autoclaved and lyophilized *S. aureus* Oxford or RS1/19 cells (1 mg/ml) in 100 mM Tris-HCl (pH 8.0), and incubated at 37°C with shaking (150 rpm). Cell wall hydrolysis was measured as a decrease in turbidity at 600 nm every 30 min.

2. 13. PBP Binding Reactions

To determine the IC50 for each PBP with selected β-lactam antibiotics (penicillin G, cefotaxime, cefoxitin, and cefsolodin), approximately 100 µg/ml of solubilized membrane protein was incubated with different concentrations of β-lactam antibiotics ranging from (0.064-64 µg/ml) for 10 min at 37°C. Bocillin FL was then added, to final concentration of 10mM, and the incubations were continued for another 10 min. The proteins were then denatured at 100˚C for 3 min and centrifuged for 1 min to pellet cell debris and insoluble material. 10µl of each reaction mixture (approx. 15µg of protein was subjected to SDS-PAGE analysis (10% polyacrylamide gel, Bio-Rad laboratories). The protein gels were rinsed with water immediately then subjected to fluorography. The 50% binding affinity (IC50) was calculated by measuring the density of each PBP band for different amount of each antibiotic used.

Subsequently, the data was represented as a graph of relative density versus concentration of antibiotic used.

2. 14. Effect of Cefoxitin or Cefotaxime on Penicillin G Sensitivity of Oxford and RS1/19 Types of *S. aureus*

Iso-sensitest plates were prepared and mixed with 25% of MICs of cefoxitin, cefotaxime, or cefsoludin. Antibiotics were added into agar media after they cooled to 50°C, then left for minimum period of time before inoculation.

Bacteria were inoculated into BHI broth media and incubated at 37°C in an orbital shaker (set to 150 rpm) overnight. Standard inoculums were adjusted by diluting overnight cultures with the same used liquid medium to turbidity equal to the 0.5 McFarland standards. Sterile cotton tipped swabs were dipped into the standard inoculum suspensions and excess fluid removed from the soaked swab prior to the entire dried agar surface of Iso-sensitest media being wiped by the swab in three directions to cover the plate by thin layer of inoculum. The plates were left for 10 min to dry and E-test strips applied to the agar surface with sterile forceps. Agar plates were incubated at 37°C overnight (18-20 h) and MICs of penicillin G determined according to E-test reading guide of AB BIODISK.

2. 15. Effect of Cefoxitin on Cefotaxime Sensitivity of Oxford and RS1/19 Types of *S. aureus*

In this experiment the macro broth dilution method was used; 12 tubes containing (10 ml) of Iso-sensitest broth media were prepared and mixed with 25% of MICs of S. aureus variants of cefoxitin. The inoculum suspension densities of Oxford or RS1/19 were adjusted to be equal that of 0.5 McFarland standards (bioMerieux, Basingstoke, UK) (Andrew JM, 2001). Antibiotic dilutions (cefotaxime) were transferred to each tube making a range of antibiotic concentrations of 0.016 to 64µg/ml. Subsequently, one ml of bacterial suspensions were added. Tubes were mixed thoroughly and incubated for 18-20 h at 37ºC in air and then checked for visible growth. The MIC was determined as the lowest concentration of antibiotic at which there was no visible growth

2. 16. Molecular Biology

2. 16. 1. Preparation of Genomic DNA

Total chromosomal DNA was prepared using the method by Palomares et al (Palomares, Torres et al. 2003) with some modifications. Cells were grown in 5 ml of LB liquid medium overnight and centrifuged. The pellet was resuspended in 1.8 ml of TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) containing lysostaphin(100 µg/µl), mutanolysin (250 µg/ml) , and incubated at 37°C for one hour. Then 300 µl of 10% sodium dodecyl sulphate (SDS) and proteinase K (250 µg/ml) were added and the suspension incubated at 65°C for one hour. Afterwards 500 µl of 5 M sodium chloride (NaCl) and 500 µl of 10% Cetyl Trimethyl Ammonium Bromide (CTAB)/0.7 M NaCl were added after being heated in a water bath at 65°C for 10 min to aid release of DNA due to increased solubility of the suspension. A chloroform-phenol extraction was then performed by adding equal volumes of Phenol/ Chloroform/ Isoamylalcohol 25: 24: 1 (V: V: V), the mixture were then centrifuged at 4000rpm/5minutes. The upper, aqueous phase was recovered and subjected to two further phenol/chloroform/ isoamylalcohol extractions. A final chloroform extraction was carried out to remove residual phenol. The DNA was precipitated with an equal volume of isopropranol and centrifuged at13, 000 rpm for 10 min, and washed with 70% ethanol before being air dried and resuspended in 100 µl TE buffer and stored at 4˚C overnight and -20˚C for longer term storage.

2. 16. 2. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) DNA amplifications were performed as described by (Sambrook et al., 1989). Screening PCR and some cloning PCR reactions were carried in a total volume of 50µl and included (2.0 ng/ µl) of target DNA, 1x buffer (Bioline), 1.5mM of $MgCl_2$, 200 nM of dNTP, 400 nM of each primer and 0.02 u/ µl of Taq DNA polymerase (Bioline). The PCR machine was set for one cycle of 5min at 95ºC, 30 cycles of 45 sec at 50ºC and 5min at 72ºC, finishing with one cycle of 10 min at 72ºC, following the PCR reaction, the products were separated by agarose gel electrophoresis using a *Hae*III molecular weight marker. Gels, containing ethidium bromide at a final concentration of 0.5µg/ml, were made at the appropriate agarose concentration to separate the size of the DNA samples. DNA samples were prepared by adding DNA loading buffer (30% sucrose, 100mM

EDTA, pH 8; 0.05% bromophenol blue) to the sample prior to electrophoresis. DNA bands were viewed by illumination with UV light and images recorded by photography.

PCR reactions for cloning and mutagenesis were carried out in 50 µl volumes using 1 U of Phusion™ High-Fidelity DNA polymerase (Finzymes supplied by NEB UK), 1 x HF Buffer, 200µM dNTPs (Promega), 0.5-1 µM of each primer, 1 pg – 10 ng DNA template and sterilized distilled water to the final volume of 50 µl. Reaction mixtures were heated to 98°C for 30 sec to denature template. Primers were annealed at 54- 60°C for 20s and the product extended at 72°C for 20 sec per 1 kb DNA to be amplified. Denaturation, annealing and extension cycles were repeated 25-35 times. A final extension time of 5 min at 72°C ensured completion of all strands.

2. 16. 3. Purification of PCR Products

PCR products were purified by either agarose gel electrophoresis or using GFX PCR DNA and gel band purification kit (Amersham Biosciences, UK). 1 x volume of PCR product was mixed with 5 x volume of capture buffer (supplied by manufacture), the mix were transferred to a spin column and spun at 14,000 rpm for 30 sec and the flow though discarded. 500 µl of wash buffer (supplied by the manufacture) were added to the column and it was spun for 30 sec at 14,000 rpm and the flow through discarded. The column was then spun for 1 min at 14,000 rpm to remove residues of the wash buffer and the dry column were inserted to a 1.5 ml microcentrifuge tube and 30 – 50 µl of sterile distilled water was added. The column was incubated at room temperature for 1 min and spun at 14,000 rpm for 1 min. Elute contains the purified PCR product.

2. 16. 4. Purification of DNA From Agarose Gels

DNA was extracted from agarose gels using GFX PCR DNA and gel purification kit (Amersham Biosciences, UK) as described by the manufacturer. Briefly, the desired bands were cut out of the agarose gel and put in a 1.5 ml micro centrifuge tube. To the gel slice add 10 µl of capture buffer for each 10 mg of gel slice. The gel dissolved in a capture buffer by heating it to 60°C for 10 min. The dissolved gel was transferred to filter spin columns incubated at room temperature for 1 min and spun

at 13,000 rpm for 30 sec. The flow through was discarded and 700 µl of washing buffer and the columns were spun at 13.000 rpm for 30 sec and the flow through discarded. Columns were spun for 1 min to remove the last residues of the wash buffers and transferred to new labeled 1.5 ml microcentrifuge tubes. Subsequently, 30 – 50 µl of sterile destilled water was added directly to the membrane and the columns were incubated at room temperature for 1 min and spun at 13,000 rpm for 1 min. The eluted DNA was stores at 4°C.

2. 16. 5. RNA Extraction

RNA extraction was performed using RNeasy mini kit (Qiagen). Briefly cells were grown and centrifuged at 4000g for 15 minutes, pellets then resuspended in TE 1.8 ml of TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) containing 200 g/ ml of lysostaphin, 400 U/ml mutanolysin, and 40 g/ ml proteinase K (final concentrations) and incubated at 37 C for 1-2 hours with occasional gentle mixing. A volume of 350 µl from lysed cells was used and proceeds to elution of RNA, followed manufacturer's instructions. DNase1 (Qiagen) was used by mixing 10 µl of enzyme with 70 µl of the DNase I supplied buffer and added to each RNA sample tubes according to the manufacturer's instructions. RNA samples were eluted 50µl volumes, analyzed using a NanoDrop1000 spectrophotometer (Lab tech), and stored at -80°C for longer terms.

2. 16. 6. RNA Agarose Gel Electrophoresis

Electrophoresis tanks and gel combs were soaked in 0.1M NaOH for 15-30 min, and rinsed with RNease free water several times. Total RNA 500ng in 1x MOPS (3-N-morpholino propane sulfonic acid) buffer, 1.0 µl of ethidium bromide and 1.0 µl of RNA loading buffer (50% glycerol, 1mM EDTA and few grains of bromo phenol blue) made up to 10.0µl by RNase free water were mixed and boiled for few minutes and loaded on RNA agarose (1.2% in 1x MOPS buffer) mini gels.

2. 16. 7. cDNA Synthesis

First strand of cDNA was generated using the Superscript III transcriptase (Invitrogen) system. For this one µg of total RNA was reverse transcribed in a total volume of 20µl containing random hexamer primers (150 ng/µl) (Invitrogen), made

up to 11.0µl with sterilized MQ RNase free water, the mixture was heated at 65ºC/ 5 minutes, put on ice for 5 minutes and spun briefly. Then 1 x RT buffer (Invitrogen), 10nM DTT (Invitrogen), dNTP (0.5 mM) (Bioline), RNase inhibitor (Promega 2.0u/ µl), and Reverse Transcriptase (RT III) (10u/ µl, Invitrogen) as final concentrations, were added. The suspension of 20 µl was incubated at 42ºC for 90 minutes, followed by 72ºC for 15 minutes. After confirming that cDNA synthesis was successful by normal PCR, samples were then stored at -20ºC.

2. 16. 8. Real-Time Quantitative PCR (RT-qPCR)

A cocktail of following components was made in 20µl reaction mixes. The cDNA synthesized from the total RNA was diluted in a 1:4 retio with sterile nuclease free water and was used as a template for the real-time reaction.

Syto9 (5µM)	0.25µl
Forward primer (10pmol/ µl)	0.25 µl
Reverse primer (10pmol/ µl)	0.25 µl
5x reaction Buffer	2 µl
$MgCl_2$ (1.5mM)	0.6 µl
dNTP mix (10mM of each dNTP)	0.4 µl
Taq DNA polymerase (Bioline)	0.08 µl
Sterile dH_2O	15.67 µl
Total	19.5 µl

0.5 µl of template was added and mixed with the rest of the reaction mix in flat topped real-time PCR tubes, and spun briefly. Samples were then run in the RotorGene 3000 (Corbett Research) using the following parameters: 95ºC for 10min followed by 95ºC for 20sec, 54-56ºC (annealing tempreture) for 20sec, and at 72ºC for 20sec, with data collection in each cycle at 72ºC.

Melt:

55ºC-99ºC, hold 30 seconds on the 1st step, and 5 seconds on subsequent steps. The data from the run were acquired the annealing tempreture using the multi channel, emission at 470nm and detection at 510 nm.

2. 16. 9. Data Analysis Using (REST-2009)

The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle. Individual reaction are characterized by the PCR cycle at which fluorescence first rises above a defined or threshold background fluorescence, a parameter known at the threshold cycle (Ct) value. The more target there is in the starting material, the lower the Ct value. This correlation between fluorescence and amount of amplified product permits accurate quantification of target molecules over a wide dynamic range (Nolan et al., 2006). Ct values and reaction efficiency were produced in comparative quantification analysis generated by Rotorgen 3000 and used for data analysis using REST-2009 software (Corbett Research). The Relative Expression Software Tool (REST) was used (Pfaffl et al., 2006). REST software is used to determine changes in target gene expression standardised by non-regulated reference genes (Corbett Research 2006). The purpose of this software tool in this study was to verify the results of microarray data of RS1/19 with the wild-type Oxford strain.

2. 16. 10. DNA Sequencing

PCR products for the genes (or part of the genes) required for sequencing was purified using Amersham Biosciences GFX purification kit, and quantified using NanoDrop1000 spectrophotometer (Labtech). The PCR products were then sequenced (Durham sequencing service) with the same primers used for amplification, details of which are given in the appropriate chapter section.

2. 16. 11. DNA Restriction Enzymes

Restriction enzymes were obtained from Promega, UK. DNA restriction digest contained 0.5-1 µg of DNA, 1U of restriction endonuclease and 1 x buffer (supplied

by manufacture) made up to a final volume of 30-40 µl with sterile distilled water. Digests were incubated for between 2 to 18 h at the appropriate temperature. Restriction enzymes and cut off ends from PCR products were removed from the reaction using GFX DNA purification kit as described in the manufacturer's instructions (Amersham Biosciences, UK). For digested plasmid desired fragment was isolated on a 0.7-1% wt/vol agarose gel and gel purified as described in section 2. 16. 4.

2. 16. 12. Dephosphorylating Vector

If a single enzyme is used (nondirectional cloning), terminal 5'-phosphates must be removed with heat inactiveted alkaline phosphatase (Invitrogen) after the digestion to prevent self-ligation (recircularization) of the plasmid.1 µL (5U) of enzyme was added to the restriction digestion reaction, then incubated for 15 minutes at 37°C to remove 5' phosphates from vector. Heat inactivate for 15 minutes at 65°C.

2. 16. 13. DNA Ligation

Ligation of DNA was carried out using either T4 DNA ligase supplied by
Promega UK. Purified DNA inserts were ligated to purified vector DNA in a ratio of 3:1. The ligation reaction mix also contained 1 x T4 DNA ligase buffer (supplied by manufacture) and 1 U T4 DNA ligase in a final volume of 10 µl made up with sterilized distilled water. Reactions were incubated overnight at 16°C.

2. 17. Manipulation of *Escherichia coli*

2. 17. 1. Electro-competent Cells

Electro-competent *E. coli* XL-1 Blue cells were prepared as described by (Ausubel et al., 1988). Briefly, 1 L of LB broth was inoculated with 10 ml of an overnight *E. coli* culture. The culture was incubated at 37°C, shaking at 200 rpm until an OD_{600} of 0.5 – 0.8 was reached. Following incubation on ice for 15 – 20 min, the cells were harvested by centrifugation at 5000 x g, 4°C for 10 min. Cells were then washed in 1 L of ice-cold sterile distilled water, recentrifuged, washed with 500 ml ice-cold sterile distilled water, then resuspended in 20 ml ice-cold 10 % vol/vol glycerol. Cells were centrifuged once again prior to resuspension in a final volume of 1 ml of ice-cold 10 % vol/vol glycerol. Aliquots of 40 µl were transferred to cold

microcentrifuge tubes and flash frozen in liquid nitrogen before storage at -80°C.

2. 17. 2. Heat Shock Transformation of *E. coli* Cells

Cells were removed from the freezer and thawed at room temperature until just liquid, and placed back on ice. 40 µL aliquots were mixed with 1-10 µL of DNA and incubated on ice for 20 min. Cells were heat shocked by placing tubes in a 42°C water bath for 90s, and then chilled by returning to ice immediately.

Cells were recovered by adding 500 µL of LB and incubated shaking at 37°C for approximately 1 h and plated on LB plates containing appropriate antibiotic.

2. 18. Manipulation of *S. aureus*

2. 18. 1. Preparation of Plasmid DNA

For purification of plasmid DNA from *S. aureus* a Wizard®Plus SV Minipreps DNA Purification System (Promega UK) was used with modification to the protocol. Briefly, 2-3 ml of *a S .aureus* overnight culture was harvested in a by centrifugation 14,000 rpm 1 min. The pellets was resuspended in 250 µl of cell resuspension solution (supplied by the manufacturer) and 5 µl of a 5 mg/ml Lysostaphin (Sigma) was added, mixed and incubated at 37°C for 30 min. 250 µl cell lysis solution (supplied by the manufacturer) was added and mixed by gently inverting the tube 3-4 times, 350 µl neutralization solution was added and tube gently inverted 3-4 times to facilitate total mixing. Mixture was centrifuged for 10 min at 14,000 rpm to precipitate genomic DNA and cell debris. Supernatant was gently poured on to a spin column and spun at 14,000 rpm for 1 min and flow through was discarded. 750 µl of wash solution (supplied by manufacturer, Ethanol added) was added and column centrifuged again for 1 min at 14,0000 rpm. Flow through discarded and the same step repeated again this time with 250 µl of wash solution. Flow through and the column spun again at 14,000 rpm for 1 min to get rid of wash buffer residues. Spin column was transferred to a new micro centrifuge tube and 30 – 50 µl nuclease free water was added. Columns were incubated at room temperature for 1 min and plasmid DNA recovered by centrifugation at 14,000 rpm for 1 min.

2. 18. 2. Preparation of Electro-competent *S. aureus*

Electro-competent *S.aureus* RN4220, cells were prepared as described by (Schenk and Laddaga, 1992). Briefly, an overnight culture of the desired strain was grown in

30 ml of B2 broth for no more than 15 h. This overnight culture was used to inoculate 200 ml of pre-warmed BHI broth to an OD_{600} of approx. 0.25. The culture was incubated at 37°C, shaking at 200 rpm until an OD_{600} of 0.35 – 0.4 was reached (approx 1 – 1.5 h). All of the washes and incubations were done a room temperature unless otherwise stated. The cells were harvested by centrifugation at 10.000 x g for 10 min. Cells were then washed in 30 ml of sterile distilled water, recentrifuged, washed with 30 ml sterile distilled water. Centrifuged again and resuspended in 10 ml 10 % vol/vol glycerol. This step was repeated with resuspension in 5 ml of 10 % vol/vol glycerol and incubated at room temperature for 15 min. Cells were transferred to 1.5 ml microcentrifuge tubes and centrifuged once again at 10.000 x g prior to resuspension in a final volume of 1.5 ml of 10 % vol/vol glycerol. Aliquots of 80 µl were stored at -80°C.

2. 18. 3. Electroporation of *S. aureus* Cells

Electrocompetent cells were removed from the freezer and thawed at room temperature prior to use. The cells were mixed with approx. 1 µg of plasmid DNA was added to 70 µl of electrocompetent *S. aureus* cells and transferred to a 0.2 mm electroporation cuvette (BioRad). A BioRad Gene Pulsar was used, according to manufacturer's instructions, to deliver an electric pulse of 2.3 kV (25µF, 100Ω).
The cuvette was immediately removed and 1 ml of SMMP broth was added. After incubation for approx. 2 h at 37°C (unless otherwise stated) 100 – 200 µl of the bacterial suspension was spread on to an appropriate selective plate, the rest of the cells were spun at 13.000 rpm for 1 min, supernatant discarded and resuspended in 200 µl SMMP broth and spread in an appropriate selective plate.

2. 19. 80α Transduction

Phage 80α for transduction of *S. aureus* was carried out as described by Lindisay, J. (2006) as follows:

2. 19. 1. Phage Lysate Preparation

To get a high titer of phage lysates, an overnight plate of the *S. aureus* 8325-4 cells was inoculated into LK broth and incubated at 37ºC with shaking for approximately 3h. A 7 ml aliquot of bacterial cells were then spun down, and resuspended in 7ml phage buffer plus 7ml BHIB. 1 ml of phage 80α stock (kindly supplied by Dr. Jodi

Lindsay) was added, mixed gently and incubated at room temperature for 10min. The suspension was then incubated at 30 ºC with slow shaking (70rpm), mixed gently after 2h incubation, then checked and mixed again each hour. Phage 80α causes partial lyses and even after it has been left overnight on the bench the suspension remains clouldy. Lysates were spun down and filter sterilized using 0.2µm pore size filter and stored at 4 ºC.

2. 19. 2. Determination of Phage Titres

Titres of 80α lysates were determined as follows. An overnight plate of the recipient cells *S*. *aureus* 8325-4 was inoculated into LK broth and incubated at 37ºC for approximately 3h. Phage lysates were diluted in phage buffer to 10^{-8} and 100µl of each dilution mixed with 400 µl of recipient cells and 30 µl of 1M $CaCl_{2,}$ this mixture was left on bench for 15 min. Each dilution was mixed with 7 ml top agar (tempered to 50˚C) and poured over phage bottom plates; the top agar was allowed to set and incubated at 30 ºC for 18-24h.

2. 19. 3. Phage Transduction

S. *aureus* (Oxford, 8325-4, RS1/19, and SH1000) cells were transduced as described by (Lidisay,J. 2006). An overnight culture of RN4220 (pGL485-*pbp4*) was grown in 20 ml LK broth. The cells were harvested by centrifugation for 10 min at 10000 rpm and resuspended in 1 ml LK broth. Both a sample and a control were mixed; the sample consisted of 500 µl cells, 1 ml LK with 10 mM $CaCl_2$ and 500 µl phage 80α lysate. The control consisted of 500 µl cells and 1.5 ml LK with 10 mM CaCl2. Both sample and control was incubated first for 25 min stationary and 15 min, with shaking at 37°C. After incubation, the sample and control was transferred to an ice bucket and 1 ml of ice-cold 0.02 M sodium citrate was added. Both Sample and control was centrifuged for 10 min at 10,000 rpm at 4°C and resuspended in 1 ml ice cold 0.02 M sodium citrate and incubated on ice for 2-3 h. Aliquots of 100 µl was spread on selective LK plate's containing 0.05% wt/vol sodium citrate. Plates were incubated at 37°C for 12-72 h and transduction of the desired DNA was verified by testing the expression of *pbp4* by RTq-PCR and overproduction of PBP4 in membrane preparations.

The same method was used with to transduce (Oxford & RS1/19) cells with RN4220(pMAD-Δ*pbp4*), expect that all the incubation were performed at 30°C instead of 37°C, because pMAD is a temperature sensitive vector.

CHAPTER THREE

Characteristics of *in vitro* Generated Penicillin Resistant *S. aureus*

3. 1. Introduction

Cell wall deficient CWD bacteria can be obtained *in vitro* by growing cells on solid or liquid media, with increased osmolality, in the presence of penicillin (Fuller et al., 2005). CWD bacteria have altered morphology and cultural characteristics due to damaged or absent cell wall structures (Onwuamaegbu et al., 2005). However, the loss of the cell wall may be permanent (stable L-forms) or temporary, as some may regain their wall and grow normally (unstable L-forms) (Yong, 2007). It has been reported that survival under conditions preventing normal cell wall formation promotes complex adaptations that acquire a fitness cost, (Hubscher et al., 2007). The ability of bacteria to survive, grow and reproduce as CWD forms has been reported for a number of bacterial species like *B. subtilus, E. coli* and *L. monocytogenes* (Leaver et al., 2009, Siddiqui et al., 2005, and Dell Erra et al., 2009). CWD forms of *S. aureus* have been identified clinically in patients with recurrent skin lesions, and also in some cases of meningitis, endocarditis, arthritis and other diseases (Stoitsova et al., 2000).

β-lactam antibiotics exhibit differential affinities for the four PBPs in *S. aureus* (Georgopapadakou et al., 1982). For example, PBP4, has a remarkably low affinity and high deacylation rate for penicillin analogues compared to other PBPs; this results in only 10-46% of the protein being bound by penicillin at a given time, depending on the strain of *S. aureus* (Chambers et al., 1994), and also explains the early observation that PBP4 has penicillinase activity, turning over 25 penicillin molecules per minute *in vitro* (Navartna et al., 2010; Kozarich et al., 1978). However, PBP4 has a very high affinity and low deacylation rate for the β-lactam cefoxitin, resulting in effective PBP4 inhibition (Wyke et al., 1981; Sumita et al., 1990). It has been observed that *S. aureus* strains with different genetic background have relatively different affinities for the same antibiotics used (Table 3. 1) (Kosowska-Shick et al., 2010).

Table 3. 1. Binding affinities of β-lactams for *S. aureus* PBPs

PBP	Strain	IC50 (μg/ml)*a*			
		ceftaroline	cefotaxime	ceftriaxone	penicillin G
PBP1	ATCC 29213	0.5	0.5	0.25	4
	873	8	4	2	0.5
	510	0.5	2	0.5	128
	2149A	1	0.5	4	2
	1287	0.125	4	1	4
	25	0.5	1	16	0.5
PBP2	ATCC 29213	0.25	1	0.25	8
	873	0.5	0.5	0.5	0.5
	510	0.125	0.5	0.25	64
	2149A	1	2	1	>128
	1287	4	1	1	4
	25	0.25	0.5	2	1
PBP2a	ATCC 29213	NP	NP	NP	NP
	873	0.5	>128	>128	64
	510	0.25	>128	1	64
	2149A	1	>128	>128	2
	1287	1	4	2	4
	25	0.01	0.5	0.25	4
PBP3	ATCC 29213	0.125	1	1	1
	873	0.125	0.125	0.25	0.03
	510	0.125	0.25	0.25	4
	2149A	0.5	4	2	2
	1287	0.1	1	1	0.25
	25	0.25	0.25	1	0.5
PBP4	ATCC 29213	>8	>8	>8	>8
	873	>128	>128	>128	>128
	510	>128	>128	>128	64
	2149A	>128	>128	>128	>128
	1287	64	>128	>128	4
	25	ND	ND	ND	ND

a NP not possible, ATCC 29213, as a MSSA strain, lacks PBP2a. ND, no demonstrable binding of Bocillin FL to PBP4 was observed. (Table taken from Kosowska-Shick et al., 2010).

Study of the CWD variants by transmission electron microscopy showed thickened and diffuse cell wall, whereas CWC strains had a cell wall which was microscopically indistinguishable from wild type cell wall (Fuller et al., 2005).

The CWD cells were resistant to lysis by lysostaphin, which suggests that they are not dependent on the presence of the cell wall for their intactness or have altered lysostaphin targets. CWD cells were very sensitive to lysis by TritonX-100. This is likely due to more accessibility of the detergent to their membranes than the membranes of parental strains. On the other hand, CWC (RS1/19) cells show a slight increase in resistance to lysis by lysostaphin in comparison to the Oxford strain and a significant increase in lysostaphin sensitivity when compared to CWD cells. It has been previously reported that the five glycine cross-bridge on peptidoglycan is the target for lysostaphin (Kline and Blackburn, 1994). This may suggest that RS1/19 cells have intact peptidoglycan structure which is essential for their integrity. In addition, RS1/19 cells were resistant to the action of Triton X-100, indicating that recovery of cell walls is important to prevent access of the detergent into the cell. Furthermore Fuller et al., also performed HPLC analysis that indicated that the muropeptide in the RS1/19 contains shorter glycan chain and a larger number of pentaglycine cross-bridges (Fuller et.al. 2005).

In order to better understand how *S. aureus* responds to exposure to the cell wall active antibiotics, penicillin G, *S. aureus* Oxford (ATCC9144) was passaged in the presence of increasing concentrations of the antibiotic in liquid medium with a raised osmolality. The aims of this chapter are to determine the kinetics of acquisition of penicillin resistance and to investigate the relationship between loss of cell wall integrity and inheritance of high-level penicillin resistance. Additionally, experiments were designed to determine if the activity of PBP4 is important in the resistance mechanism by using antibiotics with different affinities for PBPs as selective inhibitors

3. 2. Results and Discussion

3. 2. 1. Selection for Penicillin Resistant Mutants by Successive Cycling in Liquid Media and its Correlation to a Gram Negative Phenotype

The aim of this experiment was to generate CWD bacteria that exhibit resistance to penicillin G, study the characteristics of CWD cells and investigate the relation between acquisition of penicillin resistance and their Gram staining characteristics.

Induction of CWD bacteria can be carried out using both solid and liquid media. Strain RS1/19 was generated by successive sub-cultures on solid media containing increasing concentrations of penicillin G (Fuller et al., 2005). Cells within colonies on agar plates are metabolically heterogeneous, whereas those in liquid cultures are all in the same metabolic state; additionally, liquid medium led to more equivalent penicillin G concentration. It is likely therefore that penicillin resistant strain would be induced more quickly in liquid culture.

In this experiment, a series of CWD variants of *S. aureus* ATCC 9144 (Oxford) were generated in liquid medium with elevated osmotic potential in the presence of sub-lethal levels of penicillin G. Initially, a series of cultures with different, sub-lethal levels of penicillin G were grown and subsequently analyzed for their Gram reaction. The percentage of Gram negative cells in each transfer were determined by counting the number of Gram negative cells in five microscopic slides, and then the percentage of the average were calculated. The culture with the highest percentage of Gram negative cells was used to inoculate fresh cultures in CWD medium with a penicillin concentration range between 100% and 400% compared to which the cells were previously grown in. This process was repeated for a further 9 serial passages, during which time the MIC rose from 0.064 to 32µg/ml and near 100% of the cells in the culture stained Gram negative. At the end of the selection procedure 10 strains with different MIC to penicillin G concentrations ranging from (0.064 to 32µg/ml) were obtained and stored at -80˚C; each strain was named according to the number of transfers for example CWD (AM1) refers to the CWD cells obtained from 1st transfer of the serial passage.

Growth of the cells on BHI agar, or in LB medium, or CWD medium without addition of penicillin caused them to revert to CWC forms for example T1 is the revertant from 1st transfer that stained 100% Gram positive.

On incubation of the Oxford strain in high osmolality medium in the presence of penicillin, the majority of cells exhibited a cell wall defective phenotype, implied by Gram-negative staining of the cells (Figure 3. 1. C, D). Penicillin resistance rose gradually until the 6th and 7th transfer where it suddenly rose 2 fold from 0.5 to 2µg/ml and then from 2 to 8µg/ml respectively. After this time it continued to rise steadily to reach 32µg/ml (Figure 3. 2). On first exposure to penicillin G 90% of the cells stained as Gram negative, however this dropped on further transfers in the same amount of penicillin, and even dropped when grown in medium containing significant increases of penicillin concentration. Throughout this experiment, the maximum production of CWD cells which was characterized by Gram negative staining in the 3rd, (AM3) and 10th (AM10) transfer (Figure 3. 3). Cells grown in CWD media with penicillin showed profuse Gram negative staining early in the experiment, with further transfers a mixture of Gram negative and Gram positive cells were seen which may indicate reversion of CWD variants to the CWC phenotype. This may suggest that CWD are unstable and will revert back to a cell wall competent state very easily even in the presence of penicillin G. Similar results were observed from CWD lines which has been generated in CWD liquid media (CL3/19) and on CWD solid media (CS1/19) (Fuller et al., 2005).

Although CWD bacteria may be considered similar to the bacterial protoplast (Markova et al., 2008), when they are observed microscopically they show variable morphological characterization: pleomorphic cells with indistinct margins and loss of the typical cell arrangement characteristic of *S. aureus*. Interestingly, following growth of CWD forms for many generations in the absence of penicillin G, which led to reverting to CWC phenotype, the cells remain resistant to high concentrations of penicillin G. This might be achieved by these cells gaining stable resistance to penicillin G through either mutations in PBPs or alterations in gene regulation. CWC (T12) colonies also differ from the wild-type strain because they exhibit smaller colonies with less pigmentation as compared to the wild-type Oxford strain (Figure 3. 1. A, and B).

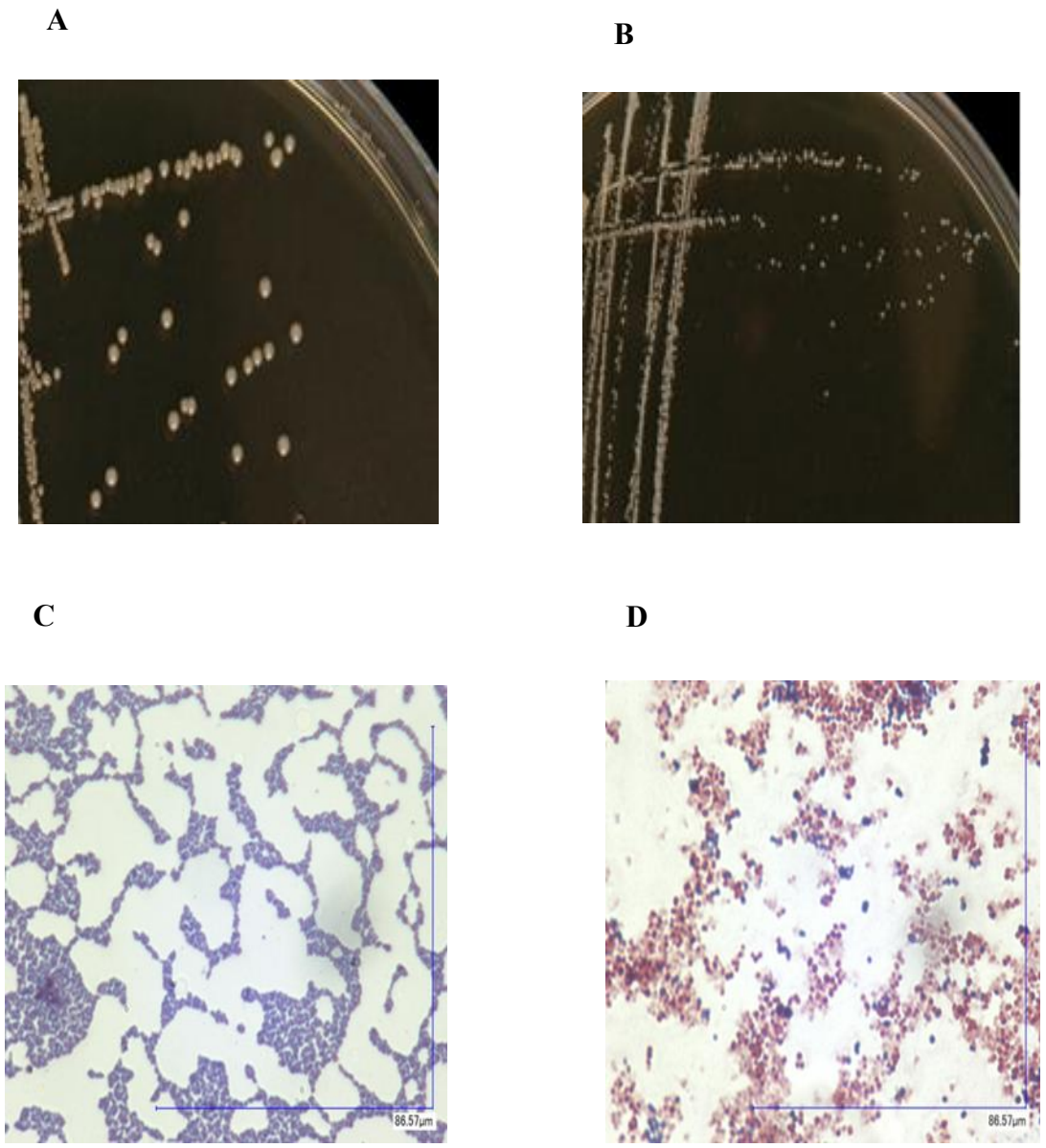


Figure 3. 1. Cultural and growth characteristic of *S. aureus* variants. Colonies of *S. aureus* grown on BHI agar plates, wild- type Oxford (A) and revertant strain T12 (B). Colonies of RS1/19 are smaller in size and less pigmented. Gram stain of the *S. aureus* strains at high magnification power 1,000X. Typical wild type *S. aureus* strain (C), CWDB in the presence of sub-inhibitory concentration of penicillin G (D). Wild-type cells stains uniformly Gram positive, while cell wall deficient cells AM3 shows 95% Gram negative cells with indistinct margins.

From this experiment it is clear that acquisition of penicillin resistance is achieved in a small incremental steps and correlates with specific mutations occurred in the bacteria to allow the antibiotic-resistant, cell-wall deficient strain to be generated. The results suggest that loss of cell wall and antibiotic resistance are two separate processes; growth in sub-letahl levels of antibiotic causses loss of cell wall, but mutation is required for a resistance phenotype to be established. On first exposure, 90% of the cells exhibit a lose cell wall integrity without cell lysis occurring. On subsequent exposure to penicillin G part of the population revert to a Gram positive reaction and continue to grow even in the presence of high concentration of penicillin G. This suggests that these cells have adapted to grow in the presence of the antibiotic, perhaps due to peptidoglycan alteration. This may be an indication that Gram negative cells can participate in the development of antibiotic resistance, by giving time to develop gene mutations and cell adaptation.

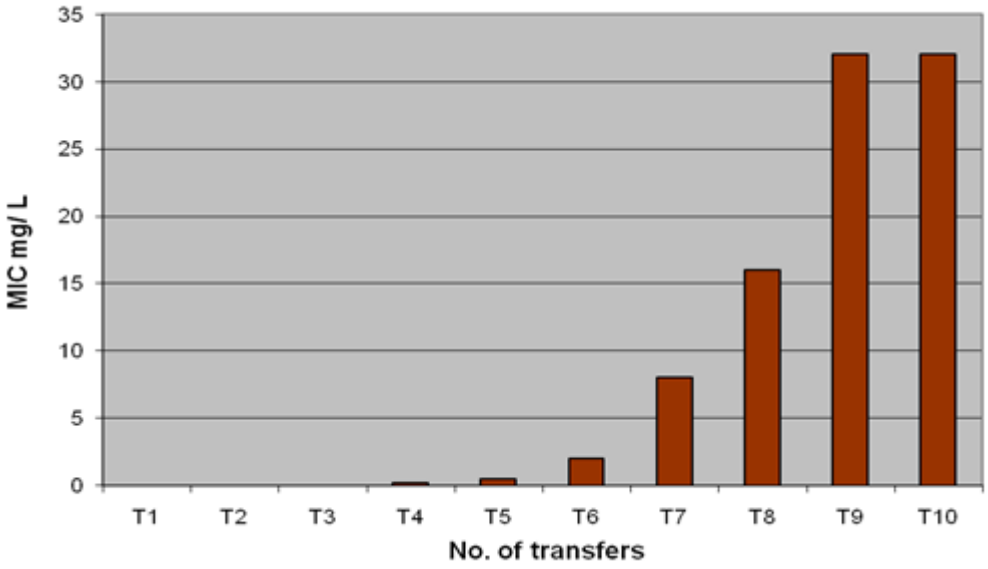


Figure 3. 2. Penicillin G MIC of *S. aureus* Oxford after each serial passage with increasing concentration of penicillin G. Penicillin resistance increased gradually from 0.032 µg/ml in 1st transfer to 32µg/ml in the 10th transfer.

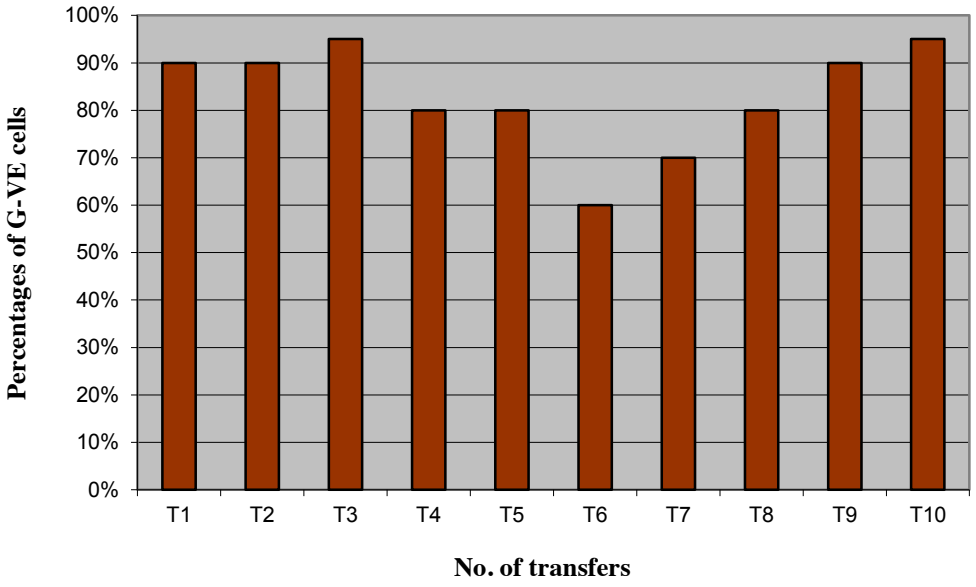


Figure 3. 3. Percentages of Gram-negative cells after each transfer. In each transfer 5 fields on the microscopic slid were counted, and then the percentage of the average represented in each bar. Majority of *S. aureus* cells become Gram negative when challenged with penicillin.

3. 3. PBP Assays Reveal a Correlation Between Increased Binding of Labeled Penicillin to PBP4 and Reduced Antibiotic Susceptibility.

It has been reported that overproduction of PBP4 is responsible for intrinsic β-lactam resistance in *S. aureus* strains exposed to high level of penicillin G (Henze and Berger-Bächi, 1996), and overproduction of PBP4 has been implicated in the resistance seen in cells generated on media with high osmolality (Fuller et al., 2005). In order to understand the kinetics of the development of high levels of PBP4 and see if there is a correlation to resistance acquisition, in CWD and CWC derivatives, PBPs profile of these strains were studied.

3. 3. 1. Optimization of Membrane Protein Preparation

S. *aureus* has four PBPs, ranging between 85 to 45 KDa in size, on the basis of migration of the labeled PBPs on SDS-PAGE. Initial analysis of PBPs in membrane preparations identified more than the four expected polypeptides. This was likely to have been caused by either proteolysis of membrane proteins during preparation or non-spesific binding of labeled penicillin to protein bands, resulting in many labeled proteins. It was therefore necessary to optimize the preparation of membranes from this particular strain of *S*. *aureus*.

Initially the method of Nigel et al., (1980) was followed. Cells of *S*. *aureus* strains were lysed by Lysostaphin and incubated at 30°C for 30mins. Membrane proteins were then labeled with Bocillin FL, separated by SDS-PAGE and scanned using the Typhoon Imager (Figure 3. 4A). Only one band was clearly visible, which may be due to incomplete lysis of cells using lysostaphin alone. Subsequently, cells were lysed using Lysostaphin as well as sonication, following the method of Zhao et al (1999). In this method in addition to incubation of cells with lysostaphin at 4°C for 30mins, cells were sonicated to lyse the cells completely. The samples were then labeled with Bocillin FL and proteins separated by SDS-PAGE (Figure 3. 4B). Several extra bands, to those expected to represent the four PBPs in *S*. *aureus*, were present on the SDS gel, which could have resulted from proteolysis of PBPs.

To test if these extra bands were due to non-specific binding to the Bocillin FL or were present because of proteolysis of the PBPs, two separate experiments were

performed. In the first experiment the method of DeLoney and Schiller (1999) was followed, 15μg of membrane fractions were labeled with penicilloic acid with increasing concentration ranging from (0.5 – 50U). Penicilloic acid is the product of the β-lactamase reaction and does not bind to the active site of PBPs; therefore, pre-treatment with this does not prevent Bocillin FL from binding to PBPs. After incubation of the membrane preparations with penicilloic acid, at 37°C for 15 min., Bocillin FL was added and incubated for another 20 min prior to the membrane protein being analyzed using SDS gels (Figure 3. 4C). Since the intensity of protein bands did not decreased in the presence of penicilloic acid it is likely that additional bands are due to proteolysis of PBPs. This hypothesis was confirmed because the bands completely disappeared when membrane protein fractions were pre-incubated with high concentration of penicillin G., prior to addition of Bocillin FL. These data indicate that the additional bands present were probably proteolytic fragments of PBPs, which still contain the active- site serine residue of penicillin-binding proteins that interacts covalently with β-lactam antibiotics (Dargis and Malouin, 1994) (Figure 3.4D). Galleni et al., 1993 have also reported the detection of additional unidentified PBPs in electrophoresis profile of *E. coli*, but in contrast to our results, they suggested that these bands may be nonspecific since they did not completely disappeared in competition experiments with high concentrations of penicillin G.

Several modifications of the membrane preparation method were made out in an attempt to prevent proteolysis. The serine protease inhibitor protease inhibitor PMSF (phenylmethylsulphonyl fluoride 0.5mM was added before addition of lysostaphin, to prevent proteolysis during cell lysis (Sieradzki et al., 1999). Samples were then run on SDS gels along with fluorescent rainbow markers (Amersham ECL Plex) (Figure 3. 4E). The rainbow fluorescent marker used was composed of proteins linked to two dyes, Cy3 and Cy5; therefore the gels were scanned via the Cy3 and Cy5 channel and then overlaid onto the labeled PBP scan which was detected using the Cy2 channel (Figure 3.4F). This experiment was useful because the molecular weight of each PBPs band was demonstrated by using the rainbow marker. From the results of this experiment it was obvious that there was overproduction of PBP4 according to its molecular weight, but PMSF was not totally effective in preventing proteolysis.

Therefore, another approach to prevent proteolysis was to add bacterial Protease Arrest™ (Biosciences) at a concentration of 10µl/ml of membrane suspension. Protease arrest contains both irreversible and reversible protease inhibitors to inhibit serine, cysteine and other proteases, which is reported to inhibit over 90% of protease activities (GE Health Care Life Sciences). The protease-inhibitor was added before sonication of the cells and another 10µl was added during solubilization of membrane protein with 2% Triton-X100. Then the membrane proteins (15µg) from wild type and RS1/19 were labeled with Bocillin FL, the labeled PBPs were separated on SDS-PAGE and detected by a Typhoon scanning (Figure 3.4G). These results shows four clear bands with a minor band labeled with Bocillin FL positioned between PBP2 and PBP3 may be due a proteolysis product.

Overproduction of PBP4 in RS1/19 compared to Oxford strain is clearly evident and confirms that the high levels of *pbp4* mRNA, observed by DNA microarray (Chapter four), leads to high levels of PBP4 protein in membranes.

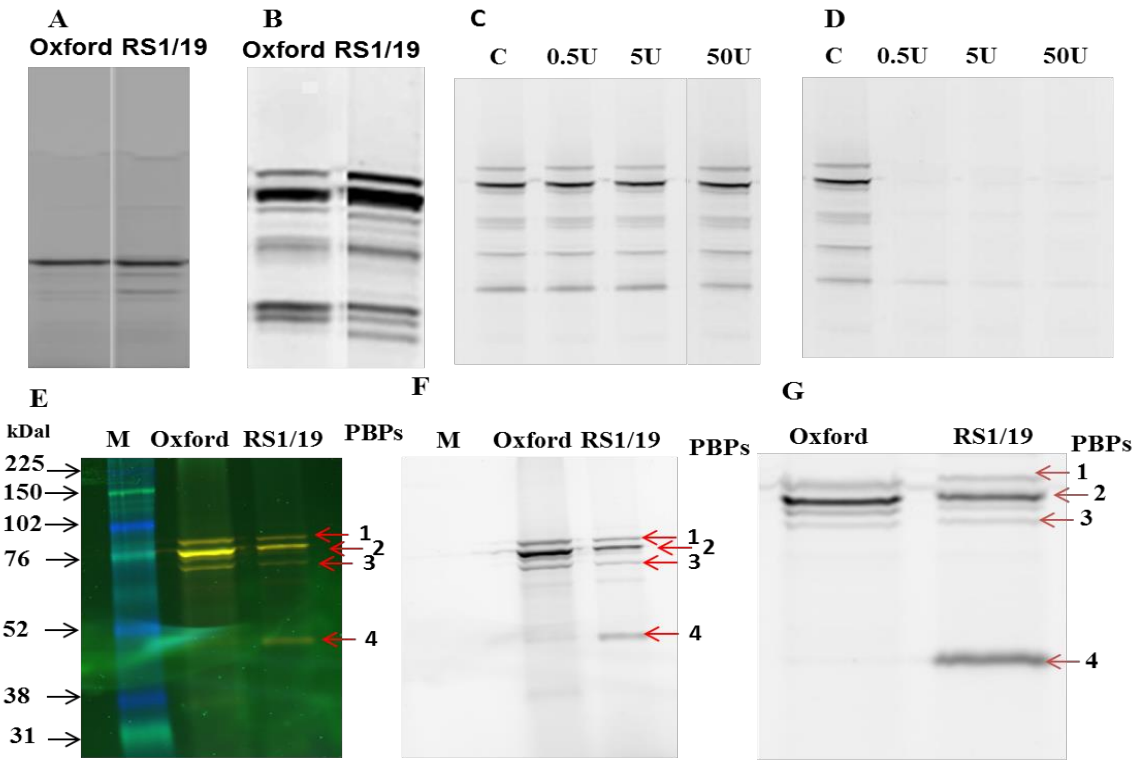
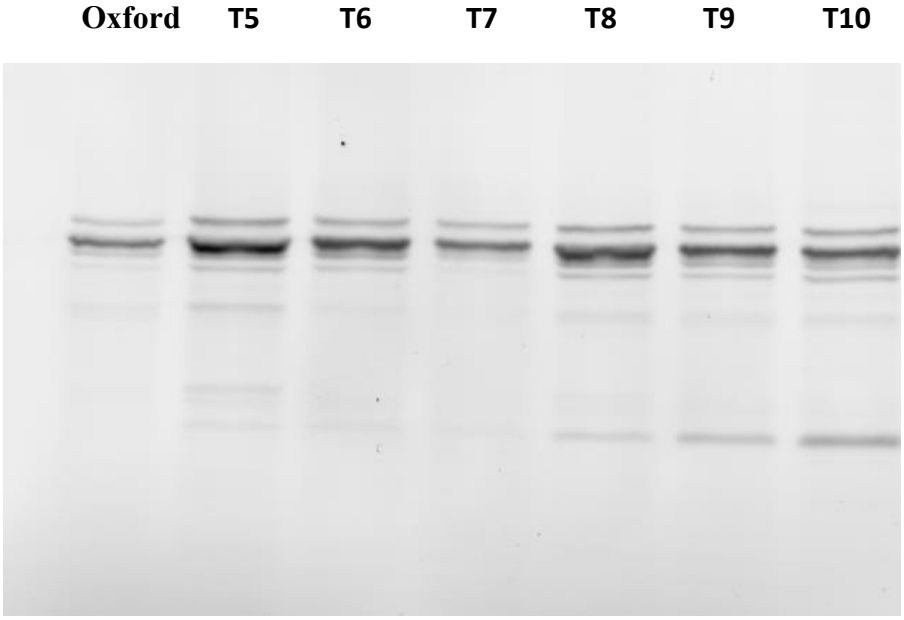


Figure 3. 4. Stages of optimization of membrane protein proteins in wild type Oxford and revertant strains. (A) Membrane prepared without sonication showing unclear bands, **(B))** showing proteolysis of the bands. Membrane proteins were pre-treated with increasing concentration of either penicilloic acid (C) or penicillin G (D) before being labeled with Bocillin FL. (C) membrane proteins pre-treated with increasing amount of penicilloic acid (C control, 0.5 U , 5U and 50 U). It is clear that the intensity of proteins bands remained the same in all lanes confirming that these bands are non specific. (D) Membrane proteins pre-treated with increasing concentrations of penicillin G (C control, 0.5U, 5U, and 50U) all the bands disappeared which means that the extra bands are the proteolysis product of PBPs. (E) Rainbow molecular weight marker (kDa) as scanned by a fluorImager using the Cy3 and Cy5 channel then overlaid, the positions of the single PBPs are indicated by arrows. (F) Shows the same image when scanned only with Cy2 channel therefore, the molecular weight marker disappears. **(G)** Bacterial protease arrest was used during lysis and solubilisation of membrane protein which leads to a reduction in proteolysis of membrane proteins. The positions of the PBPs are indicated by arrows. A minor band labelled with Bocillin Fl positioned between PBP2 and PBP3 may be a proteolysis product.

3. 3. 2. Relationship Between MICs, Gram Reaction and Penicillin Binding Profile of *S. aureus* after Each Transfer

MIC of an antibiotic is defined as the lowest concentration (maximum dilution) of antimicrobial that will inhibit the visible growth of micro-organisms after overnight incubation (Andrews, 2001). MIC determination was carried out for penicillin G using the macro broth dilution method following the British Society for Antimicrobial Chemotherapy (BSAC) standard methods (Andrews, 2001). For PBP analysis membrane proteins were prepared from CWC derivatives of each transfer from T5 to T10 strains. CWD strains were not used because they are unstable and need special requirments (CWD media supplemented with penicillin G), for this resean CWC strains were used. PBPs binding capacity of CWC strains was visualized by fluorescent penicillin in order to compare their PBPs profiles and also to investigate the relationship between changes in PBP4 activity and retention of penicillin G resistance in CWC variants.

In view of the fact that comparing penicillin-labeled bands of different strains can be affected by any changes in protein volumes loaded onto gels, PBPs ratios within a sample was considered to be a more sensitive and accurate assessment of PBP binding capacity. Assays of PBP ratios of CWC lines (Figure 3. 5), revealed great alteration in the penicillin binding capacity of PBPs. Scanning membrane proteins of T5, T6, T7, T8, T9, and T10 strains showed a gradual increase in PBP4 bands in comparison to the Oxford strains. This result is consistent with a gradual increase in the resistance to penicillin G. Interestingly, the increased PBP4 binding capacity and low PBP2/PBP4 ratio persisted in CWC derivatives when grown in antibiotic free media and was associated with stability of penicillin G resistance. These data may indicate that alteration in PBP4 activity plays an important role in obtaining high penicillin resistance stabilization in CWC derivatives. It is likely that the cell walls in CWD cells were disrupted, as indicated by Gram-negative staining and electron microscopy (Fuller et al., 2005). Together these data strongly imply that in addition to overexpressed PBP4, an altered cell wall might be associates with obtaining this form of penicillin resistance.



PBP2/PBP4 ratio	53.8	11.3	8.2	6.81	6.72	3.88	2.3
MIC µg/l	0.064	0.5	2	8	16	32	32

Figure 3. 5. Correlation between overexpression of PBP4 with decreased sensitivity to penicillin G. Bocillin FL was added to membrane proteins prepared from wild-type Oxford and CWC (T5 to T10) cells. PBPs were separated by SDS-PAGE electrophoresis and visualized using a Typhoon scanner. The intensity of PBP2 and PBP4 bands were measured, in each lane, and the ratios show increasing penicillin binding capacity of PBP4. MICs were determined by E-test.

To summarize, it can be concluded, from these experiments, that the decrease in susceptibility to β-lactam antibiotics in T10 is developed gradually, and is correlated with a variety of changes happened in these cells. The first step in developing resistance is through disruption of the cell wall, which was indicated by a Gram negative staining reaction. Meanwhile the cells are able to develop cell adaptation changes (likely through the gene mutations) needed for stable resistance to penicillin G. One change identified is an increase in penicillin binding capacity of PBP4 in membranes, which might indicate a role for this protein in resistance to penicillin G. Regarding RS1/19, the CWC strains which have been generated following each transfer in penicillin G was not available; consequently experiments were done only CWC strains (T5-T10).

3. 4. Characterization of Penicillin Resistant Lines

In previous experiments it was shown that increases in β-lactam resistance correlated with overexpression of PBP4 in membranes. Importantly this confirms the data for RS1/19, reported of Fuller et al. (2005), with an independently isolated strains. To investigate other modifications that occurred in these cells relating to stable resistance, both RS1/19 (Fuller et al., 2005) and T12 (T10 after two extra passage in CWD media in the absence of penicillin) were compared to wild-type Oxford strain. The logic for using two independent strains is that characteristics relating to the penicillin resistance mechanism should be consistently recognized in them.

3. 4. 1. Growth Rate Determination

Modification or alteration of any cell wall components by either mutation or environmental factors could potentially affect the total chemical, physical, and structural integrity of the cell wall, which may result in alterations in colonial morphology and growth characteristics. Growth rates give a simple indication of whether the revertant strains (RS1/19 and T12) behave differently compared with the wild-type Oxford strains.

The growth curve of the wild-type Oxford and the revertant strains RS1/19 and T12 were determined by measuring the absorbance readings at 600nm. Figure 3. 6 shows a growth curve that was established for these strains using three biological replications. BHI broth media was inoculated with an overnight culture to an A600

of 0.03. The culture were incubated at 37 ºC with shaking (150 rpm); growth was monitored for each of the three replicates for about 18hrs by recording the absorbance every 30 min for 5 h, then the culture left overnight and the last absorbance reading recorded after 18hrs. The mean generation times for each strain was determined from the exponential growth phase and the lag phase were also determined. For the wild-type the lag phase was 62 min and mean generation time was 40 min, in comparison the lag phases for RS1/19 and T/12 were 142 min and 135 min and the mean generation time were 64 min and 48 min respectively. The revertant strains feature much slower growth compared with the parental *S*. *aureus* cells, as the lag phase was over twice as long and the mean generation time was slower than the wild –type strain. Reduced growth rate, may be a further strategy to cope with the cell wall defects upon exposure to inhibitory concentrations of penicillin G. These cells might have a decreased metabolic rate due to the thicker cell wall, previously reported (Fuller et al., 2005).

After 18h, the absorbance reading was 2.36 for the Oxford strain and 2.26 and 2.29 for RS1/19 and T12, respectively, indicating that they grew to the same extent. Similar results have been reported for growth of cells with mutations in *femA;* after selection for lysostaphin resistance, the clones demonstrated a slow growth rate (Kusuma et al., 2007). Similarly, Hübscher et al., showed that inactivation of *femAB* resulted in a phenotypic adaptation (slow growth rate), which might be evidence for a common survival strategy in *S*. *aureus* (Hübscher et al., 2007).

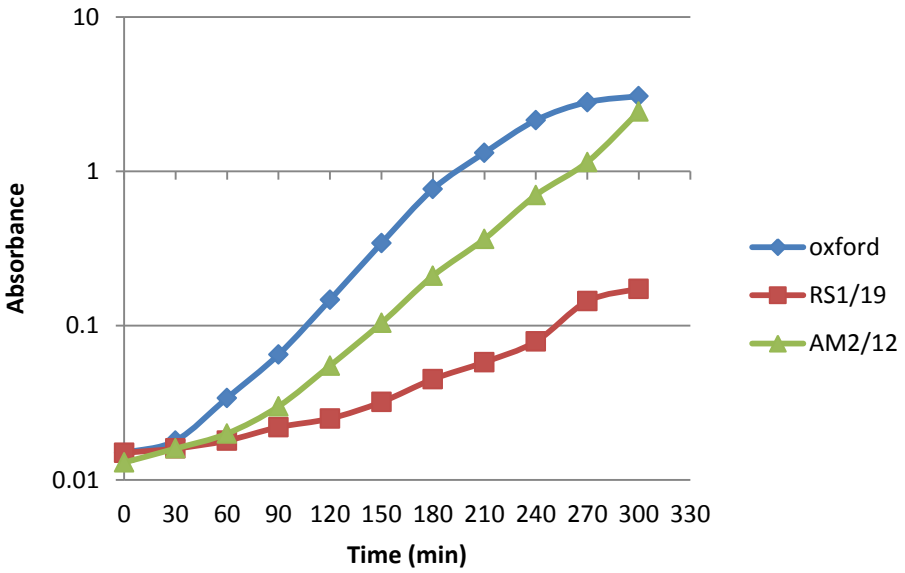


Figure 3. 6. Representative growth curve of wild type (Oxford) and revertant strains (RS1/19 and T12). Growth curves of the wild-type Oxford and revertant strains grown in BHI liquid medium at 37 °C showing that the revertant strains has a longer lag phase, slower mean generation time in comparison to the wild-type strain.

3. 4. 2. Overexpression Of PBP4 Correlates With Resistance To Lysostaphin.

Lysostaphin is an antimicrobial agent belonging to a major class of antimicrobial peptides and proteins known as the bacteriocins. It is an extracellular enzyme secreted by strains of *Staphylococcus simulans biovar staphylolyticus* (Schindler et al., 1964). The target of lysostaphin is the pentaglycine cross-bridge of peptidoglycan, where it cleaves specifically between the third and the forth glycine residues of the pentaglycine (Figure 3. 7).

PBP4 is a transpeptidase and carboxypeptidase which forms peptide bonds between adjacent stem peptide via a pentaglycine bridge; overexpression of PBP4 has been reported to lead to more pentaglycine cross-bridge of peptidoglycan in another strain (Pereira et al., 2007). Therefore lysostaphin susceptibility test were carried out to test whether this is also the case in RS1/19 and T12. According to this test cells with overexpressed PBP4 would be expected to lyse more slowly than cells with unchanged expression of PBP4 due to more pentaglycine bridge in the peptidoglycan. Lysostaphin susceptibility tests were carried out on cells grown to the exponential phase in BHI medium (Figure 3. 8). As anticipated, the revertant strains RS1/19 and T12 were more resistant to lysostaphin in comparison to the wild-type Oxford strain; 50% of cell lysis occurred after 6 min in wild-type cells and after 14.3 min and 14 min for the revertant strain RS1/19 and T12 respectively. These results confirm that the increased levels of PBP4 protein in membranes corresponds to increased PBP4 activity, correlates with increased lysostaphin resistant.

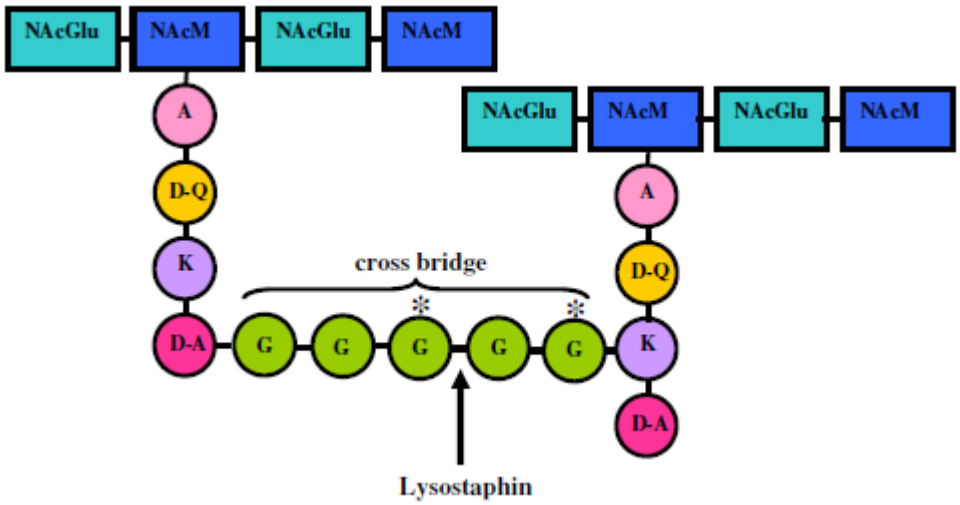


Figure 3. 7. Peptidoglycan structure in *S. aureus* showing the primary site of lysostaphin hydrolysis. NAcGlu, *N*-acetylglucosamine; NAcM, *N*-acetylmuramicacid; A, L-alanine; D-Q, D-glutamine; K, L-lysine; D-A, D-alanine; G, L-glycine.* indicates site of cleavage Adapted from (Bastos et al., 2010)

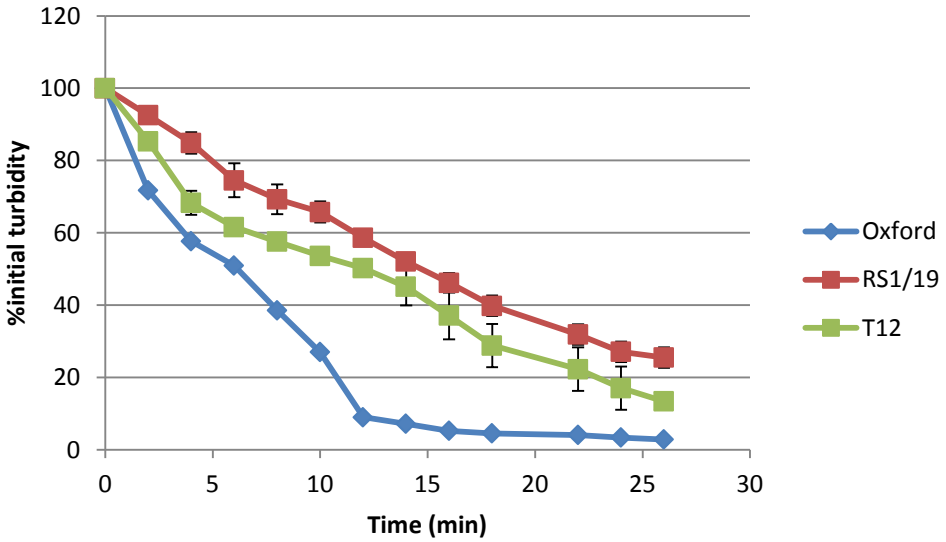


Figure 3. 8. Lysostaphin susceptibility of the wild-type (Oxford) and revertant strains RS1/19 and T12. Cells were grown to exponential phase at 37˚C then treated with lysostaphin (1U/ml). The revertant strains are more resistant to lysostaphin compared to the wild-type strain. Values are the mean of three experiments and the bars represents standard error of the mean.

3. 4. 3. Reduced Autolysis with Triton X-100 in Revertant Strains

Non-ionic detergents, like Triton X-100, have been used commonly to extract membrane associated enzymes without loss of activity (Komatsuzawa et al., 1994). This reagent also induces the release of acylated Lipoteichoic acid (LTA) and bacterial autolysis (Koch 2001). To do this, Triton X-100 has to penetrate the cell wall and therefore if the cell walls are altered in the revertant strain a difference in the rate of autolysis might be expected. Triton X-100-induced autolysis assays were performed on whole cells of the wild-type Oxford and revertant RS1/19 and T12 strains, following the method of Fournier and Hooper (2000) with minor modifications. Three biological replicates for each strain were tested.

The results show that 50% lysis occurred at 32.0 min for the wild-type strain while the revertant strain RS1/19 and T12 were more resistant, as 50% lysis occurred after 87 and 57 min respectively (Figure 3. 9),. This indicates an altered cell wall in the revertant lines, which is consistent with the earlier findings that suggest the cell walls of the revertant cells RS1/19 are thicker (Fuller et al., 2005) and this may explain the reduced autolysis in revertant strains. Abnormal wall thickness can be related to either lower rates of cell wall degradation or increased rates of cell wall biosynthesis. Increased thickness of the cell wall and slow growth rate has been shown to inhibit wall turnover and autolysis by blocking the access of murein hydrolases to their cell wall substrate (Sieradzki and Tomasz 1997). On the other hand, it has been reported that a similar substrate-arresting mechanism related to some quantitative or qualitative alterations in the wall teichoic acid led to reduced autolysis and thickened cell wall (Sieradzki and Tomasz 2003; McAleese et al., 2006). To conclude, these observations imply that reduction in the rate of autolysis is most probably related to an altered cell wall structure.

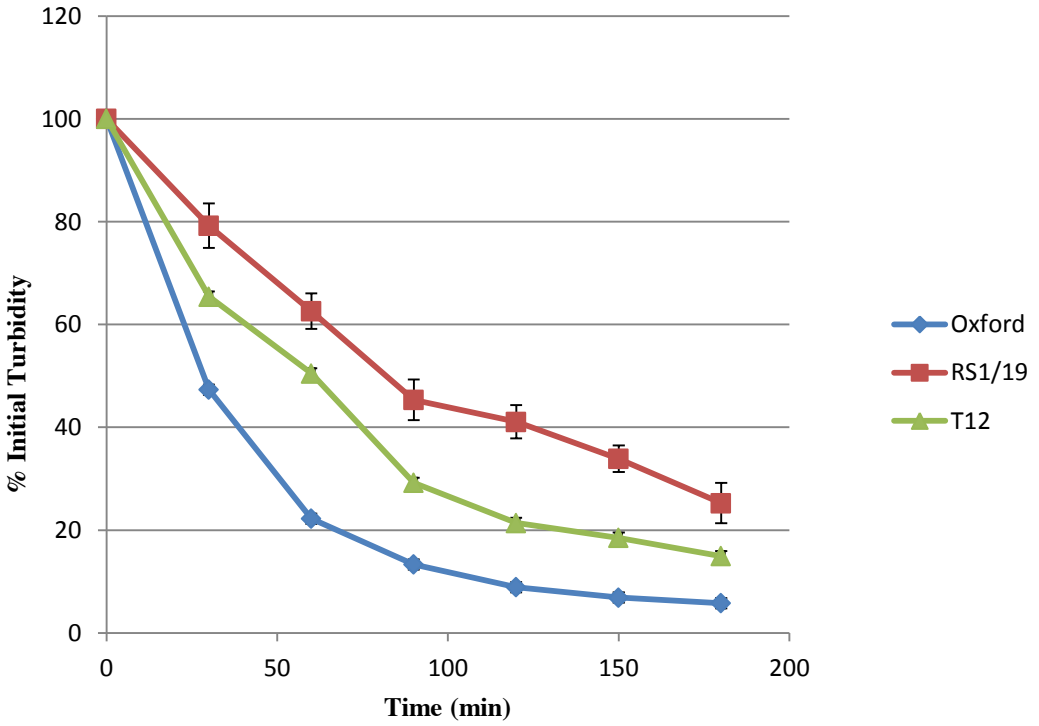


Figure 3. 9. Autolysis assay of Oxford strain and revertant strains RS1/19 and T12 in the presence of Triton X-100. Bacterial cells were collected from early exponential phase (OD_{600}= 0.7), washed twice with ice-cold water and re-suspended in an equal volume of Tris-HCl (pH 7.2), containing 0.05% (vol/vol) Triton X-100. The rate of autolysis was measured as the decline in optical density. Bars represent standard error of the mean.

3. 4. 4. Decreased Autolysis of Heat Killed Cells of RS1/19 Compared to Oxford Cells.

The *atl* gene encodes a bifunctional autolysin that can be purified from the culture supernatant as two extracellular peptidoglycan hydrolases (Liu et al., 2011)); the C-terminal domain of the protein has glucosaminidase activity and the N-terminal domain with the amidase activity (Foster, 1995). Quantitative peptidoglycan hydrolase analysis was carried out for both Oxford and RS1/19 strains to determine any reduction or absence of autolysin activity. It is thought that most of the autolysins of *S. aureus* are the products of one gene (*atl),* which can be visualized as a multiple bands during gel electrophoresis due to preform processing (Bose et al., 2012).

The autolysins extracted from both Oxford and RS1/19 cells were tested for activity using both strains as substrate, to identify any differences between these cells (Figure 3. 10). Firstly autolysins extracted from each type of cell (Oxford and RS1/19) were used to degrade the peptidoglycan from the heat-killed cells of the same line. After 4h of incubation it was obvious that the autolysins extracted from the Oxford strain hydrolyzed the peptidoglycan of the Oxford strain more efficiently as 60% lysis occurred at the 5h of incubation compared with only 10% lysis after 6h incubation when RS1/19 cells were treated with autolysin from RS1/19. This could be because either RS1/19 expressed reduced whole-cell autolytic activity, which results in less degradation of the peptidoglycan of RS1/19 cells or the cell wall of RS1/19 is thicker and more complex due to the accumulation of mutations during selection of this strain, which led to reduced susceptibility to autolytic activity.

To test whether the component of the cell wall in Oxford and RS1/19 has any effect on the autolysis activity, heat killed cells of Oxford strain used as a substrate for RS1/19 autolysins, and heat-killed cells of RS1/19 strains were used as substrate for Oxford strain (Figure 3. 10). The results showed that RS1/19 autolysins degrade peptidoglycan of the Oxford strain efficiently; however, Oxford autolysins do not have a significant effect on RS1/19 peptidoglycan. This means that the autolysins from both cells are highly active and the differences seen in rates of autolysis must therefore be related to the cell wall structures in the Oxford and RS1/19 strains. It is

possible that the thicker cell wall in RS1/19 leads to decreased autolytic activity, which is in agreement with the results reported by Utaida et al., 2006 who showed that reduced autolytic activity is a common phenotype to the glycopeptide-intermediate *S. aureus* (GISA) as a result of thickened cell wall after growth in the presence of vancomycin. In conclusion, data show that both crude autolysin preparations are nearly equally active but activity against different cell types varies (presumably reflecting differences in call wall architecture).

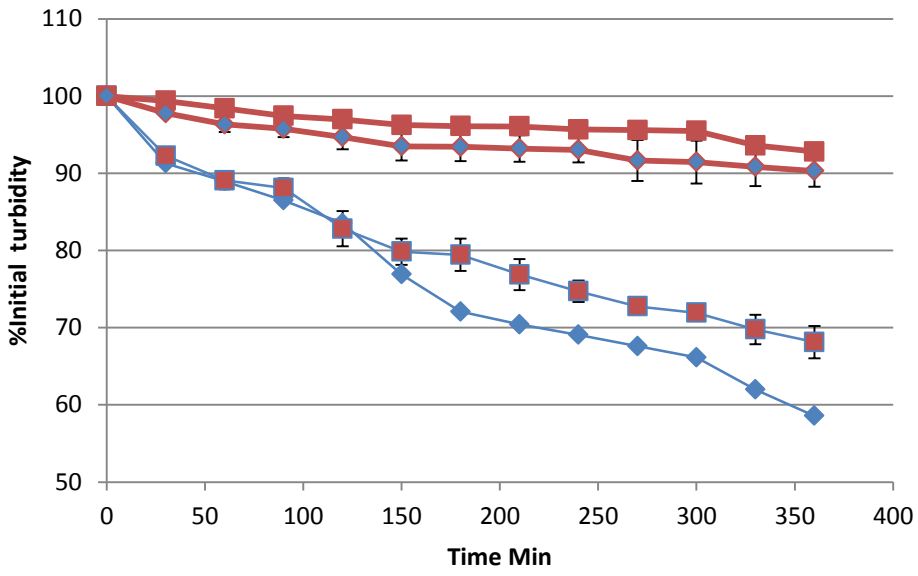


Figure 3.10. Whole cell autolysis activity profile of Oxford and RS1/19 Strains. 100µg of extracellular autolysin were added to a suspension of heat-killed and lyophilized cells and the decreased turbidity were measured over time. Extracellular autolysin extracted from Oxford cells (♦). Extracellular autolysin extracted from RS1/19 cells (■). Heat-killed cells prepared from Oxford strain (—), Heat-killed cells prepared from RS1/19 (—). Bars represents standard error of the mean.

3. 5. Analyzing the Role of PBP4 in Resistance Using ß-Lactam Antibiotics Which Have Different Affinities to PBPs.

Analysis of PBPs in a previous experiment (Section 3. 3. 1. & 3. 3. 2) showed an increase in binding of Bocillin FL to PBP4 in RS1/19 and T12. As RS1/19 also shows increased expression of the *pbp4* gene as indicated by microarray data (Appendix 1), the increased binding is likely due to an increase in the amount of PBP4 in the membranes of these strains.

It is known that PBPs differ in their binding affinity according to the penicillin derivative used (Chamber et al., 1992). Therefore differences in binding affinities to a variety of penicillins can be used as probes for PBPs activity (Sieradzki et al., 1997). For example, cefsoludin has a relatively high affinity for PBP1 and PBP3 (Georgopapadakou et al., 1980; Farha et al., 2013), penicillin G and cefotaxime have a high affinity for PBP2 (Farha et al., 2013) and cefoxitin has a high affinity for PBP4 (Farha et al., 2013). The antibiotic concentration required to block 50% of the binding of Bocillin FL to each PBP is defined as the IC50 (μg/ml) (Moison et al., 2010), which can be tested by direct labelling of PBPs with Bocillin FL after being pre-treated with each β-lactam antibiotic.

3. 5. 1. Comparison of the MIC of the Wild-Type and CWC Types of *S. aureus* to Different B-Lactam Antibiotics.

This experiment was designed to study the comparative susceptibilities of the wild-type and the revertant strain RS1/19 to different ß-lactam antibiotics. MIC determinations were carried out using both E-tests and macro broth dilution assays, following the British Society for Antimicrobial Chemotherapy (BSAC) standard methods (Andrew et al., 2001). E-test was performed to determine the MIC of *S. aureus* for penicillin G, while the macro broth dilution method was used for cefoxitin, cefotaxime and cefsoludin. All sensitivity tests used Iso-sensitest broth (Andrew et al., 2001).

Analysis of the comparative susceptibility of the *S. aureus* variants to β-lactam antibiotics showed dramatic differences in penicillin G susceptibility of 0.064 μg/ml in the wild-type (Oxford strain), and 24 μg/ml in the CWC derivative RS1/19 (about

375 fold). Significant elevation in cefotaxime resistance from 2 µg/ml in the parental strain to 128 µg/ml in the generated CWC types (about 64 fold) was also measured. Compared to the parental strain, CWC *S. aureus* variants were only slightly affected by cefoxitin and cefsoludin with two fold reduced susceptibility from 2 µg/ml and 4 µg/l to 4 µg/ml and 8 µg/ml, respectively (Table 3.2).

The observed increase in penicillin G resistance in the revertant strain is consistent with the results obtained by Elmer et al., (2002) and Fuller et al., (2005) who also observed stable retention of increased penicillin G resistance in CWC variants of *S.aureus* after reversion from CWD forms. Interestingly, the increased cefotaxime resistance of CWC types of *S. aureus* compared to the wild-type is a similar picture to MIC analysis with penicillin G and both of these antibiotics have a high affinity for PBP2.

The most likely explanation for drastically reduced susceptibility to penicillin G and cefotaxime, in RS1/19, is the low affinity of these β-lactam antibiotics to PBP4 (Georgopapadakou et al.1982). Penicillin G and cefotaxime have high affinities for PBP2 (Farha et al., 2013), this means that the transpeptidase of PBP2, which is inhibited by penicillin G and cefotaxime in the Oxford strain makes them sensitive to low concentrations of these antibiotics. In the case of the RS1/19 strain, the transpeptidase activity of overexpressed PBP4 may compensate for the inhibited transpeptidase activity of the other PBPs. In another words, higher penicillin G or cefotaxime concentrations are needed to bind to PBP4 to inhibit CWC bacterial growth, due to overexpression of PBP4 in RS1/19. This possibility is supported further by sensitivity of the revertant cells to cefoxitin and cefsoludin, which have high affinities for PBP4 (Georgopapadakou et al.1982).

Table 3. 2. β-lactam antibiotic susceptibilities of wild-type and resistant CWC cells grown on Iso-sensitest media.

Antibiotic	Oxford	RS1/19
	MIC (µg/ml)	
penicillin G	0.064	24
cefoxitin	2	4
Cefotaxime	2	128
cefsoludine	4	8

3. 6. Binding of Different β-lactam Antibiotics to Penicillin Binding Proteins in Oxford and RS1/19 Cells.

Fluorography of PBPs labeled with Bocillin FL has been the primary experimental method used to assay binding of β-lactam antibiotics. PBPs differ in their binding affinity among different species and according to the antibiotic used. Therefore, the binding affinity of PBPs in the wild- type and revertant RS1/19 strains to each β-lactam antibiotics were measured, in order to help understand the role of these proteins in the elevated resistance to penicillin G.

The association between resistance and altered binding affinity has been based largely on quantitative differences in binding to Bocillin FL to PBPs for susceptible compared with resistant strains. Binding has been measured by determination of IC50 (Chamber, 1994).

3. 6. 1. PBP Binding Studies: Competition Between Various β-Lactams and Bocillin FL.

To estimate the IC50 for each antibiotic, different amounts of β-lactam antibiotics (0.032 µg/ml to 64 µg/ml) were mixed with 100µg/ml of membrane proteins. This mixture was incubated for 30 min. prior to the addition of Bocillin FL.

Reduction of the detection of labeled PBPs with Bocillin FL by increasing antibiotic concentrations represents competition by that specific antibiotic.

Figure 3. 11 represent the affinities of each antibiotic for the PBPs in the wild-type Oxford strain. The 50% binding affinity was calculated by measuring the density of each PBPs for different amount of each antibiotics used. Figure 3. 12 exemplify the density measurements for penicillin G. Subsequently, the data was represented as a graph of relative density versus concentration of penicillin G used (Figure 3. 13). Similar analysis was carried out to calculate affinities of each antibiotic for the individual PBPs in the wild-type Oxford and RS1/19 isolates and is reported as IC50s (Figure 3. 14; Table 3. 3).

Penicillin G has a high affinity to PBP1, PBP2, and PBP3 in the Oxford strain with IC50s of 0.064, 0.4, and 0.1 μg/ml respectively. However, it shows a low affinity to PBP4 with an IC50 of more than 4μg/ml (Figure 3. 11; Table 3. 3). A very low concentration of this antibiotic (MIC 0.064 μg/ml) is sufficient to inhibit the growth of the Oxford strain. The same pattern was measured for cefotaxime, which possessed high binding affinity to PBPs 1, 2, and 3, while it has a low affinity to PBP4. In contrast cefoxitin and cefsoludin have high affinities for PBP4 with IC50s of 0.1 μg/ml; this means that in addition of inhibition of other PBPs, PBP4 is also inhibited in the presence of low concentration of these antibiotics.

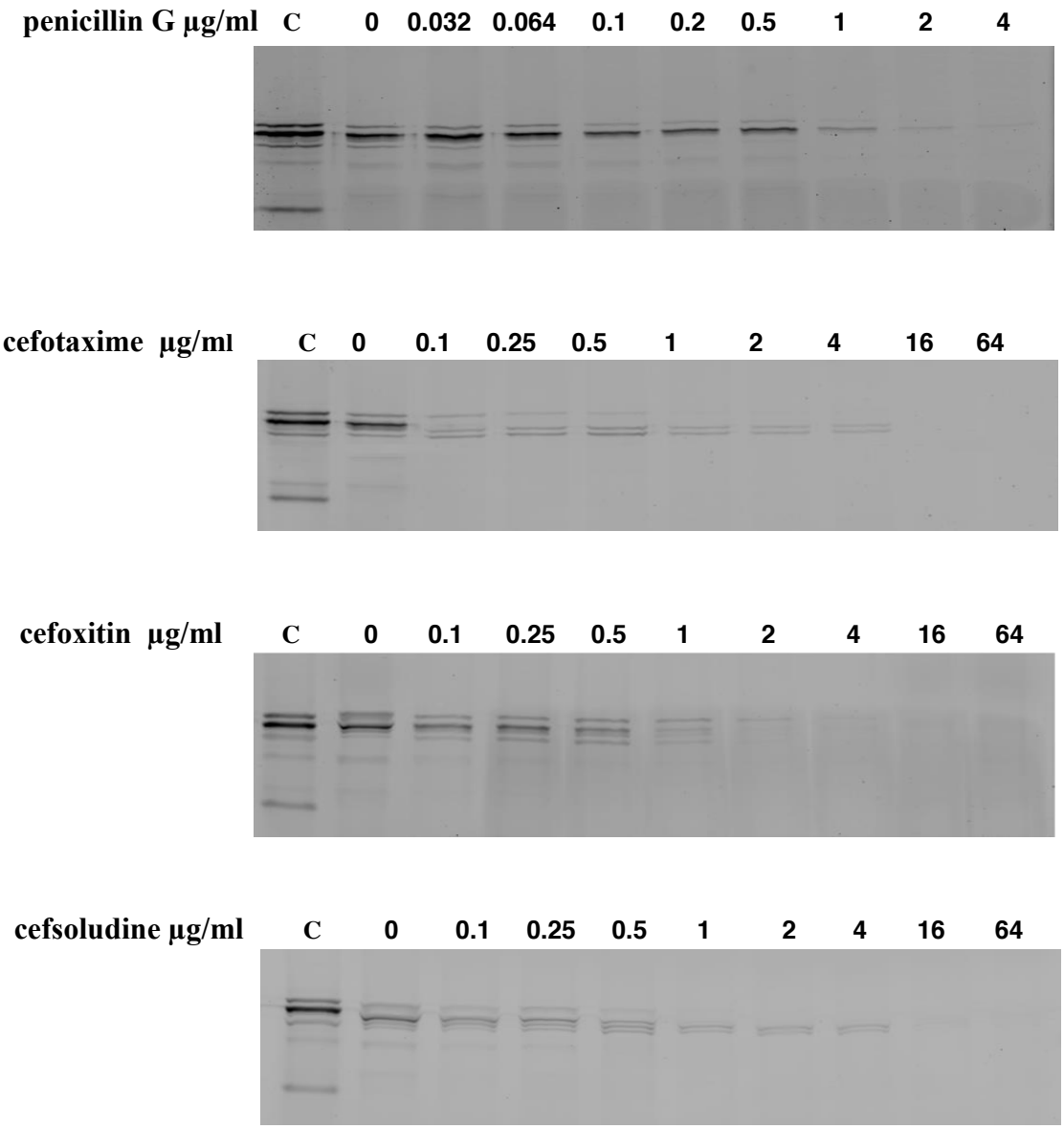


Figure 3. 11. Membrane proteins of wild-type Oxford strain pre-incubated with increasing concentrations of different β-lactam antibiotics (µg/ml). PBP profiles were detected by Bocillin FL. Antibiotic concentrations (µg/ml) adjusted in all samples to a standard amount of loaded proteins (100µg). First lane (C) is control sample of RS1/19 to show the position of PBP.

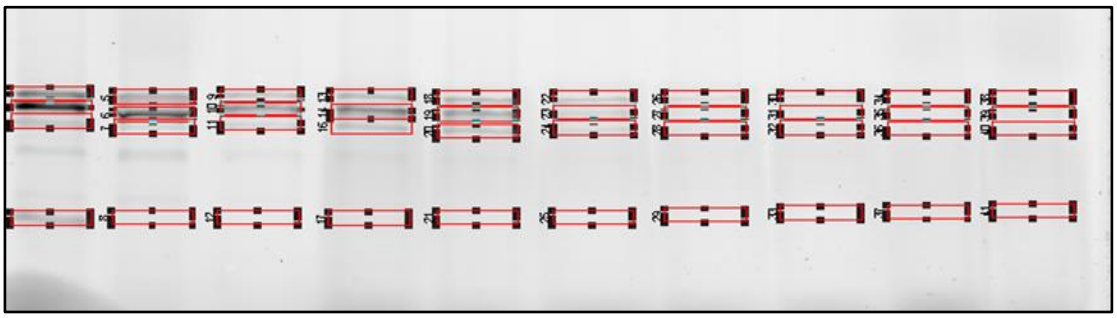


Figure 3. 12. Cell membrane of wild-type Oxford strain. The intensity of each penicillin binding protein band (PBP1-4) was measured within each square and for every concentration of antibiotic used in each lane.

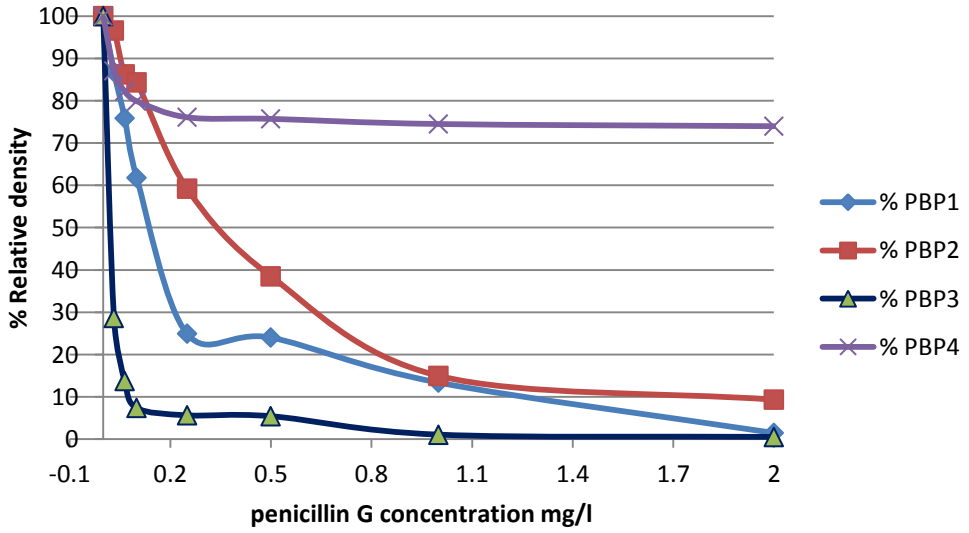


Figure 3. 13. Graphic representation to determine IC50 for each PBPs for membrane protein of wild-type Oxford strain after being pre-treated with penicillin G. the 50% binding affinity was calculated by measuring the density of each PBPs to determine IC50.

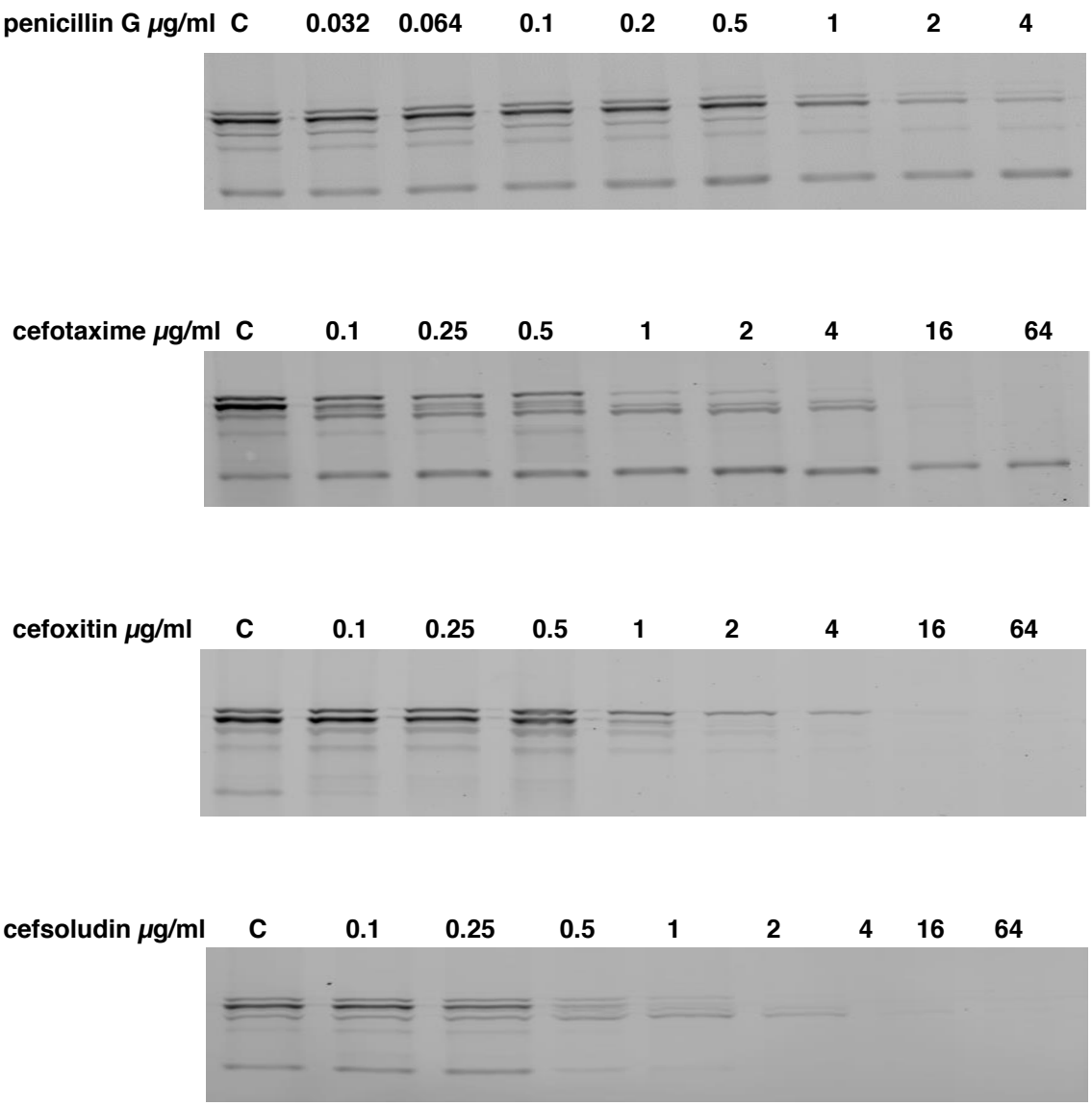


Figure 3. 14. Cell membranes of strain RS1/19 pre-incubated with increasing concentrations of different β-lactam antibiotics (μg/ml). PBP profiles were detected by Bocillin FL. Antibiotic concentrations (μg/ml) adjusted in all samples to a standard amount of loaded proteins (100μg). First lane (C) is control sample of RS1/19 to show the position of PBP.

Table 3. 3. Binding affinities of β-lactam antibiotics for *S. aureus* PBPs

Antibiotic	IC50(*μ*g/ml)							
	Oxford				RS1/19			
	PBP1	PBP2	PBP3	PBP4	PBP1	PBP2	PBP3	PBP4
penicillin	0.2	0.4	0.1	>4	0.2	0.5	0.1	>64
Cefotaxime	0.1	<0.1	0.5	64	0.5	0.1	0.2	>64
cefoxitin	0.5	0.2	<0.1	0.1	1.0	0.5	1.0	0.1
Cefso-ludin	0.1	0.1	0.5	0.1	0.2	0.2	1.0	0.5

Binding assay for each individual PBP in RS1/19 demonstrated that penicillin G and cefotaxime have a low affinity to PBP4 compared to PBP2 (Figure 3. 14). Therefore, significant increases in the amount of these antibiotics were required for PBP4 inhibition, with IC50s of >64μg/ml (Table 3. 3). This result is in agreement with the results -obtained by Kosowska-shick et al., 2010 who showed that cefotaxime inhibits 50% of binding affinity of PBP4 at about 8 fold higher than it inhibits of PBP2 and those of Miller et al., 2005, who observed that the penicillin G IC50 value was 32μg/ml for PBP4 and 0.2 μg/ml for PBP2. Moreover, from results obtained in the present study, it is obvious that cefoxitin has a relatively high affinity to PBP4 with an IC50 of 0.1 μg/ml (Table 3.3), which means that the over expressed PBP4 is inhibited in a very low concentration of cefoxitin. Likewise, cefsoludin inhibits PBP4 at a very low concentration, as it has an IC50s of 0.5μg/ml.

In summary, RS1/19 cells have a moderately increased resistance to cefoxitin and cefsoludin, but a marked increase in resistance to cefotaxime and penicillin G. This supports the proposal that overproduction of PBP4 plays important role in reduced cefotaxime and penicillin G sensitivities. Therefore, it is likely that these cells can withstand inhibition of the transpeptidase activity of PBP2 because of the high activity of PBP4. This is confirmed by an increase in the IC50 of penicillin G and cefotaxime for PBP4 in revertant types with almost no change IC50 for PBP2. MIC values of antibiotics for the revertant cells correlated directly with increases in the IC50 for PBP4.

3. 6. 2. Effect of Cefoxitin, Cefotaxime, and Cefsoludin on Penicillin G Sensitivity of Wild-Type Oxford and Revertant RS1/19 Strain.

To test the hypothesis that PBP4 is an essential PBP, in RS1/19, the penicillin G MIC was determined when in combination with β- lactam antibiotics that have different affinities for each PBP. The antibitoics used were cefoxitin, which preferentially binds PBP4 (Chambers et al., 1994), Cefotaxime, which preferentially binds PBP2 (Sieradzki et al., 1997), and Cefsoludin, which binds PBP1, PBP2 and PBP4 at low concentration (IC50 0.1μg/ml) (Table 3. 4). These β-lactam antibiotics were used to inhibit selected PBPs and assess the effect of this interference on sensitivity to penicillin G.

MICs of Penicillin G were determined for S. *aureus* strains grown in ISA media containing 25% of the MIC of the different β-lactam antibiotics (cefoxitin, cefotaxime, and cefsoludin). Expression of penicillin G resistance of parental and CWC forms of *S .aureus* altered variably under these conditions. Cefoxitin was the most effective in reducing the MIC of penicillin G in RS1/19 (Table 3. 5); it also decreased penicillin G resistance of wild-type cells, but not as significantly as with RS1/19 cells (4 fold with 25% of MIC of cefoxitin).

PBP4 seems to have an important role in *mecA* negative methicillin-resistant strains. Increased susceptibility to penicillin G in the presence of cefoxitin and reduced binding of Bocillin FL to PBP4 following pre-treatment with cefoxitin indicate that inhibition of PBP4 has an important role in penicillin G resistance of CWC types. However, if the reduction in penicillin G resistance is due to the synergistic activity of both cefoxitin and penicillin G only, reduction in resistance might be expected to be similar in both wild-type and revertant cells. The molecular and biochemical basis by which overexpressed PBP4 might confer resistance is not entirely clear though it may be that PBP4 overexpression in RS1/19 cells can replace an essential transpeptidase function of one or more high molecular mass PBPs. It has been shown that β-lactam resistance might be changed by targeting PBP2 and PBP4 with combinations of β-lactam antibiotics that have different affinities for these PBPs. For instance, using a combination of antibiotics which have high affinity to PBP4 (cefoxtin) and the PBP2-selective β-lactams cefuroxime, ceftizoxime, oxacillin, and penicillin, decreased the MICs of these antibiotics between 32- to 128-fold. Conversely, combining cefoxitin with β-lactams having a low affinity for PBP2 led to only a 2- to 8-fold change in the MIC (Farha et al., 2013).

Table 3. 5. Analysis of penicillin G sensitivities of *S.aureus* variants grown in the presence of 25% of the MICs of cefoxitin, cefotaxime, and cefsoludin.

	penicillin G(*μ*g/ml)						
Strains	**PG MIC**	**25%MIC Fox**	**fold decrease**	**25%MIC cef**	**Fold decrease**	**25%MIC cso**	**fold decrease**
Oxford	0.064	0.016	4	0.016	4	0.016	4
RS1/19	24	0.094	255	6	4	8	3

*PG, penicillin G; fox, cefoxtin; cef, cefotaxime; and cso, cefsolodin.

Additionally, cefotaxime also caused an increase in penicillin G sensitivity of both the wild-type and revertant RS1/19 strains (4 fold with 25% of MIC of cefotaxime). Hence cefotaxime had similar effects on penicillin sensitivity of wild-type and CWC variants. Accordingly, reduction of MICs of penicillin G of *S.aureus* forms with cefotaxime could be due to synergistic activity of both antibiotics. However, other reasons for alteration of penicillin susceptibility of *S. aureus* variants by cefotaxime cannot be ruled out from results obtained in these experiments. Compared to cefoxitin, when cefotaxime was included in the ISA medium at a concentration 25% MIC, the MIC for penicillin G dropped from 24 to 6, because both of these antibiotics has a greater affinity for PBP2 than PBP4 and in combination did not dramatically alter the MIC. Cefsoludin led to an increase in penicillin G sensitivity of both wild-type (4 fold with 25 % MIC of cefsoludin) and (3 fold with 25% MIC of cefsoludin) in the revertant RS1/19 strain (Table 3. 5).

In summary, the reduced sensitivity to penicillin G in CWC cells is likely related to PBP4 overproduction, as antibiotics that selectively inhibit PBP4 activity cause the greatest reduction in penicillin G MIC, when used in combination. This is evidenced by a remarkable rise of penicillin sensitivity of cells following treatment with the PBP4 selective antibiotic, (cefoxitin).

3. 6. 3. Effect of sub-inhibitory concentrations of cefoxitin on cefotaxime MIC in the wild-type and revertant RS1/19 strains.

Cefotaxime like penicillin G has a higher affinity for PBP2 than PBP4 (>200 fold and 1000 fold) respectively (Georgopapadakou et al., 1982). Like penicillin G, the MIC of cefotaxime rose dramatically in CWC cells (64 fold). Therefore, an experiment were carried out to investigate whether cefotaxime behave like penicillin G if it is used in combination with 25% MIC of cefoxitin.

The cefotaxime MIC was tested for *S. aureus* wild-type and RS1/19 strains, grown in ISA media mixed with 25% MIC of cefoxitin (Table 3. 6); interestingly, the results were very similar to results with penicillin G. The RS1/19 strain exhibited a 32 fold reduction in MIC to cefotaxime while, the wild-type strain revealed only 4 fold reduction in their MIC to cefotaxime.

Table 3. 6. Cefoxitin increases Cefotaxime sensitivity of the wild-type Oxford and the revertant RS1/19 strains.

Strains	cefotaxime MIC(μg/ml)		
	Cefotaxime MIC	**25% MIC Cefoxitin**	**fold decrease**
Oxford	2	0.5	4
RS1/19	128	4	32

These data again suggest that the increased activity of PBP4 is crucial for resistance in RS1/19, possibly because the transpeptidase activity it encodes is able to replace for cefotaxime inhibited PBP2 transpeptidase activity.

To conclude, the data obtained in this chapter indicate that the reduction in susceptibility to penicillin G in CWC cells (RS1/19 and T12) is developed gradually. The resistant cells were growing slower than the wild-type Oxford strain and more resistant to lysostaphin and Triton X-100 induced autolysis. Moreover, Membrane protein prepared from the cells of each transfer showed a gradual increase in the PBP4 band in comparison to Oxford strain. Interstingly, the reduced sensitivity to penicillin G in CWC cells is likely related to PBP4 overproduction, as antibiotics that selectively inhibit PBP4 activity cause the greatest reduction in penicillin G MIC, when used in combination. These data suggest a role for PBP4 overproduction in the penicillin resistance observed in RS1/19 and T12 strains, therefore it is necessary to overexpress *pbp4* in *S. aureus* of different genetic background to check if PBP4 overproduction is necessary and sufficient for the observed resistance in RS1/19 and T12 strains.

CHAPTER FOUR

Effect of Overexpression of PBP4 on the Susceptibility of *S. aureus* to β-lactam Antibiotics

4. 1. Introduction

It is well accepted that cell wall-active antibiotics inhibit bacterial growth by inhibiting peptidoglycan biosynthesis (Walsh, 2003). *S*. *aureus* and other bacteria undergo an extensive program of gene and protein expression when treated with cell wall-active antibiotics (Singh et al., 2001) and cell wall stress stimulon is the term used to describe altered gene expression triggered by antibiotics that inhibit cell wall biosynthesis (Utaida et al., 2003).

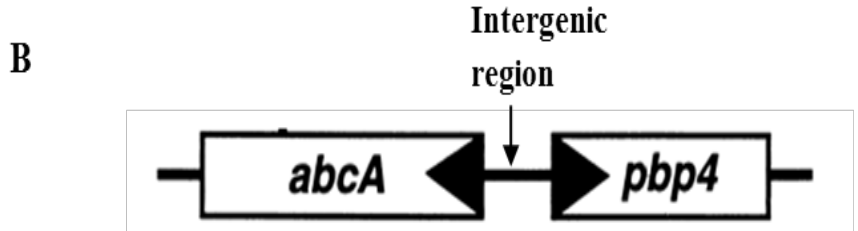
The effect of antibiotics on global bacterial gene expression is dose dependant. At low concentration, the number of transcripts regulated by the antibiotic is low, increasing until around 5% of total bacterial transcripts at concentrations close to those that inhibit growth. The number of regulated transcripts is reduced at higher concentration of antibiotics. This curve reflects the different effect of antibiotics on bacterial gene expression, at low concentration antibiotics might act a signalling molecules regulating specific gene expression. Upon increase in concentration stress responses sum up to the panel of regulated genes and at high inhibitory concentrations the observed changes mainly deal with the mechanism of action of antibiotic and cell growth inhibition (Fajardo et al., 2008).

Increased microbial resistance to penicillin has been widely reported, and a number of resistance mechanisms have been observed. Amongst them is resistance that is mediated through a plasmid-encoded β-lactamase (*blaZ*), which hydrolyses the β-lactam ring of penicillin and many of its derivatives (Bondi and Dietz, 1945; Novick, 1963). While, in some strains of *S*. *aureus* methicillin resistance is due to the production of a low affinity PBP2a, which is encoded by the *mecA* gene (Song et al., 2004). Methicillin resistance relies upon a number of other genes, *fem*, for the expression of methicillin resistance; these housekeeping genes have a direct or indirect role in peptidoglycan synthesis or turnover and their correct function is essential for full expression of resistance (Berger-Bächi and Rohrer, 2002), because in *mecA* positive MRSA strains mutations in *fem* genes lead these strains to be oxacillin sensitive (Giannouli et al., 2010). Another class of borderline methicillin-resistance is due to an alteration in the targets of these drugs, resulting in a decrease in or loss of affinity for β-lactams of crucial PBPs (Malouin and Bryan 1986). An

example of altered methicillin-binding affinities of PBP2 occurred as a result of a point mutation near the penicillin-binding motif of the transpeptidase active site (Hackbarth et al., 1995).

In *mecA* and β-lactamase negative *S. aureus* increased levels of PBP4 in the membrane following growth in increased concentration of penicillin, have been shown to be related to methicillin resistance (Fuller et al., 2005). In another example, the methicillin-resistant mutant strain PV1 was selected *in vitro* with increasing concentrations of penicillin. The increase of resistance was correlated with changes in the amount of PBP4. The subsequent Tn551-mediated insertional inactivation of PV1 yielded a susceptible mutant strain, and no PBP4 was visible in membrane preparations. This was due to integration of the transposon into the *pbp4* gene itself or into a region controlling or interacting with its expression. The *abcA-pbp4-taqD* sequences of wild-type *S. aureus* and of PV1 showed that the *in vitro* resistant strain PV1 had a single adenosine insertion and a 90 nucleotide deletion in the putative promoter region of *abaA* and *pbp4* (Figure 4. 1. A) (Henze and Berger-Bächi, 1995). The *pbp4* gene is transcribed from a single promoter, the -10 region of which is reported to lay 264 base pairs upstream of the translational start site, resulting in a single transcript. The promoter region for *pbp4* overlaps with the promoter for a divergently transcribed gene, *abcA*, by about 80 base pairs (Figure 4. 1. B) (Domanski et al., 1997). The *abcA* gene encodes an ABC transporter, which is involved in the transport of antibiotics, heavy metals, hemolysins, and metabolites (Domanski and Bayles., 1995; Remy et al., 2012). Interestingly, a *S. aureus* strain with a deletion of *abcA* results in increased expression of *pbp4* and increased peptidoglycan cross-linking, as well as increased resistance to PBP4-specific antibiotics such as imipenem and cefoxitin (Domanski et al., 1997).

Adapted from Henze and Berger-Bachi. Antimicro. Agents Chem. 1996, 40(9): 2121-2125



Adapted from Schrader-Fischer and Berger-Bachi. Antimicro. Agents Chem. 2001, 45(2): 407-412

Figure 4.1. A. Nucleotide sequence of the intergenic region of *abcA* and *pbp4* from *S. aureus* PV1, including portions of each open reading frame. The direction of transcription of *pbp4* and *abcA* is indicated by head arrows. Putative ribosome binding sites are underlined. The 90 nucleotide deletion and an adenosine insertion which occurred between GA of the mutant strain PV1 are indicated by small letter case. **B**. Schematic diagram of the locus containing *abcA* and *pbp4*

Microarrays are one of the most powerful techniques that allow researchers to analyse expression of many genes in a single experiment quickly and efficiently. The simplest way to calculate the fold change is to divide the expression level of a gene in the sample by the expression level of the same gene in the control. A value of one indicates unchanged expression, less than one indicates a down-regulated gene, and more than one an up-regulated gene (Knudsen, 2004).

The initial aim of the experiments described in this chapter is to verify the results of microarray data of RS1/19 cells, using qRT-PCR to examine the steady-state mRNA levels of selected genes. Another part is to study the effect of PBP4 over-expression on the sensitivity of *S*. *aureus*, with different genetic background, to penicillin. T12 was considered phenotypically similar to RS1/19, therefore only one strain RS1/19 was taken forward.

4. 2. Results and Discussion

4. 2. 1. Microarray Data Revealed Overexpression of Genes Related to Cell Wall Biosynthesis.

Comparison of the transcriptional profile of the Oxford parental strain and the *in vitro* selected penicillin resistant strain, RS1/19, was performed previously in our lab (T. Fawcett, unpublished) in order to identify genes that may be contributing to penicillin resistance. As many as 145 genes were found to be up-regulated and 91 genes were down regulated in RS1/19 compared to the Oxford strain. In RS1/19 stable transcript reprograming of several cell wall related genes occurred following the challenge with penicillin; among them were *pbp4*, *murA*, *murG*, and *lytM*. These alterations in the cell wall are believed to prevent access of penicillin G (Fuller et al., 2005).

The gene encoding penicillin binding protein 4 (*pbp4*) displayed the highest relative expression level in RS1/19 (Appedix 1), of about 18.6 fold changes. However, the levels of the genes encoding the other PBPs (1-3) were unchanged. Other genes that encode enzymes involved in cell wall biosynthesis showed significant changes in expression in RS1/19; UDP-N- acetylglucosamine1-carboxyvinyltransferase (*murA*), which is the enzyme that catalyze the first step of peptidoglycan biosynthesis by the formation of UDP- Glc-NAc from fructose-6-phosphate (Barreteau et al., 2008) was up-regulated by 2.5 fold. Similarly, beta-N-acetylglucosaminyltransferase (*murG*), which is a transferase that adds GlcNAc from UDP-N- acetylglucosamine to lipid linked Mur-NAc pentapeptide to form undecaprenyl pyrophosphate linked disaccharide pentapeptide (lipid II) (Schneider and Sahl, 2010) was up-regulated 2.31fold. Up-regulation of these enzymes may be a response by the cell wall to increase the rate of peptidoglycan synthesis. *murA* appears to be the primary enzyme utilized in the staphylococcal cell for catalyzing the first step in peptidoglycan biosynthesis (Figure 4. 2); in *murA* inactivated cells the peptidoglycan content was reduced by approximately 26% and cells exhibited a decreased growth rate and final culture density (Blake et al., 2009). The Overexpression of *murA*, *murG*, and *pbp4*, may be responsible for catalyzing more substrate needed for peptidoglycan biosynthesis in the cell wall. This may explain why the RS1/19 peptidoglycan is thicker than that in the Oxford strain (Fuller et al., 2005). Additionally, expression of

peptidoglycan hydrolase (*lytM*) mRNA increased 2.16 fold in RS1/19. LytM is suggested to be a lysostaphin-type endopeptidase with a glycyl-glycine target (Singh et al., 2010). This protein has been shown to have enzymatic activity that reduces muropeptide cross-linkage and glycan chain length (Pieper et al., 2006). However, overexpressed LytM is suggested to cause destruction of the cell wall and enhance cell lysis (Ingavale et al., 2003). LytM overexpression in RS1/19 may be required to promote septal peptidoglycan splitting and eventual daughter cell separation due to a thicker cell wall. On the other hand, reports indicated that overexpression of *lytM* in vancomycin resistant *S*. *aureus* (VRSA) plays some role in resistance to vancomycin and probably other cell wall inhibitor antibiotics (Pieper et al., 2006).

To conclude, the *pbp4* gene had the highest relative expression level in RS1/19 and reports from other studies indicate that high levels of PBP4 can have a role in penicillin resistance (Henze and Berger-Bächi, 1996, Fuller et al., 2005, and Banerjee et al., 2010). Therefore, experiments in this chapter were designed to study the effect of this protein on the sensitivity to penicillin in RS1/19.

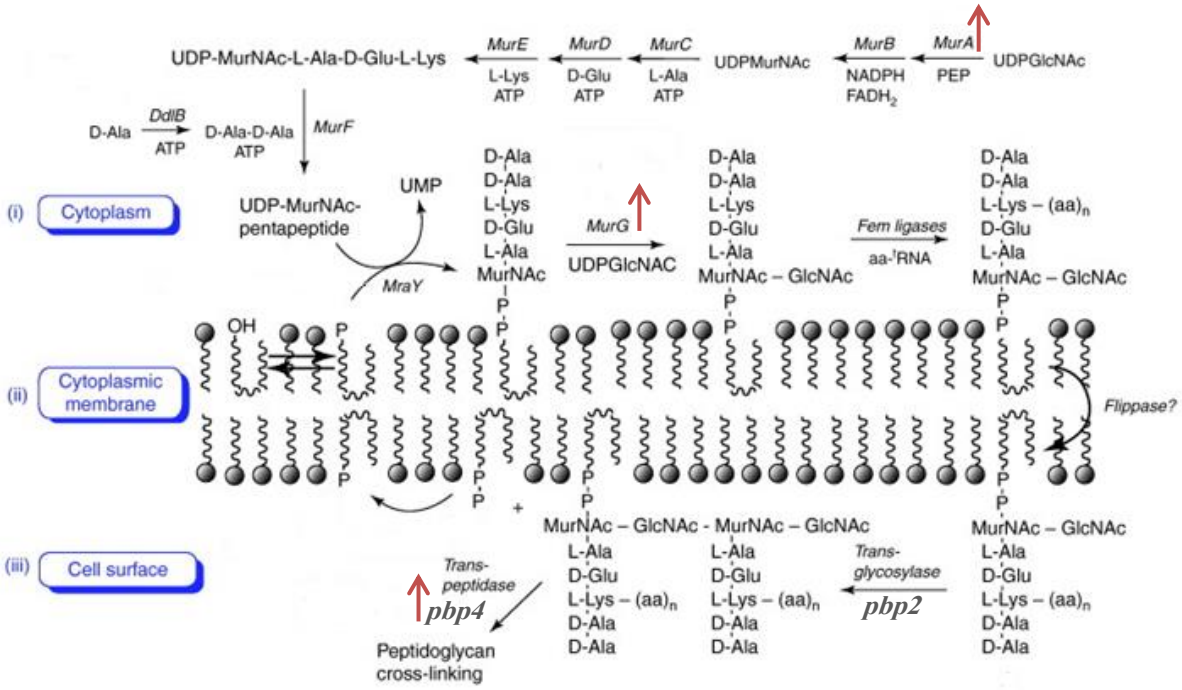


Figure 4. 2. Schematic diagram of cell wall biosynthesis in *S. aureus* showing genes up-regulated during treatment with penicillin G. The synthesis of peptidoglycan precursors is initiated in the cytoplasm, where 6 ligases (*MurA-F*) catalyze the formation of the peptidoglycan subunit, lipid II. Interestingly, the *murA* and *murG* genes, which encode two of these ligases have been up-regulated according to microarray data. Lipid II subunits are then flipped to the membrane-cell wall interface, where they are cross-linked by the penicillin-binding proteins (PBPs). Bifunctional protein PBP2 mediates both transglycosylation and transpeptidation, while other PBPs, especially PBP2 and PBP4, can crosslink the lipid II pentapeptide to a pentaglycine cross-bridge via transpeptidation activity. PBP4 was also up-regulated according to the microarray data. The up-regulated genes are indicated as red arrows. (Images adapted from Bugg et al., 2011).

4. 2. 2. Validation of Microarray Assay by Real-Time RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was performed to validate microarray data. As the original RNA used for the microarray analysis was not available, new RNA samples were extracted using the same conditions that were used for that experiment. Cells were grown in CWD medium until the early exponential phase then RNA was extracted, quantified by NanoDrop 1000 Spectrophotometry and cDNA synthesis carried out using 1µg of total RNA, using random hexanucleotide primers. PCR primers were designed for each gene to be quantified (Table 4.1) and the specificity of each primer was analysed by standard PCR and agarose gel electrophoresis, to establish that they gave PCR products of the expected sizes (Appendix 1). Additionally, three primer pairs, for *gyrA*, *nuc* and *rho* (DNA gyrase subunit A, Nuclease and Transcription termination factor respectively) were used to represent fragments of three internal control genes that had previously been used for a similar purpose (Theis et al., 2007). qRT-PCR experiments were used to test these internal gene primers and the stability of their expression in Oxford compared to RS1/19 strains. *gyrA* was found to be the most reliable primer as it showed stable expression in both strains; therefore this gene was chosen as a control reference gene to use in qRT-PCR experiments. PCR was carried out using a Rotor-Gene 3000 (Corbett Research) machine using thermal cycling condition as follows: at 95ºC for 10min followed by 95ºC for 20sec, 54-56ºC (according to the primers melting temperature) for 20sec, and at 72ºC for 20sec. The data were then subjected to analysis using the Relative Expression Software Tool (REST 2009) program, which is a software tool used to determine changes in the target gene expression standardized by non-regulated reference gene (Corbett Research 2006).

A selected group of five genes showing up- or down- regulation was chosen to test the validity of the microarray data (Figure 4. 3). In general, there was a good correlation between microarray data and real-time RT-PCR data (Table 4. 2), as data using the two methods. Some variation between the two methods was seen for *narG* which was measured as having a 10-fold reduction in expression level in RS1/19 using the microarray and only 2-fold reduction when measured by qRT-PCR.

Table 4. 1. Primers used for qRT-PCR experiments

Gene	Primer sequence forward (F) and reverse (R)	Amplicon size	Reference
Nuc	F: tgtagtttcaagtctaagtagctcagcaa R: tgcactatatactgttggatcttcagaa	96bp	Alarcon et al., 2005
Rho	F: cgacgttcagacaatttacg R: gaaatattgaagcgggtgg	145bp	Balaky, 2010
gyrA	F: gttgatggccaaggtaactttgg R: gatacctgatgcaccattgg	210bp	Balaky, 2010
pbp4	F: aagtatggacaatcgcagacc R: aaaccatctgtacccggcaaac	405	This study
murG	F: tcctaagcttccacccataacg R: ctgacttaacaccaggattagcg	210	This study
lytM	F: aacagcagcagcgattgaac R: tcaggtgtgtgatgataatcccc	230	This study
narG	F: tatcccagtctgaacgcgattc R: acttcagaatgacggcaacacc	207	This study
tpi	F: atcaatatcagtttgtgcca R: tggcacaaactgatattgatgg	86	This study

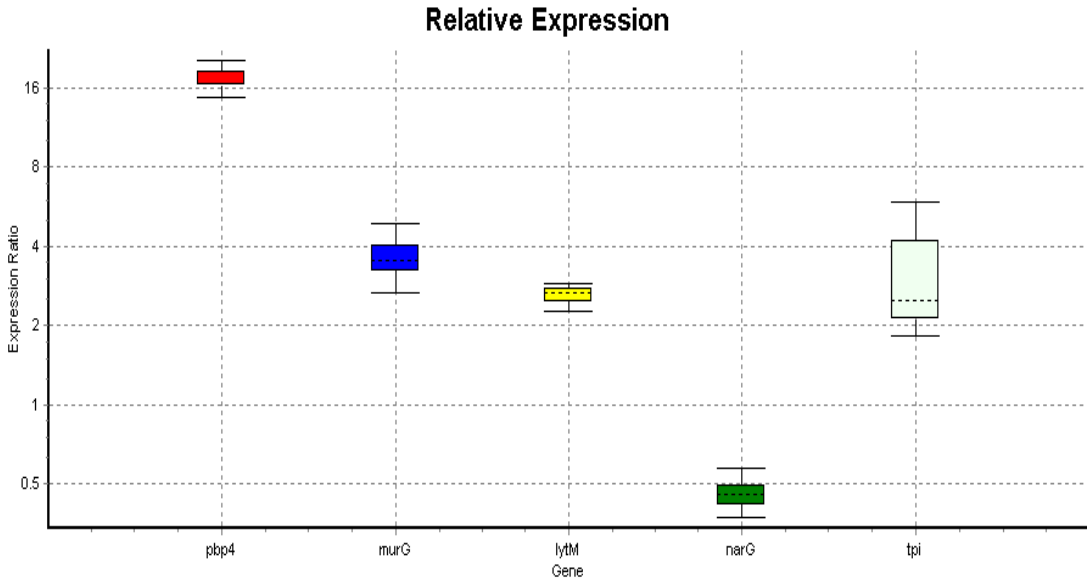


Figure 4. 3. Relative expression ratio of a selection of genes in RS1/19 compared to Oxford. Genes used for RT-PCR analysis were *pbp4*, *murG*, *lytM*, *narG*, and *tpi* each with three replications. *gyrA* used as internal control gene ($p < 0.05$).

Table 4. 2. Comparison of expression levels determined by microarray by real-time RT-PCR analysis

Gene	Fold-change RT-PCR	Fold-change Microarray data
pbp4	17.832 ↑	18.56 ↑
murG	3.586 ↑	2.31 ↑
LytM	2.608 ↑	2.16 ↑
narG	0.458 ↓	0.097 ↓
tpi	2.953 ↑	2.055 ↑

Green downward arrows indicate significant reduction and red upward arrows indicate significant induction ($p<0.05$)

This difference may be reflective of the greater dynamic range of the RT-PCR assay. In addition to that sometimes in microarray assay poor or absent hybridization signals were generated for one of the tested samples, and number of fold induction values can be under- or overestimated (Liu et al., 2005).

4. 2. 3. Sequencing of *pbp4* and its Intergenic Region Between *pbp4* and *abcA* Confirms no Mutations

It has been reported that mutations in the promoter region (upstream of the *pbp4* gene, in the putative promoter region of *abcA* and *pbp4*) are responsible directly or indirectly for the overproduction of PBP4 and penicillin resistance (Henze and Berger-Bachi, 1996). Microarray data and real-time RT-PCR confirmed that *pbp*4 is the gene with highest expression level in RS1/19 compared to wild-type. Additionally strain T12, generated in this study, also has increased levels of PBP4 in membranes. The working hypothesis is that high PBP4 levels contribute to reduced antibiotic sensitivity in RS1/19 and T12 and experiments were designed to determine the reason for this over-expression.

To check if there are any mutations in the intergenic region between *abcA* and *pbp4*, PCR was performed to amplify the promoter operator region of both genes (*abcA* and *pbp4*) from RS1/19, T12 and the parent Oxford strains using the primers DS1 and DS2 (Table 4. 3). The PCR products were analyzed by agarose gel electrophoresis, purified using Amersham Biosciences GFX purification kit and directly sequenced using forward primer DS1. The data was then analyzed using multiple sequence aligment software (http://bioinfo. genotoul. fr/ multalin/multalin.html), which showed that the sequences of strains RS1/19 and T12 are identical to the parent strain and no mutations were found (Appendix1).

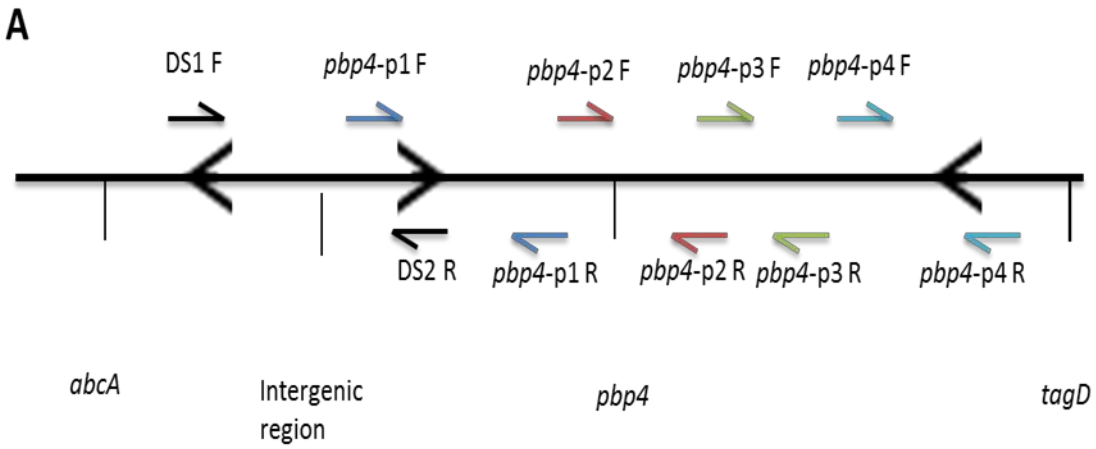
Recently, Banerjee at al., reported that methicillin resistance in an *in vitro* generated strain is due in part to an amino acid change at the entrance to the binding pocket of PBP4 (Banerjee et al., 2010). Therefore, it was important to establish whether the *pbp4* gene has any mutations that could lead to reduced penicillin susceptibility, as seen in other systems. PCR was performed to amplify the structural gene of *pbp4* from RS1/19, T12 and the parent Oxford strain; four primer pairs, were designed to

cover the whole area of *pbp4* (Table 4. 3). The PCR product was analyzed by agarose gel electrophoresis (Figure 4. 4). Then purified and sequenced using forward primers *pbp4*-p1, *pbp4*-p2, *pbp4*-p3, *and pbp4*-p4. No differences were found between the sequences of the parent Oxford and the revertant RS1/19 and T12 strains (Appendix 1).

To summarize, these experiments confirmed that the penicillin resistance observed in RS1/19 and T12 is not due to mutations in the promoter or the structural gene of *pbp4*. However, the data in chapter three showed correlation of PBP4 overexpression to resistance and therefore it remains likely that increased PBP4 is a contributory factor and therefore an important focus for further investigation.

Table 4. 3. Primers used for sequencing of *pbp4* gene.

Primer name amplified	**Primer sequences forward primer (F) reverse primer (R)**	**Amplicon size (bp) region relative to *pbp4*start codon**
DS1	F:gcaacgataagacccactgg	597
DS2	R: gccgacaaacctgcataacc	
pbp4-p1	F:agggaagattaacgctttatg	498 (-18 to 481)
	R***:***aaatcaacgaaatcgctcgtg	
pbp4-p2	R***:***aaatcaacgaaatcgctcgtg	402 (371to 773)
	F: aagtatggacaatcgcagacc	
pbp4-p3	F: accaacaacgcatgcagttacg	405 (690 to1095)
	R: ttctcttggatagtccgcgtg	
pbp4-p4	F: aaggtgagcaaaggataaatgg	384 (967 to1351)
	R: aaacggacaagtttcgcagcaa	



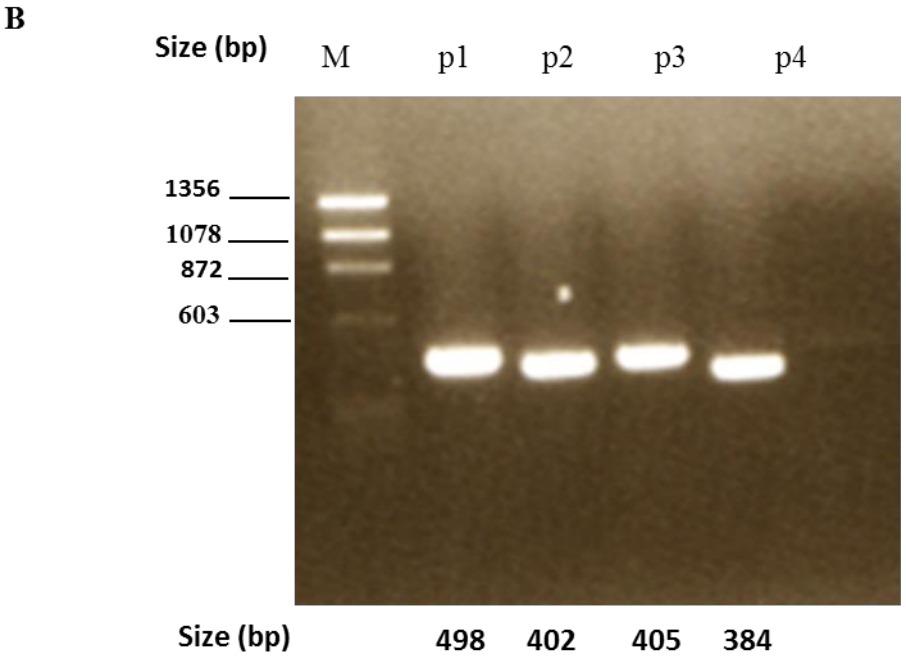


Figure 4. 4. A . A scale diagram showing the abcA-pbp4-tagD locus. Head arrows indicates the direction of gene transcription, forward and reverse primer pair positions marked with the same colour**. B**. Amplification of *pbp4* from parent Oxford strain using four pairs of primers to cover the whole area of *pbp4* gene. Agarose gel electrophoresis of PCR amplicons for *pbp4* region. M is DNA moleculr weight marker (M) øX Hae III indicated on the left p1, p2, p3, p4 is the amplicon of the *pbp4* regions, the size of each product in bp is indicated underneath.

4. 3. Over-expression of PBP4 is not Sufficient for β-lactam Resistance

There is more than one potential explanation for the reduced sensitivity to β-lactam antibiotics observed in RS1/19, because there are many genes in this strain with changed expression levels, as confirmed by microarray data analysis. Notable amongst these data is that *pbp4* has the highest relative expression. Testing the sensitivity of RS1/19 to β- lactam antibiotics with different binding affinities for the four PBPs indicated that *pbp4* is necessary for the observed β- lactam antibiotic resistance (Chapter 3). Therefore a series of experiments were designed to test if over-expression of PBP4 alone is sufficient for β-lactam resistance. To test this, the *pbp4* gene was cloned into the high copy number plasmid pGL485 and the recombinant plasmid transformed into a number of *S*. *aureus* strains.

4. 3. 1. Cloning of the *pbp4* Structural Gene

In order to overexpress the *pbp4* gene in *S*. *aureus,* a high copy plasmid pGL485 with a size of ~ 10kb was used. This plasmid is a shuttle vector containing two origins of replications, one is from pGB2 that allows replication in *E*. *coli* that are selected on spectinomycin (100μg/ml), and the second origin of replication is from pE194 that allows replication in *S*. *aureus* with a selection marker of chloramphenicol (10μg/ml) (Figure 4. 5) (Cooper 2009). Approximately 1.3 kilobases (kb) of sequence, covering the *pbp4* structural gene and including 18bp upstream of the ATG was amplified by PCR using forward and reverse primers FB1 and FB2, then cloned downstream to the promoter region of pGL485 vector (Figure 4. 6). The sequence of *pbp4* in the wild-type Oxford and RS1/19 strains were previously shown to be identical, therefore the structural gene of *pbp4* was amplified from *S*. *aureus* Oxford strain genomic DNA using the Phusion Hot Start II polymerase (High-Fidelity DNA Polymerase, Thermo Scientific). First the restriction site of *Apa*I and *Bam*HI were incorporated to the 5' end of the forward and reverse primers respectively and three extra nucleotides were added at the end of the restriction site to increase the efficiency of restriction enzyme cutting (New England Biolabs).

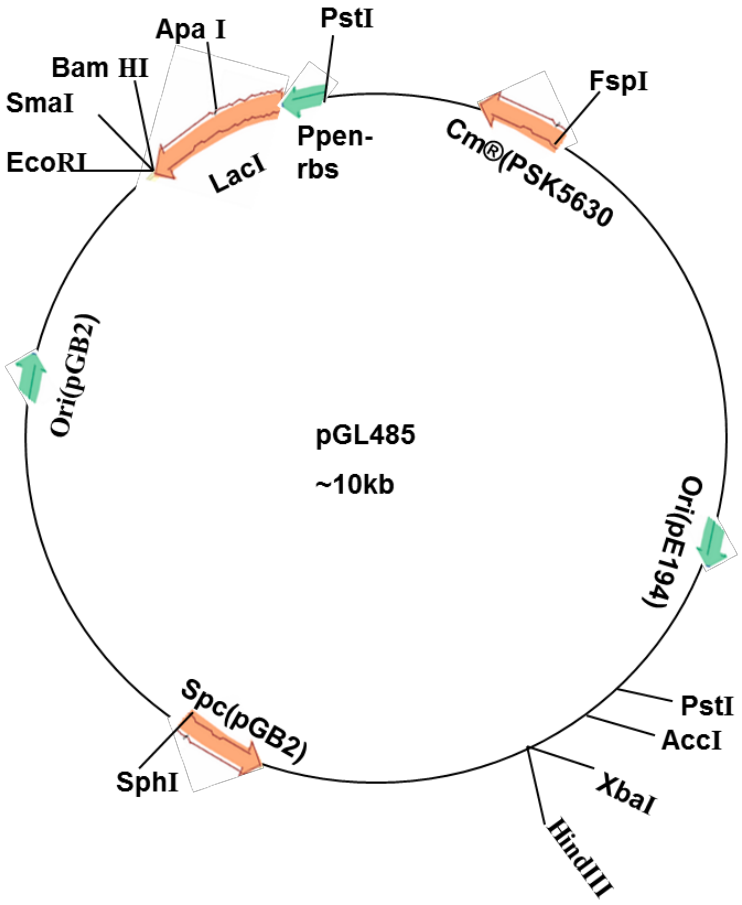


Figure 4. 5. Schematic diagram of the pGL485 vector. This shuttle vector have two origins of replication pGB2 in *E.coli* and pE194 in *S. aureus.*

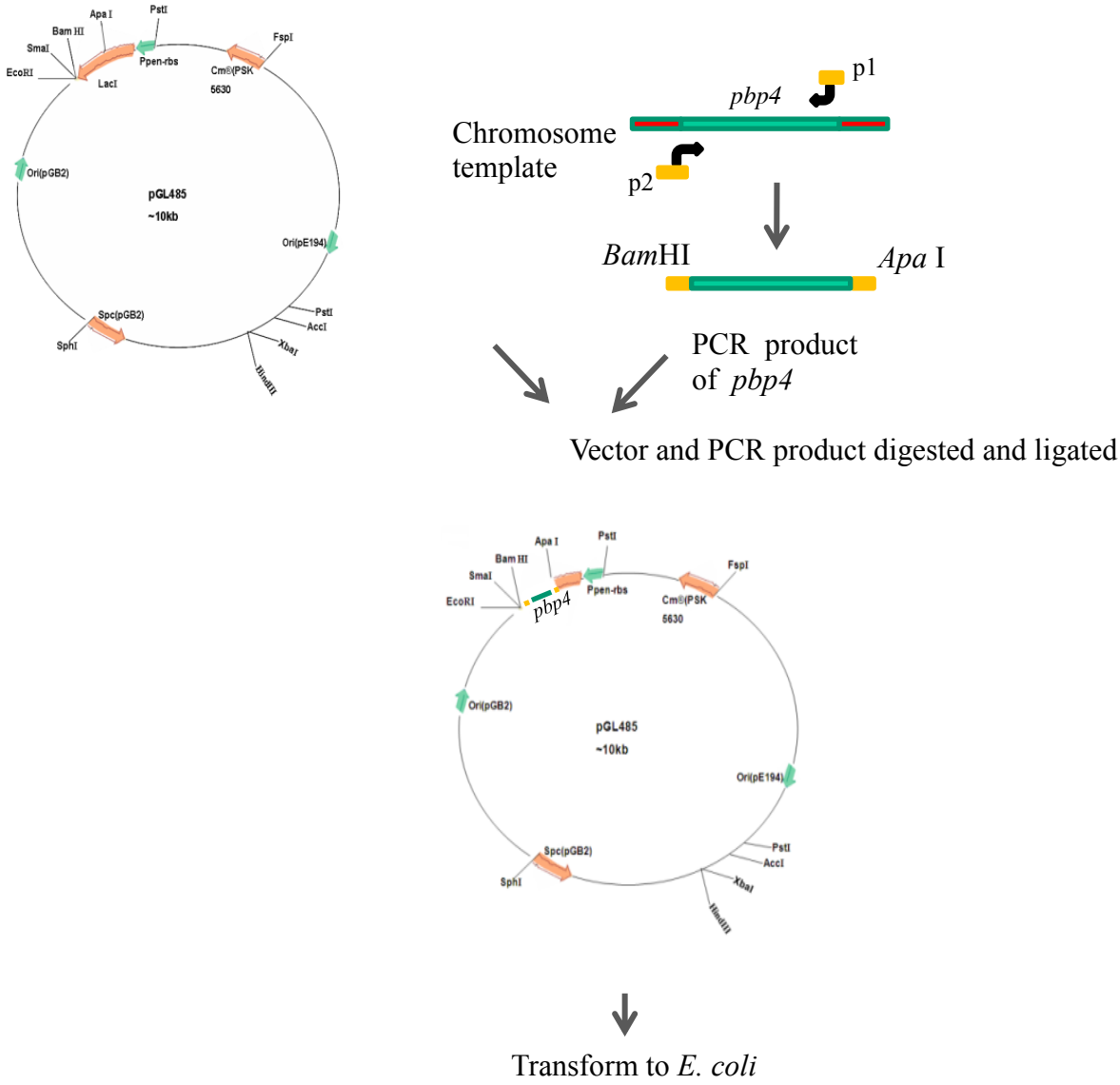


Figure 4. 6 . Diagramatic scheme for cloning the *pbp4* gene into high copy plasmid pGL485. A DNA fragment containing the structural gene of *pbp4*, generated by PCR amplification using primers FB1 and FB2, was restricted with *Apa*I and *Bam*HI. pGL485 vector was restricted with the same restriction enzymes, followed by ligation of these fragments, *pbp4* and pGL485. The constructed vector pGL485-*pbp4* was transformed to *E. coli* competent cells using electroporation. Transformed cells then identified by colony PCR using FB1 and FB2 primers.

The primers were as follows: Forward *Apa*I primer (FB1) with the sequence acgggcccagggaagattaacgctttatg, and the reverse *Bam*HI primer (FB2) with the sequence caaggatcctttataatgtgcaacttgtcc (restriction site underlined). The PCR machine was set for one cycle of 3min at 98ºC, 30 cycles of 30 sec at 98 ºC, 30 sec at 57ºC and 30 sec at 72ºC, finishing with one cycle of 5 min at 72ºC. Following the PCR reaction, the products were resolved by agarose gel electrophoresis and a band of 1.3 kb was obtained (Figure 4. 7). The PCR product was purified with the Roche *High Pure* PCR kit. The *pbp4* fragment and pGL485 were both digested with *Bam*HI for 4h at 37C˚and then purified with Roche *High Pure* PCR kit. The product was suspended in 30µl dH_2O. A second digestion was carried out with *Apa*I at 37C˚ for 4h, and then it was cleaned again with PCR cleaning kit and suspended in 30µl of dH_2O. Following overnight ligation of the digested *pbp4* gene and pGL485 at 4˚C the construct pGL485-*pbp4* was transformed into competent *E .coli* cells (XL1-Blue) and selected on LB plates containing spectinomycin (100µg/ml). Colonies containing recombinant plasmids were identified by PCR. The sequence of the *pbp4* gene was confirmed by sequencing with four forward primers *pbp4*-p1, *pbp4*-p2, *pbp4*-p3 and *pbp4*-p4 which was used earlier for *pbp4* sequencing (Table 4. 3). The DNA sequence analysis confirmed that no mutations had been introduced into the *pbp4* sequence during the cloning into pGL485-*pbp4*.

Both the empty vector and the construct pGL485-*pbp4* were transformed into competent *S*. *aureus* RN4220 cells by electroporation and selected on BHI agar with chloramphenicol (10µg/ml). A negative control of RN4220 without vector was also incubated overnight at 37˚C. As expected the control RN4220 did not grow because of the absence of selectable marker, but both RN4220 that contained the empty vector and the recombinant vector grew on the plates supplemented with chloramphenicol (10 µg/ml).

Plasmids were extracted from RN4220(pGL485-*pbp4*) (plasmid encoded *pbp4)* and RN4220(pGL485) (empty vector) then digested with the restriction enzyme *Bam*HI, and *Apa*I to compare the size of the empty and constructed vector Figure 4. 8 shows that the recombinant plasmid has a band of 1.3kb in size, corresponding to the expected size fragment of the *pbp4* gene, while in the empty vector there is not a band of this size.

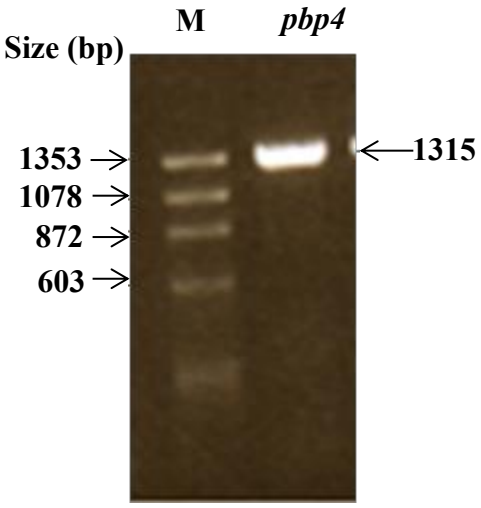


Figure 4. 7. Amplification of *pbp4* from genomic DNA of the wild-type. Agarose gel electrophoresis of PCR amplicon for *pbp4*. DNA molecular weight marker (M) øX *Hae* III indicated on the left. The size of *pbp4* product in bp is indicated on the right.

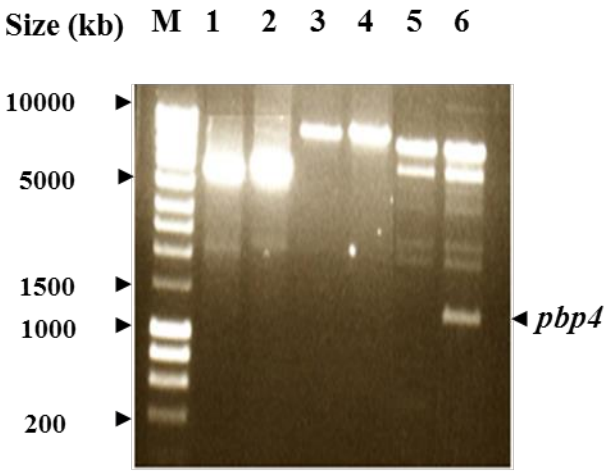


Figure 4. 8. Enzymatic digestion of the constructed and empty vector pGL485. (Lane 1-6) shows agarose gel electrophoresis of the degested empty and constructed vector pGL485. uncut vector pGL485 (1), uncut constructed vector pGL485-*pbp4* (2), vector pGL485 digested with BamHI (3), pGL485-*pbp4* digested with BamHI (4), pGL485 digested with BamHI and ApaI. Showing two bands the upper one is digested vector and the lower band is the uncut vector as a result of incomplete digestiion (5), pGL485-*pbp4* digested with BamHI and ApaI which shows in addition to the bands in lane 5, a band of 1351 bp of the *pbp4* (6), M is a molecular marker hyperladder I

4. 3. 2. Expression of *pbp4* in RN4220(pGL485-*pbp4*) is Higher Relative to Wild-Type Strain RN4220

The *pbp4* gene was successfully cloned into a high copy plasmid vector and transformed into RN4220. Before the hypothesis that increased levels of PBP4 protein are sufficient for decreased sensitivity to penicillin G could be tested, it was necessary to determine the expression level of *pbp*4 in RN4220(pGL485-*pbp4*) relative to the wild-type RN4220 and whether there is an increase in PBP4 in membranes of the recombinant line. The results indicated that the steady-state mRNA levels of the target gene *pbp4* in RN4220(pGL485-*pbp4*) was 228 fold higher that in RN4220, indicating a very high expression level from the plasmid encoded copy (Figure 4. 9).

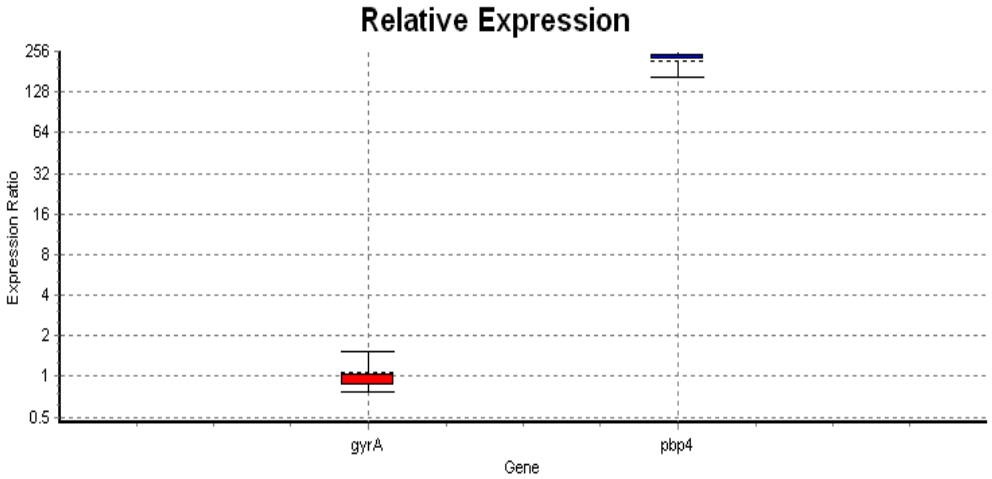


Figure 4. 9. Relative expression ratio of *pbp4* RN4220(*pbp4)* relative to the wild-type RN4220 strain. It is clear that the *pbp4* is up-regulated in RN4220(*pbp4)* compared to the wild-type, *gyrA* used as a reference gene. p value <0.05%. Three replication of the tested gene were used.

4. 3. 3. Membrane Protein Profile Confirms Increased PBP4 Production in RN4220(pGL485-*pbp4)* Strain

This experiment was carried out to test whether the high level of *pbp4* mRNA in the recombinant strain results in an increase in the level of PBP4 protein in cell membranes. Membrane proteins were prepared following the method of (Zhao et al., 1999). Membrane proteins (15µg) from RN4220 and RN4220 (pGL485-*pbp4)* were labeled with Bocillin FL. The labeled PBPs were then separated on SDS-PAGE and detected by Typhoon scanning (Figure 4.10). PBP profile showed a detectable PBP4 band in RN4220 (pGL485-*pbp4)* but not in the wild-type RN4220 membrane fractions. This result clearly shows an increase in PBP4 protein in RN4220 (pGL485-*pbp*4), indicating that the increase in expression of *pbp*4 mRNA resulted in increased PBP4 protein in the membrane.

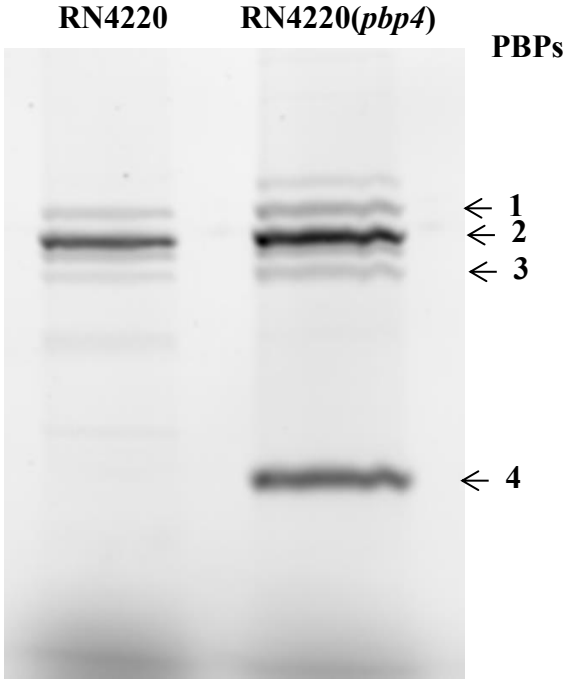


Figure 4. 10. Bocillin FL binding capacity of membrane proteins. Fluorescently labelled Bocillin FL was added to membrane preparations and the proteins separated by SDS-PAGE electrophoresis and visualized using Typhoon scanner. The first lane shows the binding capacity of the membrane proteins prepared from the wild-type RN4220, while the second lane from RN4220 containing the constructed vector pGL485(*pbp*4). Arrows indicate the positions of the single PBPs.

4. 3. 4. RN4220(pGL485-*pbp4*) Contains More Pentaglycine Cross-Links Than the Parent Strain, RN4220

Experiments, this far, clearly showed that the high level of *pbp4* mRNA in the recombinant strain results in an increase in the level of protein in cell membrane. PBP4 is a transpeptidase, which forms peptide bonds between one stem peptide and the pentaglycine cross bridge of an adjacent stem peptide (Navratna et al., 2010). Overexpression of functional PBP4 would be predicted to lead to more pentaglycine cross-bridge in the peptidoglycan (Pereira et al., 2007). The enzyme lysostaphin is a peptidase that targets the pentaglycine cross bridge and so can be used to compare the extent of cross-linking in cell wall preparations (Sieradzki et al., 1999; Memmi et al., 2008 and Bastos et al., 2010). Therefore lysostaphin susceptibility tests were carried out determine the extent of cross-linking.

Lysostaphin susceptibility of the wild-type and RN4220 (pGL485-*pbp4*) were tested during the exponential phase of growth in BHI medium. RN4220 cells were grown in BHI medium, while BHI medium containing chloramphenicol 10µg/ml used to maintain the plasmid in RN4220(pGL485-*pbp4*). The collected bacterial pellets were washed three times in 20 mM KPO_4 buffer (pH 7.5), and after the final wash re-suspended in the same buffer. The absorbance reading at wavelength 620 nm was adjusted to approximately 0.25. The spectrophotometer was temperature controlled at 37 °C and the experiment started by the adding one unit of lysostaphin the cuvette shaken and returned to the spectrophotometer for continuous reading. The data was plotted as a percentage of the initial $A_{620\ nm}$.

The results show that RN4220(pGL485-*pbp4*) cells are more resistant to lysostaphin compared to the wild-type (Figure 4. 11), as 50% of lysis occurred after 8 min in wild-type and after 13.8 min in RN4220 (pGL485-*pbp4*), which is a 1.7 fold difference and suggests that the there is a greater level of cross-linking in the recombinant strain, likely due to an increase in functional PBP4 protein in the membranes of RN4220 (pGL485-*pbp4*).

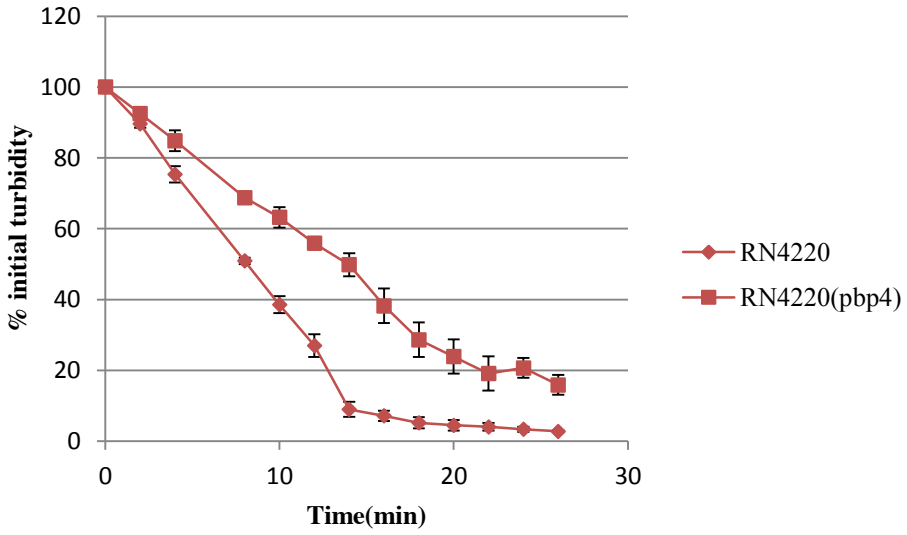


Figure 4. 11. Lysostaphin susceptibility of the wild-type (RN4220) and recombinant RN4220(pGL485-*pbp4*) strains. Cells were grown to exponential phase at 37˚C then treated with lysostaphin, The RN4220 (pGL485-*pbp4*) strain is more resistant to lysostaphin in comparison to the wild-type strain. Bars represent standard error of the mean of three independent experiments.

It is possible that more cross-linking results in delayed peptidoglycan hydrolysis, or may be the increased in cross–linking prevented lysostaphin access to all pentaglycine target. Therefore, It can be concluded that PBP4 overexpression is one of the most important causes of resistance to lysostaphin, probably as a result of more cross-linking in the RN4220 (pGL485-*pbp4*).

4. 3. 5. Effect of PBP4 Expression on Autolysis Rate

RS1/19 was resistant to lysis by Triton X-100, which is an agent that removes inhibitors of endogenous autolysins (Boyle-Vavra et al., 2001). To assess whether increased pentaglycine bridge in the transduced strain RN4220 (pGL485-*pbp4*)) has any effect on peptidoglycan hydrolase activity, Susceptibility to Triton X-100 was performed.

Autolysis of the wild-type RN4220 and the transformant cell RN4220 (pGL485-*pbp4*) were tested during the exponential phase of growth in BHI medium. RN4220 cells were grown in BHI medium until early exponential phase, while BHI medium containing chloramphenicol 10µg/ml used to maintain the plasmid pGL48(*pbp4*) in RN4220(pGL485-*pbp4*). Cells were collected by centrifugation and the pellets washed twice in ice cold water, and resuspended in 0.05M Tris -HCl buffer (pH 7.2) containing 0.05% (vol/vol) Triton X-100. The cell suspensions were incubated with shaking and150rpm and 30°C. Cell lysis was measured as a decrease in $A_{580\ nm}$ over a period of 3h and was expressed as a percentage of the initial $A_{580\ nm}$ (Xiong et al., 1999). This analysis revealed that there was no difference in the observed rate of autolysis between RN4220 and RN4220(pGL485-*pbp4*) strains (Figure 4. 12). Both strains exhibited a high autolysis rate, with about a 50% reduction in absorbance readings in approximately 30 min. This data suggests that PBP4 over-expression has no effect on the autolysis rate.

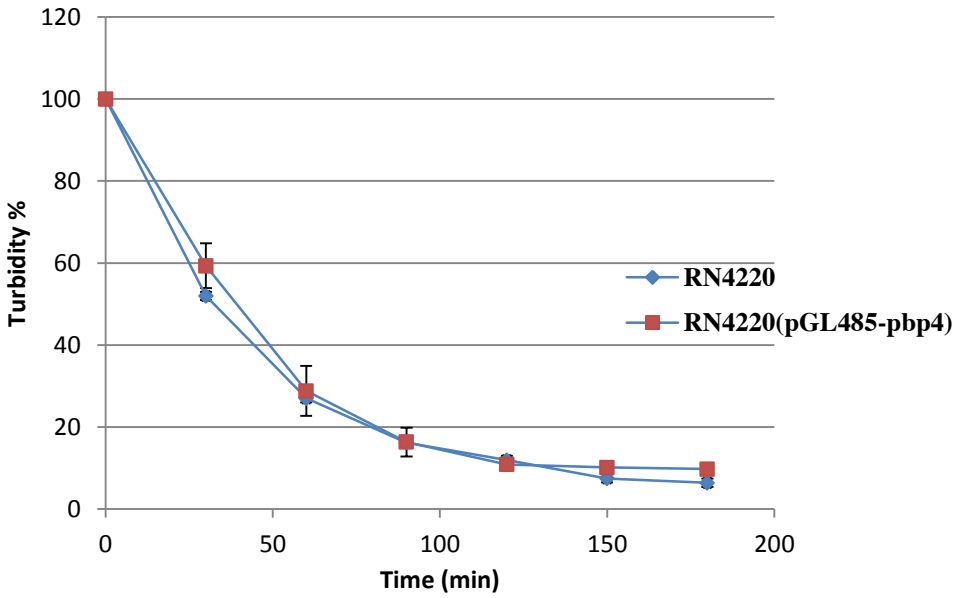


Figure 4. 12. Autolysis assay of RN4220 and RN4220(pGL485- *pbp4*) in the presence of Triton X-100. Bacterial cells were collected from early exponential phase ($A_{580\ nm}$ of 0.7), washed twice with ice-cold water and re-suspended in an equal volume of Tris-HCl (pH 7.2) containing 0.05% (vol/vol)Triton X-100. The rate of autolysis was measured as the decline in optical density $A_{580\ nm}$. Bars represent standard error of the mean for three independent experiments.

4. 3. 6. Over-expression of PBP4 is not Sufficient for the Reduced Penicillin G Sensitivity

Previous experiments have clearly established that RN4220(pGL485-*pbp4*) has increased levels of functional PBP4 protein. To determine if the change in the level of PBP4 alone is sufficient to cause reduced susceptibility to β-lactam antibiotics, antibiotic sensitivity tests were carried out using penicillin G, oxacillin and vancomycin, which is a glycopeptid (Table 4. 4). *S*. *aureus* (Oxford) was used as the control for RS1/19 and RN4220 was used as the control for RN4220(pGL485-*pbp4*).

The Oxford strain was highly sensitive to both penicillin G and oxacillin while RS1/19 showed much decreased susceptibility to both antibiotics, with MICs of 32 and 64μg/ml respectively. Conversely, the Oxford strain was less sensitive to vancomycin compared to RS1/19 with MIC values of 2 μg/ml and 1μg/ml respectively. These data show that RS1/19 has altered susceptibilities to all three antibiotics tested (Table 4. 4). Interstingly, It has been noted that the component of the media has an effect on the MIC determination of peniciilin G. The MIC for Oxford and RS1/19 was 0.064 and 64 μg/ml respectively when CWD media was used (Table 3. 2), while the sensivity of cells increased to 0.016 and 32 μg/ml for Oxford and RS1/19 when ISS was used (Table 4. 4).

Altered vancomycin susceptibility in RS1/19, and RN4220 (pGL485-*pbp4*), could be explained by a higher percentage of completed cross bridges in their peptidoglycan, as a result of overproduction of PBP4. Vancomycin can bind to two types of targets in *S*. *aureus* cells; binding to free D-Ala-D-Ala dipeptides in the completed peptidoglycan layers interferes with cross bridge formation mediated by PBPs, but does not inhibit peptidoglycan synthesis in the nascent peptidoglycan (Cui et al., 2006). The second target is on the nascent peptidoglycan chain, therefore the monomer that is located in the cytoplasmic membrane serves as the main target for vancomycin (Finan et al., 2001). PBP4 has been shown to be involved in secondary cell wall remodeling (Wyke et al., 1981). It has transpeptidase activity and appears also to act as a D, D-carboxypeptidase, by cleaving terminal D-alanine residues from un-cross linked muropeptides (Kozarich and Strominger 1978, and Navratna, V. 2010). An increase in PBP4 activity would decrease the number of surrogate

vancomycin targets by decreasing the total cell wall content of D-Ala-D-Ala containing muropeptide as a result of increasing D, D-carboxypeptidase activity.

It has been estimated that in wild-type strains of *S. aureus* about 20% of D-Ala-D-Ala residues remain unprocessed by PBPs (Hiramatsu, 2001and Boyle-Vavra et al., 2001). While in cells overproducing PBP4 cross-linking there could be more than 90% processing of the cross-links, causing the diffusion rate of vancomycin to its target (lipidII) to be increased (Pereira et al., 2007), because it passes through about 20 layers of peptidoglycan without being trapped by the first target (Hiramatsu, 2001). This may explain the increased sensitivity of RS1/19 and RN4220 (pGL485-*pbp4*) to vancomycin, because more pentaglycine bridge are present in these strains, leading to increased diffusion rates of vancomycin without being blocked by the first target.

The antibiotic sensitivity profiles of RN4220 and the recombinant strain RN4220 (pGL485-*pbp4*), showed no differences in the MIC of penicillin G. However, the MIC for vancomycin was reduced from 4 μg/ml to 1 μg/ml in the recombinant strain, supporting the contention that there is additional PBP4 activity in RN4224(pGL485-*pbp4*). It is therefore clear that, PBP4 over-expression alone is insufficient to cause the β-lactam resistance phenotype observed in RS1/19. This leads to two possible conclusions; the first is that additional PBP4 activity is not required for the reduced susceptibility phenotype and the second is that it is necessary, but is in itself not sufficient. The antibiotic sensitivity data, presented in chapter three, indicates a role for PBP4 and therefore it is more likely that PBP4 plays a role in the resistance phenotype, but is not solely responsible for it, meaning that modification of additional, unknown, host factor(s) is required for full expression of resistance to penicillin.

Furthermore, reduction in vancomycin sensitivity in RN4220 (pGL485-*pbp4*) confirms that more pentaglycine cross-bridges, due to PBP4 over-expression, leads to increased sensitivity in the recombinant strain. Therefore it is clear that factors, other than PBP4 overexpression, may be involved in resistance to β-lactam resistance in RS1/19, whilst over-expression of *pbp4* alone is enough to alter vancomycin sensitivity in the recombinant, RN4220 (pGL485-*pbp4*), strain.

Table 4. 4. MIC determinations for cell wall antibiotics, against wild-type cells and strains with increased PBP4 activity.

Antibiotic	MIC (*μ*g/ml)			
	Oxford	**RS1/19**	**RN4220**	**RN4220(pGL485- *pbp4*)**
penicillin G	0.016	32	0.016	0.016
oxacillin	0.25	64	0.25	0.25
vancomycin	2	1	4	1

MICs determined by E-test for penicillin G, and by broth microdilution for oxacillin and vancomycin.

4. 4. Testing PBP4 Expression in Other *S. aureus* Strains

S. *aureus*, RN4220 is a cloning intermediate in widespread use because it is the only *S*. *aureus* strain that can be efficiently transformed with foreign DNA (Atilano et al., 2010). However, it has been recently shown, by whole genome sequencing, that it contains a number of genetic polymorphisms that affect virulence and general fitness (Nair et al., 2011) and therefore results obtained in this strain should be verified in a variety of other well characterized strains. For this purpose the recombinant vector was transduced, using bacteriophage 80α, to a number of genetic backgrounds *S*. *aureus* strains (Oxford, 8325-4, and SH1000), which were then tested for their PBP profile and antibiotic sensitivities.

4. 4. 1. Generalized Transduction Using Phage 80α

This experiment was carried out to transfer the vector pGL485 (*pbp4*) from RN4220 (pGL485-*pbp4*) to other strains of *S*. *aureus*. Initially phage lysates were made on recombinant strain RN4220 (pGL485-*pbp4*), grown in LK broth containing chloramphenicol 10μg/ml, to select for strains that contain the vector pGL485(*pbp4*). Following incubation of the phage lysate (titre of 10^7 /ml) with the recipient strains, at a multiplicity of infection (MOI) of 10, plates were incubated for 12-72 h at 37ºC and the resulting colonies used to calculate the number of transductants per ml of culture (Table 4. 5).

Transduction frequency was calculated as the ratio of the number of transductants (CFU) obtained to the number of plaque-forming units (PFU) (Varga et al., 2012). Data showed that *S. aureus* strains, 8325-4 and SH1000, transduced more efficiently at frequencies of 2×10^{-4} and 7×10^{-5} respectively ompared to Oxford (Table 4. 5).Transductants from each strain were selected and re- streaked on BHI plates containing chloramphenicol (10μg/ml) to use in the following experiments.

Table 4. 5. Transduction Frequencies of pGL485 (*pbp*4) into different *S. aureus* genetic backgrounds.

Recipient	Plasmid	Selection	MOI	Transduced cells CFU/ml	Transduction Frequency (CFU/PFU)
8325-4	pGL485(*pbp4*)	CmR	10	220	2×10^{-4}
SH1000	pGL485(*pbp4*)	CmR	10	72	7×10^{-5}
Oxford	pGL485(*pbp4*)	CmR	10	5	1×10^{-12} *

***** This relates to 2 attempts before any transductants were obtained.

4. 4. 2. Over-expression of *pbp4* in Three Different Genetic Backgrounds of *S. aureus* Results in Increased PBP4 Protein in Membrane Fractions

To confirm the transfer of the constructed vector pGL485 (*pbp4*) to the different *S. aureus* strains, membrane proteins were prepared from wild-type strains Oxford, 8325-4, and SH1000 and transduction strains, Oxford (pGL485-*pbp4*), 8325-4 (pGL485-*pbp4*), and SH1000 (pGL485-*pbp4*). Analysis of membrane protein fractions showed increased binding of Bocillin FL to PBP4 in cells where the plasmid was successfully transferred compared to the wild-type. Additionally, investigation into the comparative PBP2/PBP4 ratios of wild-type and transduced cells showed a significant reduction in PBP2/PBP4 ratios in transduced cells due to PBP4 overexpression in these cells (Figure 4. 13). This result, in addition to altered vancomycin susceptibility in the transduced cells (Table 4. 6), indicate that this vector pGL485 (*pbp4*) has been successfully transduced to different *S. aureus* background strains.

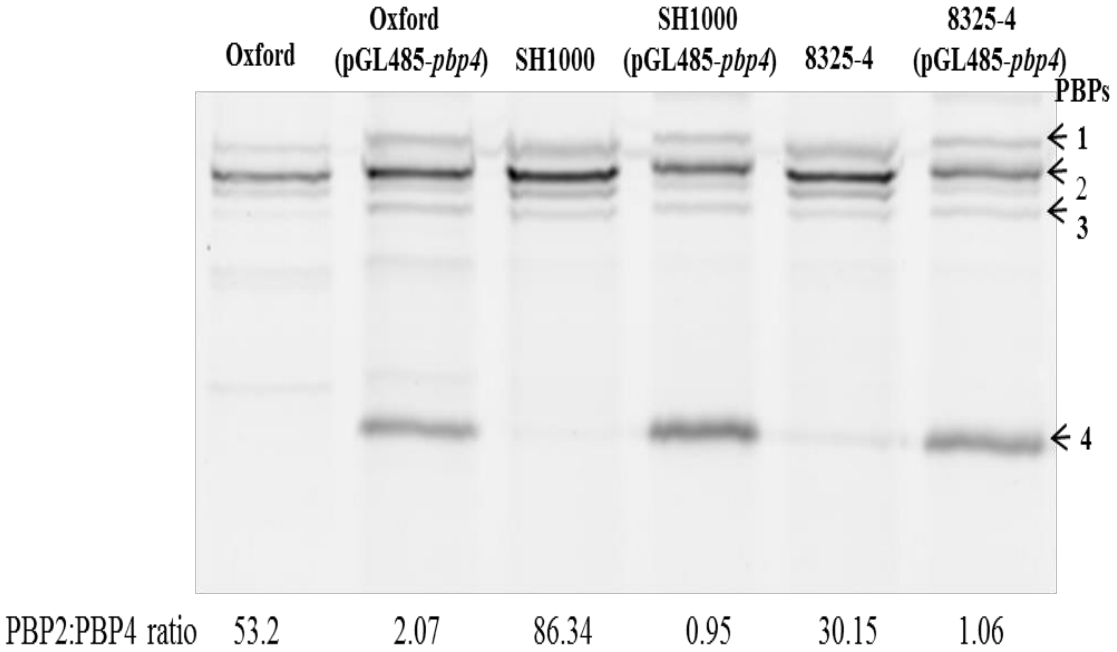


Figure 4. 13. penicillin binding capacity of membrane proteins. Bocillin FL was added to membrane preparations and the proteins separated by SDS-PAGE electrophoresis. Membrane proteins visualized using Typhoon scanner. Lane (1, 3,and 5) shows the binding capacity of the membrane proteins prepared from the wild-type Oxford, 8325-4, and SH1000 no PBP4 can be detected. While transductant strains in lane (2, 4, and 6) shows increased binding capacity. PBP2:PBP4 ratio indicated under each lane.The positions of the single PBPs are indicated by arrows.

4. 4. 3. Overproduction of PBP4 is Not Sufficient for the Observed Reduced Antibiotic Sensitivity

A previous experiment (section 4. 3. 6) showed that RN4220 (pGL485-*pbp4*) exhibits the same sensitivity to β-lactam antibiotics (penicillin G and oxacillin) but show increased susceptibility to vancomycin as the parent, RN4220 cells. It was important to determine the effect of overexpression of PBP4 on cell wall inhibitor antibiotics in different genetic backgrounds of *S*. *aureus*. Therefore strains 8325-4 (pGL485-*pbp4*), SH1000 (pGL485-*pbp4*), and Oxford (pGL485-*pbp4*) were tested (Table 4. 6). The MICs of each strain with and without the *pbp4* expression plasmid remained the same for penicillin G and oxacillin, confirming that overproduction of PBP4 alone is insufficient to cause a change in the sensitivity to to β-lactam antibiotics, irrespective of the genetic background of the *S*. *aureus* strain.

In each genetic background tested, the MIC for vancomycin in the transductants was lower than for the corresponding parental strain (Table 4. 6). In each case the change was small, but consistent. This means that increased vancomycin susceptibility was uniformly associated with increased peptidoglycan cross-linking in transductants. Therefore it indicates a possible relationship between the degree of cross-linking and vancomycin susceptibility.

Table 4. 6. MIC determinations for cell wall antibiotics, against wild-type and transductant strains with increased *pbp4* activity.

Antibiotic	MIC (μg/ml)					
	8325-4	8325-4 (pGL485-*pbp4*)	SH1000	SH1000 (pGL485-*pbp4*)	Oxford	Oxford (pGL485-*pbp4*)
Penicillin	0.064	0.064	0.064	0. 064	0.016	0.016
Oxacillin	0.25	0.25	0.25	0.25	0.25	0.25
vancomycin	4	2	4	2	2	1

MICs determined by E-test for penicillin G, and by broth microdilution for oxacillin and vancomycin.

To conclude, the results demonstrated in this chapter showed that RS1/19 cells do not contain any mutations in *pbp4* gene or in the region upstream of the *pbp*4 gene which might interfere with the resistance to penicillin G. Furthermore the data presented in this chapter do not support the hypothesis that expression of *pbp4* alone is sufficient for the observed penicillin resistance in RS1/19. This is because overproduction of PBP4 in transductant strains had no effect on the sensitivity to penicillin G or oxacillin. To test the necessity of this gene in the resistance mechanism, we aim to inactivate *pbp4* gene using allelic replacement experiment in the background of RS1/19 cells.

CHAPTER FIVE

The Role of PBP4 in β-lactam Resistance

5. 1. Introduction

In vitro selection for resistance to cell wall inhibitor antibiotics in *S. aureus* has been associated with a variety of gene mutations, in particular genes related to cell wall biosynthesis (Utaida et al., 2003). It has been reported both in laboratory investigations and clinical studies that a major role of PBP4 over-expression (Henze and Berger-Bachi, 1996; and Fuller et al., 2005) or mutations in the *pbp4* structural gene (Banerjee et al., 2010) is involved in methicillin resistance. On the other hand, studies in some methicillin resistant strains COL, N315, and Mu50, β-lactam susceptibility is unaffected by the deletion of *pbp4* (Katamaya et al., 2003; Memmi et al., 2008). However, in some other strains MW2 and USA300 the loss of PBP4 resulted in 16 fold reduction in oxacillin and nafcillin MICs, and dramatically decreased the expression of *pbp2* (Memmi et al., 2008). These data appear to be conflicting and indicate that the full expression of methicillin resistance varies in different strains.

There are different ways in which bacteria can naturally undergo mutagenesis; it may happen spontaneously (Beale, G., 1993), or be due to exposure to mutagens such as chemicals (Odete et al., 2011). Mutations can also be obtained experimentally in the laboratory by using transposons, signature-tagged mutagenesis, and allelic replacement experiments (Lindsay, 2008).

The most commonly used technique to alter the *S. aureus* genome is allelic replacement using homologous recombination experiments. O'Reilly et al., were the first to use allelic replacement to generate a mutation in a structural gene in the chromosome of *S. aureus* (O'Reilly et al., 1986). Allelic replacement mutants are normally generated by cloning the gene of interest and then inserting an antibiotic selectable marker into the gene, prior to introducing the interrupted allele into the genome by homologous recombination. Practically, 1kb of the mutated gene is required for gene replacement, however longer pieces of DNA seem to increase the efficiency of the allelic replacement (Lindasy, 2008).

The allelic replacement vector pMAD, which is a thermo-sensitive *E.coli*/*S. aureus* shuttle vector (Arnaud et al., 2004) was used in this study. In this method, the vector

containing the mutated gene is introduced into *S*. *aureus* by electroporation, and the bacteria are then grown at the permissive temperature to allow replication of the plasmid. Following introduction of the vector the cultivation temperature is increased to the non-permissive temperature to block autonomous replication of the vector. Loss of the vector sequence from *S. aureus* can be carried out using a unique system. The pMAD vector encodes a constitutively expressed thermo-stable β-galactosidase. This can be used in combination with the chromogenic substrate 5-bromo 4-chloro-3-indolyle-β-D-galactopyranoside (X-gal) to identify bacterial colonies in which the plasmid has been excised. In addition, inserting a selectable marker (gene disrupting marker) into the target gene to be deleted makes the screening easier. The use of this vector enables screening for bacteria that have lost the vector instead of selecting the allelic replacement mutants. The ability to screen for bacteria that have lost the plasmid increases the efficiency of mutant isolation compared to the classical method of allelic replacement (Arnaud et al., 2004).

The experiments in Chapter Four proved that overexpressed PBP4 alone is not sufficient for the β-lactam resistance, as over-expression of PBP4 in *S. aureus* with different genetic backgrounds did not alter the antibiotic sensitivity to a range of β-lactam antibiotics tested. To study the necessity of this gene for decreased sensitivity to penicillin G in RS1/19, it is important to knock-out this gene in RS1/19. For this purpose, mutants of *S. aureus* were constructed using the chromosomal integration-excision approach described by Arnaud et al., 2004. This method was used to create allelic replacement mutants by cloning *pbp4* gene and inserting a selectable spectinomycin resistant marker (*spc)* into the gene.

5. 2. Results and Discussion

5. 2. 1. Construction of Δ*pbp4*

Construction of Δ*pbp4* was carried out utilizing chromosomal DNA from the Oxford strain as a template, DNA primers were designed with flanking restriction sites (Table 5. 1). The restriction site of *Eco*RI and *Bam*HI were incorporated to the 5' end of the forward and reverse primers of the first *pbp4* fragment respectively. Similarly, the restriction site of *Hin*dIII and *Bgl*II were incorporated to the 5' end of the forward and reverse primers of the second *pbp4* fragment. Three extra nucleotides were added at the end of all restriction sites to increase the efficiency of restriction enzyme cutting (New England Biolabs).

The first fragment consisted of 490bp from the upstream part of the gene (including the start codon and 5' part of the coding sequence) and the second fragment was 644bp (including the 3' part of the gene and the stop codon). These two fragments flank a 213bp deletion from the coding sequence of *pbp4;* DNA fragments (5' *pbp4* and 3' *pbp4*) were amplified by PCR. At the same time, primers were designed to amplify a 1.54 kb DNA fragment of a PCR product containing the entire spectinomycin resistance gene *spc* was used as a template. The forward primer spc1 F contained the *Bam*HI restriction site while the reverse primer spc1 R contained the *Hin*dIII restriction site. Following PCR and purification of the amplicons. These three fragments (5' *pbp4, 3' pbp4,* and *spc)* were digested with the corresponding restriction enzymes then purified.

The amount of DNA in each fragment was quantified by NanoDrop 1000 Spectrophotometry. These purified PCR DNA fragments were mixed in equal amounts (molar ratio 1:1:1) and ligated. The ligation mixture was used as templates to amplify the final fusion product, using the outside primers of (pbp4P1 F and pbp4P2 *R*). A single band of 2.7 kb was expected when run on agarose gel, but extraneous products were produced (Figure 5. 1. A). To reduce non-specific bands step wise PCR was used (IPGRI and Cornell University, 2003), with a raised annealing temperature to reduce the non-specific bands. The PCR reaction was set as follows: one cycle of 3min at 98ºC. Then three steps of cycling were used. First step was, 5 cycles of 30 sec at 98 ºC, 30 sec at 65ºC and 40 sec at 72ºC. Second step was

5 cycles of 30 sec at 98 ºC, 30 sec at 60ºC and 40 sec at 72ºC. Third step was 25 cycles of 30 sec at 98 ºC, 30 sec at 56ºC and 40 sec at 72ºC, finishing with one cycle of 5 min at 72ºC. However, the non-specific bands were still detected. Subsequently, a band of a 2.7 kb was purified by loading the PCR reaction and excising the correct size band from an agarose gel and re-PCR. This time a smear of PCR product appeared on the agarose gel (Figure 5. 1. B). In order to clone the mutated *pbp4* fragment, the purified band of 2.7 kb, which was expected to be the right band, was used to insert into the PCR-Blunt II-TOPO vector. The constructed vector was transformed into competent *E.coli* cells, selected on LB media with kanamycin (50µg/ml) and colony PCR performed using vector specific primers M13 forward primer 5´gtaaaacgacggccag and M13 reverse primer 3´ caggaaacagctatgac. The vector was present, but no recombinant plasmids containing the correct insert were isolated.

Table 5. 1. Sequences of primers used for *pbp4* deletion, restriction sites are bold and Italic.

Primer name	Primer Sequence (5'- 3')	Restriction site	Amplicon size in bp
pbp4P1	F: cag***gaattc***cagggaagattaacgctttatg	*Eco*RI	490
	R: caa***ggatcc***aaatcaacgaaatcgctcgtg	*Bam*HI	
pbp4P2	F: caa***aagctt***caacgcatgcagttacg	*Hin*dIII	644
	R**:** cac***agatct***gctttataatgtgcaacttgtcc	*Bgl*II	
spc1	F: ***ggatcc***caggctgggtgccaagctctcg	*Bam*HI	1548
	R***: aagctt***gtgcttagtgcatctaacgc	*Hin*dIII	

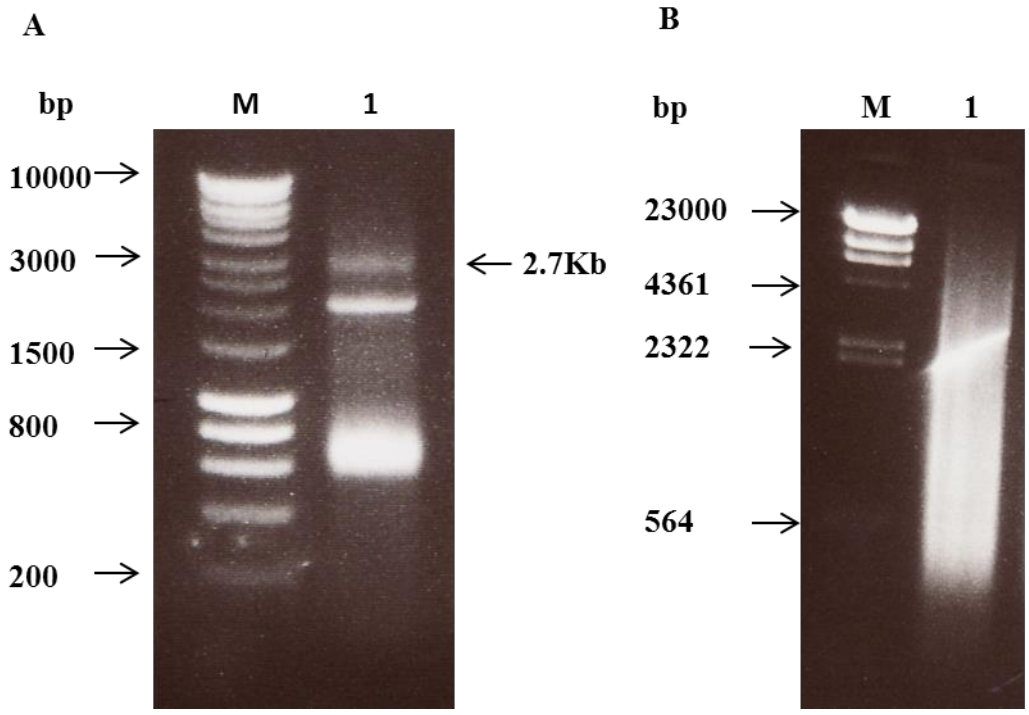


Figure 5. 1. Agarose gel of ligation mixture to construct Δ*pbp4.* *(*A) these *5´ pbp4 ,3´ pbp4,* and *spc* fragments were mixed in equal amounts and ligated. The ligation mixture was used as templates to amplify the final fusion product, using the outside primers of (*5´* pbp4 *P1* and 3´pbp4*P2*). A single band of 2.7 kb was expected when run on agarose gel, M is a DNA molecular marker Hyperladder I. (B) Amplification of purified 2.7 kb, after being excised from the agarose gel but a smear obtained when run the PCR product on the agarose gel. M is Lambda DNA/ HindIII marker.

The initial strategy was to use PCR of the ligation mixture directly to clone into the pMAD vector, but this method was unsuccessful. As all the primers were already designed, a cloning strategy was developed to be able to utilize these, and the pGEM-3Zf (+) (Promega) plasmid as an intermediate vector for sub-cloning (Figure 5. 2). This vector contains all of the same restriction sites as the primers in the correct orientation for the cloning strategy proposed except one primer, the pbp4P2 reverse primer (Table 5. 1). Therefore an additional primer with a *Hin*dIII restriction site in addition to the *Bgl*II restriction site was synthesized, for cloning into pGEM-3Zf (+) as follows: cac***aagcttagatct***gctttataatgtgcaacttgtcc.The first fragment of *pbp4* (490bp) and pGEM-3Zf (+) were restricted with *Eco*RI and *Bam*HI then ligated. This was transformed into competent *E. coli* cells that have been supplemented with ampicillin (150µg/ml) and X-gal (40 µg/ml), for easy screening for white/blue colonies. Usually vectors containing PCR products produce white colonies as a result of insertional inactivation of the α-peptide coding region of β-galactosidase. Colony PCR was used to screen the white colonies for the presence of the first insert and all the colonies tested were confirmed to contain the insert (Figure 5. 3. A). This recombinant plasmid (pGEM-3Zf (+) (*5 'pbp4*)) and the amplified spectinomycin resistance cassette (*spc*) were then restricted with *Bam*HI and *Hin*dIII and ligated together. This was transformed to *E.coli* cells on LB plates supplemented with ampicillin (150µg/ml). Colony PCR for the growing colonies confirmed the presence of the second insert in three clones (Figure 5. 3. B). Recombinant pGEM-3Zf + (*5 'pbp4+spc*) was then extracted from *E. coli* cells.

Since both the recombinant vector (pGEM-3Zf + (*5 'pbp4+spc*)) and the second fragment of *pbp4* (644bp) needed to be restricted with the same restriction enzyme *Hin*dIII, the vector was treated with alkaline phosphatase to prevent self-ligation, greatly facilitating ligation of the insert fragment into the vector. Colony PCR was used to search for positive clones. Since the third fragment had the same restriction site (*Hin*dIII) on both ends, insertion of the fragment in the right orientation was checked either by PCR using the 5′ primer of the third fragment (insert specific primer) and 3′ priming site of the pGEM-3Zf (+) vector (vector specific primer), or by extracting the vector from the positive colonies and restriction with *Eco*RI and *Bgl*II.

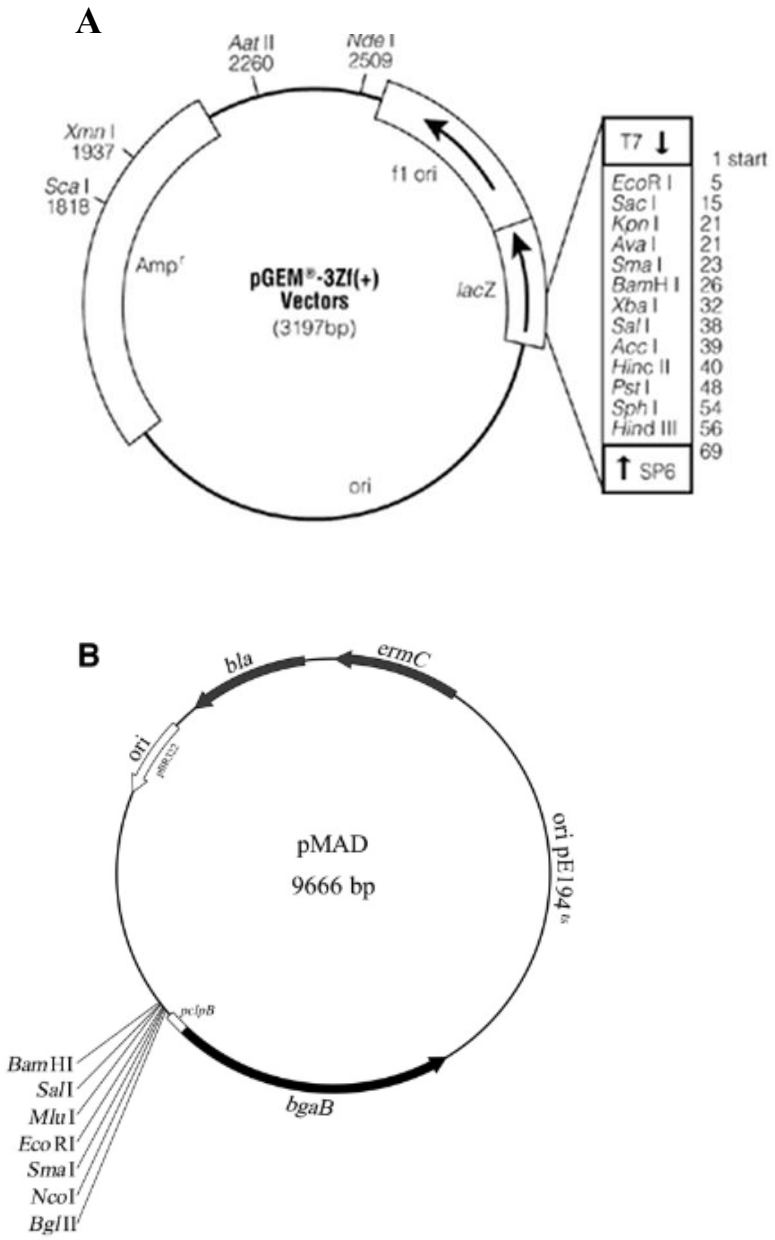


Figure 5. 2. A. Schematic diagram of pGEM-3Zf(+) vector promoter and multiple cloning region sequence. This vector serves as a standard cloning vector. Images taken from Promega. **B**. Map of the pMAD plasmid. Unique restriction sites are indicated. Images taken from (Arnaud et al., 2004).

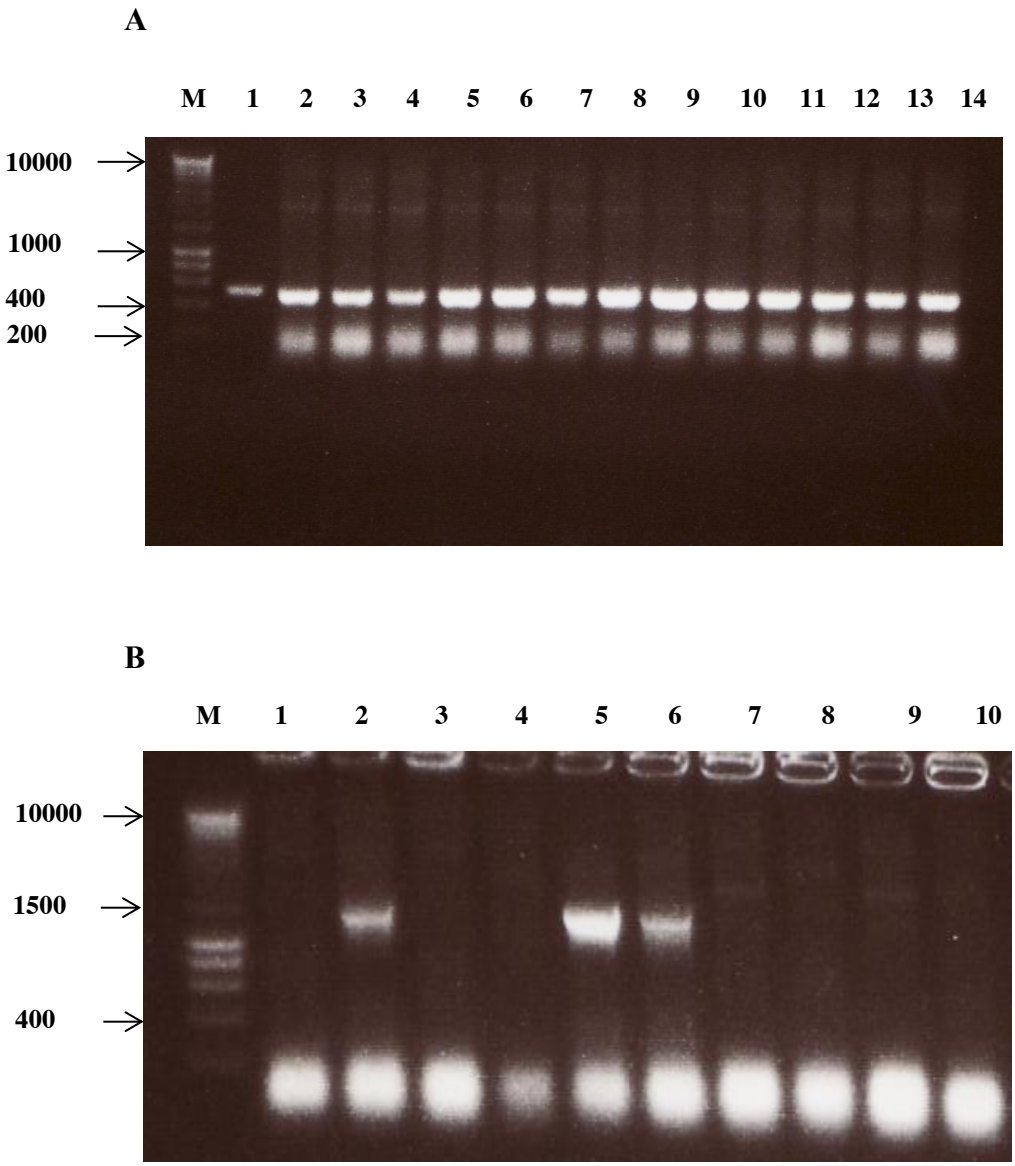


Figure 5. 3. Colony PCR for screening the presence of the insert in *E.coli* cells. A. First fragment of *pbp4* in recombinant plasmid pGEM 3Zf (+)(*5 'pbp4)* vector**.** All colonies screened contained the insert. M is Hyperladdar I DNA marker, 1 is a control PCR product the other lanes are colony PCR. **B**. Amplification of the second fragment corresponding spectinomycin cassette gene (*spc*) from *E.coli* strains using colony PCR. M is Hyperladder I DNA marker, 2, 5, and 6 lanes are desired inserts.

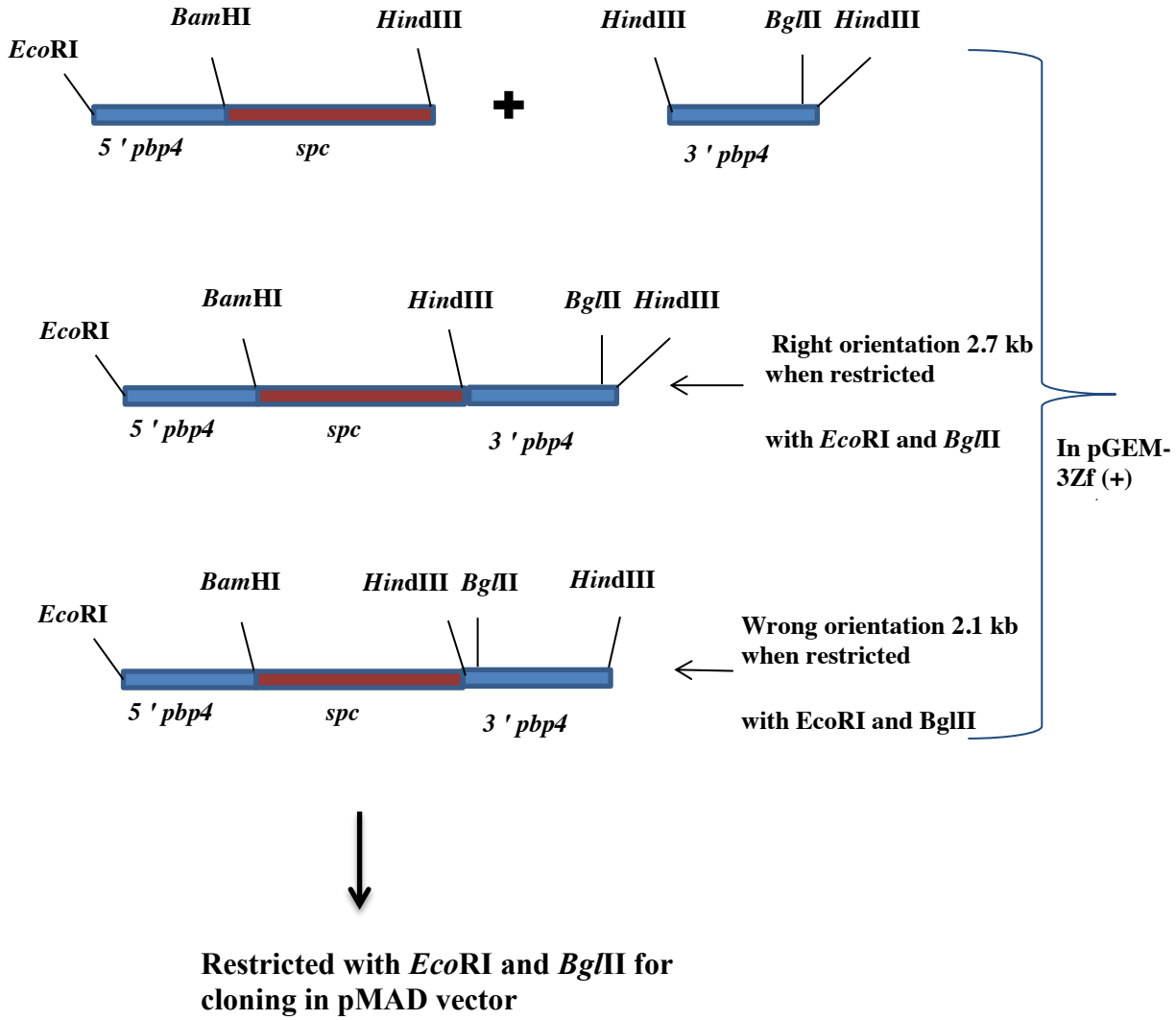


Figure 5. 4. Diagram of sub-cloning Δ *pbp4* into intermediate vector pGEM(+). Only the insert with the right orientation (when restricted with *Eco*RI and *Bgl*II) would give the band of 2.7 kb.

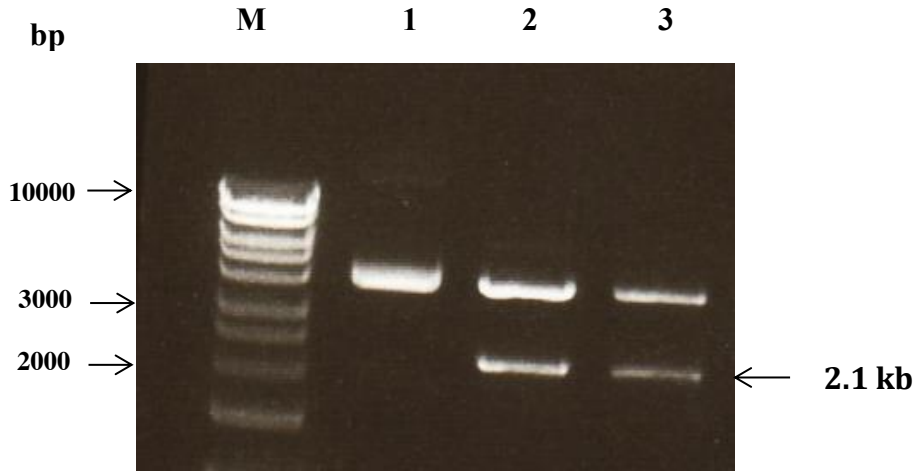


Figure 5. 5. Restriction enzyme digestion of recombinant pGEM-3zf (+) (Δ*pbp4*). The constructed plasmid pGEM-3zf (+) (Δ*pbp4)* were digested with *Eco*RI and *Bgl*II then resolved on 1% agarose gel. The marker is Hyperladder I, lane 1 is an empty vector and lane 2 and 3 are recombinant vector that contain the insert in the wrong orientation of the size of 2.1kb.

The right orientation of the final fragment would give a band of 2.7 kb, while the wrong orientations would give a band of 2.1 kb. Unfortunately, all the positive clones tested were with the wrong orientation (Figure 5. 4; Figure 5. 5).

Another approach to ligate these fragments in the right orientation was by cutting out the insert (*pbp4* and spectinomycin (*5 'pbp4+spc*)) from the constructed vector pGEM-3Zf+(*5 'pbp4+spc*) using the restriction enzymes *Eco*RI and *Hin*dIII. The correct sized fragment was excised from an agarose gel and purified, whilst the third fragment was simultaneously restricted with *Hin*dIII and *Bgl*II. These fragments were mixed in equal amounts in a molar ratio of 3:1 to the corresponding restricted pMAD vector and ligated. The constructed vector was transformed to *E.coli* XL1-Blue cells on LB plates selected with ampicillin (150µg/ml), spectinomycin (100 µg/ml) and X-gal (40 µg/ml). Colonies of *E. coli* that contain pMAD vector were blue. Colony PCR was performed on ten blue colonies using outside primers (pbp4P1F and pbp4P2R). Four positive colonies were isolated with the size band 2.7 kb (Figure 5. 6).

The recombinant pMADΔ*pbp4* vector was extracted from *E. coli* cells and transformed into competent *S. aureus* RN4220 cells by electroporation. Cells were grown on BHI plates with erythromycin (5µg/ml) as a selectable marker and X-gal (150µg/ml). A negative control of RN4220 without vector was also incubated at 30 C˚ for 48h. Blue colonies were only seen on plates containing recombinant vector. Plasmids were extracted from RN4220 (pMADΔ*pbp4)* and a PCR was performed to check the size of the insert; this was found to be 2.7 kb confirming the presence of the insert. Besides, when pMADΔ*pbp4 was* restricted with *Eco*RI and *Bgl*II, a band of 2.7 kb was visualized on agarose gel.

5. 2. 3. Generalized Transduction Using Phage 80α

This experiment was carried out to transduce the plasmid pMADΔ*pbp4* from the restriction deficient strain, RN4220 to Oxford and RS1/19 so that *pbp4* allelic replacement could be carried out in these strains. Initially phage lysates were made on recombinant strain RN4220(*pbp4*). Cells were grown in LK broth containing erythromycin (5µg/ml) and the phage lysates were used to transduce wild-type

Oxford strain using The Double-Layer Agar (DLA) technique (Santos et al., 2009). The plates were supplemented with erythromycin 5µg/ml and X-gal 150 µg/ml to select for Oxford strains that contain the vector pMADΔpbp4. Successful transduction and presence of the vector was confirmed by the presence of blue colonies and resistance to erythromycin after 12-72 h incubation at 30ºC.

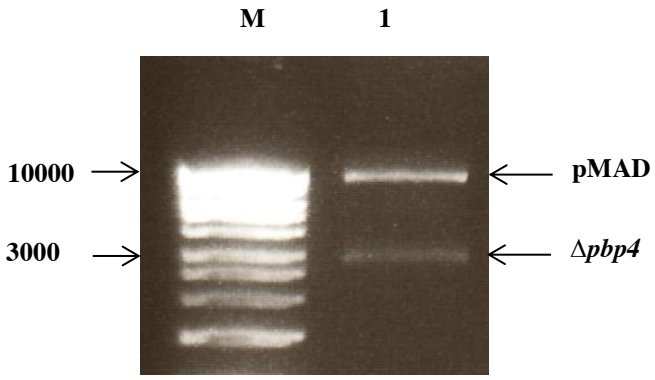


Figure 5. 6. Restriction enzyme digestion of pMAD Δ*pbp4*. The constructed plasmid vector pMAD Δ*pbp4,* isolated from *E.coli* , was digested with *Eco*RI and *Bgl*II then resolved on 1% agarose gel. The marker is Hyperladder I. Lane 2 is recombinant vector that contained the insert Δ*pbp4* of the size of 2.7 kb.

The frequency of transduction reduced dramatically in the Oxford strain in this experiment to 1×10^{-18}. The same experiment was repeated to transduce pMAD Δ*pbp4* into RS1/19, but the efficiency of transduction was even lower in methicillin resistant RS1/19, being 1×10^{-30}. Several modifications of the transduction method were used in an attempt to increase the efficiency. For this, the calcium chloride concentration was increased first from 5mM to 10 mM during transduction to increase the absorbance of the phage to receptors (Jodi Lindsay, personal communication) on the RS1/19 cells. These experiments were repeated with all the steps and incubation temperature was changed from 37˚C to 30˚C. None of the modifications of the basic transduction protocol produced any transductants.

It has been reported that some antibiotics, like β-lactams, stimulate bacteria to produce phage (Santos et al., 2009), which has been shown to result in replication and high frequency transfer of staphylococcal pathogenicity islands (Maiques et al., 2006). Therefore, in an attempt to improve the transduction efficiency, a sub-inhibitory concentration of penicillin G (final concentration of 0.5μg/ml) was added to LK plates in addition to erythromycin and X-gal. This method resulted in successful transduction, but only two colonies of RS1/19 pMAD Δ*pbp4* were obtained; this shows that this strain can be transduced, but very inefficiently (1×10^{-30}).

One of the main reasons that it has been difficult to genetically manipulate *S. aureus,* in this study, is that RS1/19 used does not belong to the same lineage as RN4220, which is derived from 8325-4 and belongs to lineage ST 8 (Lindsay and Holden, 2004). RS1/19 which was generated from the Oxford strain belongs to lineage ST 30 (Kearns et al., 2006). The major known barrier to transduction in *S. aureus* is the type 1 Restriction Modification (R-M) system, encoded by the *sau1hsdR* genes, and it is lineage specific. The R-M system serves to strictly control the mechanisms of uptake of foreign DNA. Moreover, RN4220 has a mutation in the *sau1hsdR* that allows it to accept foreign DNA (Waldron and Lindsay, 2006). It has been reported that the ten main lineages of *S. aureus* carry unique combinations of *sau1hsdR* genes that control the sequence specificity of the system. Thus, DNA from a different lineage is regarded as foreign DNA and destroyed. Therefore mobile genetic element (MGE) present in one strain will transfer horizontally to other strains of the same

lineage at a higher frequency than to strains of other lineages. In another words the R-M model suggests that genetic exchange of any kind may be more efficient between isolates with the same *sau1hsdR* profile than between those with different profiles (Waldron and Lindsay, 2006). This explains the difficulties experienced during transducing pMADΔ*pbp4* to both wild-type Oxford and RS1/19 strains.

5. 2. 4. Allelic Replacement of *pbp4* Gene to Create RS1/19Δ*pbp4* Mutants

The recombinant pMADΔ*pbp4* was successfully introduced into RS1/19 strain by transduction. Thus, to obtain cells with a chromosomally integrated copy of pMADΔ*pbp4* via a single crossover event (SCE), a single blue colony was streaked onto BHI agar. Plates were incubated at 30° C for 24h and a single blue colony, from this plate, was inoculated into BHI medium without antibiotic and incubated with shaking for 2h at 30°C, before transfer to 42°C for 6h to increase the frequency of a single cross over event. Serial dilutions of this culture were plated on BHI agar plates, containing erythromycin (5µg/ml) and X-gal (150µg/ml) and incubated at 42°C for 24-48h. One light blue colony was selected which represent cells that have a SCE due to the fact that the vector integrated as a single copy in the chromosome produces less β-galactosidase than several plasmids in the cytoplasm (Arnaud et al., 2004). The recombinant pMADΔ*pbp4* did not integrate to RN4220 (pMADΔ*pbp4)* chromosome as a single crossover event, because for this process to happen cells need to be cultivated at non-permissive tempreture to block autonomous replication of the vector.

To subsequently obtain cells that had excised pMAD vector sequences and the chromosomal copy of *pbp4* from the chromosome (double cross over events), light blue colonies were used for alternative cultures in BHI medium at 30˚C and 42˚C. For this purpose, one colony inoculated in BHI medium without antibiotic was incubated with shaking at 30°C for 6h then a 1000-fold dilution of the culture was made in fresh BHI medium and incubated overnight with shaking at 42°C. This procedure was repeated over 6 days, and then serial dilutions were made from the culture and plated on BHI plates supplemented with X-gal and spectinomycin (100µg/ml). White colonies that are resistant to spectinomycin are potential mutant

strains due to plasmid excision. Following 48h of incubation, no growth was detected, indicating that either no correct excision events had taken place or that the spectinomycin gene disrupting marker was not functional in *S. aureus*. To determine if the spectinomycin gene was functionally expressed in these cells, the MIC of spectinomycin was tested for both RS1/19 and RS1/19(pMADΔ*pbp4*). The values obtained showed no difference, with both strains having an MIC of 8µg/ml, regardless of the pMADΔ*pbp4* construct. This suggests that the spectinomycin gene was not functional in RS1/19 cells and an alternative selection strategy was required. Therefore blue/ white colony screening was performed on BHI plates supplemented with X-gal, as erythromycin-sensitive white colonies would represent candidate clones resulting from a double crossover event and loss of the erythromycin resistance and β-galactosidase genes, encoded on the vector (Figure 5. 7).

To confirm the gene deletion and integration of the Δ*pbp4* into the chromosome, genomic DNA was prepared from each of the candidate colonies. PCR was performed using flanking primers approximately 100bp upstream and downstream of *pbp4* gene. The sequence of the forward primer was *pbp4*F1 F (acacctttagctacacacg*)* and the sequence of the reverse primer was *pbp4*F2 R (acgtacagaaggcatttcgac); the size of PCR product for the wild-type parent strain would be 1.6 kb, while the size of PCR product for the mutant RS1/19 Δ*pbp4* was expected to be 2.9 kb. To further confirm the deletion and the presence of *spc* gene in the chromosome, PCR was performed using flanked forward primer of pbp4 F1 and reverse primer of spc which gave the size of 2298 bp and another PCR with forward primer of spc and reverse primer of pbp4 F2 with a size of 2335 bp (Figure 5. 8). From approximately 100 colonies tested only one clone contained a deleted *pbp4*, whilst the remainder of the colonies all had the original genomic DNA configuration of the *pbp4* gene.

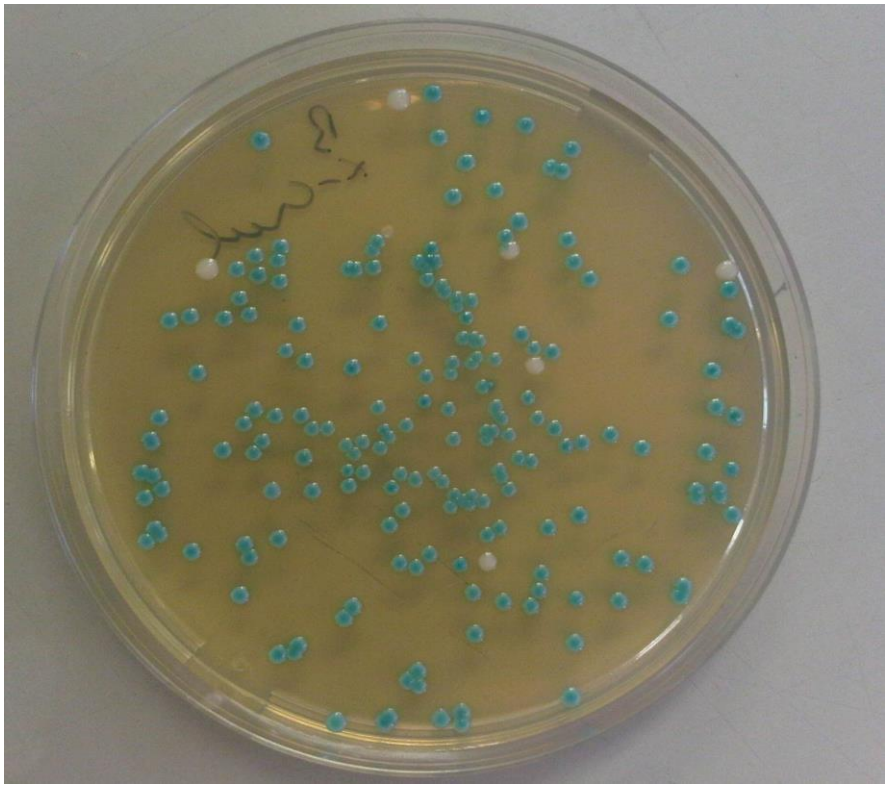


Figure 5. 7. RS1/19 colonies following transformation with the pAMDΔ*pbp4* vector. Cells were cultivated at 30° C for 2h then transferred to 42° C for 6h; dilutions were plated on BHI agar containing X-gal in the absence of the antibiotic. Then one single blue colony was used for alternative sub-cultures at 30° C and 42° C for 6 continuous days; dilutions were then plated on BHI containing X-gal without antibiotics. White colonies have undergone the excision and loss of the vector; whereas blue colonies retain a copy of the plasmid integrated in the chromosome.

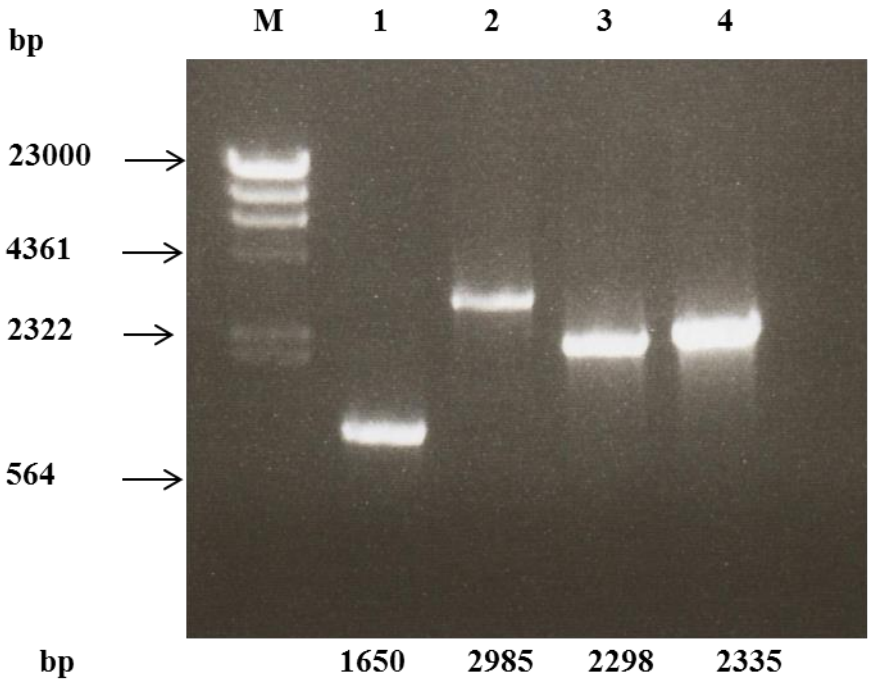


Figure 5. 8. PCR product of *pbp4* to confirm the allelic replacement in RS1/19. PCR products using flanking primers upstream and downstream region of *pbp4* in RS1/19 Lane 1 PCR products using flanking primers upstream and downstream region of Δ*pbp4* in RS1/19 Lane 2. PCR product of RS1/19Δ*pbp4* using flanked forward primer of *pbp4* F1 and reverse primer of *spc* lane 3, while lane 4 with forward primer of *spc* and reverse primer of *pbp4* F2. M is Lambda/*Hin*dIII DNA marker. The size of each PCR product is indicated.

When a suicide pMADΔ*pbp4* vector inserts in the upstream region of *pbp4* as a single event, the entire plasmid is inserted into the chromosome (SCE). Two copies of the downstream region will be present on the chromosome until a second homologous recombination event has taken place between the two downstream regions. This second recombination event excises the pMAD vector. The resulting products are either the original *pbp4* allele or a strain that now has the mutant Δ*pbp4* allele, within the chromosome and the wild-type allele on the plasmid. This is determined by the location of the recombination events; if the second crossover occurs on the opposite side of the Δ*pbp4*, the chromosome of the resulting bacteria will contain the mutant allele of the *pbp4* gene (Figure 5. 9).

To further confirm allelic replacement, the PCR product of the flanking region and the regions that had undergone recombination were sequenced (Figure 5. 10). The sequencing confirmed the insertion-deletion event. This analysis showed the presence of some point mutation in the sequence of the first fragment of *pbp4 (5′ pbp4)*; four point mutations at positions 32, 70, 297, and 302 were present. In the same manner, three point mutations occurred in the second cloned *pbp4* fragment (3′ *pbp4)* in the position 1068, 1334 and 1366. There is no effect of these mutations because *pbp4* is already none-functional in the RS1/19Δ*pbp4*. These mutations may have occurred during amplification of PCR fragments, or during exposure to UV light to cut out fragments from the sub-cloning vector pGEM-3Zf (+) to clone it to pMAD vector.

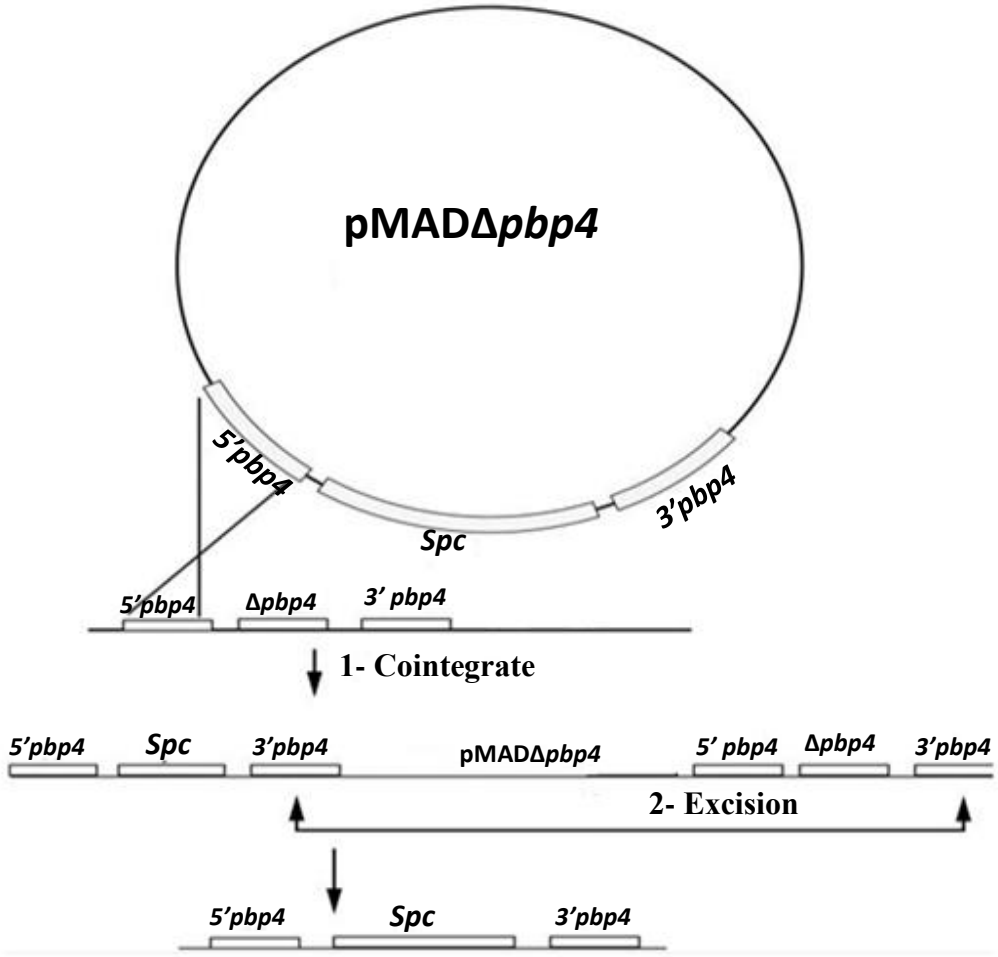


Figure 5. 9. Schematic representation of the two step allelic exchange procedure. Areas labelled *5'pbp4* and *3'pbp4* represent DNA sequences of two fragments of *pbp4* flanking a 213 bp deletion of the *pbp4* gene. The crossed lines lines indicate crossover events. The integration of pMAD via homologous sequences can take place in *5'pbp4* or *3'pbp4***.** The co-integrate undergoes a second recombination event, such that the spectinomycin resistance marker will either remain in the chromosome *3'pbp4* area or be excised along with the plasmid *5'pbp4* area. Gene replacement occurs only if the second recombination event occurs in *3'pbp4* area.

Figure 5. 10. Schematic diagram for the nucleotide sequence in RS1/19Δ*pbp4*. Nucleotide sequence of the *pbp4* region is indicated by its corresponding ATG starting codon which is underlined. Nucleotides of recombinant regions of the RS1/19Δ*pbp4* (*pbp4*) allele that differ from the presented RS1/19 sequence are indicated above the corresponding nucleotide position in lowercase letters. The 213 nucleotides deleted from strain RS1/19Δ*pbp4* which has been substituted by *spc* gene are highlighted. The flanking primers used for sequencing are indicated by arrows, while the primers used for amplification of *pbp4* gene for homologous recombination are indicated as dashed arrows.

5. 3. Characteristic of RS1/19Δ*pbp4* Strain

Experiments in this section were carried out to study the effect of the *pbp4* gene deletion on RS1/19, testing whether PBP4 over-expression is necessary for the reduced sensitivity to β-lactam antibiotics, the effect on the degree of cross-linking indirectly using lysostaphin susceptibility and the rate of autolysis.

5. 3. 1. Characterization of PBP Profile in the Membrane of RS1/19Δ*pbp4*.

This experiment was carried out to confirm the deletion of the *pbp4* gene by homologous recombination via double crossover event from RS1/19Δ*pbp4* resulted in an absence of PBP4 in membranes, as detected by binding of fluorescently labeled penicillin. Single colonies from overnight plate cultures of RS1/19 and RS1/19Δ*pbp4* were grown in BHI medium and the pellets were used for membrane preparation. Membrane proteins were analyzed by SDS-PAGE, where PBPs were identified by their ability to bind to Bocillin FL. Figure 5. 11 shows a detectable PBP4 band in RS1/19, but no evidence of binding to a protein of the size of PBP4 in RS1/19Δ*pbp4*. This result clearly shows disrupting of the *pbp4* gene resulted in no PBP4 production in the membrane fractions. This is another substantial indication for *pbp4* deletion in RS1/19Δ*pbp4*.

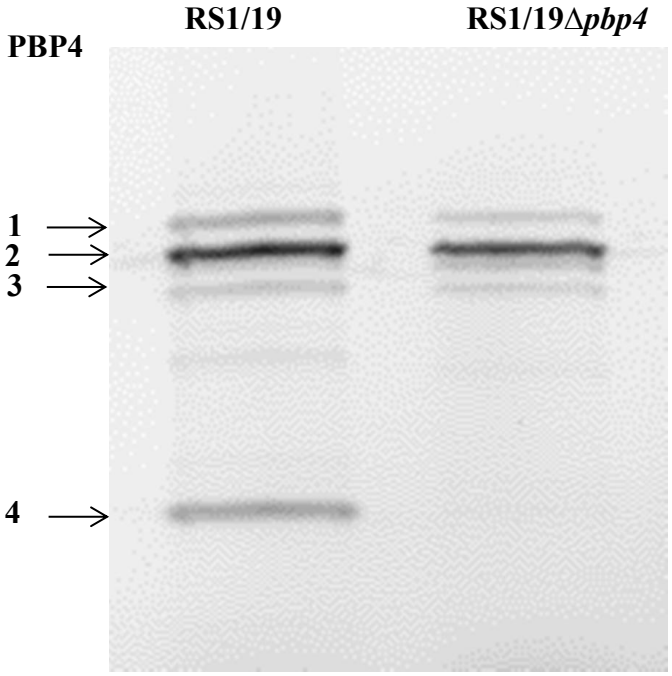


Figure 5. 11. Bocillin FL binding capacity of membrane proteins. Bocillin FL was added to membrane preparations and the proteins separated by SDS-PAGE electrophoresis and visualized using Typhoon scanner indicating the absence of PBP4 from RS1/19Δ*pbp4* strain. The positions of the single PBPs are indicated by arrows.

5. 3. 2. Antibiotic Sensitivity Tests show that PBP4 is Necessary for the Reduced Sensitivity to ß-lactam Antibiotics in RS1/19

It has been confirmed previously that overproduction of PBP4 alone is not sufficient for reducing the sensitivity of RS1/19 to penicillin G (section 4. 4. 3). Therefore macro-dilution antibiotic sensitivity tests were performed, with a number of β-lactam antibiotics and vancomycin, to determine if deletion of *pbp4* had any effect on antibiotic sensitivity in RS1/19 (Table 5. 2). Penicillin G, oxacillin, and cefotaxime all bind relatively poorly (higher dissociation constants) to PBP4, while cefoxitin and cefsoludin have high affinities (lower dissociation constants) for PBP4 (Georgopapadakou et al., 1982).

Deletion of *pbp4* led to a significant increase in sensitivity to penicillin G in RS1/19Δ *pbp4*, compared to RS1/19 (Table 5. 2). The MIC reduced dramatically from 32µg/ml to 0.008µg/ml - a 4000 fold reduction), which is lower than in the Oxford strain. This clearly confirms that *pbp4* is a key element in the network of genes associated with the resistance to penicillin G in RS1/19. The same affect was noted with oxacillin and cefotaxime, as the MICs for these antibiotics reduced from 64µg/ml and 128µg/ml, in RS1/19, to 4µg/ml and 0.5µg/ml, in RS1/19Δ *pbp4*, respectively. The MIC for cefoxitin and cefsoludin were changed only slightly because PBP4 is inhibited at very low concentrations of cefoxitin and cefsoludin in RS1/19. Since cefoxitin and cefsoludin have high affinities for PBP4, deletion of PBP4 has little effect on the MIC for these antibiotics. The increased Bocillin FL binding capacity in RS1/19 is due to over-production of PBP4 and deletion of this in RS1/19Δ *pbp4* results in a reduction in the concentration of antibiotic required for inhibition of growth. Therefore, it can be concluded that *pbp4* is vital for the expression of β-lactam resistance in RS1/19, but it is not sufficient on it is own.

Vancomycin susceptibility testing demonstrated a reduction in vancomycin susceptibility, from (1 µg/ml) in RS1/19 to (16 µg/ml) in RS1/19Δ *pbp4* following *pbp4* inactivation. This result is in agreement with the results obtained by (Finan et al., 2001) who showed that loss of PBP4 led to increased vancomycin resistance in VSSA. In another report, inactivation of overproduced PBP4 was shown to decrease the MIC for vancomycin resistant isolates (Sieradzki et al., 1999). Inactivation of

pbp4 is associated with a lower degree of cross-linking of the peptidoglycan network, which can be measured using HPLC (Finan et al., 2001) and leads to an increase of free D-Ala-D-Ala residues in the cell wall (Sieradzki and Tomasz 2003). The lethal targets for vancomycin molecules are located at the sites of cell wall synthesis on the plasma membrane (Sieradzki et al., 1999). The free D-Ala-D-Ala residues in the mature peptidoglycan strands constitute a potential non-lethal target for vancomycin (Sieradzki and Tomasz, 1997). Binding of large vancomycin molecules to non-lethal targets blocks the movement of the vancomycin molecules to their lethal target, D-Ala-D-Ala residue of the lipid linked peptidoglycan precursor (Hiramatsue., 2001). This explains the reduction in vancomycin sensitivity in RS1/19Δ*pbp4* as reduction in secondary transpeptidase in this strain would lead to less cross-linking and additional non-lethal targets in the mature peptidoglycan structure.

In summary, RS1/19Δ *pbp4* tested in this study has a moderately increased sensitivity to cefoxitin and cefsoludin, but a marked increase in sensitivity to cefotaxime, oxacillin, and penicillin G compared to RS1/19. The data presented in this chapter lead to the conclusion that inactivation of *pbp4* played an important role in increased cefotaxime and penicillin G sensitivities in RS1/19Δ *pbp4* and is therefore necessary for the penicillin and methicillin resistance phenotype of RS1/19.

Table 5. 2. Antibiotic sensitivity test declare that PBP4 is necessary for the reduced sensitivity to β-lactam antibiotics.

Antibiotic	**MIC (*μ*g/ml)**		
	Oxford	**RS1/19**	**RS1/19Δ*pbp4***
penicillin G	0.064	24	0.008
oxacillin	0.25	64	1
Cefotaxime	2	128	0.5
cefoxitin	2	4	2
cefsoludin	4	8	4
vancomycin	2	1	16

5. 3. 3. Inactivation of PBP4 Affects the Level of Peptidoglycan Cross-linking

PBP4 is a transpeptidase, which forms peptide bonds between adjacent stem peptide via a pentaglycine bridge. Previous reports related loss of PBP4 to a reduction in peptidoglycan cross-linking in methicillin sensitive *S*. *aureus*, MRSA COL, MW2 and glycopeptide resistant *S*. *aureus* (Memmi et al., 2008 and Sieradzki et al 1999). It seems likely, therefore, that inactivation of PBP4 would lead to less cross-linking in RS1/19Δ*pbp4*. Lysostaphin is a protease that targets pentaglycine bridges and lysostaphin susceptibility has been used as an indirect way to detect decreased cross-linking observed in GISA strains (Koehl et al., 2004). For this reason, lysostaphin susceptibility tests were performed for the wild-type, RS1/19, and RS1/19Δ*pbp4* cells (Figure 5. 12). These results show that the RS1/19Δ*pbp4* was more sensitive to lysostaphin compared to RS1/19, with 50% of cells lysing after 14 min in RS1/19, and only 4 minutes in RS1/19Δ*pbp4*.

From the results obtained in this experiment it is clear that lysostaphin resistance correlates with overexpression of PBP4. In RS1/19 PBP4 overexpression correlates with reduced sensitivity to lysostaphin and inactivation of the *pbp4* gene, in RS1/19 Δ*pbp4*, correlates to increased sensitivity to lysostaphin. This is likely due to variations in cross-linking, because PBP4 acts as a secondary transpeptdase in the mature peptidoglycan.

To summarize, the data presented support the contention that overexpression of *pbp4*, leading to an increase in functional PBP4 protein in membranes, was the main cause of resistance to lysostaphin in RS1/19. Inactivation of this gene in RS1/19Δ*pbp4* caused it to be more sensitive to lysostaphin, likely due to a decrease in the degree of peptidoglycan cross-linking.

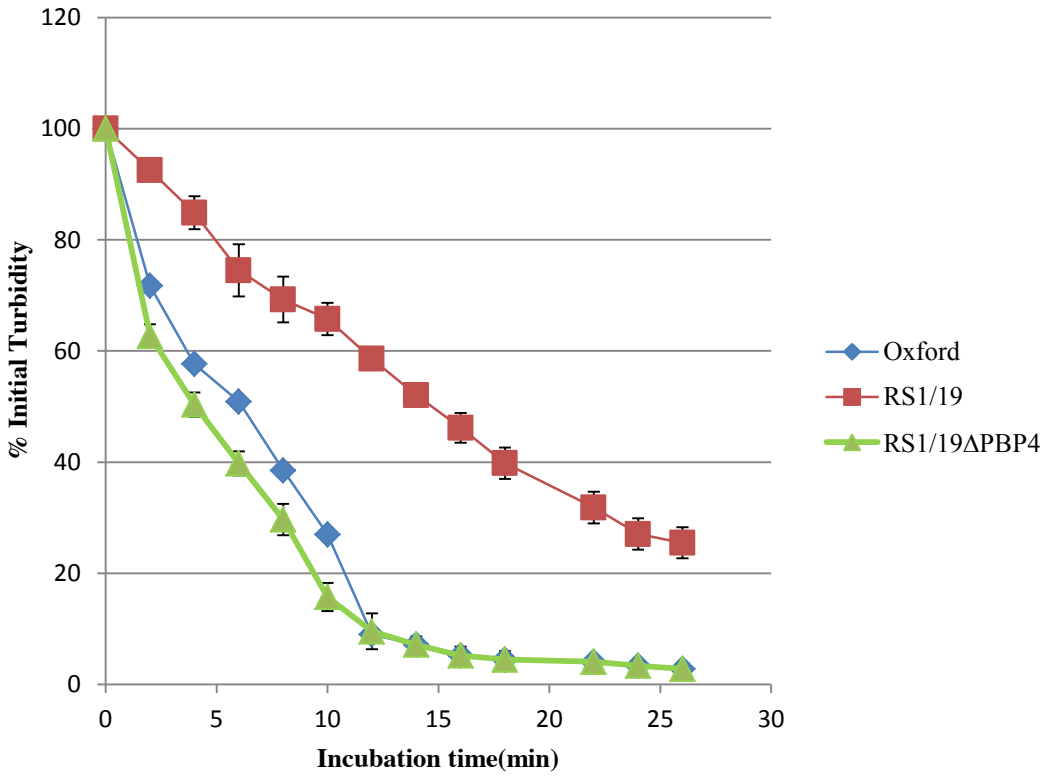


Figure 5. 12. Lysostaphin susceptibility of the wild-type (Oxford), RS1/19 and RS1/19Δ*pbp4*. Cells were grown to exponential phase at 37˚C then treated with lysostaphin (1U/ml); RS1/19 is more resistant to lysostaphin in comparison to the wild-type and RS1/19Δ*pbp4* strain. Values are the mean of three experiments and the bars represents standard error.

5. 3. 4. Autolysis with Triton X-100

To assess whether the reduction of cross-linking in RS1/19Δ *pbp4* had any effect on murein hydrolase activity, induction of autolysis with non-ionic detergent Triton X-100 was analysed. However, no differences in ODs were observed between the RS1/19 and RS1/19Δ *pbp4* strains exposed to 0.05% Triton X-100 at 30˚C with shaking (Figure 5. 13).

In common with RS1/19, RS1/19Δ*pbp4* exhibited a low autolysis rate, with 50% lysis taking approximately 95min for each strain, which is significantly longer than the Oxford strain (50% lysis in approximately 30 minutes), suggesting that *pbp4* inactivation has no effect on the autolysis rate. This and the previous data showing that PBP4 overexpression in Oxford did not change the autolysis rate in Oxford(*pbp4*) (section 4. 3. 5) indicates that autolysis is independent of PBP4 activity. Therefore, other factors must be responsible for the reduced autolysis in RS1/19 and RS1/19Δ*pbp4*, such as a thickened cell wall, which may be a by-product of the antibiotic resistance (Fuller et al., 2005). This result is consistent with the results reported by Memmi et al. (2008) who observed no differences in the autolysis rate between methicillin resistance *S*. *aureus* MW2 and *pbp4* mutated MW2 strain. On the other hand, Utaida et al., 2006 showed that reduced autolytic activity is a common phenotype to the glycopeptide-intermediate *S*. *aureus* (GISA) as a result of thickened cell wall after growth in the presence of vancomycin (Utaida et al., 2006). In addition, alterations in the teichoic acid component of the cell wall have also been reported to be responsible for reduced autolytic activity in GISA (McAleese et al., 2006). Therefore it can be concluded that inactivation of *pbp4* has no effect on autolysis rate in RS1/19Δ*pbp4*.

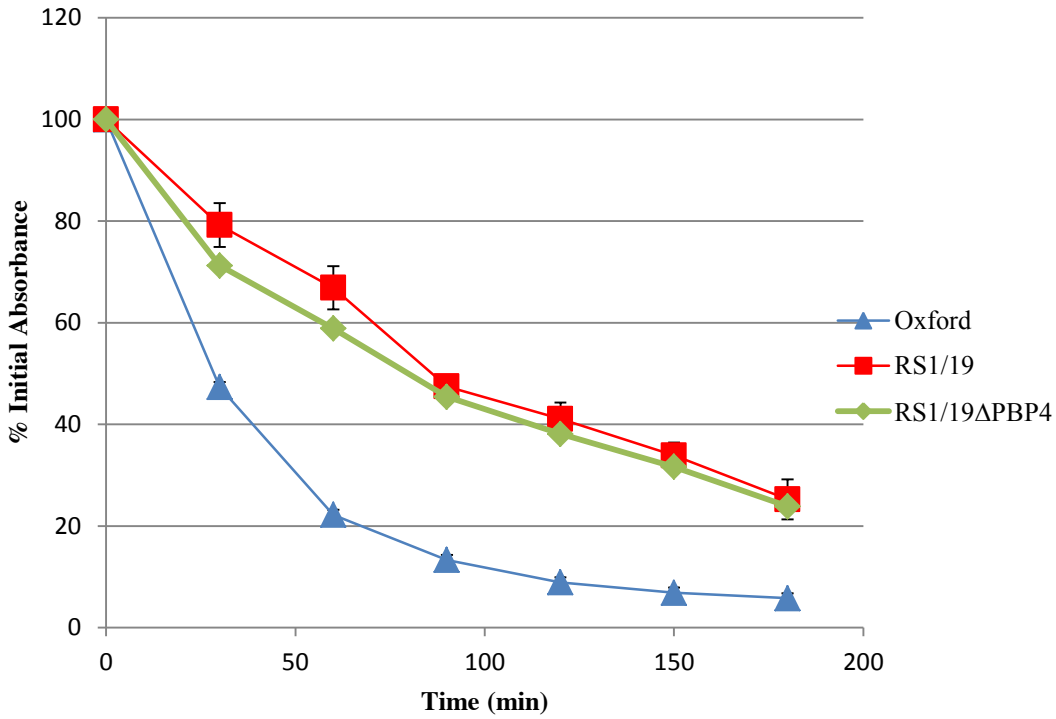


Figure 5. 13. Autolysis assay of Oxford strain, RS1/19 and RS1/19 Δ*pbp4* with 0.05% Triton X-100. After adding Triton X-100 (0.05%), the autolysis of mid-exponential phase cultures were determined at 30˚C with shaking by serial OD_{580} measurments. Bars represent standard error of the mean for three independent experiments.

Taken together, the results presented in this chapter shows that RS1/19Δ*pbp4* is more sensitive to lysostaphin, likely due to a decrease in the degree of peptidoglycan cross-linking, also inactivation of *pbp4* gene contributes with reduced sensitivity to vancomycin as a result of reduction in the cross-linking and increase in the degree of non-lethal targets in the mature peptidoglycan structure. Interestingly, RS1/19Δ*pbp4* has a moderately increased sensitivity to cefoxitin and cefsoludin, but a marked increase in sensitivity to cefotaxime, oxacillin, and penicillin G compared to RS1/19. This suggests that PBP4 is not solely responsible for the resistance and other factors must also play a role. We aim to identify these changes in resistant cells. Therefore a number of genes that participate in cell wall biosynthesis were selected to be inactivated using allelic replacement experiments to test their association with the resistance mechanism in RS1/19 cells.

CHAPTER SIX

The Role of MGT in β-lactam Resistance

6. 1. Introduction

S. *aureus* produces four native PBPs, designated PBP1-4, which differ in their peptidoglycan biosynthetic functions and affinity to β-lactam antibiotics (Chambers et al., 1994). In addition two monofunctional transglycosylases, namely MGT and SgtA, have been shown to be involved in peptidoglycan synthesis (Reed et al., 2011). It is clear that the functions of PBPs are critical for cell morphology (Paul et al., 1995) and also for the development and maintenance of antibiotic resistance (Leski and Tomasz, 2005). PBP1 has been shown to be essential in several studies, because its function cannot be replaced by the other PBPs. Inactivation of this protein cause a loss of septum formation, a dramatic increase in cell mass, and a block of cell division leading to rapid lysis (Pereira et al., 2007). Penicillin analogous and the carbapenem antibiotics have a very high affinity to PBP1, and it is over-expressed by the *S*. *aureus* when exposed to these antibiotics (Dumitrescu et al., 2011). PBP2 is the only bifunctional (traspeptidase and trasglycosylation activities) HMM protein. In MRSA strains the essential function of PBP2 (transpeptidase acivity) can be replaced by PBP2a; however point mutations made in the active site of the TGase domain caused a dramatic increase in methicillin sensitivity as well as the presence of very short peptidoglycan chain. On the other hand it has been shown that TPase activity of PBP2 is essential for growth of MSSA lacking the *mecA* gene (Pinho et al., 2001).

PBP3 has a monofunctional TPase activity, however deletion of the *pbp3* gene results in no gross phenotypic change, though it does cause a considerably reduced autolysis rate (Pinho et al., 2000). However, in some strains of *S*. *aureus* which developed a spontaneous resistance to methicillin a loss of *pbp3* expression is observed due to a premature stop codon in the gene (Pinho et al., 2003). The acyl-enzyme intermediate between penicillin and PBP3 is very stable, with a half-life of up to 115 minutes (Chambers et al., 1994), that is why long exposure to penicillin can result either in PBP3 with considerably reduced affinity for β-lactam antibiotics or totally loss of PBP3 expression (Georgopapadakou et al., 1982). Additionally, some membrane bound enzymes, MGT and SgtA which catalyzes the elongation of peptidoglycan chains in a metal-ion dependent manner, were found to be correlated

to transglycosylase activity *in vitro* and to antibiotic resistance in *S*. *aureus* (Heaslet et al., 2009; Reed et al., 2011).

The initial aim of this part of the project was to inactivate the other native PBPs (1-3), in addition to the monofunctional transglycosylases MGT and SgtA in RS1/19 to test whether these genes have a role in reduced penicillin sensitivity. This approach could highlight any involvement of these genes, alongside with overexpressed PBP4 in developing the novel mechanism of resistance in RS1/19. Furthermore, as PBP2 transpeptidase activity has been reported to be essential for viability of *S*. *aureus* strains lacking *mecA* (Pinho et al., 2001), if it proved possible to isolate a deletion of Δ*pbp2* in RS1/19 that would suggests an important role for overexpressed PBP4 in this strain. This would mean that the transpeptidase activity of overexpressed PBP4 in RS1/19 had the ability to replace the transpeptidase activity of PBP2, with the TGase activity perhaps being provided by MGTand/or SgtA. For these experiments allelic replacement, using homologous recombination, was chosen to delete the selected genes in RS1/19.

6. 2. Results and Discussion

6. 2. 1. Role of Monofunctional Transglycosylase *mgt* in ß-lactam Resistant Strain RS1/19

Inactivation of PBP4 in RS1/19 showed an important role of PBP4 overproduction in the observed β-lactam resistance in RS1/19. On the other hand it also proved that it is not the sole determinant of decreased sensitivity to penicillin G. Therefore other genes along with *pbp4* must participate in the β-lactam resistance.

The final stage in peptidoglycan polymerization involves the synthesis of glycan strands catalyzed by transglycosylase enzymes of PBP2, MGT, and SgtA (Terrak and Nguyen- Distěche, 2006). Although microarray data shows no change in the relative transcript level of *mgt* in RS1/19, to date there are few data regarding the relation of MGT to antibiotic resistance in *S*. *aureus*. Therefore experiments in this section were carried out to delete *mgt* in wild-type Oxford and RS1/19 strain and determine whether it plays a role in β-lactam resistance in RS1/19.

6. 2. 2. Inactivation of *mgt* in RS1/19 Strain

mgt was deleted in RS1/19 strain using the same strategy used to delete the *pbp4* gene in RS1/19, except that the gene used to interrupt the *mgt* structural gene encoded chloramphenicol resistance. External fragments of *mgt* were amplified from genomic DNA of RS1/19 flanking a 780bp deletion in the coding sequence of the gene. A chloramphenicol resistance gene was amplified from the vector pGL485, using PCR primers (Table 6. 1) and cloned into pMAD with the *mgt* gene fragments flanking it on either side. An intermediate cloning vector pBlue-script (SK+) was used for sub-cloning these fragments (Figure 6. 1). The mutated *mgt* fragment was cloned to the final pMAD vector, using DNA ligase. pMADΔ*mgt* was transformed to *E. coli* XL-1 Blue and colonies selected on LB plates containing Ampicillin (150 µg/ml) and X-gal (40 µg/ml). Colony PCR was performed for ten blue colonies using outside primers (mgtP1F and mgtP2R) and four positive colonies were identified with a band of 2.5 kb. The recombinant pMADΔ*mgt* vector was extracted from *E. coli* cells transformed by electroporation to *S*. *aureus* RN4220 competent cells, which were selected on BHI agar plates supplemented with chroramphenicol (10µg/ml) and X-gal (150µg/ml). Plasmids were extracted from RN4220 pMADΔ*mgt* and PCR was performed to check the size of Δ*mgt* 2.5 kb to confirm the presence of the insert. pMADΔ*mgt* were transduced to Oxford and RS1/19 strains. Allelic replacement was then carried out via double a cross over event to delete the chromosomal copy of the *mgt* gene, Screenings for the required clones was depended on the counter selection of chloramphenicol. Five white colonies that were resistant to chloramphenicol and sensitive to erythromycin were selected. To confirm allelic replacement and integration of the mutated *mgt* into the chromosome, PCR was performed on the genomic DNA using flanking forward and reverse primers mgtF1 (Table 6. 1), the size 2.55 kb were obtained, while the size of wild-type *mgt* gene is 2.63 kb (Figure 6. 2).

Table 6. 1. Sequences of primers used for *mgt* deletion, restriction sites are bold and Italic

Primer name	Primer Sequence (5'- 3')	Restriction site	Amplicon size in bp
mgtP1	F: caa*gtcgac*aggtgatttgcaatgac	*Sal*I	896
	R: caa*ggatcc*agtttgagtacctatcgcttc	*Bam*HI	
mgtP2	F: caa*ctaga*aagtaacggggtgatccag	*Xba*I	722
	R: aa*gagctcagatct*tgttgcgtgtacttctttcg	*Sac*I *Bgl*II	
cm*1*	F: caa*ggatcc*aataatggtttcttagacg	*Bam*HI	866
	R*:* caa*ctaga*agtcggcattatctc	*Xba*I	
mgtF1	F: tgccacataagatgtgttagg	-	2637
	R: ctcataaggccttcgatag	-	

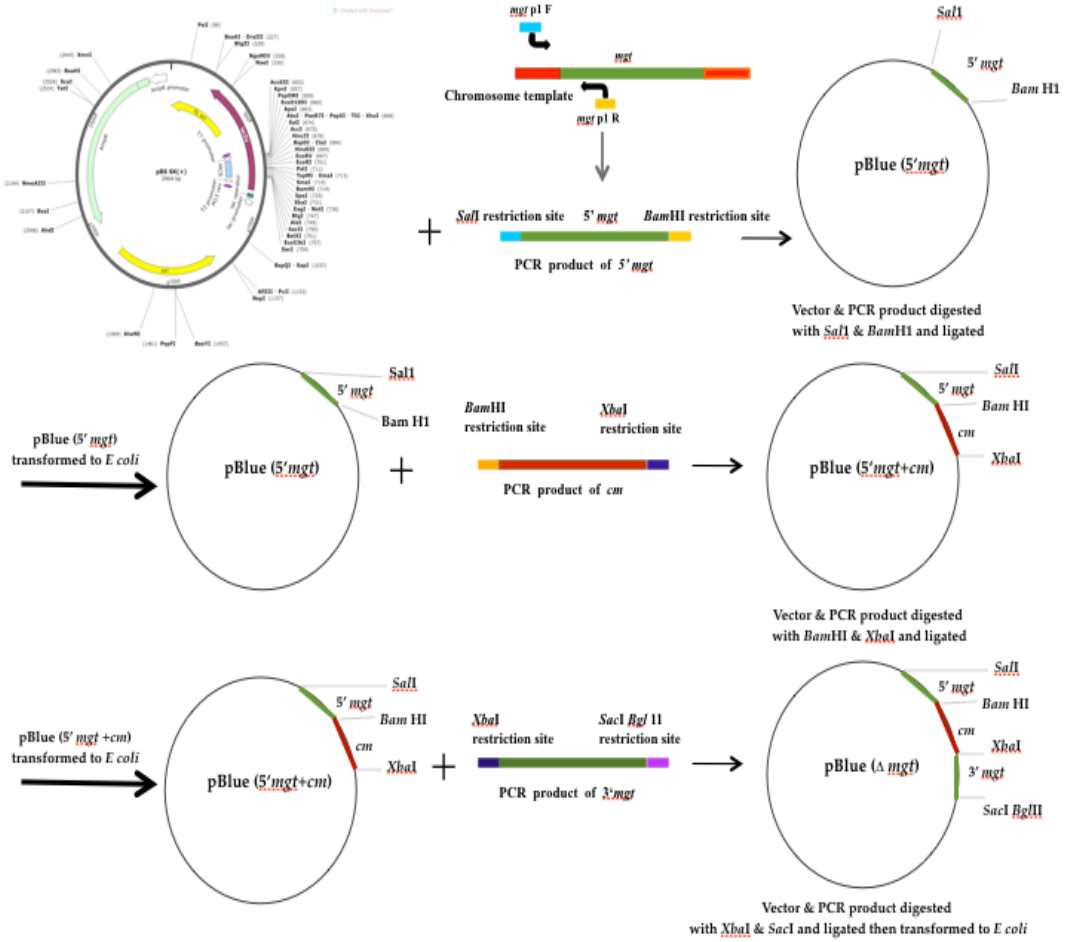


Figure 6. 1 Schematic diagram of sequential cloning of the target gene fragments into pBlue-script (SK+) cloning vector. First DNA fragment (5'*mgt*) amplified by PCR flanked by *Sal*l and *Bam*HI. Both 5'mgt fragment with pBlue-script were restricted by *Sal*l and *Bam*HI. The restricted DNA fragments were ligated and transformed to *E. coli* cells. Subsequently, the recombinant vector pBlue (5'*mgt*) was extracted. The second chloramphenicol gene (*cm*) with the pBlue (5'*mgt*) were restricted with *Bam*HI and *Xba*I and ligated, followed by transformation to *E. coli* cells. The recombinant vector pBlue(5'*mgt+cm)* was extracted from *E. coli* cells, then ligated with the third fragment 3'*mgt* restricted with *Xba*I and *Sac*I. The recombinant vector which contains the mutated *mgt* pBlue(Δ*mgt*)) was transformed to *E. coli* cells and the then extracted. Finally the recombinant vector pBlue(Δ*mgt*) were restricted with *Sal*l and *Bgl*ll and sub-cloned to the final vector pMAD for allelic replacement experiments.

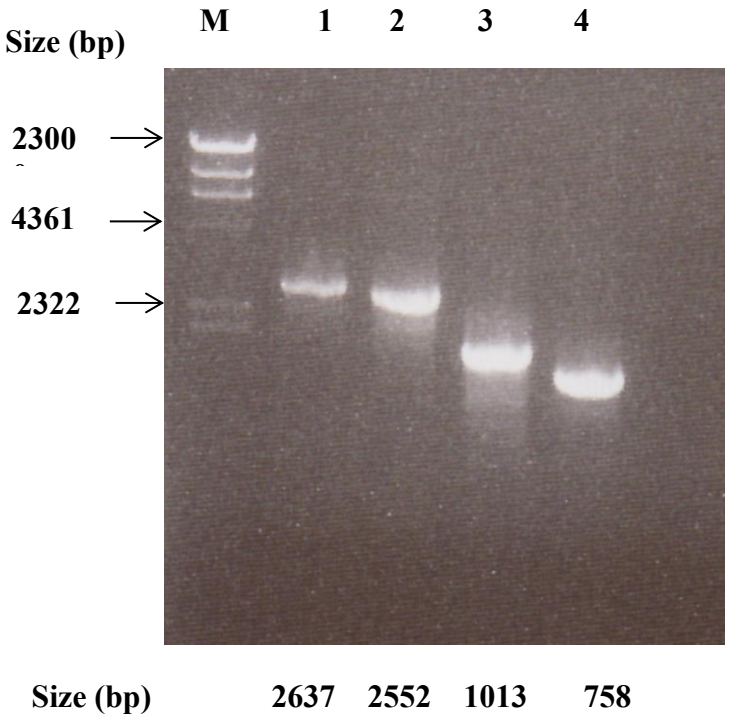


Figure 6. 2. PCR to confirm the allelic replacement of *mgt* in RS1/19. PCR products using flanking primers upstream and downstream region of *mgt* in RS1/19 lane 1. PCR products using flanking primers upstream and downstream region of *mgt* in RS1/19Δ*mgt* lane 2. PCR products using flanked forward primer of mgt F1 and reverse primer of cm1 in RS1/19Δ*mgt* lane 3, while lane 4 with forward primer of *cm1* and reverse primer of mgt F1. M is Lambda/*Hin*dIII DNA marker. The size of each PCR product is indicated.

6. 2. 2. Inactivation of Monofunctional TGase *mgt* has no Effect on Resistance to Cell Wall Targeting Antibiotics

Antibiotic sensitivity tests were performed, using a number of β-lactam antibiotics and the glycopeptide antibiotic vancomycin, to show if deletion of *mgt* has any effect on antibiotic sensitivity in RS1/19Δ*mgt* (Table 6. 2). MGT mutant strains in Oxford and RS1/19 showed no reduction in sensitivity to β-lactam antibiotics or vancomycin. The results obtained in this experiment is in agreement with those reported for the COL strain, which showed that inactivation of TGase activity of PBP2 caused a 16-fold increase in sensitivity to oxacillin, but no change was measured for the MGT deleted mutant (Reed et al., 2011). Accordingly, the authors indicated that even though MGT can replace the TGase activity of PBP2 in terms of cell viability, it cannot do so in terms of antibiotic resistance (Reed et al., 2011). It can be concluded from the data obtained in this experiment that although MGT activity may be important for peptidoglycan biosynthesis, it is not critical for cell viability because RS1/19Δ*mgt* continued to grow normally. This is likely because the TGase domain of PBP2 and SgtA can replace the absence of TGase activity of MGT. In addition, it is not essential for the expression of resistance in RS1/19 or Oxford, as the MICs for β-lactam antibiotics remain the same in RS1/19Δ*mgt* as in RS1/19 cells.

Table 6. 2. Antibiotic sensitivity test show that inactivation of *mgt* has no effect on reduced sensitivity to β-lactam and vancomycin antibiotics.

	MIC (mg/l)			
Antibiotic	Oxford	Oxford Δ*mgt*	RS1/19	RS1/19 Δ*mgt*
penicillin G	0.064	0.064	24	24
oxacillin	0.25	0.25	64	64
cefotaxime	2	2	128	128
cefoxitin	2	2	4	4
cefsoludin	4	4	8	8
vancomycin	2	2	1	1

6. 3. Attempted Deletion of Other Penicillin Binding Proteins (*pbp1*, *pbp2*, *pbp3*) and Monofunctional TGase *SgtA*

In order to understand the role of other cell wall synthesis enzymes, PBPs and SgtA, in expression of β-lactam resistance in the background of RS1/19 cells, it is necessary to knock-out these genes. Furthermore, in *S. aureus* the transpeptidase of PBP2 is essential for viability in MSSA and MRSA exposed to β-lactam antibiotics (Pinho et al., 2001). Therefore the aim was to inactivate PBP2 in the background of RS1/19 to test if the transpeptidase domain of overexpressed PBP4 can replace the transpeptidase of *pbp2* at the same time that the TGase activity of Mgt and/or SgtA replaces the TGase activity of PBP2. If transductants with Δ*pbp2* could be obtained it would mean that PBP2 is not essential for survival of RS1/19 and would show that the transpeptidase activity of overexpressed PBP4 with the TGase activity of MGT and SgtA would be sufficient for the survival in the *pbp2* mutated background.

The same strategy that was used to generate the *mgt* deletion was also used to delete the *sgtA* gene, except that the selectable marker was *spc* instead of chloramphenicol. For the other *pbp* genes (1, 2, and 3), minor modifications in sub-cloning fragments in the intermediate vector, pBlue-script (SK+), were used (Figure 6. 3). Genes with approximately a 1kb flanking region of the coding sequence were amplified using genomic DNA from RS1/19 and the primers shown in Table 6. 3. These were then restricted with *Sal*I and *Bgl*II and sub-cloned to the intermediate vector pBlue-script (SK+). Constructs were transformed to *E. coli* competent cells and selected on LB plates supplemented with ampicillin (150 μg/ml) and X-gal (40μg/ml). The presence of the insert was confirmed in white colonies by colony PCR and plasmids were extracted from positive clones. One or more restriction sites for *Hin*dIII were found in each *pbp* gene, which is not present in pBlue-script (SK+); therefore recombinant vectors containing the *pbp* genes were restricted with *Hin*dIII to cut-out fragments of around 366bp and 316bp from *pbp1* and *pbp3* respectively. The *pbp2* gene contained only one *Hin*dIII site, so no deletion took place in this case. The selectable marker (gene-disrupting marker, *spc*) was restricted with *Hin*dIII and ligated into each of these genes prior to transformation into competent *E. coli* cells, which were selected with spectinomycin (100 μg/ml). Colony PCR reactions were carried out with spc2 primers to screen for positive clones. Plasmid DNA was extracted from these cells,

restricted with *Sal*I and *Bgl*II and cloned to the final vector pMAD. The four constructed vectors: pMAD(Δ*pbp1*), pMAD (Δ*pbp2*), pMAD (Δ*pbp3*), and pMAD (Δ*sgtA*) were transformed into *E.coli* The recombinant pMAD vectors was extracted from *E. coli* cells and transformed by electroporation into *S. aureus* RN4220 competent cells and selected on BHI agar plates supplemented with erythromycin (5µg/ml) as a selectable marker and X-gal (150 µg/ml). Recombinant plasmids were extracted from RN4220; pMAD(Δ*pbp1*), pMAD (Δ*pbp2*), pMAD (Δ*pbp3*), and pMAD (Δ*sgtA*) were restricted with *Sal*I and *Bgl*II and run on agarose gels to check the size of the inserts (Figure 6. 4). Each vector was transduced to wild-type Oxford and RS1/19; this took several repeated attempts with the difficulty of transduction once again being more pronounced in RS1/19. Allelic replacement by homologous recombination was attempted for each of the target genes. Unfortunately, it was very difficult to screen for the allelic replacement mutants as the gene-disrupting marker (*spc*) was not functional in *S. aureus*. Therefore blue/white colony selection using X-gal in the media to screen for the absence of the vector in the mutant strains was the only available option. White colonies were selected following the second recombination event; these could contain a chromosomal copy of either a mutant or wild-type allele of the target gene. Counter selection (spectinomycin) will only allow the mutant cells (single cross over and double cross over event) to grow on plates containing (100µg/ml) spectinomycin, but *spc* gene was not functional in these cells, therefore the only option was to isolate genomic DNA from all white colonies to screen for the mutated cells by PCR. Despite considerable efforts, the frequency of allelic replacement was too low to identify a mutated target allele using this approach and all of the white colonies tested contained the wild-type allele of the target gene and therefore must have had excised the vector during the attempted second cross over procedure.

In conclusion, it was not possible to isolate deletion mutants of the *pbp1*, *pbp2*, *pbp*3 or *sgt*A genes due the time constraints. Spectinomycin selection did not work as expected in RS1/19 or Oxford and despite considerable effort; it was not possible to identify the required strains using the PCR analysis method. However, all of the tools needed to continue this part of the project are available and by swapping the *spc* gene with another antibiotic resistance selectable marker e.g. chloramphenicol it

should be possible to obtain deletions in the target genes and determine whether they are necessary for full expression of β-lactam resistance in RS1/19.

Table 6. 3. Sequences of primers used for *pbp1, pbp2, pbp3,* and *sgtA* deletion. Restriction sites are bold and Italic

Primer name	Primer Sequence (5'- 3')	Restriction site	Amplicon size in bp
pbp1P1	F: caa*gtcgac*tacgcgaggaaagattgc	*Sal*I	1726
	R: cac*ggatcc*acttgtgctgtaccagtc	*Bam*HI	
pbp2P1	F: caa*gtcgac*acaaaggatcttctcagcc	*Sal*I	2087
	R: cac*ggatcc*tgctgatgtgtcactacc	*Bam*HI	
pbp3P1	F: caa*gtcgac*acaaatcgcacaaggctcac	*Bam*HI	1880
	R*:* cac*ggatcc*tacctaagtctccacctg	*Xba*I	
spc2	F: aa*aagctt*caggctgggtgccaagctctcg	*Hin*dIII	1548
	R: caa*aagctt*gtgcttagtgcatctaacgc	*Hin*dIII	
sgtAP1	F: caa*gtcgac*acaaaggatcttctcagcc	*Sal*I	901
	R: caa*ggatcc*cgatagcaacgatggcaatg	*Bam*HI	
sgtAP2	F: caa*tctaga*atggctatacaccctacg	*Xba*I	777
	R*:*caa*gagctcagatct*agcgttggtatgtcatc	*Sac*I *Bgl*II	
spc3	F: *ggatcc*caggctgggtgccaagctctcg	*Bam*HI	866
	R*:* caa*tctaga*gtgcttagtgcatctaacgc	*Xba*I	

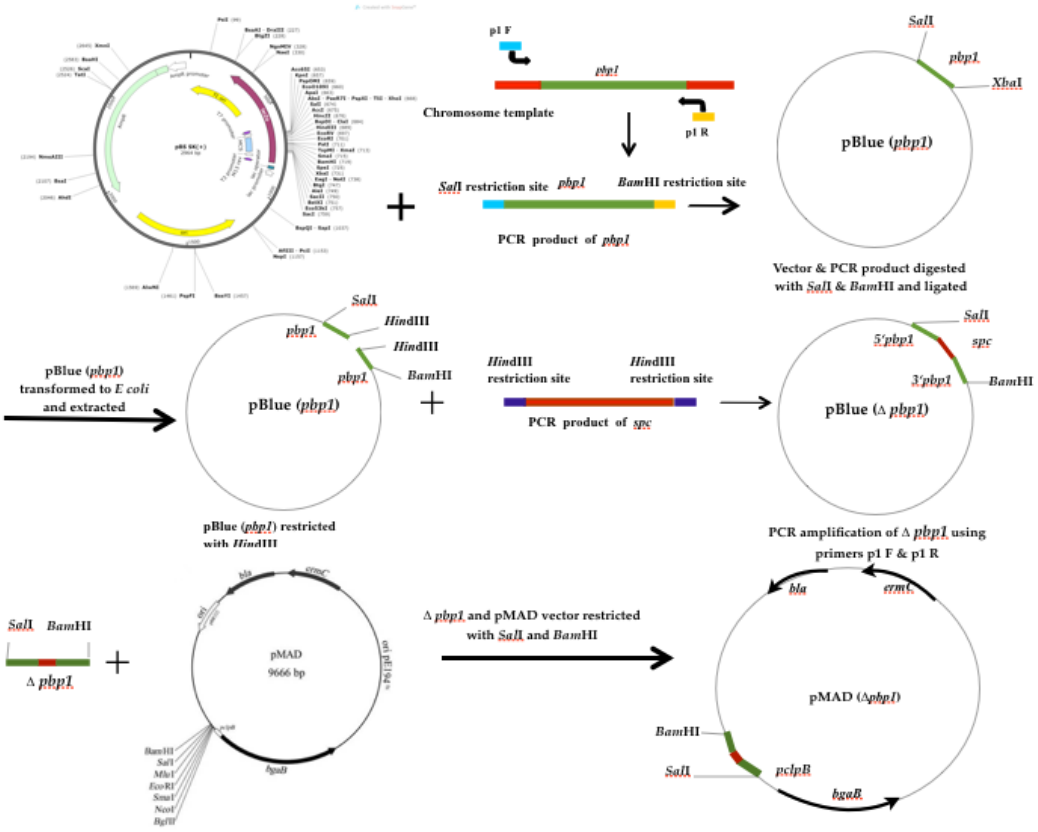


Figure 6. 3. Schematic diagram of sequential cloning of the target gene fragments into pBluE-script (SK+) cloning vector. First DNA fragment *pbp1* amplified by PCR flanked by *Sal*I and *Bam*HI. Both *pbp1* and pBlue-script were restricted by *Sal*I and *Bam*HI. The restricted DNA fragments were ligated and transformed to *E. coli* cells. Subsequently, the recombinant vector pBlue (*pbp1*) were extracted, and restricted with *Hin*dIII. Digested pBlue(*pbp1*) and *spc* gene which is restricted with the same enzyme were ligated. The recombinant vector pBlue(Δ*pbp1)* transformed to *E. coli* cells and then extracted. Δ*pbp1* were amplified and along with pMAD vector restricted with *Sal*I and *Bam*HI, then ligated and transformed to *E. coli*.

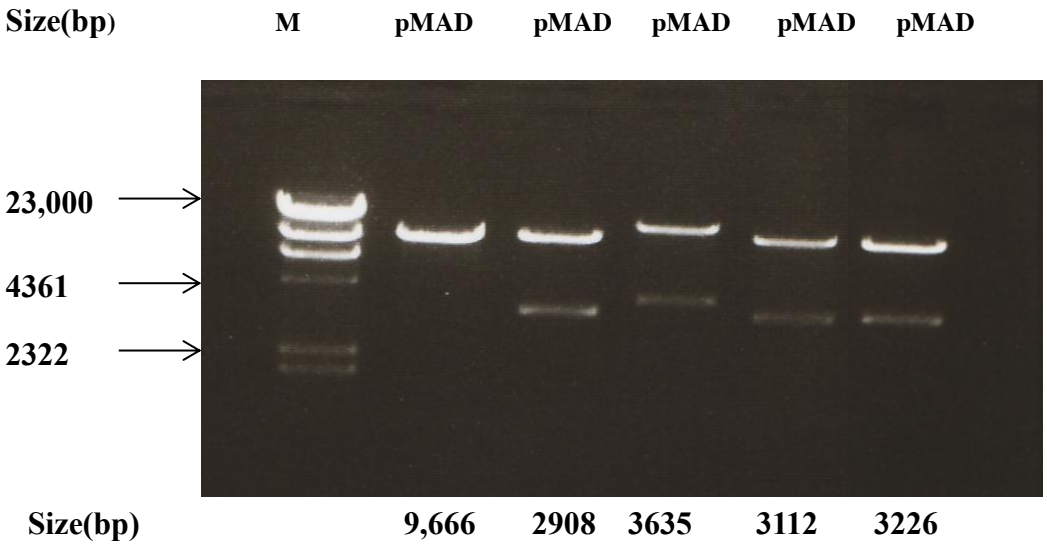


Figure 6. 4. Restriction enzyme digestion of recombinant pMAD vector. The recombinant vector (isolated from *E.coli* were digested with *Sal*I and *Bgl*II to cut-out the insert. The digestion product resolved on 1% agarose gel. M is a DNA marker Hyperladder I. The size of each insert is indicated in bp.

CHPTER SEVEN

General Discussion

7. 1. Kinetics of Acquisition of Penicillin Resistance

In the present study CWD bacteria from Oxford strain were generated by passaging in liquid medium, with a high osmolality, that contained increasing concentration of penicillin G. Following 10 serial passages, the MIC rose from 0.064 to 32 μg/ml. The cells were then reverted to CWC forms by sub-culturing them in antibiotic free media several times. Of special interest was the observation that revertant CWC (T12) cells exhibited stable tolerance to penicillin G with an MIC of 24 μg/mL. Acquisition of penicillin resistance was achieved in a small incremental step and correlated with changes in the Gram staining characteristics of a large proportion of the cells in the population. These data are contrary to a previous report showing step-wise acquisition of resistance to penicillin G on the fifth passage of an *in vitro* selection procedure (Henze and Berger- Bächi., 1995). The major difference in these two cases was the osmolality of the medium used. In this study a media with a high osmotic potential CWD was used which support the formation of CWD bacteria, while Henze and Berger- Bächi selected out methicillin resistance mutant PV1 by growth on increasing concentrations of penicillin in standard LB medium which did not allow the formation of CWD bacteria. Therefore it is likely that the composition of the media has a crucial role on the development of resistance mechanism.

On the first exposure to penicillin G, Gram staining showed that about 90% of the cells gave a Gram-negative reaction and the cells had a diffuse edge. On continued exposure to the antibiotic, part of the population showed a Gram-positive reaction and continued to grow even in the presence of high concentration of the antibiotic. As the CWD medium, used for generating resistant strains, had a high osmolality, it is likely that bacteria with disrupted cell walls were protected from lysis. CWD bacteria and L-forms are osmotically fragile, requiring electrolytes, sucrose, or other osmotic stabilizers to survive in the medium, (Onoda and Oshima, 1988) which is one of the factors that increased the chance of the surviving cells while introducing the mutations needed for developing increased penicillin resistance. Fuller et al. (2005) used the same method using both LB medium instead and high osmolality (CWD) medium. The results showed that even though increasing penicillin concentrations in LB media caused an increase in penicillin resistance, it was much less than the level of resistance seen in CWD medium and was also quickly lost

when the penicillin pressure was removed. Besides, in the presence of penicillin, cells did not exhibit any Gram negative staining, which means that the content of the media had a very important role in induction and survival of CWD bacteria (Fuller et al., 2005).

Reports in the literature suggest that the antibiotic sensitivity profile of revertant cells depends on the way that the L-form was induced (e.g. depending on the presence of stabilizer in the medium, period of incubation, and concentration of the antibiotic). Consequently, some revertant cells have susceptibility profiles similar to their parent cells, while others remain resistant to cell wall active antibiotics (Bertolani et al., 1975; Fuller et al., 2005).

S. *aureus* Oxford, which is mecA-*negative,* was previously used to generate a methicillin resistant strain (RS1/19), on media with a high osmolality and was one of the strains used in this study. RS1/19 has small colonies, which are paler in appearance and has a slower growth rate compared to the wild type (Fuller, 2005). Like RS1/19, T12 cells small in size, lack the golden pigmentation, typical of *S*. *aureus* Oxford, and has a slow growth rate. RS1/19 cells have undergone extensive transcript reprogramming, resulting in different gene expression patterns, as confirmed by microarray data (Appendix 1). The ability to form "variants" under stress, both *in vivo* and *in vitro,* is one obvious character of *S. aureus*. Although *S. aureus* is non-motile and cannot form spores, it can adapt to environmental changes including changes to cell architecture. This includes L-forms, lack of pigmentation, small colony variants (SCVs), and slow growth rate (Marcova et al., 2008).

The formation of variants also occurs in other bacterial strains. For example, Dell'Erra et al. (2009) reported that stable L-forms of *Listeria monocytogenes* generally feature much slower growth rates compared to parental cells. They suggested that multiple phenotypic abnormalities could be as a result of alterations in global gene expression and /or cell signaling pathways in the mutant strains. Array-based transcriptomics of parent and L-form cells revealed differences in expression of genes associated with morphological and physiological functions, leading to the conclusion that the L-forms featured down-regulated metabolic functions correlating with the dramatic shift in surface to volume ratio, whereas up-regulation of stress

genes reflects the difficulties in adapting to this unusual, cell wall-deficient lifestyle (Dell'Erra et al., 2009).

The colonies of the Oxford strain produce yellow pigmentation, while both RS1/19 and T12 were paler in colour. The cell membrane contains a carotenoid pigment, Staphyloxanthin responsible for yellow or orange pigmentation. The gene for Staphyloxanthin biosynthesis (*crtM*, and *crtN*) are under the control of *SigB*, which is expressed as a general stress response (Wieland et al., 1994). However microarray data shows no change in the expression of *SigB*, therefore other factors might be responsible for this phenomenon in T12 and RS1/19. Another possibility for the lack of pigmentation is due to mutation of the *crt* gene (Bornstein et al., 2010), but no data is available for the gene sequence in resistant cells.

T12 and RS1/19 also exhibit small sized colonies, which look like SCVs and have slower growth rates compared to the wild type. In has been reported that SCVs are deficient in the electron transport system, such as the pathways for hemin, menadione and thiamine and thereby exhibit slow growth rate. Indeed, the disruption of genes involved in hemin or menadione biosynthesis generates SCVs (Morikawa et al., 2010). Similar results were obtained when other areas of *S*. *aureus* were affected; for example, cold shock caused slower growth of *S*. *aureus* due to activation of stress responses that decrease membrane fluidity and reduce enzymatic activity within the cell (Ooi et al., 2009). *S*. *aureus* and other bacteria grow more slowly in biofilms and diminished growth rates are an important factor in the increased resistance of bacteria within biofilms to many antibiotics (Fux et al., 2005).

A common feature of alteration of peptidoglycan metabolism in RS1/19 and T12 is decreased sensitivity to lysis by both lysostaphin and Triton X-100. RS1/19 cells have a thicker cell wall (Fuller et al., 2005); thickening of the cell wall could be due to either a lower rate of peptidoglycan degradation or an increased rate of peptidoglycan biosynthesis. In other systems, increased thicknesses of the cell wall and slow growth rate have been shown to inhibit wall turnover and autolysis by blocking the access of murein hydrolases to their cell wall substrate (Sieradzki and Tomasz 1997).

The regulation of autolytic activity is generally complex and can occur at a number of different levels including autolysin enzymatic activity, post-translational processing of autolysins, and autolysin gene transcription. Alteration in these characteristics would contribute to a thicker cell wall, and in turn, decrease the susceptibility to lysostaphin and Triton X-100. As *atl* plays a crucial role in cell division and separation, lowered expression may produce build-up of peptidoglycan layers contributing to a thickened cell wall (Wootton et al., 2005). In RS1/19, the gene appears to be expressed normally as confirmed by Microarray data (Appendix 1) and activity assays show autolysins from RS1/19 can effectively lyse wild-type cells, but that autolyins from either Oxford or RS1/19 do not readily lyse the altered walls of RS1/19 cells.

Another possible reason for altered autolysis could be related to some quantitative or qualitative alterations in the wall teichoic acid, as this has been reported to lead to reduced autolysis and thickened cell wall (Sieradzki and Tomasz 2003; McAleese et al., 2006). AtlA has its optimal activity at neutral pH and this reduced dramatically at pH values below 6.5. WTA phosphate groups retain protons in the cell wall, which develops an acidic environment keeping the activity of the major autolysin AtlA low, therefore the activity of AtlA is affected by the presence or absence of WTA (Biswas et al., 2012). Additionally, mutations within the *S aureus* virulence factor regulatory gene *agr* results in reduced autolysis rate (Fujimoto et al., 1998). Interestingly, microarray data (Appendix 1) shows down regulation of the *agr* regulatory factor in RS1/19, which might participate in the reduced autolysis rate.

Another abnormality of peptidoglycan metabolism in RS1/19 was detected *in vitro* by quantitative autolysis rate of whole cell using extracellular autolysin. Results indicated that the peptidoglycan component of RS1/19 is more resistant to extracellular autolysin extracted from both RS1/19 and Oxford strain. This result confirms that the autolysins in Oxford and RS1/19 cells are functionally active and reduced autolysis rate might be due to an altered cell wall in RS1/19.

Assays of PBPs, during acquisition of penicillin resistance, revealed a gradual increase in the amount of PBP4 in membrane proteins, which altered the PBP2/PBP4 ratio from 53.8 to 2.3 in Oxford and T12 respectively. The decrease in susceptibility to β-lactam antibiotics also developed gradually, suggesting a causal link between the level of PBP4 and antibiotic sensitivity. In RS1/19, there are a variety of changes in gene expression, but prominent amongst these was *pbp4*, which was the gene with the highest expression level change. PBP4 is the only LMW PBP present in *S. aureus* (Wyke et al., 1981) and laboratory studies have shown that it has DD-carboxypeptidase, transpeptidase, and β-lactamase activities (Navratna et al., 2010). However the main function of LMW PBP4 in *S. aureus* is transpeptidase with little DD-carboxypetidase activity, which is evidenced by the presence of highly cross-linked peptidoglycan in the cell wall of these bacteria. High DD-carboxypeptidase activity would decreases cross-linking, by removing the terminal alanine residue and so decreasing the amount of free D-Ala-D-Ala in the peptidoglycan, which is essential for transpeptidation. This is supported by studies that have show that PBP4 inactivation leads to a lower degree of cross-linking (Sieradzki et al., 1999) and overproduction of PBP4 increases the percentage of cross-linking (Henze and Berger-Bächi, 1996).

7. 2. The Use of Penicillin Analogues to Understand the Resistance Mechanism

RS1/19 cells have a moderately increased resistance to cefoxitin and cefsoludin, (which readily inhibit PBP4 activity) but a marked increase in resistance to cefotaxime and penicillin G (which effectively inhibit the TPase activity of PBP2). These cells can likely withstand inhibition of the transpeptidase activity of PBP2, by cefotaxime and penicillin G, because of the high activity of PBP4. This contention is supported by data showing that in the presence of 25% of the MIC of cefoxitin, the MIC of penicillin G and cefotaxime, went down from 24 μg/ml to 0.094 μg/ml and 128 μg/ml to 4 μg/ml respectively. This means that reduced sensitivity to penicillin G and cefotaxime in CWC cells is likely related to PBP4 overproduction, as antibiotics that selectively inhibit PBP4 activity cause the greatest reduction in penicillin G MIC, when used in combination. This is evidenced by a remarkable rise

of penicillin and cefotaxime sensitivity of RS1/19 following treatment with selective PBP4 inhibitory antibiotic (cefoxitin). Farha et al. (2013) reported that β-lactam resistance might be reversed by targeting PBP2 and PBP4 with combinations of existing β-lactams. This was tested by using the PBP4-selective β-lactam cefoxitin. When cefoxtin was combined with PBP2-selective β-lactams cefuroxime, ceftizoxime, oxacillin, and penicillin, the MICs of these antibiotics decreased 32- to 128-fold. Conversely, combining cefoxitin with β-lactams having a low affinity for PBP2 led to only a 2- to 8-fold change in the MIC. They showed that in the absence of wall teichoic acid leads to mislocalization of PBP4 away from division septum, for that it is unable to perform its role of cross-linking in peptidoglycan. When these MRSA cells treated with β-lactams which binds PBP2 selectively sensitizes these cells. This shows the importance cooperation between PBP2 and PBP4 in mediating resistance to β-lactams antibiotics (Farha et al., 2013). This supports our conclusion that PBP4 overproduction has an important role in the observed resistance.

Others have demonstrated that cefoxitin, which binds PBP4 irreversibly, was found to be synergistic with oxacillin at killing CA-MRSA strains compared to oxacillin alone. In the presence of one-fourth the MIC of cefoxitin, the MIC of oxacillin went down 64-fold (Memmi et al., 2008). Data produced in this study supports a role for PBP4 overexpression in β-lactam resistance, via molecular function as a transpeptidase that is necessary for highly cross-linked peptidoglycan. This hypothesis is based on a number of key observations, relating to high relative expression of *pbp4* in RS1/19 strains*;* increasing the amount of PBP4 in CWC lines gradually is consistent with the rise in MICs and the synergistic effect of β-lactams. Which selectively inhibit PBP2 and PBP4, indicates an important role for PBP4 in the observed resistance.

7. 3. The Role of PBP4 in Penicillin and Methicillin Resistance

To test the role of PBP4 in mediating β-lactam resistance in RS1/19, PBP4 was overexpressed in *S. aureus*. Surprisingly, PBP4 overproduction in *S. aureus*, with different genetic backgrounds, did not alter the MIC to β-lactam antibiotics. However, it produced a twofold decrease in the vancomycin MIC. The antibiotic sensitivity data presented in (Chapter Three) implicates the involvement of PBP4

overproduction in the resistance mechanism, leading to the conclusion that PBP4 overproduction, alone, is not sufficient for full expression of resistance. In a previous report, high-level expression of *pbp4* in *mecA* negative isolates, caused by a deletion in the region upstream of this gene, resulted in an increase in the amount of PBP4 in cell membranes and a reduction in susceptibility to penicillin and methicillin (Henze and Berger-Bächi, 1996). Recently, Banerjee at al., reported that methicillin resistance in an *in vitro* generated strain is due in part to an amino acid change at the entrance to the binding pocket of PBP4 (Banerjee et al., 2010). Therefore, methicillin resistance in strains lacking *mecA* have different underlying bases, indicating that the genome is able to respond in different ways to subtly different selection pressures.

*mec*A-mediated meticillin-resistance in *S. aureus* requires the concerted action of several host factors for full expression. In addition to factors essential for methicillin resistance (*femAB*). *mecA* positive strains have hetero-resistance to methicillin, with the majority of cells expressing low resistance to methicillin and a minority of cells express resistance to much higher concentrations. It has been demonstrated that this high level resistance is due to chromosomal mutations outside of the *mecA,* gene, and is also not related to the *femAB* operon. When the highly resistant cells were cured of the vector containing the *mecA* gene, the result was a methicillin sensitive strain and when the vector was introduced to these cells again it became highly resistant to methicillin. On the other hand transduced cells, derived from the original parents, showed a lower level of resistance to methicillin which was heterogeneous resistance (Ryffel et al., 1994). Arbeloa et al., demonstrated that expression of the *mecA* of *S. aureus* in *E. faecalis* and *E. faecium* can confer the resistance phenotypes in subpopulations of the bacteria, therefore, there are be other factors required for full expression of resistance (Arbeloa et al., 2004).

PBP4 over-expression slightly decreased the vancomycin MIC; this was evident in strains with different genetic backgrounds and, although small, this effect was consistent. This means that increased susceptibility was uniformly correlated with increased peptidoglycan cross-linking in transductants. Therefore it indicates a relationship between the degree of cross-linking and vancomycin susceptibility. Increased PBP4 activity leads to a concomitant reduction in the number of free D-Ala-D-Ala motifs in the mature peptidoglycan which serves as a first target for

vancomycin molecules, leading to increased diffusion rates of vancomycin to its second target on lipid II. Similarly, Finan et al. reported that PBP4 overproduction produces two-three folds decrease in the sensitivity to vancomycin in the VISA isolates (Finan et al., 2001).

Allelic replacement via a double cross-over event was used to delete *pbp4* in RS1/19. The constructed vector pMAD(Δ*pbp4*) was transduced from RN4220 pMAD(Δ*pbp4*) into the RS1/19 strain. Interestingly deletion of *pbp4* led to a dramatic decrease in the MIC to penicillin G 4000 fold, oxacillin 64 fold, and cefotaxime 256 fold. It has been proposed that the transpeptidase activity of overproduced PBP4 correlates with the resistance mechanism in RS1/19 and therefore depletion of PBP4 would sensitize these cells. This result is in agreement with the results of Curtis et al. (1980) who isolated a mutant of *S*. *aureus* H lacking PBP4, selected on the basis of increased sensitivity to cefotaxime and a range of β - lactam antibiotics. PBP4 inactivation in this strain led to two-fold reduction of resistance in penicillin G, methicillin, and cefotaxime and one fold for cefoxitin. In another study, a *S*. *aureus* COL *pbp4* knockout strain has been reported to be more susceptible than the parent strain to penicillin and ampicillin (Katamaya et al., 2003).

Further, Henze and Berger Bächi (1996) reported that a mutant strain UT-39-1 generated by Tn551-mediated insertional inactivation of *pbp4* in a methicillin resistant strain, showed a reduction in MIC from 8µg/ml to 2.5µg/ml. It has been reported that a mutation in PBP4 in two strains of CA-MRSA led to a significant decrease in high-level resistance to oxacillin and nafcillin. *mecA* expression remained unchanged in PBP4 mutants and PBP2a did not contribute to oxacillin resistant in the absence of PBP4. Thus the authors concluded that PBP4 is an important factor in β-lactam resistance in CA-MRSA but not in HA-MRSA isolates (Memmi et al., 2008). However, more recently Navratna et al., showed that MRSA COL strain, which have been selected for *in vitro* resistance to vancomycin, challenged with (1× MIC) of either ampicillin or cefotaxime. Unlike other PBPs, the expression of *pbp4* didn't alter under these conditions (Navratna et al., 2010).

There is evidence that PBP2, PBP2A and PBP4 act cooperatively in cell wall synthesis under antibiotic pressure and that PBP4 is essential for β-lactam resistance

in community-acquired MRSA strains (Leski and Tomasz, 2005). This might suggest that when the PBP2 transpeptidase activity is inactivated in RS1/19, by penicillin G, the transpeptidase activity of overproduced PBP4 would replace the transpeptidase activity of PBP2. This is supported by data which shows that overexpressed PBP4 is necessary for the expression of penicillin and methicillin resistance, in this strain, though it is not sufficient for the observed resistance on its own.

The results obtained by Sieradski and Tomasz, showed that *in vitro* selection for vancomycin and teicoplanin caused an altered peptidoglycan composition due to decrease in highly cross-linked peptidoglycan (Sieradski and Tomazs, 1997; Sieradski and Tomazs, 1998). Subsequently they showed that the disrupted peptidoglycan was due to inactivation of PBP4 in the mutant strains. Inactivation of PBP4 leads to increased monomeric muropeptide in the cell wall (Sieradzki et al., 1999). The free D-Ala-D-Ala residues in the mature peptidoglycan strands constitute a potential nonlethal target for vancomycin (Sieradzki and Tomasz, 1997). Binding of large vancomycin molecules to nonlethal targets would further blocks the movement of the vancomycin molecules to their lethal target, D-Ala-D-Ala residue of the lipid linked peptidoglycan precursor (Hiramatsu, 2001). This is explains decreased sensitivity of RS1/19(Δ*pbp4*) to vancomycin antibiotics with a four-fold increase in MIC. It has been reported that only a thickened cell wall with inactivated PBP4 lead to vancomycin resistance (Cui et al., 2003).

To find out what proteins, in addition to PBP4 overproduction, contribute to this mechanism of resistance a number of allelic replacement studies were initiated. Monofunctional TGase *mgt* which is responsible for Mgt production (Terrak and Nguyen- Distěche, 2006) was deleted; To date there is little information about the relationship of this gene to antibiotic resistance. Although microarray data shows no change in the relative expression of this gene, but it was necessary to remove the activity to determine if it plays an active part in the resistance mechanism. Following inactivation of this gene, antibiotic sensitivity showed no alteration in the MIC to the antibiotics used in this study. This result is similar to the results demonstrated by Reed et al., who indicated that MGT inactivation has no effect on oxacillin sensitivity. They suggested that TGase activity of PBP2 and SgtA can replace the

monofunctional TGase activity of MGT (Reed et al., 2011). Therefore it was concluded that monofunctional TGase MGT has no effect on the resistance in RS1/19.

7. 4. Implications for understanding resistance in MRSA strains

Experiments performed in this study indicate that the underlying resistance mechanism in RS1/19 is different to previously reported β-lactam resistance mechanisms and appears to involve mutations in multiple genes (or regulatory regions). Data presented here prove that PBP4 over-production is an essential component of this resistance. These data are consistent with reports from other workers showing that PBP4 can work co-operatively with PBP2 and PBP2A in building cell walls (Leski and Tomasz, 2005) and that it contributes to the resistance seen in CA- but not HA-MRSA strains (Memmi et al., 2008). Recent work by Farha et al., shows a dual function of PBP2 and PBP4 in mediating β-lactam resistance (Farha et al., 2013), confirming a central role for PBP4 in metnicillin resistance in *mecA* negative *S. aureus* strains. The data presented here clearly shows that PBP4 is not the only factor involved in resistance in RS1/19, but that it is necessary for the full expression of resistance. Future studies of RS1/19 are likely to uncover other factors that are essential for full expression of methicillin resistance in *mecA* negative strains of *S. aureus*.

Future Work

Further studies are needed to answer the question about the role of the other cell wall biosynthesis enzymes like PBPs (1-3), and the mono-functional TGase SgtA in expression of β-lactam resistance in the background of RS1/19. Transductants of RS1/19 pMAD(Δ*pbp1*), RS1/19 pMAD (Δ*pbp2*), RS1/19 pMAD (Δ*pbp3*), and RS1/19 pMAD (Δ*sgtA*) were made for deletion of these genes in RS1/19. However, each of these relied upon spectinomycin selection (Arnaud et al., 2004), which did not work as expected in these cells. In spite of considerable effort; it was not possible to identify the required strains using the PCR analysis method. However, all of the tools needed to continue this part of the project are available and by swapping the *spc* gene with another antibiotic resistance selectable marker e.g. chloramphenicol it should be possible to obtain deletions in the target genes and determine whether they are necessary for full expression of β-lactam resistance in RS1/19.

It would also be interesting to determine if RS1/19(Δ*pbp2*) could be isolated, which would highlight an important role of overproduced PBP4 that can replace the transpeptidase activity of PBP2, which prove a coordinating function of PBP2 and PBP4, but in the time available it was not possible to analyse.

Another important area that requires further experimentation is comparison of the genomes of Oxford and pencillin resistant lines by genome re-sequencing and bioinformatics to identify mutations potentially involved in resistance.

We have repeatedly been able to isolate stable penicillin and methicillin resistant lines from the Oxford strain (ATCC9144), in liquid cultures (T12) and Fuller et al., isolated the strain from solid media (RS1/19). However, the methods used in generating these lines may introduce changes to the genome, which are not directly related to the resistance mechanism. Therefore sequencing two independently generated lines (RS1/19 and T12) and compares these to the sequence of the Oxford parental line to identify candidate genes for further study.

Another interesting point for future work would be replacement of mutated regions in strain RS1/19 with wild-type equivalents and replacement of wild-type DNA in

Oxford with mutated regions from RS1/19. The contribution of each potential mutation, identified from genome re-sequencing, would be investigated by swapping that genome region between RS1/19 and the parental line. That is to say, a mutated region identified in RS1/19 be transferred into Oxford and the equivalent region from Oxford would be transferred into RS1/19. In this way it would be possible to build up a library of strains of RS1/19 and Oxford to determine the contribution of each mutation to overall resistance. Regions that show an effect could then be combined to see if any additive or synergistic effect is achieved with multiple regions. This approach would be equally useful in determining the effect of mutations in non-coding regions of the genome as it is in investigating mutations in structural genes.

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Appendix 1

Genes up-regulated by penicillin G at least twofold in RS1/19 compared to Oxford strain ($P < 0.05$)

Gene	Fold change	Product or putative function	Functional category
pbp4	18.56	penicillin-binding protein 4	Translation, ribosomal structure and biogenesis
sdrE	10.06	bone sialoprotein-binding protein [conserved region]	Translation, ribosomal structure and biogenesis
clfB	9.256	fibrinogen and keratin-10 binding surface anchored protein	Translation, ribosomal structure and biogenesis
SAR2532	5.207	hypothetical protein	Translation, ribosomal structure and biogenesis
sdrD	4.676	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	Translation, ribosomal structure and biogenesis
argG	4.646	putative argininosuccinate synthase	Translation, ribosomal structure and biogenesis
thiI	4.128	putative thiamine biosynthesis protein	Translation, ribosomal structure and biogenesis
pyrAA	4.11	putative carbamoyl-phosphate synthase, pyrimidine-specific, small chain	Translation, ribosomal structure and biogenesis
pyrC	4.105	putative dihydroorotase	Amino acid transport and metabolism
rpsS	4.081	30S ribosomal protein S19	Translation, ribosomal structure and biogenesis
pyrAB	4.054	putative carbamoyl-phosphate synthase, pyrimidine-specific, large chain	Translation, ribosomal structure and biogenesis
rpsN	4.023	30S ribosomal protein S14	Translation, ribosomal structure and biogenesis
icaA	4.006	glucosaminyltransferase	Translation, ribosomal structure and biogenesis
SAR0841	3.888	putative acetyltransferase	Translation, ribosomal structure and biogenesis
rplN	3.841	50S ribosomal protein L14	Translation, ribosomal structure and biogenesis
SAR0343	3.738	putative Sec-independent protein translocase protein	Translation, ribosomal structure and biogenesis
SAR0345	3.737	conserved hypothetical protein	Translation, ribosomal

			structure and biogenesis
rpsC	3.703	30S ribosomal protein S3	Translation, ribosomal structure and biogenesis
SAR2083	3.683	putative single-strand DNA-binding protein	Translation, ribosomal structure and biogenesis
rplR	3.603	50S ribosomal protein L18	Translation, ribosomal structure and biogenesis
secY	3.57	preprotein translocase SecY subunit	Translation, ribosomal structure and biogenesis
rpsQ	3.549	30S ribosomal protein S17	Translation, ribosomal structure and biogenesis
rpmC	3.466	50S ribosomal protein L29	Translation, ribosomal structure and biogenesis
rplX	3.459	50S ribosomal protein L24	Translation, ribosomal structure and biogenesis
rplP	3.433	50S ribosomal protein L16	Cell envelope synthesis or location
SAR1794	3.425	aminotransferase class-V protein	Cell envelope synthesis or location
rplO	3.36	50S ribosomal protein L15	Nucleotide transport and metabolism
rpsH	3.334	30S ribosomal protein S8	Carbohydrate transport and metabolism
rplF	3.304	50S ribosomal protein L6	Amino acid transport and metabolism
rplV	3.255	50S ribosomal protein L22	Translation, ribosomal structure and biogenesis
icaD	3.234	intercellular adhesion protein D	Cell envelope synthesis or location
SAR2082	3.23	putative phage regulatory protein (pseudogene)	Cell envelope synthesis or location
rpsE	3.225	30S ribosomal protein S5	Methyl group transfer
rnhB	3.209	putative ribonuclease HII	Poorly characterised
infA	3.204	translation initiation factor IF-1	Poorly characterised
SAV0855	3.18	hypothetical protein	Poorly characterised
rpmD	3.173	50S ribosomal protein L30	Poorly characterised
rpsM	3.168	30S ribosomal protein S13	Poorly characterised
rplW	3.141	50S ribosomal protein L23	Poorly characterised
rpsK	3.065	30S ribosomal protein S11	Poorly characterised
SAR0773	3.039	ABC transporter ATP-binding protein	Poorly characterised
SAR0344	3.012	putative Sec-independent protein translocase protein	Poorly characterised
rplD	2.987	50S ribosomal protein L4	Poorly characterised
rpmJ	2.977	50S ribosomal protein L36	Cell envelope synthesis or location
SAR2207	2.963	putative thymidine kinase	DNA replication, recombination and repair

adk	2.923	adenylate kinase	DNA replication, recombination and repair
sdrC	2.921	putative surface anchored protein	DNA replication, recombination and repair
rplC	2.918	50S ribosomal protein L3	DNA replication, recombination and repair
clfA	2.852	clumping factor	DNA replication, recombination and repair
glpD	2.844	aerobic glycerol-3-phosphate dehydrogenase	Cell envelope synthesis or location
prfA	2.804	peptide chain release factor 1	Cell envelope synthesis or location
SAR1635	2.754	putative helicase	Cell envelope synthesis or location
SAR2189	2.745	putative membrane protein	Cell envelope synthesis or location
SAR0271	2.744	putative transport protein	Lipid metabolism
SAR0554	2.741	putative peptidase	Poorly characterised
SAR2086	2.74	hypothetical phage protein	Poorly characterised
SAR2205	2.69	conserved hypothetical protein	Poorly characterised
SAR0212	2.684	putative membrane protein	Carbohydrate transport and metabolism
SAR2168	2.672	putative helicase	Poorly characterised
SAR1702	2.655	putative cysteine desulfurase	Poorly characterised
8325-2691	2.648	hypothetical protein	Poorly characterised
icaB	2.646	intercellular adhesion protein B	Poorly characterised
argH	2.645	putative argininosuccinate lyase	Poorly characterised
rplE	2.615	50S ribosomal protein L5	Poorly characterised
rpoA	2.614	DNA-directed RNA polymerase alpha chain	Poorly characterised
SAR1024	2.562	putative membrane protein (pseudogene)	Poorly characterised
def	2.556	putative polypeptide deformylase	Poorly characterised
fnb	2.551	fibronectin-binding protein precursor [conserved region]	Cell envelope synthesis or location
rpsJ	2.541	30S ribosomal protein S10	Cell envelope synthesis or location
rplQ	2.525	50S ribosomal protein L17	Cell envelope synthesis or location
murA1	2.522	putative UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Amino acid transport and metabolism
SAR1083	2.516	BipA family GTPase	Poorly characterised
mutS	2.511	DNA mismatch repair protein MutS	Cell envelope synthesis or location
SAR2170	2.462	D-alanine--D-alanine ligase	Translation, ribosomal structure and biogenesis

SAR1219	2.443	putative GTPase protein	Amino acid transport and metabolism
8325-2694	2.413	hypothetical capsid protein	Cell envelope synthesis or location
coa	2.4	staphylocoagulase precursor [conserved region]	Secretion
SAR1192	2.39	methionyl-tRNA formyltransferase	Carbohydrate transport and metabolism
SAR1701	2.387	conserved hypothetical protein	Amino acid transport and metabolism
8325-2689	2.381	hypothetical protein	Poorly characterised
SAR1429	2.378	putative membrane protein	Poorly characterised
SAR0918	2.372	NADH:flavin oxidoreductase / NADH oxidase family protein	Amino acid transport and metabolism
fnb	2.368		Amino acid transport and metabolism
SAR1404	2.351	ABC transporter ATP-binding protein	Energy production
SAV1997	2.349	hypothetical protein	Energy production
8325-2690	2.341	hypothetical protein	Amino acid transport and metabolism
grlB	2.34	topoisomerase IV subunit B	Amino acid transport and metabolism
glpK	2.323	glycerol kinase	Poorly characterised
SAR1653	2.315	conserved hypothetical protein	Energy production
SAR0120	2.31	putative ornithine cyclodeaminase	Nucleotide transport and metabolism
murG	2.31	putative UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide)pyr ophosphoryl-undecaprenol N-acetylglucosamine transferase	Poorly characterised
SAR2076	2.309	hypothetical phage protein	Poorly characterised
sdrD	2.308		Cell envelope synthesis or location
SAR1431	2.303	putative acetyltransferase	Poorly characterised
upp	2.302	uracil phosphoribosyltransferase	Poorly characterised
mnaA	2.251	UDP-GlcNAc 2-epimerase	Poorly characterised
SAR1173	2.245	putative RNA pseudouridylate synthase	DNA replication, recombination and repair
SAR1634	2.238	putative endonuclease	DNA replication, recombination and repair
SAR2505	2.238	putative transport system protein	Lipid metabolism
SAR2542	2.237	putative transport protein	Cell envelope synthesis or location
murA2	2.207	putative UDP-N-acetylglucosamine 1-	Cell envelope synthesis or location

		carboxyvinyltransferase	
8325-2692	2.202	hypothetical protein	Cell envelope synthesis or location
grlA	2.199	topoisomerase IV subunit A	Cell envelope synthesis or location
polC	2.193	DNA polymerase III PolC-type	Cell envelope synthesis or location
SAR2297	2.177	putative acetolactate synthase	Cell envelope synthesis or location
rplB	2.171	50S ribosomal protein L2	Cell envelope synthesis or location
fabZ	2.162	putative hydroxymyristoyl-(acyl carrier protein) dehydratase	Amino acid transport and metabolism
lytM	2.16	peptidoglycan hydrolase	Amino acid transport and metabolism
SAR1193	2.159	hypothetical protein	Protease
SAR1675	2.153	conserved hypothetical protein	Poorly characterised
SAR0276	2.143	putative membrane protein	Poorly characterised
SAR2202	2.138	conserved hypothetical protein	Poorly characterised
SAR2368	2.135	putative ferrichrome-binding lipoprotein precursor	Poorly characterised
fus	2.135	translation elongation factor G	Nucleotide transport and metabolism
atpH	2.132	putative ATP synthase delta chain	Nucleotide transport and metabolism
sbi	2.125	IgG-binding protein	Poorly characterised
SAR0893	2.123	putative membrane protein	Poorly characterised
topA	2.121	DNA topoisomerase I	Poorly characterised
pgm	2.121	putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Secretion
SAR1674	2.119	putative GTPase	DNA replication, recombination and repair
SAR1059	2.115	putative cytochrome ubiquinol oxidase	Cell envelope synthesis or location
SAN1789	2.112	hypothetical protein	Amino acid transport and metabolism
dnaG	2.101	DNA primase	Nucleotide transport and metabolism
SAR1484	2.099	putative GTPase	Cell envelope synthesis or location
aroA	2.098	3-phosphoshikimate 1-carboxyvinyltransferase	Cell envelope synthesis or location
SAR1654	2.09	conserved hypothetical protein	Cell envelope synthesis or location
SAR0623	2.09	putative membrane protein	Aminosugar metabolism
SAR2151	2.087	putative RNA binding protein	Cell envelope synthesis or location

gatC	2.082	glutamyl-tRNA amidotransferase subunit C	Cell envelope synthesis or location
SAR1265	2.081	putative pyruvate flavodoxin/ferredoxin oxidoreductase	Cell envelope synthesis or location
SAR1209	2.08	putative ribonuclease III	Amino acid transport and metabolism
SAR1227	2.067	conserved hypothetical protein	Cell envelope synthesis or location
8325-2686	2.066	hypothetical protein	DNA replication, recombination and repair
glyA	2.063	serine hydroxymethyltransferase	DNA replication, recombination and repair
tpiA	2.055	triosephosphate isomerase	Translation, ribosomal structure and biogenesis
SAR0186	2.034	putative ornithine aminotransferase precursor	Translation, ribosomal structure and biogenesis
SAR2139	2.029	conserved hypothetical protein	Carbohydrate transport and metabolism
SAR2175	2.014	conserved hypothetical protein (pseudogene)	Aminosugar metabolism
SAR1194	2.013	conserved hypothetical protein	DNA replication, recombination and repair
SAR0277	2.012	putative exported protein	Cell envelope synthesis or location
SAR1195	2.012	putative protein phosphatase	Cell envelope synthesis or location
msrA2	2.005	peptide methionine sulfoxide reductase II	Cell envelope synthesis or location
atpF	2.005	putative ATP synthase subunit b	Cell envelope synthesis or location

Genes down-regulated by penicillin G in RS1/19 compared to Oxford strain ($P < 0.05$)

Gene	Fold change	Product or putative function	Functional category
narT	0.0921	nitrite transport protein	Amino acid transport and metabolism
narG	0.0976	nitrate reductase alpha chain	Amino acid transport and metabolism
narH	0.129	nitrate reductase beta chain	Amino acid transport and metabolism
nasD	0.182	nitrite reductase large subunit	Amino acid transport and metabolism
cudA	0.224	putative betaine aldehyde dehydrogenase	Amino acid transport and metabolism
SAR0523	0.286	SNO glutamine amidotransferase family protein	Amino acid transport and metabolism
cudB	0.301	putative choline dehydrogenase	Amino acid transport and metabolism
SAR2641	0.397	putative aminotransferase	Amino acid transport and metabolism
SAR2640	0.45	D-isomer specific 2-hydroxyacid dehydrogenase	Amino acid transport and metabolism
SAR2679	0.451	putative alpha-acetolactate decarboxylase	Amino acid transport and metabolism
SAR1337	0.452	putative aspartate kinase	Amino acid transport and metabolism
thrC	0.453	threonine synthase	Amino acid transport and metabolism
ilvD	0.453	putative dihydroxy-acid dehydratase	Amino acid transport and metabolism
SAR0131	0.16	putative sugar transferase	Carbohydrate transport and metabolism
SAR0130	0.174	NAD dependent epimerase/dehydratase family protein	Carbohydrate transport and metabolism
SAR0558	0.403	conserved hypothetical protein	Carbohydrate transport and metabolism
ldh2	0.444	L-lactate dehydrogenase 2	Carbohydrate transport and metabolism
SAR0129	0.476	putative short chain dehydrogenase	Carbohydrate transport and metabolism
SAR0924	0.492	putative glucose-6-phosphate isomerase	Carbohydrate transport and metabolism
SAR0133	0.22	putative membrane protein	Cell envelope synthesis or location
SAR0954	0.233	transport system extracellular binding lipoprotein (pseudogene)	Cell envelope synthesis or location
SAR0132	0.268	galactosyl transferase	Cell envelope synthesis or location
SAR2743	0.297	putative capsule synthesis protein	Cell envelope synthesis or location
SAR2745	0.297	putative capsule synthesis protein	Cell envelope synthesis or location
SAR2744	0.299	putative capsule synthesis protein	Cell envelope synthesis or location
SAR0199	0.301	putative transport system permease	Cell envelope synthesis or location

SAR0145	0.341	putative lipoprotein	Cell envelope synthesis or location
SAR0390	0.348	putative lipoprotein	Cell envelope synthesis or location
SAR2651	0.349	putative membrane protein	Cell envelope synthesis or location
lrgB	0.361	holin-like protein	Cell envelope synthesis or location
opuCB	0.394	putative glycine betaine/carnitine/choline transport system permease protein	Cell envelope synthesis or location
opuCA	0.394	putative glycine betaine/carnitine/choline transport ATP-binding protein	Cell envelope synthesis or location
SAR0139	0.403	putative transport system protein	Cell envelope synthesis or location
SAR1066	0.438	putative lipoprotein	Cell envelope synthesis or location
SAR2462	0.441	putative membrane protein	Cell envelope synthesis or location
rlp	0.443	RGD-containing lipoprotein	Cell envelope synthesis or location
lrgA	0.447	holin-like protein	Cell envelope synthesis or location
SAR0182	0.449	putative membrane protein	Cell envelope synthesis or location
SAR0794	0.471	putative lipoprotein	Cell envelope synthesis or location
yycJ	0.471	metallo-beta-lactamase superfamily protein	Cell envelope synthesis or location
SAR0781	0.474	putative proton-dependent oligopeptide transport protein	Cell envelope synthesis or location
SAR0637	0.487	sodium/hydrogen exchanger family protein	Cell envelope synthesis or location
SAR0013	0.495	putative membrane protein	Cell envelope synthesis or location
opuCC	0.496	putative glycine betaine/carnitine/choline-binding lipoprotein precursor	Cell envelope synthesis or location
ribD	0.209	bifunctional riboflavin biosynthesis protein	Cofactor biosynthesis
ribA	0.299	riboflavin biosynthesis protein	Cofactor biosynthesis
SAR0522	0.35	putative pyridoxine biosynthesis protein	Cofactor biosynthesis
ribE	0.415	riboflavin synthase alpha chain	Cofactor biosynthesis
SAR0917	0.315	putative S1 RNA binding domain	DNA replication, recombination and repair
recR	0.378	putative recombination protein	DNA replication, recombination and repair
SAR0452	0.483	putative NADH-Ubiquinone/plastoquinone (complex I) protein	Energy production
plc	0.405	1-phosphatidylinositol phosphodiesterase	Lipid metablism
SAR2351	0.262	hypothetical protein	Poorly characterised
SAR2520	0.334	hypothetical protein	Poorly characterised
SAR1326	0.337	conserved hypothetical protein	Poorly characterised

SAR2113	0.339	hypothetical protein	Poorly characterised
SAR2768	0.341	conserved hypothetical protein	Poorly characterised
SAR2115	0.343	hypothetical protein	Poorly characterised
SAR0383	0.371	hypothetical protein	Poorly characterised
SAR1688	0.394	conserved hypothetical protein	Poorly characterised
SAR1683	0.406	putative membrane protein	Poorly characterised
SAR2498	0.422	conserved hypothetical protein	Poorly characterised
SAR0111	0.427	putative myosin-crossreactive antigen	Poorly characterised
SAR0405	0.428	hypothetical protein	Poorly characterised
SAR1784	0.435	putative universal stress protein	Poorly characterised
SAR2482	0.439	hypothetical protein	Poorly characterised
SAR0012	0.443	putative hydrolase	Poorly characterised
SAR1914	0.45	hypothetical protein (pseudogene)	Poorly characterised
SAR2688	0.45	hypothetical protein	Poorly characterised
SAR2381	0.451	hypothetical protein	Poorly characterised
SAR0985	0.452	conserved hypothetical protein	Poorly characterised
SAR0969	0.457	conserved hypothetical protein	Poorly characterised
SAR2380	0.46	hypothetical protein	Poorly characterised
SAR2497	0.466	conserved hypothetical protein	Poorly characterised
SAN0930	0.472		Poorly characterised
SAR1279	0.481	conserved hypothetical protein	Poorly characterised
SAR2120	0.484	conserved hypothetical protein	Poorly characterised
SAR0666	0.49	hypothetical protein	Poorly characterised
SAR1684	0.495	conserved hypothetical protein	Poorly characterised
SAR0342	0.497	putative membrane protein	Poorly characterised
SAR2487	0.213	tetrapyrrole (corrin/porphyrin) methylase family protein	Porphyrrin metabolism
sodM	0.496	superoxide dismutase	Reactive oxygen species metabolism
SAR2382	0.384	putative transcriptional regulator	Signal transduction mechanisms
srrB	0.413	sensor kinase protein	Signal transduction mechanisms
SAR2026	0.432	GntR family regulatory protein	Signal transduction mechanisms
SAR2481	0.465	putative histidine kinase	Signal transduction mechanisms
icaR	0.486	ica operon transcriptional regulator	Signal transduction mechanisms
SAR2480	0.488	putative response regulator	Signal transduction mechanisms
agrA	0.493	autoinducer sensor protein response regulator protein	Signal transduction mechanisms
SAR0806	0.233	putative S30EA family	Translation, ribosomal structure and

		ribosomal protein	bogenesis
miaA	0.481	putative tRNA delta 2-isopentenylpyrophosphate transferase	

Alignment of *pbp4* gene sequence between wild-type Oxford and mutant strain RS1/19 and T12. No mutations have been detected. Wild-type, RS1/19, T12 are indicated on the left

Alignment of intergenic region between *pbp4* and *abcA* genes sequences, including portions of each open reading frame. No mutations have been detected. Wild-type, RS1/19, T12 are indicated on the left